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

Part 1: Analysis of Agilent 6210 TOF data with the Molecular Feature Extractor in MassHunter Workstation software

Application Note

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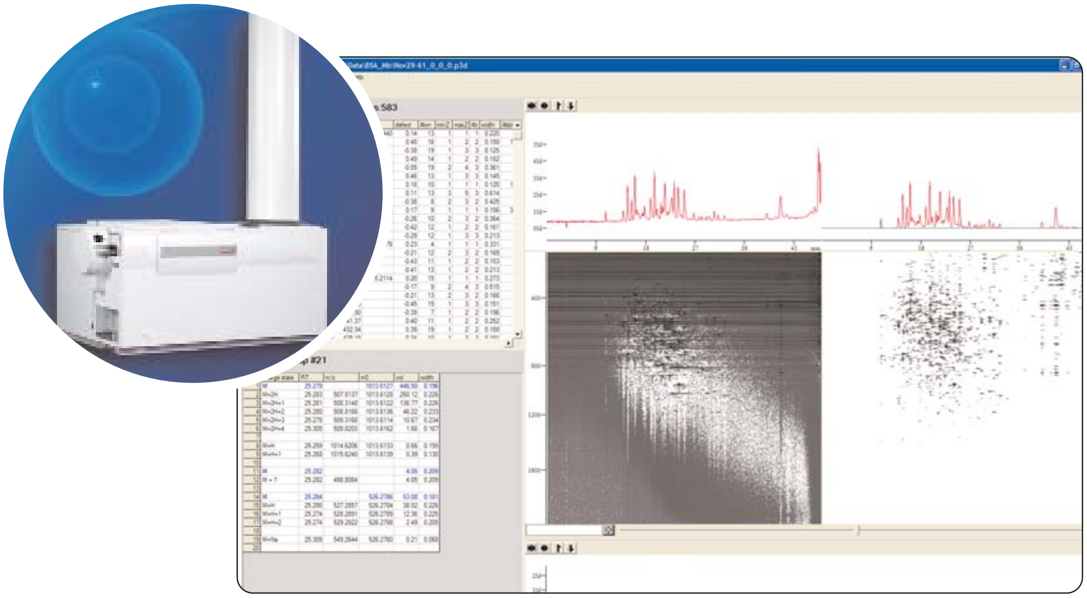

Abstract

This Application Note describes:
- Accurate mass measurement with LC/ESI-TOF for the determination of complex natural compound structures
- Fast computer aided identification and elucidation of chemical structures of new natural product compounds
- 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 Molecular Feature Extractor in the MassHunter Workstation software, to generate molecular features, which are discrete molecular entities defined by the combination of retention time and mass.

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

Application Area:
Natural product analysis in drug discovery

Agilent Technologies

Introduction

Since prehistoric times, herbal extracts have been used for the treatment of disease. Their effects on humans were found by 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 potential. But Western pharmaceutical quality standards require a deep knowledge about the ingredients in medicine based on natural products. A famous Asian herb, which has been used in herbal medicine for more than 5000 years, is the ginseng root (Panax species). Pharmacological effects of ginseng that have been reported are, for example, stimulatory and inhibitory effects on the central nervous system (CNS), anti-stress, antihyperglycemic, antineoplastic and immunomodulatory effects[1]. The main active compounds of the ginsenosides are triterpene saponins, of which more than 80 have been isolated and characterized during the past years.

A lot 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 the ginseng root, is high performance liquid chromatography (HPLC)[2]. For the determination of the complex and similar structures of ginsenosides, modern LC/MS equipment such as the LC/ESI-TOF 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[3]. However the time consuming bottleneck is the examination of the acquired MS data to identify and elucidate chemical structures of new natural product compounds.

This Application Note demonstrates the use of the Agilent 1200 Series Rapid Resolution LC (RRLC) system with rapid resolution high throughput (RRHT) columns for the separation of ingredients in a complex ginseng root extract, in combination with the Agilent 6210 ESI TOF mass spectrometer for accurate measurement of their molecular masses. The TOF data obtained were processed by the Molecular Feature Extractor (MFE) of the MassHunter Workstation software to generate molecular features, which are discrete molecular entities defined by the combination of retention time and mass. Natural product compounds were identified from this simplified MS data. The composition differences between ginseng sub-species is elucidated by software-aided data comparison[4] and an example for an automated approach to analyze complex natural product extracts is given[5].

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 for best resolution performance.
- Agilent 1200 Series high performance autosampler SL (h-ALS) with thermostat. This autosampler is especially designed to work together with the Agilent 1200 Series binary pump SL.
- Agilent 1200 Series thermostatted column compartment (TCC). This TCC is ready for use with the binary pump SL with optional separate heat exchangers and post column cooling under optimized delay volume conditions, as well as alternating column regeneration with an optional 2-position/10-port valve.
- Agilent 1200 Series diode array detector SL (DAD SL) which can acquire data at a sampling rate of up to 80 Hz.
- Agilent 6210 MSD TOF. Orthogonal acceleration time-of-flight (TOF) mass spectrometer with dual sprayer interface for mass calibration to acquire molecular masses with highest accuracy. This TOF mass spectrometer 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 particle size.
- Software: TOF instrument control software MassHunter Workstation A02.00 for data acquisition, Analyst software for data review and Molecular Feature Extractor (MFE) software for data processing.

In this LC/TOF instrument set-up, the binary pump SL, which is in the low delay volume configuration, is connected to the h-ALS SL with a 0.12-mm ID stainless steel capillary. To reduce delay volume, the seat capillary in the h-ALS SL has a 0.12 mm ID. 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 cell is built into the DAD SL. The outgoing capillary is directly connected to the sprayer in the electrospray source of the TOF mass spectrometer. This instrument set-up is optimized to achieve the highest possible resolution.

Sample preparation

Powdered freeze-dried Asian and American ginseng root (1g) (*Panax ginseng and Panax quinquefolius*) was ultrasonically treated for 30 minutes in 10 mL methanol, filtrated 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 for 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 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, ref. 360/100 with the 2-µL flow cell, 3-mm path length.
- The 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 or 300 V for CID
  Skimmer: 60 V
  Capillary: 3000 V

Results and discussion

The ingredients of a ginseng root extract from an Asian ginseng root (*Panax ginseng*) were separated with the Agilent 1200 Series RRLC system on a RRHT column (1.8-µm particle size) with subsequent ESI TOF mass spectrometry (Agilent 6210 TOF). The high resolution LC provided an excellent separation of the major and minor ingredients of the natural product extract (figure 1). The acquired TOF data were extracted by the MFE software and the identified molecular ions of the comprised compounds were separated from undefined

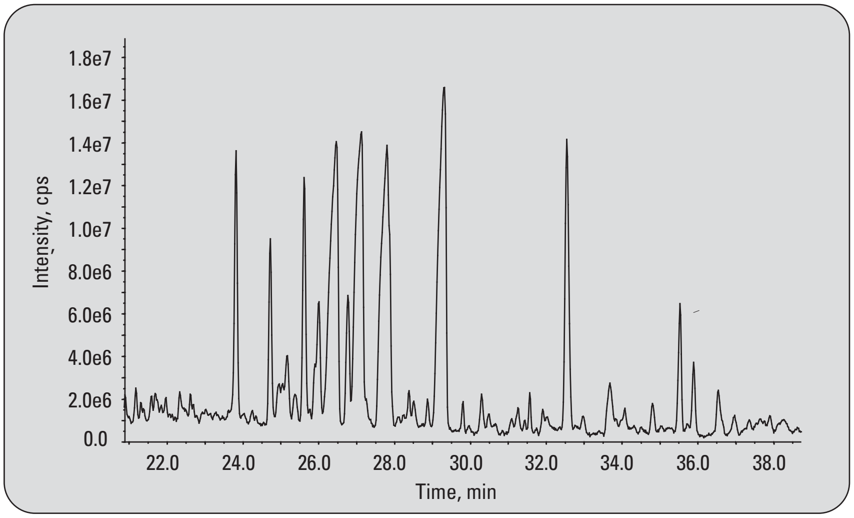

Figure 1
High resolution LC on a 1.8 µm particle column with an extract from Asian ginseng root (*Panax ginseng*) for MS analysis by ESI-TOF.

background ions (figure 2). In this process, the identified ions were clustered to molecular features comprising isotope compounds and adducts. From the processed data, the feature group #7 at the retention time of 26.3 min will be examined in more detail to identify these compounds in the ginseng extract. At this retention time, 20 different features, molecular ions together with their different adducts and isotopes, are grouped together. The processed EIC of feature group #7 at a retention time of 26.3 min is shown in figure 2B. The capability to extract groups of ions as molecular features can be optically displayed by a detailed 2D contour plot of the spectrum, which shows retention time and the mass of the ions (figure 3). The unprocessed chromatograms of the protonated ginsenoside Rb1 ion at m/z 1109.6 and the sodiated ion at m/z 1131.4, both at a retention time 26.3 min, are showing a lot of unspecific chemical noise ions (figures 3A and 3C). These unspecific ions can not be grouped to a molecular feature because of an unclear isotopic distribution and are eliminated during data processing. The ions remaining at the same retention time window are grouped together to molecular features comprised of adducts and iso-

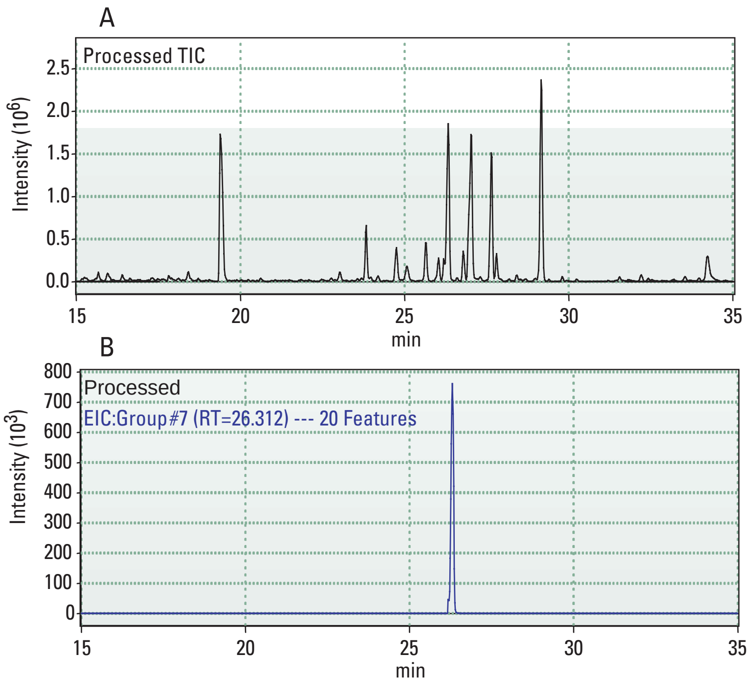

Figure 2
A) Software processed TIC of Asian ginseng extract and grouped molecular features.
B) Feature group # 7 at retention time 26.3 min groups 20 molecular features.

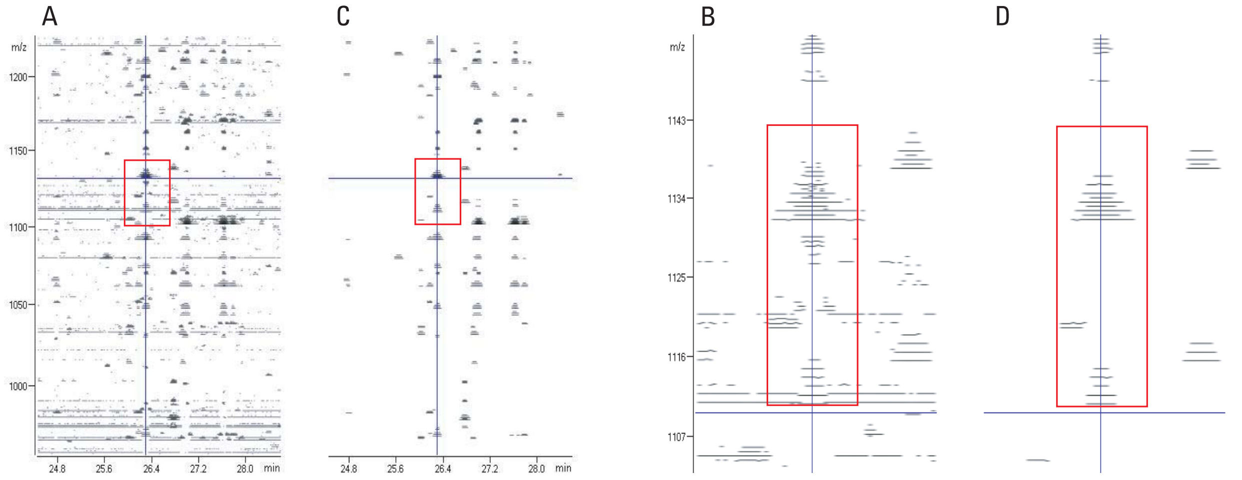

Figure 3
2D contour plot of TOF spectrum of ginseng extract, retention time vs. mass. A) Unprocessed 2D contour plot at retention time 24.8 – 28.0 min and mass 900 – 1250. B) Magnified contour plot around the unprocessed ginsenoside Rb1 ions. C and D) Contour plot of processed data of the same mass and retention time area.

topes of the basic ion (figures 3B and 3D). The magnified window (figures 3C and 3D) clearly demonstrates the simplification of the data. The data enhancement can also be demonstrated by the comparison of the original mass spectrum of ginsenoside Rb1 (figure 4A) to the processed mass spectrum (figure 4B). The significant simplification of the mass spectra resulting from the generation of molecular features and elimination of chemical noise allows an easier interpretation, especially when fragments of the molecular ion are generated by collision induced decay (CID) for structure elucidation (figure 4B). These fragments, which elute at the same time, are included in the same group of molecular features together with the molecular ion. The interpretation of the spectrum from ginsenoside Rb1 for structure elucidation is shown in figure 5. The protonated molecular ion was found at m/z 1109.6104 and the sodium adduct at m/z 1131.5926. The fragment at m/z 785.5045 is derived from the loss of one glucose moiety. An additional loss of a molecule of water results in the

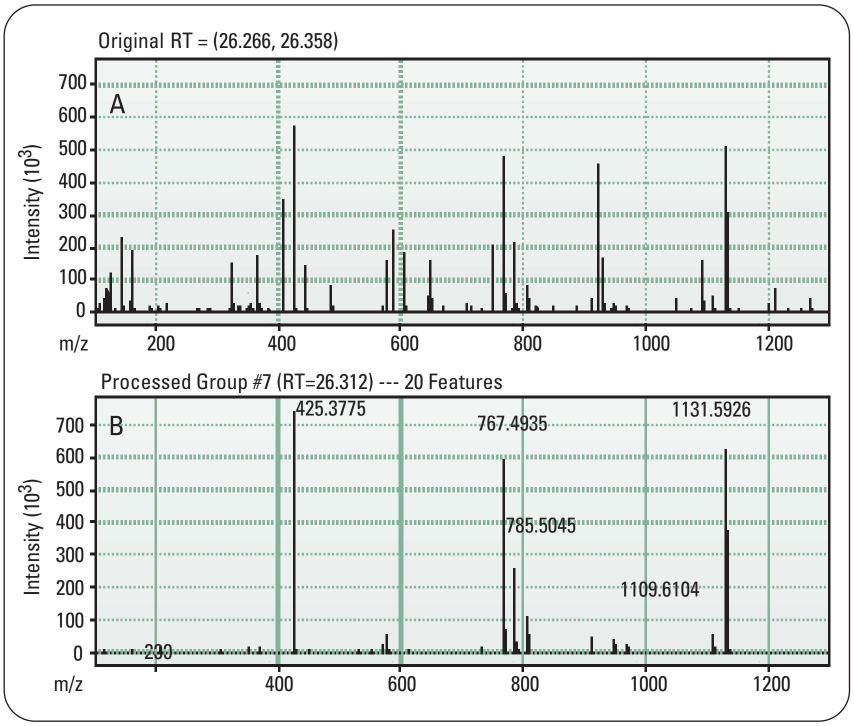

Figure 4
A) Unprocessed mass spectrum at 26.3 min. B) Processed mass spectrum at 26.3 min showing molecular features, which belong to the same group.

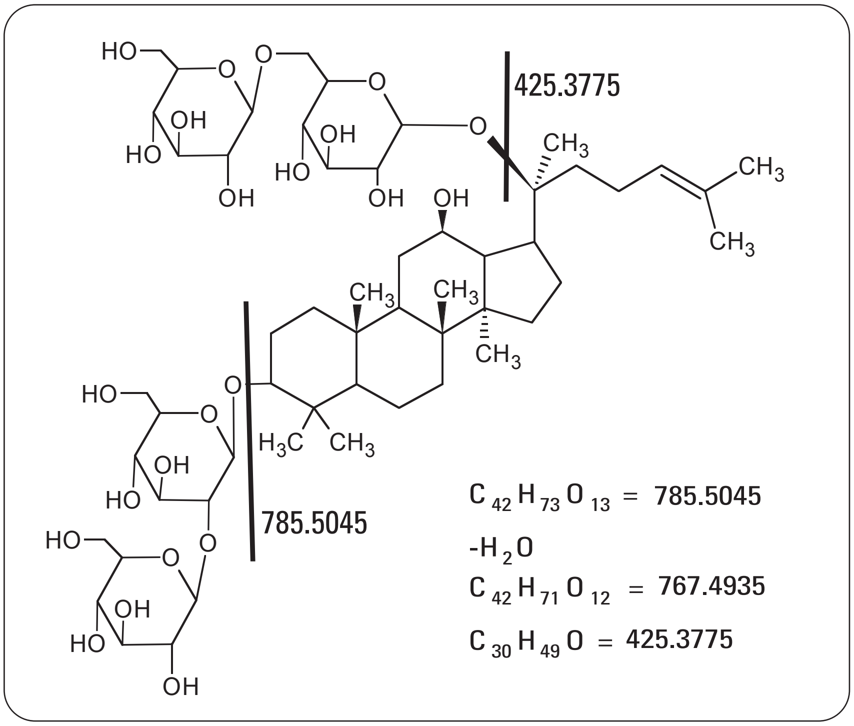

Figure 5
Structure of ginsenoside Rb1 obtained by interpretation of the processed mass spectrum with molecular features included.

fragment at m/z 767.4935. The loss of the second glucose moiety yielded the ion at m/z 425.3775 from the terpenoide core structure. The identified molecular features for ginsenoside Rb1 and its CID fragments are summarized in table 1. The protonated, sodiated and potasiated adducts are found with high mass accuracies of -0.14, -0.41 and -0.32 ppm, respectively. The calculated mass accuracy of the molecule is -0.41 ppm. In addition, the mass accuracies for the obtained CID fragments and their adducts are calculated (table 1). This confirms the empirical formula and the proposed molecular structure. The content of a natural product extract often depends on the subspecies, the growth region and growth conditions. To decide if a natural plant extract contains the right active ingredients in proper concentration, a comparison to a standard extract is necessary. For ginseng the different subspecies contain common ginsenosides as well as specific ginsenosides. A typical example is Asian ginseng (*Panax ginseng*) (figure 2) in comparison to American ginseng (*Panax quinquefolius*). The simplification achieved by the generation of molecular features unravels the differences (figure 6). Especially the high content of ginsenoside F11 eluting at 24.5 minutes and the absence of ginsenoside Rf (eluting at 24 minutes in figure 2) is typical for the American ginseng subspecies[5].

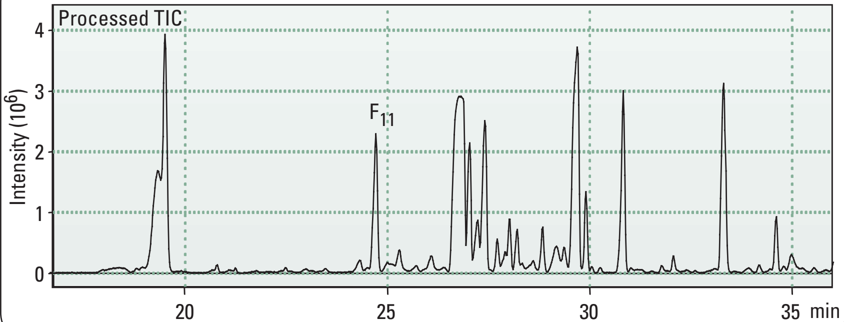

Figure 6
Software processed TIC of American ginseng extract and grouped molecular features.

Species	RT	m/z	Mass	Accuracy	RT	m/z	Mass	Accuracy	RT	m/z	Mass	Accuracy	RT	m/z	Mass	Accuracy
M+H	26.31	1109.6104	1108.6031	- 0.14 ppm	26.31	785.5045	784.4972	0.10 ppm	26.31	767.4935	766.4862	0.68 ppm	26.31	425.3775	424.3702	0.75 ppm
M+H+1	26.31	1110.6152			26.31	786.5081			26.31	768.4976			26.31	426.3813		
M+H+2	26.31	1111.6176			26.31	787.5108			26.31	769.5004			26.31	427.3850		
M+H+3	26.31	1112.6219			26.31	788.5134			26.31	770.5027			26.31	428.3882		
M+H+4	-----	-----			-----	-----			26.31	771.4965			-----	-----		
M+Na	26.31	1131.5926	1108.6034	- 0.41 ppm	26.31	807.4486	784.4976	0.39 ppm	26.31	789.4756	766.4864	0.42 ppm	-----	-----	-----	-----
M+Na+1	26.31	1132.5964			26.31	808.4902			26.31	790.4809			-----	-----		
M+Na+2	26.31	1133.599			26.31	809.4950			26.30	791.4883			-----	-----		
M+Na+3	26.31	1134.6021			26.30	810.4938			-----	-----			-----	-----		
M+Na+4	26.31	1135.5998			-----	-----			-----	-----			-----	-----		
M+K	26.30	1147.5664	1108.6033	- 0.32 ppm	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
M	**26.31**		**1108.6034**	**- 0.41 ppm**	**26.31**		**784.4972**	**+ 0.10 ppm**	**26.31**		**766.4862**	**+ 0.7 ppm**	**26.31**		**424.3702**	**+ 0.75 ppm**

Table 1
Identified molecular features and calculated mass accuracies for empirical formula confirmation. (Ginsenoside Rb1, $C_{54}H_{92}O_{23}$, M 1108.6029; Fragment $C_{42}H_{72}O_{13}$, M 784.4973; Fragment $C_{42}H_{70}O_{12}$, M 766.4867; Fragment $C_{30}H_{49}O$, M 424,3705).

Conclusion

The ingredients of highly complex natural product samples can be accurately separated by the Agilent 1200 Series RRLC system with 1.8 µm particle size columns. By connecting the ESI-TOF MS, a large amount of accurate mass data for empirical formula calculation and compound structure elucidation can be created. This data is processed by the Molecular Feature Extractor of the MassHunter Workstation software, which generates molecular features, including the molecular ion, its isotopes and adducts, to simplify the large amount of data and to help with data interpretation. The highly accurate measured masses of the different molecular species of molecular ions and CID fragments, included in the molecular features, are used for empirical formula calculation and structure elucidation.

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Published January 1, 2007
Publication Number 5989-5928EN

Agilent Technologies