Examination of the differences in related complex samples using accurate mass data from TOF and QTOF analysis

Differential analysis of Agilent 6210 time-of-flight and Agilent 6510 quadrupole time-of-flight MS data using Agilent MassHunter Profiling software

Application Note

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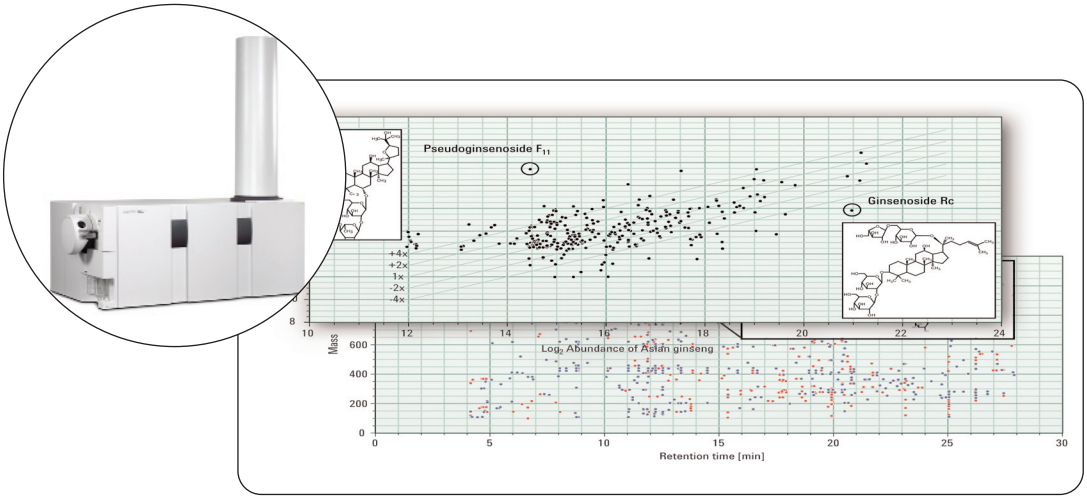

Abstract

This Application Note describes:

- Separation of compounds in complex samples using an Agilent 1200 Series Rapid Resolution LC system and Agilent ZORBAX Rapid Resolution High Throughput columns.
- Identification of unknown compounds by empirical formula calculation from high mass accuracy data acquired with the Agilent 6210 time-of-flight and teh Agilent 6510 Q-TOF mass spectrometer.
- Fast, computer-assisted analysis of complex mass spectrometry data using the Agilent MassHunter Profiling software.
- Examination of differences in related samples in different applications using the Agilent MassHunter Profiling software.

Agilent Equipment

- 1200 Series RRLC system
- 6210 time-of-flight MS
- 6510 quadrupole time-of-flight MS
- ZORBAX RRHT columns
- MassHunter workstation software

Application Area

- Drug discovery and development

Agilent Technologies

Introduction

The analysis of highly complex samples such as natural product extracts, metabolism reactions or compound degradations are easy to perform with modern analytical equipment. Today's analytical chemist operates liquid chromatography devices with sub-two-micron columns for high performance separations as well as mass spectrometer (MS) devices such as time-of-flight (TOF) or quadrupole time-of-flight (QTOF) for highest resolution and accurate mass measurement in the single-digit ppm mass error range. With these high performance LC/MS devices a large amount of data can be generated. The bottleneck in the analysis of the sample is now the analysis of the acquired MS data. A special case is the analysis of newly emerging compounds in a set of samples from different origin. These could be natural product extracts from different subspecies, compounds emerging during a metabolism reaction or during a degradation reaction. This Application Note demonstrates the differential analysis of TOF and QTOF data from different subgroups using the Agilent MassHunter Profiling software for the identification of newly emerging compounds in a single group.

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution LC system
- Agilent 6210 TOF mass spectrometer
- Agilent 6510 QTOF mass spectrometer
- Agilent ZORBAX SB C18, 2.1 x 150 mm, 1.8 µm particle size Rapid Resolution High Throughput (RRHT) column
- Agilent MassHunter Workstation software for data acquisition
- Agilent MassHunter Qualitative software, Molecular Feature Extractor software and Profiling software for data analysis

Method

- The Agilent 1200 Series binary pump SL was operated under the following conditions:
  Solvent A: Water + 0.1 % formic acid
  Solvent B: AcN + 0.1 % formic acid
  Flow: 0.5 mL/min
  Gradient: 0 min, 5 %B, up to 95 %B
  Stop time: 20 min
  Post time: 10 min.
- The Agilent 1200 Series high performance autosampler SL was used to make injections of 1 µL sample and the samples were cooled. The sample loop was switched to bypass after flushing with 20 times the injection volume to reduce delay volume.
- The Agilent 1200 Series thermostated column compartment SL was adjusted to 50 °C and equipped with the low delay volume heat exchanger.
- The Agilent 1200 Series DAD SL was operated at a wavelength of 220 nm ±4 nm, reference 360 nm ± 16nm, with the 2 µL flow cell, 3 mm path length.
- The Agilent 6210 TOF mass spectrometer was operated under the following conditions:
  Source: ESI in positive mode with dual spray for reference mass (m/z 121.05087 and m/z 922.00980)
  Dry gas: 10 L/min
  Dry Temp.: 300 °C
  Nebulizer: 50 psi
  Scan: 200-1300
  Fragmentor: 200 V
  Skimmer: 60 V
  Capillary: 3500 V.
- The Agilent 6510 QTOF mass spectrometer was operated under the same conditions. In addition, data-dependent MS/MS spectra were acquired with one MS spectrum per second and two MS/MS spectra per second, two selected precursors for three spectra and subsequent exclusion for 0.5 minutes.
- For the data analysis with the Agilent MassHunter Profiling software, five repeated injections of each extract sample were measured by the same LC/MS method. The acquired MS data were extracted by the Molecular Feature Extractor in the Agilent MassHunter Qualitative software or standalone version software. In this process, the identified ions were clustered to molecular features comprising isotope compounds and adducts. The obtained files were grouped according to the samples into two respective groups and loaded into the Agilent MassHunter Profiling software. For the differential analysis of both groups the features of each group are displayed in a $\log_2$ plot showing the abundance ratio of first sample against second sample. In the plot there are five lines for selected levels of abundance difference in the two sample

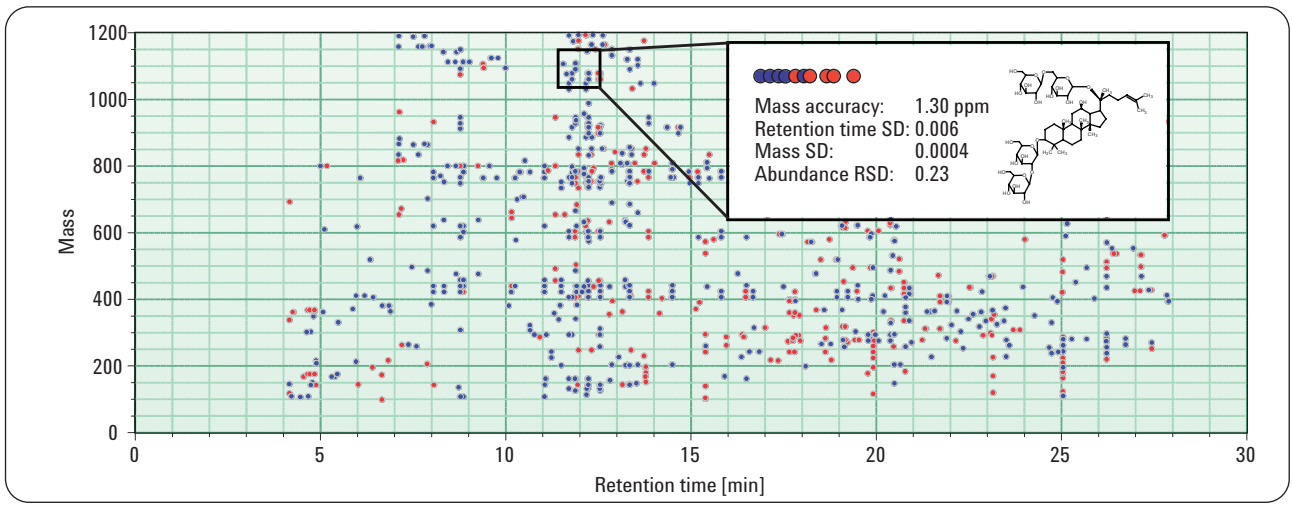

Figure 1
All 671 molecular features found in the Asian (red) and American (blue) ginseng extracts and molecular features of ginsenoside Rb1 ($C_{54}H_{92}O_{23}$ at M=1108.6029 and RT 11.90 min).

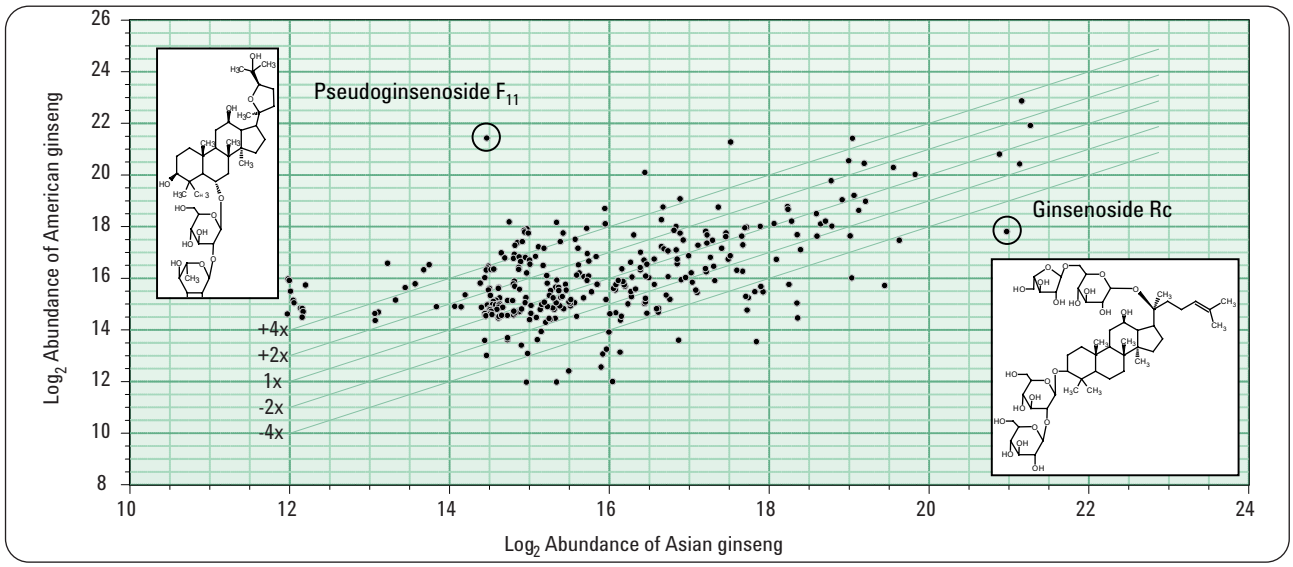

Figure 2
Differential analysis of Asian ginseng and American ginseng, showing pseudoginseoside F11 present exclusively in American ginseng and a higher concentration of ginsenoside Rc in Asian ginseng.

groups. Molecular features lying on the line in the middle (1x) are equal in both groups, molecular features in the margins of 2x are up to twice of the abundance in one group and in the margins of 4x up to fourfold. Beyond these margins a feature is nearly unique or exclusively present in one group.

Results and discussion

Identification of differences in complex natural product extracts from different subspecies[1]

From a sample of an extract from Asian ginseng (Panax ginseng) and from American ginseng (Panax quiniquefolius), 671 molecular features were identified and displayed in a mass against the retention time plot in the Agilent MassHunter Profiling software to inspect the quality of the data (figure 1). The molecular features for the known ginsenoside Rb1 ($C_{54}H_{92}O_{23}$ at M = 1108.6029, retention time 11.90 min) are magnified in the plot. The high quality of the data can be demonstrated by the standard deviations for retention time, mass and the relative standard deviation of the abundance in each group as well as by the high relative mass accuracy of 1.38 ppm for this particular compound. An example for a compound which has a higher concentration in the Asian ginseng sample group is the ginsenoside Rc (figure 2). Ginsenoside Rc in Asian ginseng has a signifi-

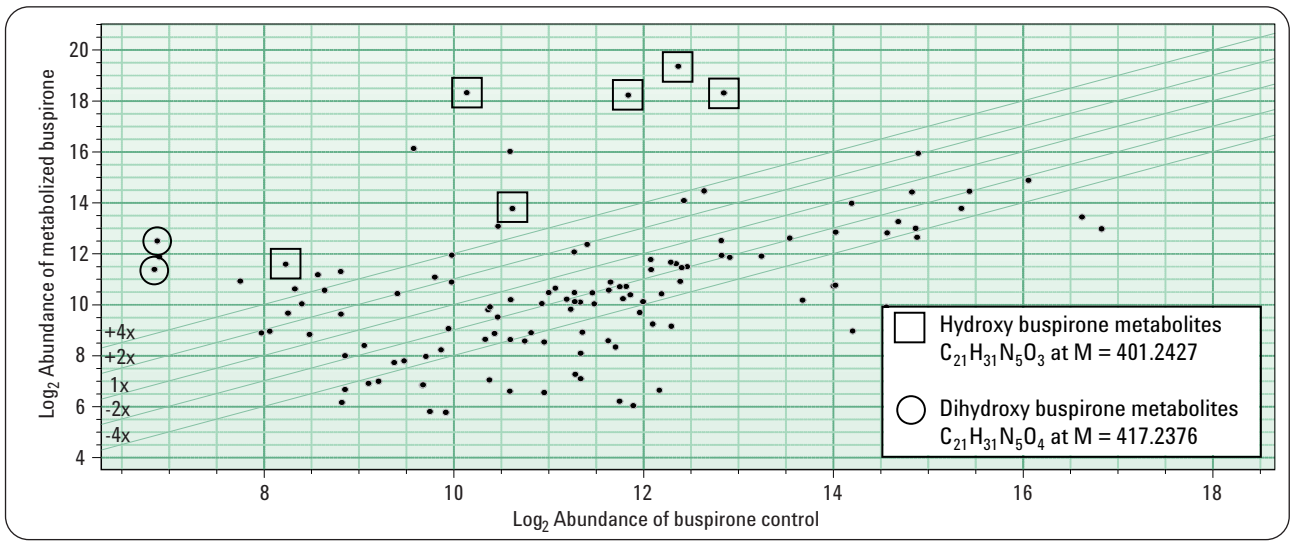

Figure 3
Differential analysis of the metabolized drug buspirone ($C_{21}H_{31}N_5O_2$ at M=385.2478) against an early-stopped metabolism control sample.

cantly higher abundance of about eigthfold than in American ginseng. The average relative mass error for this compound in the Asian ginseng sample group is 1.71 ppm and the average for the American ginseng sample group is 0.99 ppm. The molecular feature, which is easily recognizable as nearly exclusively present in the American ginseng samples, is the special compound pseudoginsenoside F11 (figure 2). This compound has an abundance of over 100-fold higher in the samples from the American ginseng root extract (table 1). For this compound, the average of relative mass accuracies in the American ginseng sample group is 1.53 ppm and for the Asian ginseng sample group it is 1.26 ppm.

Identification of metabolites of a pharmaceutical drug[2]

QTOF data from the in-vitro metabolized drug compound sample was compared to the control sample in which the metabolizing reaction was stopped immediately after incubation with microsomal S9 preparation. For this study the anxiolytic pharmaceutical drug buspirone ($C_{21}H_{31}N_5O_2$, M=385.2478) was used as a model drug compound. The differential plot clearly shows a few molecular features with higher abundance in the metabolized sample (figure 3). Examination of the molecular feature data points showed the molecular masses around M=401.2427 and at M=417.2326, which are the molecular masses for the mono and dihydroxy buspirone metabolites with the respective empirical formulas $C_{21}H_{31}N_5O_3$ and $C_{21}H_{31}N_5O_4$. As an example, the abundance of the monohydroxyl metabolite at retention time 5.5 min in the sample group with the complete metabolism reaction exceeded the abundance of this compound in the stopped control samples by about 400 times (table 2). The aver-

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	
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	1.53
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	
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	1.26
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 1
Retention times, abundancies and mass accuracies for pseudoginsenoside F11 ($C_{42}H_{72}O_{14}$ at M=800.4922) which is present nearly exclusively in American ginseng samples.

ID	Name	RT	Mass	Abundance	Mass error [mDa]	Mass error error [ppm]
1	Buspirone_metabolized-1	5.531	401.2421	409,387	-0.6	1.47
2	Buspirone_metabolized-2	5.524	401.2426	408,933	-0.1	0.22
3	Buspirone_metabolized-3	5.523	401.2426	410,559	-0.1	0.22
4	Buspirone_metabolized-4	5.523	401.2423	414,209	-0.4	0.97
5	Buspirone_metabolized-5	5.517	401.2425	409,787	-0.2	0.47
6	Buspirone_control-1	5.521	401.2426	911	-0.1	0.22
7	Buspirone_control-2	5.513	401.2421	1,008	-0.6	1.47
8	Buspirone_control-3	5.522	401.2415	887	-1.2	2.96
9	Buspirone_control-4	5.524	401.2430	934	-0.3	0.77
10	Buspirone_control-5	5.527	401.2412	1,083	-1.5	3.71

Table 2
Comparison of abundancies of the hydroxylated buspirone metabolite $C_{21}H_{31}N_5O_3$ at M=401.2427 and RT 5.55 min.

age relative mass accuracy for this particular compound at retention time 5.52 min was 0.67 ppm, which confirms the calculated empirical formula $C_{21}H_{31}N_5O_3$ for the monohydroxy metabolite. But this compound is only one possible isomer of the six identified compounds with an accurate mass of M = 401.2427. Therefore, after the described review of the sample, a more detailed data analysis on the MS/MS level in combination with accurate mass was necessary.

The first step in the manual metabolite identification was the extraction of the molecular masses of possible expected metabolites and of the parent drug from the MS data. For the example used in this study, the drug buspirone was found with excellent mass accuracy of -1.42 ppm for the protonated parent ion in the control sample. As expected metabolites the mono and dihydroxylated species were extracted. The EIC traces (not shown) indicated that there were more than one mono and dihydroxylated compounds, which were chromatographically separated. The different individual compounds were produced by oxidizing reactions, which took place at different parts of the parent drug leading to chromatographically different and separable compounds. To elucidate the structure of the metabolites a MS/MS spectrum of buspirone was extracted from the obtained QTOF data for comparison with the MS/MS spectra of the metabolites. For all assigned fragments the calculated relative mass accuracies were sufficient in the low single digit range to calculate the right empirical

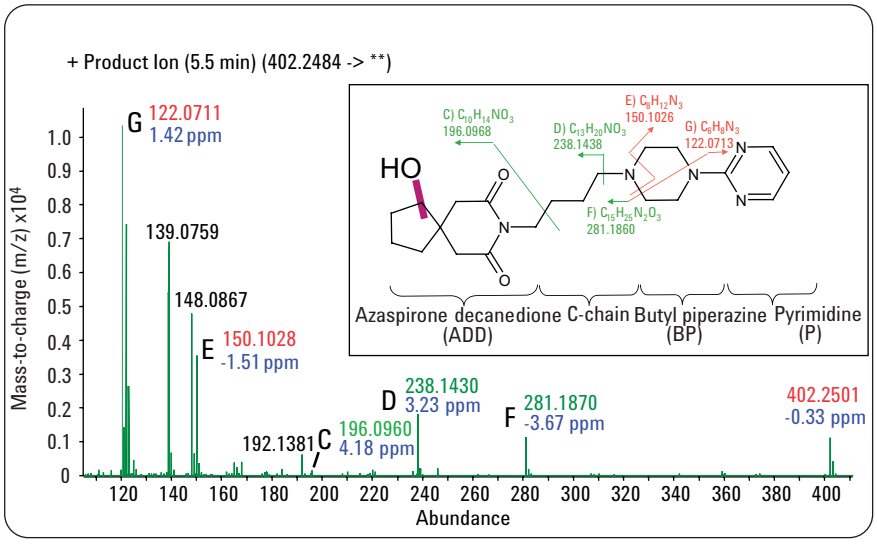

Figure 4
MS/MS spectrum of the monohydroxy metabolite of buspirone at RT 5.5 min. The mass shifts of the fragments (green) in comparison to the MS/MS spectrum of buspirone and the comparable fragments (red) indicate the hydroxylation at the ADD Group (calculated relative mass accuracies are annotated in blue).

formula of the fragments. Similar fragmentation patterns should be found for the MS/MS fragmentation of the metabolites and the mass shifts of some fragments should give information about the real structure and the modification. The MS/MS spectrum of the mono hydroxy buspirone metabolite eluting at retention time 5.5 min from the column was examined in more detail (figure 4). The shifted fragments were colored green and the remaining fragments were colored red. This identified clearly the oxygen modification by the shift of 16 for the assigned fragments and the location of the modification from the metabolism reaction on the azaspiro decane dion moiety of the molecule.

Identification of degradation products in a pharmaceutical formulation[3]

To identify the newly emerging degradation products a sample was taken from a formulation trial to optimize reaction conditions and compared to a standard of the pure drug compound amoxicillin. For the differential analysis of both groups the molecular features of each group are displayed in a $\log_2$ abundance plot showing the abundance ratio of the amoxicillin sample from formulation batch A against the amoxicillin standard sample (figure 5). The plot clearly shows a few molecular features with higher abundance in the degradated sample. Examination of the molecular feature data points shows the molecular mass at M=383.1150, M=365.1038 and at M=339.1248 with the respective calculated empirical formulas $C_{16}H_{21}N_3O_6S$, $C_{16}H_{19}N_3O_5S$ and $C_{15}H_{21}N_3O_4S$. The calculated empirical formulas were confirmed by the low relative mass errors of 0.28 ppm, 2.0 ppm and 1.4 ppm.

The empirical formulas belong to the known amoxicillin derivatives amoxicillin penicilloic acid (2), diketopiperazine amoxicillin (3) and amoxicillin penilloic acid (4).

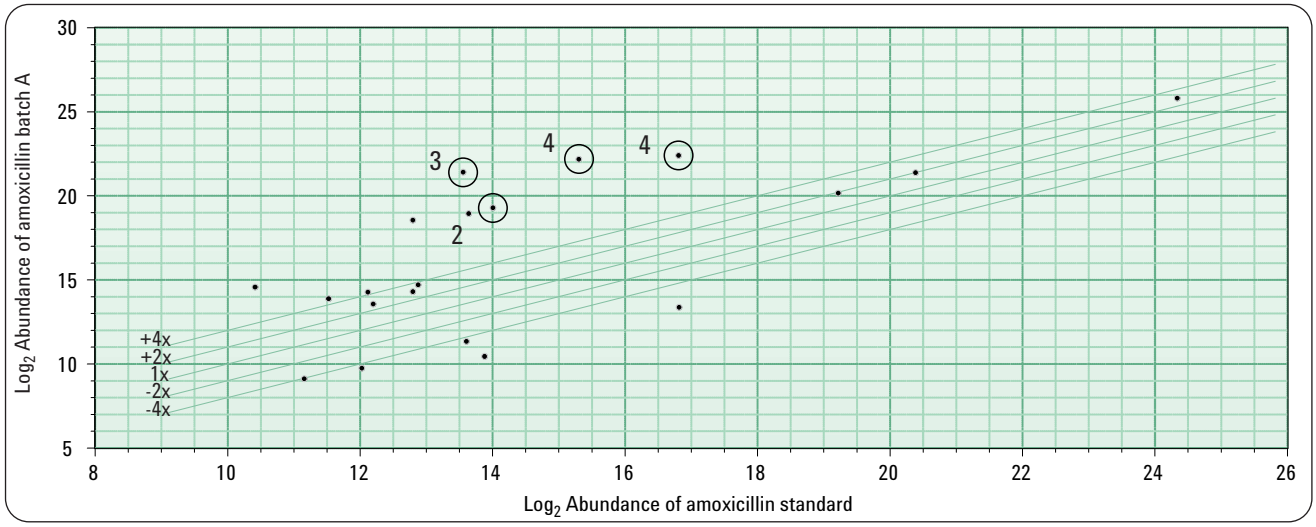

Figure 5
Comparison between a formulation batch of amoxicillin with degradation products and an amoxicillin standard using Agilent MassHunter Profiling software based on accurate TOF MS data.

The degradation reaction of amoxicillin (1), which was responsible for the generation of the impurities in the final drug formulation started with a hydrolysis reaction of the four membered beta lactame ring to yield the carboxylic acid (2) (figure 6). Rearrangement to a six membered ring structure led to the diketopiperazine derivative (3) and a decarboxylation reaction to the amoxicillin derivative (4).

The base peak chromatogram (BPC) between m/z 300–500 shows the peaks for the protonated compounds at retention times of 3.3 min for compound (2), 6.1 and 7.1 min for the diastereo isomeric compounds (4) and 8.9 minutes for compound (3). The main peak for amoxicillin was found at retention time 5.0 min. From the MS total ion chromatogram (TIC) the extracted ion chromatograms (EIC) for compound (2), (3) and (4) were extracted. The measured

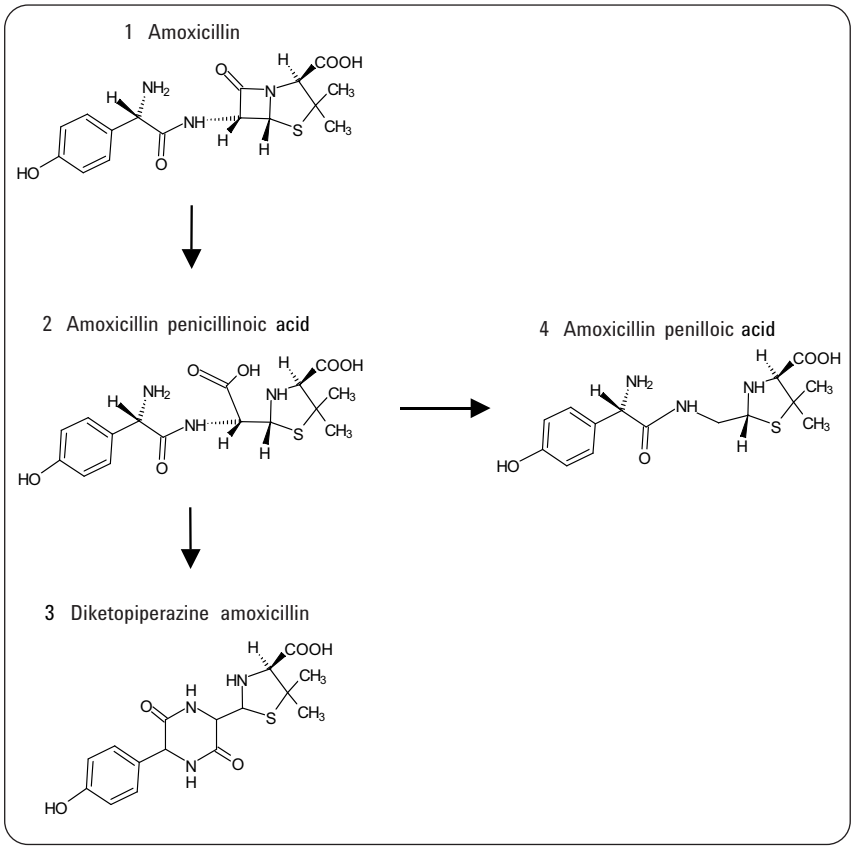

Figure 6
Degradation reactions and products from amoxicillin.

[M+H]$^+$ at m/z 366.1125 for compound (3) shows a very low relative mass error of 0.36 ppm (figure 7).

Conclusion

This Application Note demonstrated the examination of differences in related complex samples using the Agilent MassHunter Profiling software. The data was acquired by TOF or QTOF mass spectrometry. Both delivered highly accurate MS data for the extraction of molecular features and examination of the differences on the level of molecular features. Further, the QTOF measurement delivered the MS/MS fragmentation with high mass accuracy for structure elucidation. The capabilities of the Agilent MassHunter software were demonstrated on examples of pharmaceutical relevance such as metabolite identification, impurity profiling and natural product research.

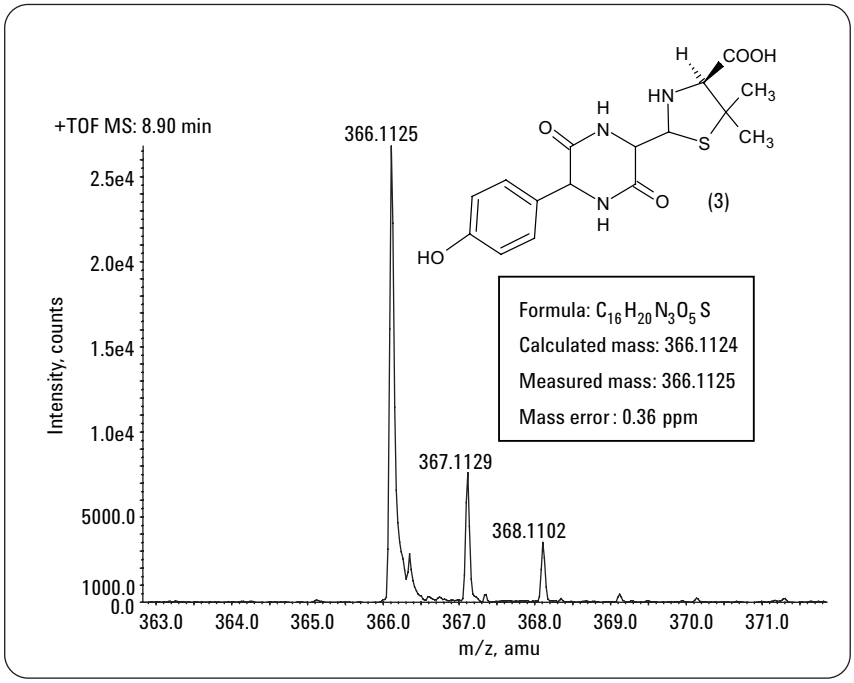

Figure 7
Mass spectrum of diketopiperazine amoxicillin (3) [M+H]$^+$ = 366.1124.

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