Analysis of Environmental Samples with Ultra High Definition LC/Q-TOF MS and Accurate Mass: How Much Resolving Power is Enough?

Application Note

Food Safety and Environmental

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Abstract

This study demonstrates the high resolving power and accurate mass analysis of sulfa antibiotics and anabolic steroids in water samples or water extracts using ultra high performance liquid chromatography (UHPLC) coupled to ultra high definition quadrupole time-of-flight (Q-TOF) mass spectrometry with the Agilent 1290 Infinity LC with an Agilent 6540 Accurate Mass Q-TOF LC/MS System. European Union regulations for identification points of banned drugs using high resolution (> 20,000 FWHM) are demonstrated for the first time with quadrupole time-of-flight mass spectrometry.

Introduction

European Union regulations for high resolution analysis of banned veterinary drugs in food and food products requires resolving power equal to or exceeding 10,000 using the 10% valley definition. This is equal to 20,000 resolving power using full width at half maximum (FWHM) [1]. Identification points of 2 or 2.5 are assigned for each accurate mass with this method by MS/MS for a total of at least four points for correct identification [1]. The concept of identification points by this system has been advocated for environmental water samples in several publications using Q-TOF LC/MS [2]. However, in all published examples of TOF the minimum requirement of 10,000 resolving power at 10% valley (20,000 FWHM) has not been shown by time-of-flight mass spectrometry. The reason is that, until recently, TOF mass spectrometers have not been able to resolve small fragment ions of pharmaceuticals (less than *m/z* 150) at 20,000 (FWHM). This application note shows that the UHD Agilent 6540 Accurate Mass Q-TOF LC/MS System can easily reach and exceed the resolving power requirement of 10,000 at 10% valley (20,000 FWHM) for antibiotics, anabolic steroids, and other pharmaceuticals in aqueous samples.

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Furthermore, it is possible to distinguish isobaric product ions of the anabolic steroid, stanazolol, that differ by either CH_2CH_2 or N_2, which is a mass difference of 0.0252 mass units [3]. This is a fragmentation pattern with resolution that was thought only to be possible with FT/MS or Orbitrap instruments [4]. In this study, the results exceeded those of both instruments in the published report [4] with a broader range of *m/z* ions from masses as low as *m/z* 69 to the protonated molecule at *m/z* 279. The results showed resolving power exceeding the 20,000 (FWHM) requirement, defining the amount of resolving power necessary for small molecule work (*m/z* less than 300). These analyses are carried out with accurate masses at less than ~1 ppm mass accuracy using both MS and MS/MS techniques. This application note discusses two examples: sulfa antibiotics in water and stanazolol fragmentation according to EU regulations for veterinary drugs [5].

Experimental Conditions

The Agilent 1290 Infinity LC System was used for high resolution chromatographic separation of a suite of sulfa antibiotics with a mobile phase of 0.1% formic acid in water and acetonitrile. An Agilent ZORBAX RRHD Eclipse Plus C18 2.1 × 50 mm, 1.8 µm column (part number 959757-902) was used with a 10-min gradient. Five to ten microliters of sample were injected into a 1 ppm solution of each of the analytes or analyte mixtures depending on the sensitivity of the analyte. The linear gradient was from 90% B to 100% A in 8 min with a hold of 2 min for 10 min total; where B is reagent grade water (Sigma Aldrich) with 0.1% formic acid and A is acetonitrile.

The mass spectrometer conditions for the Agilent Jet Stream were 375 °C for the sheath gas temperature, 11 L/min for the sheath gas flow, 250 °C for the drying gas temperature, 10 L/min for the drying gas flow rate, 45 psi for the nebulizer pressure, 4000 V for the capillary voltage in positive ion, and 0 V for the nozzle voltage. The fragmentor voltage was set from 100 to 190 V and all other mass spectrometer parameters remained at autotune conditions.

Results and Discussion

Identification of Sulfa Antibiotics in Food or Water

Figure 1 shows the UHPLC chromatogram for three sulfa antibiotics: sulfamethazine, sulfamerazine, and sulfadimethoxine. Two of these three antibiotics are used in hog feed at ppm levels (sulfamethazine and sulfamerazine) and sulfadimethoxine is used in water for caged birds; thus, they could occur in food or water samples. Furthermore, the common occurrence of sulfa antibiotics is reported in water samples impacted by wastewater effluents. It is important to show that these compounds can be easily identified using high resolution mass spectrometry under the European Union criteria of identification points for either food or water samples [1-2, 5].

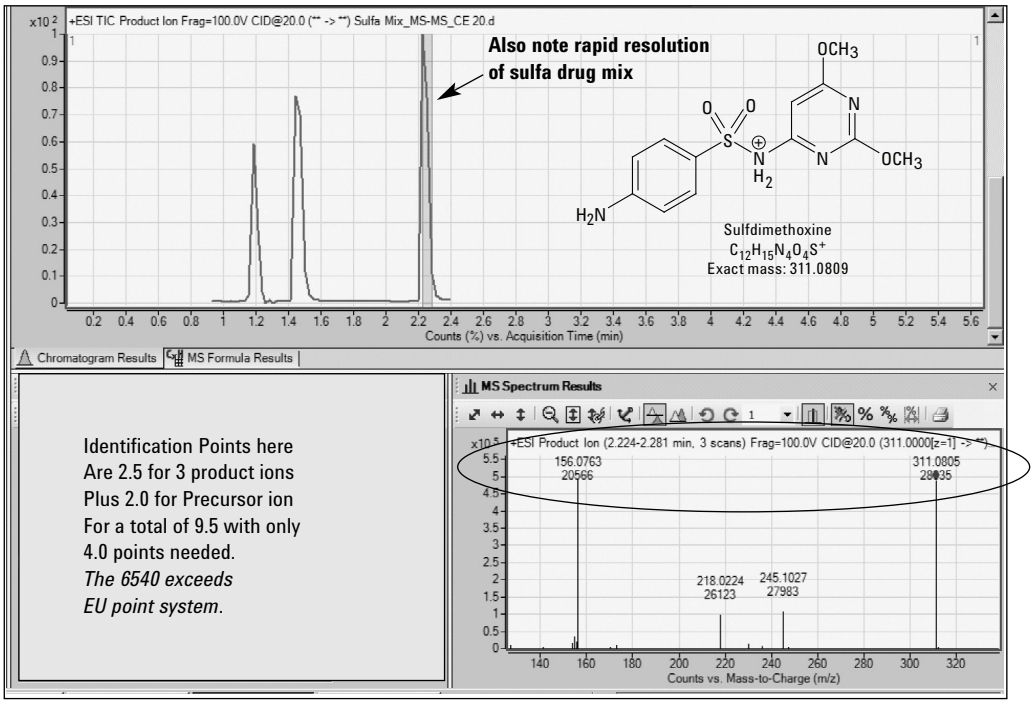

Figure 1. Sulfa drug mix found in water by UHD with Agilent 6540 Accurate Mass Q-TOF LC/MS.

The three drugs were separated in less than 3 min with a rapid gradient and peak widths of 6 s at the base (Figure 1). The accurate masses and resolving power for MH^+ ion and each of the products ions are also shown in Figure 1 for sulfadimethoxine. The accurate masses were all within 0.5 millidaltons of the exact calculated mass for each analyte, which is equal to the accurate mass of an electron. Using the European Union guidelines for identification points for banned veterinary drugs, the MH^+ ions would receive 2 points and each of the three product ions using MS/MS receives 2.5 points for a total of 9.5 identification points (Figure 1). The resolving power of greater than 10,000 (10% valley) or 20,000 (FWHM) is exceeded for all ions. There is a diagnostic ion of the sulfa drug family with an exact mass of *m/z* 156.0114, which is shown in Figure 2 and this ion occurs in each of the sulfa antibiotics. Therefore the accurate mass of this ion can be used to scan for other sulfa drugs or metabolites. The resolving power again exceeds the minimum value of 20,000 (FWHM) for this ion. The EU regulations do not stipulate an accurate mass but a simple calculation shows the value of low errors on accurate mass. It is also noted that there is another isobaric ion at *m/z* 156 with an accurate mass of 156.0768, which is the other half of the sulfadimethoxine molecule (Figure 2).

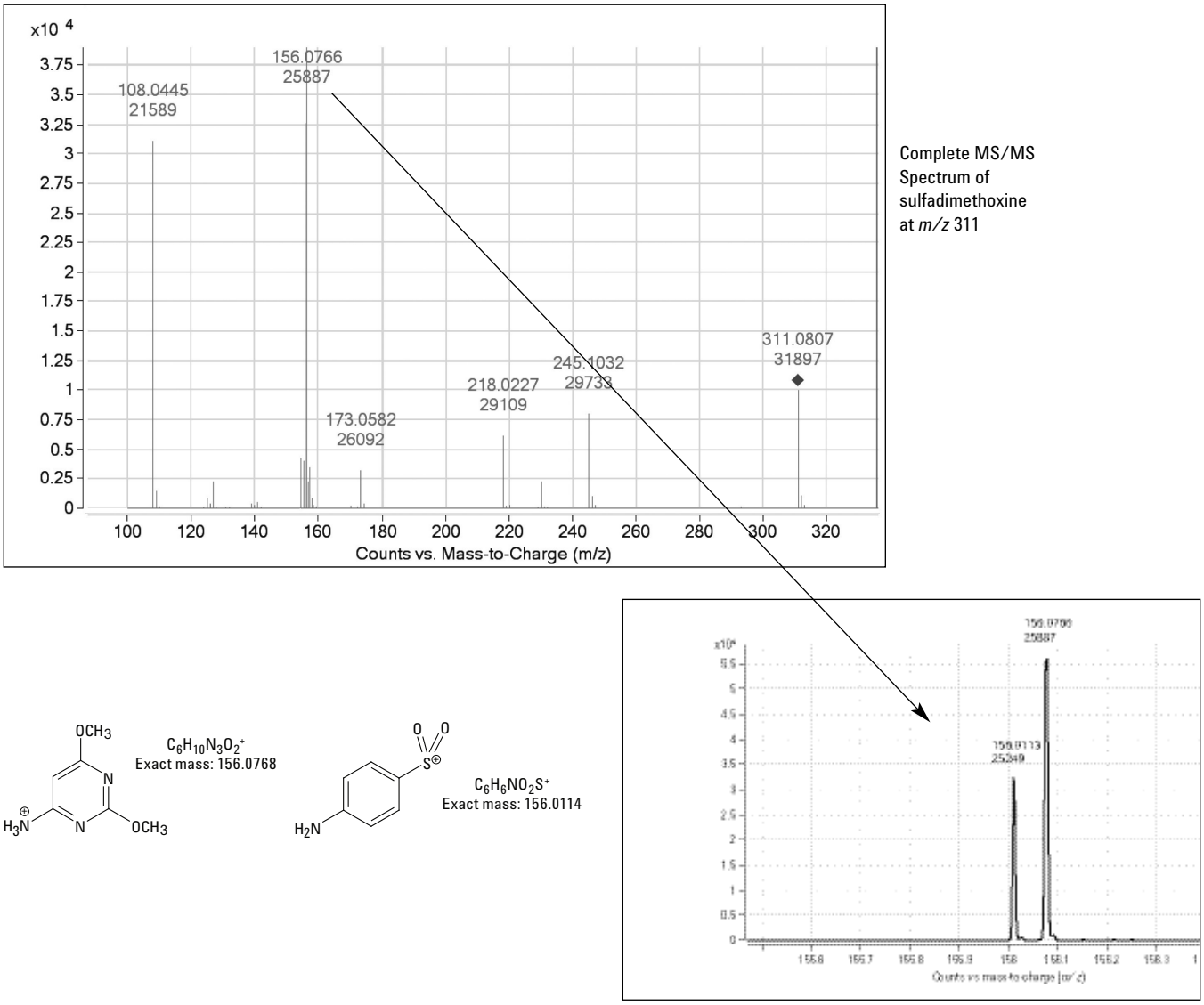

Figure 2. Isobaric products ions at m/z 156.

The number of formulas within the measured accurate mass of sulfadimethoxine at errors of 10 ppm, 5 ppm, and 2 ppm decrease from ten possible formulas at 10 ppm to six at 5 ppm to only two at 2 ppm using a composition of elements of C, H, N, O, S, and P. These are reasonable elements to enter into the calculation given the isotopic cluster shown in Figure 3 for sulfadimethoxine. In fact, the large relative isotopic mass defect for the A+2 ion in the isotopic cluster (Figure 3) demonstrates that S is present in the molecule. Therefore, only the correct formula for sulfadimethoxine is found at the 2-ppm mass error limit. For more information on the use of the relative isotopic mass defect to limit molecular formulas, see reference number 3 from our book on LC/TOF-MS analysis. Generally, the error limits on accuracy are set at 5 ppm based on publication requirements in reputable journals for unknown analysis. However, one quickly sees that it is insufficient when faced with the number of formulas that may exist. Thus, we would recommend accuracies less than 2 ppm for high resolution and accurate mass analysis, as well as the use of the accurate masses of the isotopic cluster and their intensity. These features are part of the MassHunter Software.

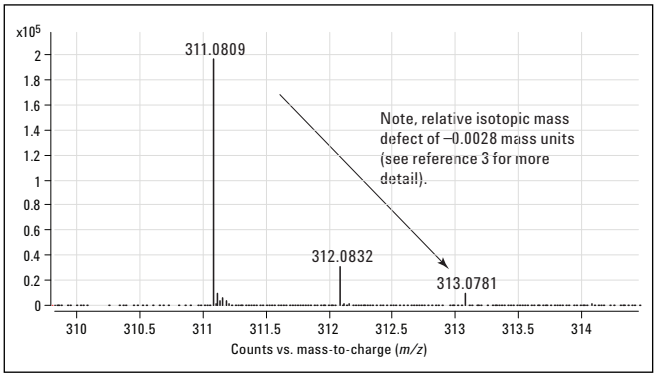

Figure 3. Isotopic cluster for sulfadimethoxine showing the A+1 and A+2 ions, and the S relative isotopic mass defect [3].

Stanazolol: Resolving Product Ions with Q-TOF LC/MS

The neutral losses of N_2, CH_2CH_2, and CO all result in a 28 mass unit difference, and perhaps, may represent one of the greatest challenges to MS/MS analysis for high resolution and accurate mass results [4]. The paper of Nielen et al. [4] examined such losses in the fragmentation of stanazolol, a banned anabolic steroid that gives a series of neutral losses, with isobaric product ions that differ in either the loss of CH_2CH_2 or N_2, which is a difference that is equal to 0.0252 mass units. In particular, they showed that both FT/MS and Orbitrap MS were capable of separating such differences at resolving powers of ~50,000 (FWHM) by Orbitrap and ~100,000 (FWHM) by FT/MS. Furthermore, they found that this separation of isobaric products ions was not possible by Q-TOF LC/MS, thereby, setting forth a separation challenge using high-resolution accurate-mass analysis with ultra high definition Q-TOF LC/MS.

In this way, we repeated their experiment with stanazolol doing both chromatography and MS/MS at > 40,000 (FWHM) maximum mass resolving power using the Agilent 6540 Accurate Mass Q-TOF LC/MS System. The product ions shown in Figure 4 at *m/z* 161.1068 and *m/z* 161.1317 represent isobaric fragments that differ by the mass difference of CH_2CH_2 and N_2. The exact calculated mass difference between these two neutral losses is 0.0252 mass units versus the measured mass of 0.00249. Table 1 shows the ten isobaric losses that occur within the stanazolol molecule when the MS/MS experiment is done. Note that the average mean loss for all of the fragments is 0.0252. This exactly matches the calculated mass difference between CH_2CH_2 and N_2, with a standard deviation of 0.0002 mass units, which is less than 1 percent. The accurate masses for all fragments is approximately 1 ppm across the mass range and resolving power is approximately 25,000 (FWHM), (Figure 5). Thus, the Agilent 6540 Accurate Mass Q-TOF LC/MS System has met the challenge presented by stanazolol and the EU regulation of high resolution mass spectrometry in both accurate mass and resolving power. The banned anabolic steroid, stanazolol, can be positively identified with these product ions and its MH^+ with high resolution, satisfying the EU requirements of greater than 4.0 points. In fact, these data are equal to 2.0 + 10(2.5) for a total of 27.0 identification points.

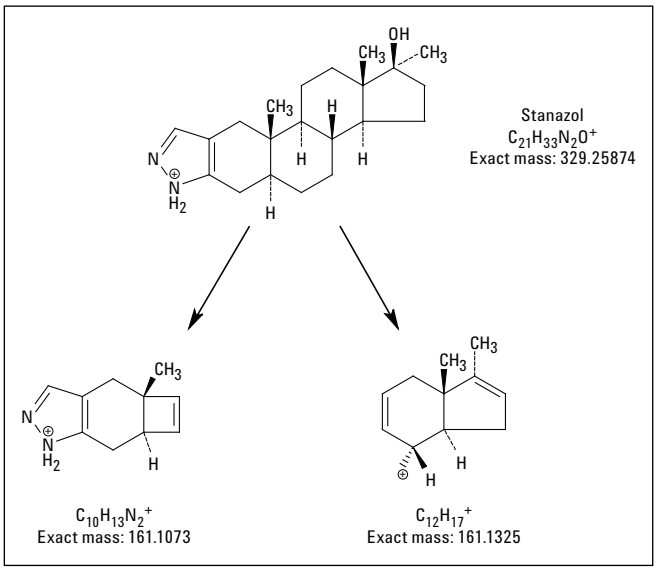

Figure 4. Isobaric product ions from the banned anabolic steroid, stanazolol, which differ in mass as an ethylene group minus diatomic nitrogen (CH_2CH_2 - N_2) or 0.0252 mass units.

Table 1. Mass Accuracies and Differences for Product Ions of Stanazolol

Product	Elemental composition MH^+	Exact calculated mass 329.2587	Measured accurate mass 329.2591	Error in ppm 1.2	Measure difference
229	C15H21N2	229.1699	229.1703	1.7	0.0252
229	C17H25	229.1951	229.1955	1.7	
189	C12H17N2	189.1386	189.1385	-0.5	0.0255
189	C14H21	189.1638	189.1640	1.1	
161	C10H13N2	161.1073	161.1068	-3.0	0.0249
161	C12H17	161.1325	161.1317	-5.0	
149	C9H13N2	149.1073	149.1072	-0.7	0.0248
149	C11H17	149.1325	149.1320	-3.4	
147	C9H11N2	147.0917	147.0914	-2.0	0.0255
147	C11H17	147.1168	147.1169	0.7	
135	C8H11N2	135.0917	135.0917	0.0	0.0252
135	C10H15	135.1168	135.1169	0.7	
119	C7H7N2	119.0604	–	–	
119	C9H11	119.0855	119.0856	0.8	
109	C6H9N2	109.0760	109.0761	0.9	0.0252
109	C8H13	109.1012	109.1013	0.9	
95	C5H7N2	95.0604	95.0604	0.0	0.0252
95	C7H11	95.0855	95.0856	1.1	
81	C4H5N2	81.0447	81.0449	2.5	0.0251
81	C6H9	81.0699	81.0700	1.2	
69	C3H5N2	69.0447	69.0448	1.4	0.0251
69	C5H9	69.0699	69.0699	0.0	
Measured loss					0.0252
Std dev					0.0002
Calculated loss					0.0252

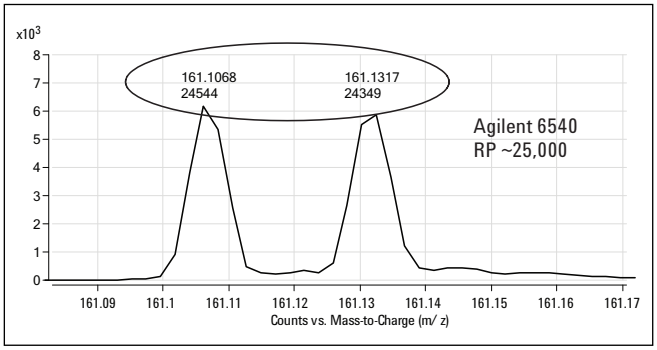

Figure 5. The m/z 161 product ions are isobaric fragments of stanazol with different formulas for each product ion. The resolving power of the Agilent 6540 Accurate Mass Q-TOF LC/MS easily separates and identifies the two product ions with 2.5 identification points for each product ion.

Conclusions

In conclusion, the Agilent 6540 Accurate Mass Q-TOF LC/MS System is capable of greater than 40,000 resolving power (FWHM) at maximum tune mass (*m/z* 2721.8948). It is also capable of fast chromatography with greater than 20,000 (FWHM) for the smallest fragment ions. This is a feature that is unique to this Q-TOF LC/MS instrumentation. These specifications allow accurate identification of banned veterinary drugs such as stanazolol, in food, and also the analysis of pharmaceuticals and antibiotics in water samples with the highest analytical confidence. This defines the amount of resolving power necessary for small molecules, and satisfies the EU requirement for high resolution mass spectrometry.

References

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