Environmental Applications of the Agilent 1290 Infinity UHPLC:
The Evolution of Chromatography

Application Note

Environmental

Authors

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Abstract

This application note presents examples of the use of UHPLC (ultrahigh performance liquid chromatography) for environmental applications using the new Agilent 1290 Infinity LC. The examples include wastewater analysis of pharmaceuticals with focus on EPA Method 1694 and the way that UHPLC makes this analysis more efficient and reliable. The second example will show that complex mixtures of pesticides may be analyzed with fast chromatography, opening the door for many types of UHPLC analyses for environmental applications. Finally, an example will show a 60-sec analysis of pharmaceuticals in wastewater using UHPLC and a ballistic gradient, which is one of the first examples in environmental analysis. The insights of efficient UHPLC analysis are discussed and illustrated.

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Introduction

The advent of UHPLC has brought two important innovations to liquid chromatography/mass spectrometry (LC/MS) analysis. First is the use of 1.8-µm columns, which gives an increase in plate number from the 5-µm columns of about a factor of two. This is important for environmental applications where complex matrices are encountered, such as pharmaceuticals in wastewater analysis. However, the use of 1.8-µm columns requires higher pressure pumps operating at pressures that exceed 600 bar on a routine basis, which is the range commonly used for UHPLC. Secondly, the 1.8-µm column allows the use of rapid resolution chromatography and even ultrafast chromatography, where runs may be less than 1 min in length. The high flow rates required for these analyses create pressures on the order of 800 to 1000 bar, or more, and are clearly in the range of UHPLC.

The demands of high sample throughput in short timeframes have given rise to high efficiency and fast liquid chromatography using the 1.8-µm reverse-phase columns. Fast chromatography has become a necessity in those labs that analyze hundreds of samples per day or those labs needing short turnaround times. Using Rapid Resolution liquid chromatography, results of a sample batch can be reported in a few hours rather than a few days. In the water quality and the food industries, regulatory labs produce validated results in less than an hour so that water treatment may proceed or vegetable shipments can be released the same day they are measured or produced. The end result is greater productivity for customers and greater cost efficiency for the reporting laboratory. Thus, productivity is improved by shortened analysis time, which typically requires UHPLC. The definition of Rapid Resolution liquid chromatography is simple. Liquid chromatographic separations that are less than 10 min are fast, and separations less than 1 min are popularly known as ultrafast [1].

The other aspect of UHPLC is the increased peak capacity available when longer columns with 1.8-µm packing are used. It is now possible to have almost 300 times greater peak capacity, which is a valuable asset to unknown analysis in wastewater and other environmental applications such as pesticide screening. Finally, the UHPLC system should be robust and capable of both high pressure and high flow (>1 mL/min at pressures up to 1200 bar) to do both rapid resolution and normal flow chromatography with high peak capacity. Agilent has 1.8-µm columns specially designed for pressures to 1200 bar (18,000 psi) and give a variety of phases (C-8, C-18 in both StableBond and ZORBAX Eclipse Plus formats). These are useful for difficult water samples, as this application note will show, including improved peak capacity for an EPA Method 1694 for pharmaceuticals in wastewater. They are also useful for rapid resolution of pharmaceuticals and pesticides using both triple quadrupole mass spectrometry as well as liquid chromatography/time-of-flight mass spectrometry.

Experimental

The work shown here was carried out at the Center for Environmental Mass Spectrometry by Drs. Imma Ferrer and Michael Thurman at the University of Colorado in Boulder, Colorado, USA, using the Agilent 1290 Infinity LC system and both the Agilent 6430 triple quadrupole LC/MS and the Agilent 6220 accurate-mass time-of-flight LC/MS system.

Columns

Two different columns were tested for rapid resolution high throughput (RRHT) analyses including the high pressure (1000 bar) 1.8-µm particle sizes. Table 1 lists the columns tested in this work and their theoretical plates.

Table 1. Columns used in this study

Column	Dimension (mm)	Particle (m)	Theoretical Plates (N)	Pressure Rated (bar)
ZORBAX Eclipse Plus-C18	2.1 × 100	1.8	21,688	1000
ZORBAX Eclipse Plus-C18	2.1 × 50	1.8	10,392	1000

Chromatographic and mass spectral conditions

The Agilent 1290 Infinity LC was used for all UHPLC chromatographic separations and the Agilent 1200 Series SL was used for the standard EPA Method 1694. The conditions were as follows for each of the figures shown in this application note.

Figure 1. Part A
The liquid chromatograph was the Agilent 1200 Series SL. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 30 min with a 5-min hold time. The flow rate was 0.6 mL/min. The column was the ZORBAX Eclipse Plus-C18, 4.6 mm × 150 mm, 3.5 µm. Peak widths at the base were 15-18 seconds and peak capacity of 100. Maximum pressure was 75 bar.

The mass spectrometer was the Agilent 6410 triple quadrupole LC/MS system in electrospray positive mode with three time segments in multiple reaction monitoring (MRM) mode. There were two transitions per compound with 10-ms dwell time for each transition. The compounds were Group 1 of EPA Method 1694. See our application note for further detail on compounds and their transitions [2].

Figure 1. Part B
The liquid chromatograph was the Agilent 1290 Infinity LC. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 10 min with a 1-min hold time. The flow rate was 0.6 mL/min. The column was the ZORBAX Eclipse Plus-C18, 2.1 mm × 50 mm, 1.8 µm. Peak widths at the base were 5–6 sec and peak capacity of 100. Maximum pressure was 375 bar.

The mass spectrometer was the Agilent 6430 triple quadrupole LC/MS in electrospray positive mode with one time segment in MRM mode. There were two transitions per compound with 10-ms dwell time for each transition. The compounds were Group 1 of EPA Method 1694. See our application note for further detail on compounds and their transitions [2].

Figure 2
The liquid chromatograph was the Agilent 1290 Infinity LC. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 20 min with a 2-min hold time. The flow rate was 0.6 mL/min. The column was the ZORBAX Eclipse Plus-C18, 2.1 mm × 100 mm, 1.8 µm. Peak widths at the base were 5–6 sec and peak capacity of 100. Maximum pressure was 750 bar.

The mass spectrometer was the Agilent 6430 triple quadrupole LC/MS in electrospray positive mode with one time segment in MRM mode. There was one transition per compound with 10-ms dwell time for each transition. The compounds were Group 1–4 of EPA Method 1694, plus 15 additional pharmaceuticals. See our application note for further detail on compounds and their transitions [2].

Figure 3A
The liquid chromatograph was the Agilent 1290 Infinity LC. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 2 min without a hold time. The flow rate was 1.2 mL/min. The column was the ZORBAX Eclipse Plus-C18, 2.1 mm × 50 mm, 1.8 µm. Peak widths at the base were 1–3 sec and peak capacity of 60. Maximum pressure was 780 bar.

The mass spectrometer was the Agilent 6430 triple quadrupole LC/MS in electrospray positive mode with two time segments in MRM mode and six compounds per segment. There was one transition per compound with a 5-ms dwell time for each transition. The compounds were a selected set of 12 compounds from EPA Method 1694.

Figure 3B
The liquid chromatograph was the Agilent 1290 Infinity LC. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 2 min without a hold time. The flow rate was 1.2 mL/min. The column was the ZORBAX Eclipse Plus-C18, 2.1 mm × 50 mm, 1.8 µm. Peak widths at the base were 1–3 sec and peak capacity of 60. Maximum pressure was 780 bar.

The mass spectrometer was the Agilent 6430 triple quadrupole LC/MS in electrospray positive mode with two time segments in MRM mode and three compounds per segment. There was one transition per compound with a 5-ms dwell time for each transition. The compounds were carbamazepine, continine, caffeine, diphenhydramine, thiabendazole, and trimethoprim from EPA Method 1694.

Figures 4 and 5
The liquid chromatograph was the Agilent 1290 Infinity LC. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 2 min without a hold time. The flow rate was 1.5 mL/min. The column was the ZORBAX Eclipse Plus-C18, 2.1 mm x 50 mm, 1.8 µm. Peak widths at the base were 1–3 sec and peak capacity of 60. Maximum pressure was 900 bar.

The mass spectrometer was the Agilent 6520 accurate-mass Q-TOF LC/MS in electrospray positive mode in 2-GHz mode with 20 scans per second at a mass accuracy of >2 ppm. The compounds included a mix of 220 pesticides from the list reported by Thurman et al. 2008.

Figure 6
The liquid chromatograph was the Agilent 1290 Infinity LC. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 2 min without a hold time. The flow rate was 1.2 mL/min. The column was the ZORBAX Eclipse Plus-C18, 2.1 mm x 50 mm, 1.8 µm. Peak widths at the base varied as a function of scans per second on the mass spectrometer. Maximum pressure was 780 bar.

The mass spectrometer was the Agilent 6430 triple quadrupole LC/MS in electrospray positive mode with one time segment in MRM mode and one compound per segment, carbamazepine. There was one transition per compound with dwell times that varied from 1 to 300 ms and were equal to a range of 3 to greater than 20 scans per second.

Figure 7
The liquid chromatograph was the Agilent 1290 Infinity LC. The gradient was from 10% acetonitrile/water to 100% acetonitrile in 2 min without a hold time, 6 min without a hold time, and 30 min without a hold time. The flow rate was 0.6 mL/min. The column was the ZORBAX Eclipse Plus-C18, 2.1 mm x 100 mm, 1.8 µm. Peak widths at the base were 2–6 sec and peak capacity of 60. Pressure maximum was 750 bar.

The mass spectrometer was the Agilent 6430 triple quadrupole LC/MS in electrospray positive mode with one time segment in MRM mode and one compound per segment, caffeine. There was one transition per compound with a dwell time of 5 ms.

Sample preparation

Pharmaceutical analytical standards were purchased from Sigma, (St. Louis, MO, USA). Individual pharmaceutical stock solutions (approximately 1000 g/mL) were prepared in pure acetonitrile or methanol depending on the solubility of each individual compound, and stored at –18 ºC. From these solutions, working standard solutions were prepared by dilution with acetonitrile and water.

Pesticide analytical standards were purchased from Dr. Ehrenstorfer (Ausburg, Germany). Individual pesticide stock solutions (1000 g/mL) were prepared in pure acetonitrile or methanol depending on the solubility of each individual compound, and stored at –18 ºC. From these concentrated solutions, working standard solutions were prepared by dilution with acetonitrile and water.

Wastewater samples were collected from an effluent site in Boulder Creek (Boulder, CO) and extracted with polymeric cartridges using a modified EPA protocol. One liter water samples were extracted directly onto a 500-mg cartridge without pH adjustment, dried for 10 minutes with air, and eluted with 8 mL of methanol. The methanol was evaporated to 1 mL and analyzed by LC/MS/MS as described below. "Blank" wastewater extracts were used to prepare the matrix matched standards for validation purposes. The wastewater extracts were spiked with the mix of pharmaceuticals at different concentrations (ranging from 0.1 to 500 ppb) then analyzed by LC/MS/MS.

Results and Discussion

This application note contains three sections discussing examples of peak capacity and rapid resolution for pharmaceuticals in wastewater using EPA Method 1694, UHPLC and LC/TOF-MS of pesticides, and some important chromatographic considerations with UHPLC/MS.

Part 1: Environmental pharmaceutical analysis by LC/MS/MS.

The EPA Method 1694 is a standard method requiring 20-min analysis times or longer to satisfy the method requirements. However, recent changes published by EPA suggest that other chromatographic conditions may be used, such as shorter analysis times and rapid resolution, if sufficient mass spectrometric analysis is used (for example, 2 transitions per compound). Figure 1 shows the use of the new Agilent 1290 Infinity with UHPLC to reduce analysis times from 30 min to 10 min, a 66% decrease in analysis times with a peak capacity of 100 in both analyses. The original EPA method called for a ZORBAX Eclipse Plus-C18, 3.5 µm column and the rapid resolution is with the ZORBAX Eclipse Plus-C18 2.1 mm × 50 mm, 1.8 µm column.

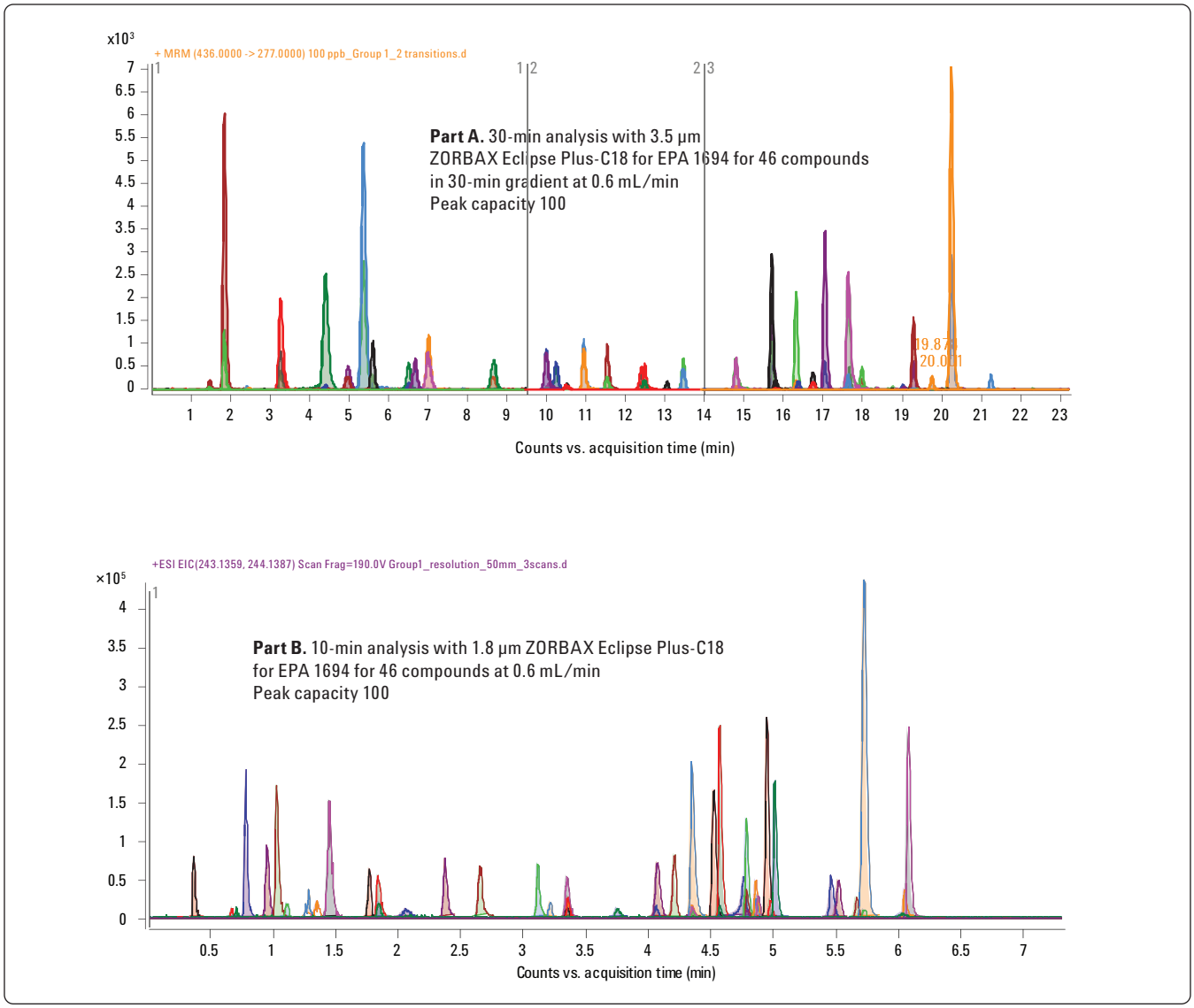

Figure 1. Shows the reduction of time of analysis from a 30-min analysis to a 10-min analysis using the Agilent 1290 Infinity with UHPLC for Group 1 pharmaceuticals in EPA Method 1694.

Because the pressure is at 375 bar it is possible to easily increase peak capacity and the number of pharmaceuticals that may be separated by substituting a longer column (2.1 mm x 100 mm) and maintaining the same flow rate of 0.6 mL/min. This doubles the pressure from 375 to 750 bar. The results are shown in Figure 2.

It is also possible to do ultrafast chromatography with the Agilent 1290 Infinity LC for pharmaceuticals using a triple quadrupole LC/MS. In the example shown in Figure 3, the number of compounds have been reduced to 12 compounds in order to obtain at least 20 scans across each peak. By obtaining 20 scans or more, the peak width may be reduced to 1 – 2 sec and the result is ultrafast chromatography. The column is a ZORBAX Eclipse Plus-C18, 2.1 mm x 50 mm with a flow rate of 1.2 mL/min and a 1.5 min gradient. The 12 pharmaceuticals eluted in less than 60 seconds. This, to our knowledge, is the first example of ultrafast chromatography applied to an environmental sample. In this case, it is a wastewater from Boulder, Colorado, that contains the following pharmaceuticals: carbamazepine, continine, caffeine, diphenhydramine, thiabendazole, and trimethoprim.

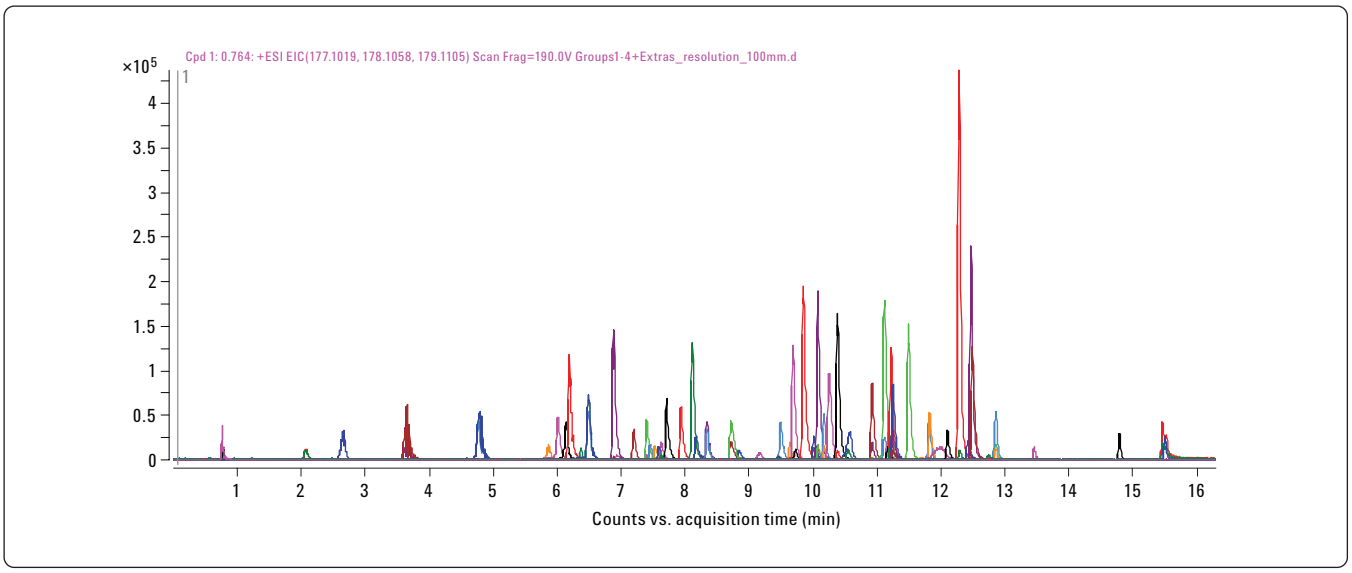

Figure 2. Increased peak capacity showing the separation of the entire list of EPA Method 1694 pharmaceuticals plus 15 new compounds for a total of 90 pharmaceuticals in less than 20 min by using a ZORBAX Eclipse Plus-C18, 2.1 mm × 100 mm, 1.8-µm packing material with UHPLC using the Agilent 1290 Infinity LC. Peaks are 5 to 6 sec wide and peak capacity is 200.

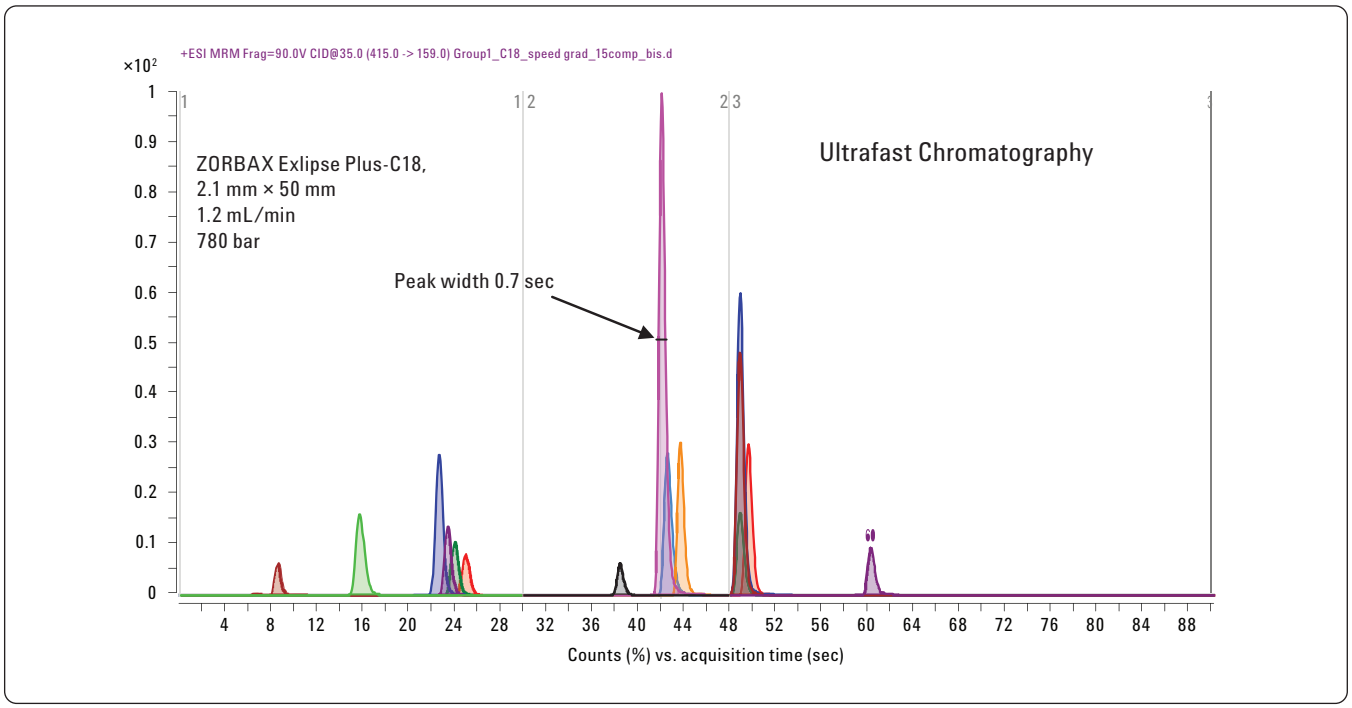

Figure 3A. The "ultrafast" gradient for 12 pharmaceutical standards.

It is important to realize that when using ultrafast gradient conditions, quality assurance and quality control data are required. Therefore, all sample purification measures must be taken during sample preparation to minimize suppression. It is also necessary to use labeled internal standards for quantitation since the entire sample matrix is eluting in a very narrow window. The reproducibility of the 1290 Infinity was within one second making it easy to obtain reliable data. Also important is the use of at least two transitions by LC/MS/MS, one for quantitation and the other as a qualifier ion. See our application note on the EPA Method for further examples. [2]

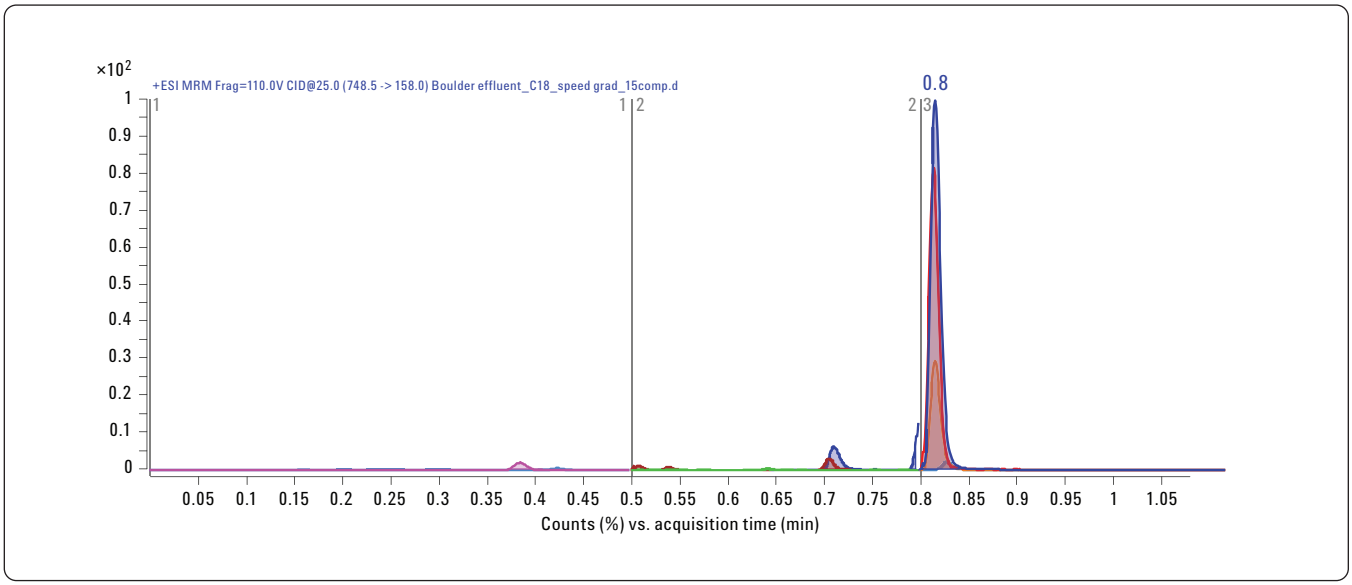

Figure 3B. Ultrafast gradient for wastewater from Boulder, CO, USA. The compounds detected included: carbamazepine, continine, caffeine, diphenhydramine, thiabendazole, and trimethoprim.

Part 2: UHPLC and LC/TOF/MS

One of the major concerns when doing rapid resolution is obtaining good sampling across the narrow peaks of 1–2 sec using mass spectrometry. In the previous examples, we showed how this is done using triple quadrupole LC/MS and maintaining 20 scans across each peak. The use of LC/TOF-MS and LC/Q-TOF-MS makes this task easy for as many compounds as one would like to monitor. This is because the TOF-MS instruments are obtaining data in full spectrum mode at all times. It is merely necessary to set the software to obtain the 20 spectra per second for the TOF-MS instruments. Figure 4 shows how effective this strategy is when doing UHPLC with either LC/TOF-MS or LC/Q-TOF-MS. More than 100 pesticides were analyzed in less than 80 sec using the Agilent 1290 Infinity LC with a ZORBAX Eclipse Plus-C18, 2.1 mm × 50 mm column at a flow rate of 1.5 mL/min. The UHPLC was required as pressures reached 900 bar. The separation included peak widths at half-height of only 0.7 seconds. See Figure 5 for the herbicide, terbutryn.

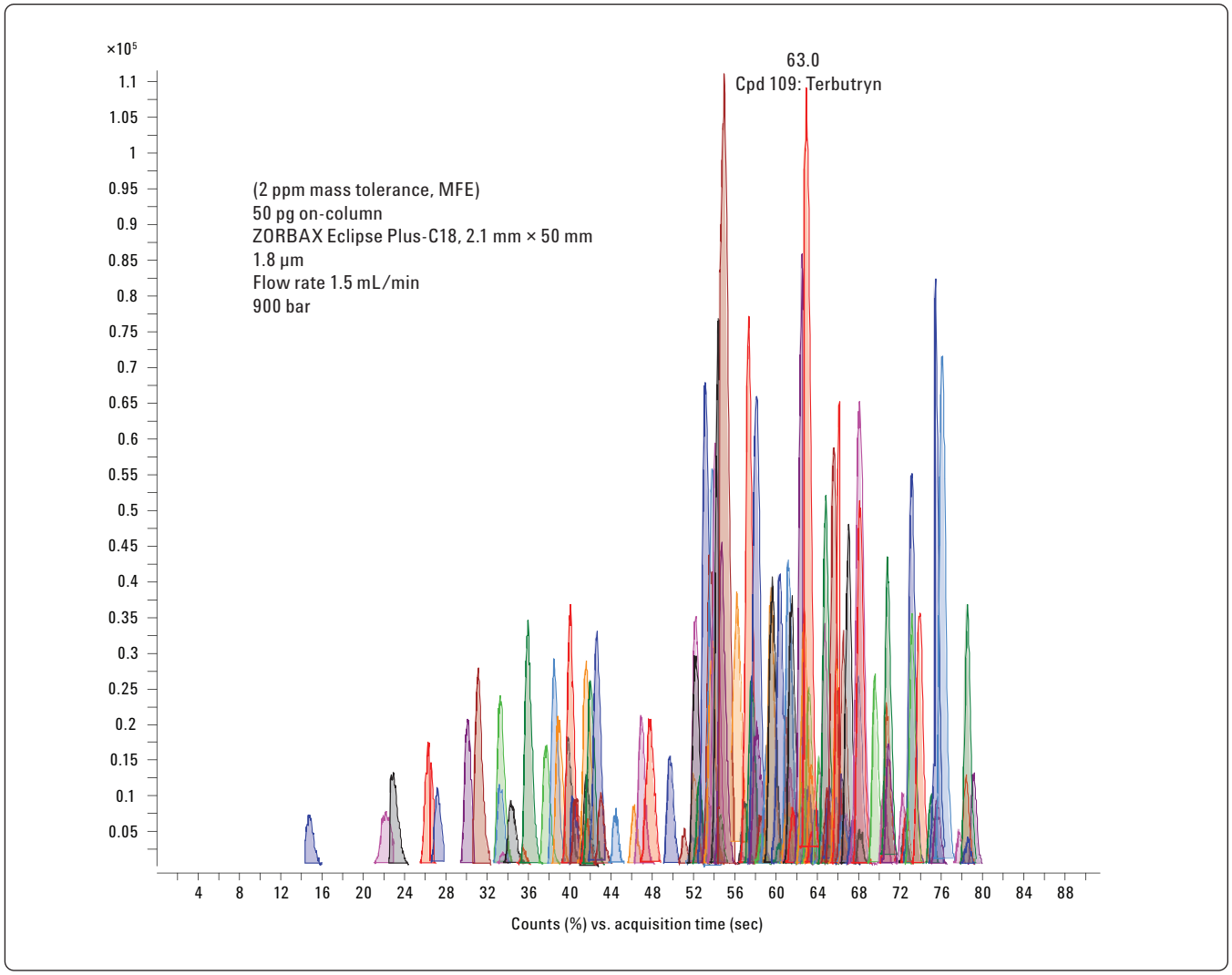

Figure 4. Pesticide analysis of over 100 compounds by LC/TOF-MS in less than 2 min.

The chromatographic speed, separation, and power of LC/TOF-MS makes it possible to analyze complex mixtures of pesticides from food or water matrices in a few minutes. This allows the rapid analysis used in the food monitoring industry in Europe, where shipments of vegetables and fruits are not unloaded until the analysis of pesticides are complete. Therefore, rapid analysis is important in these cases.

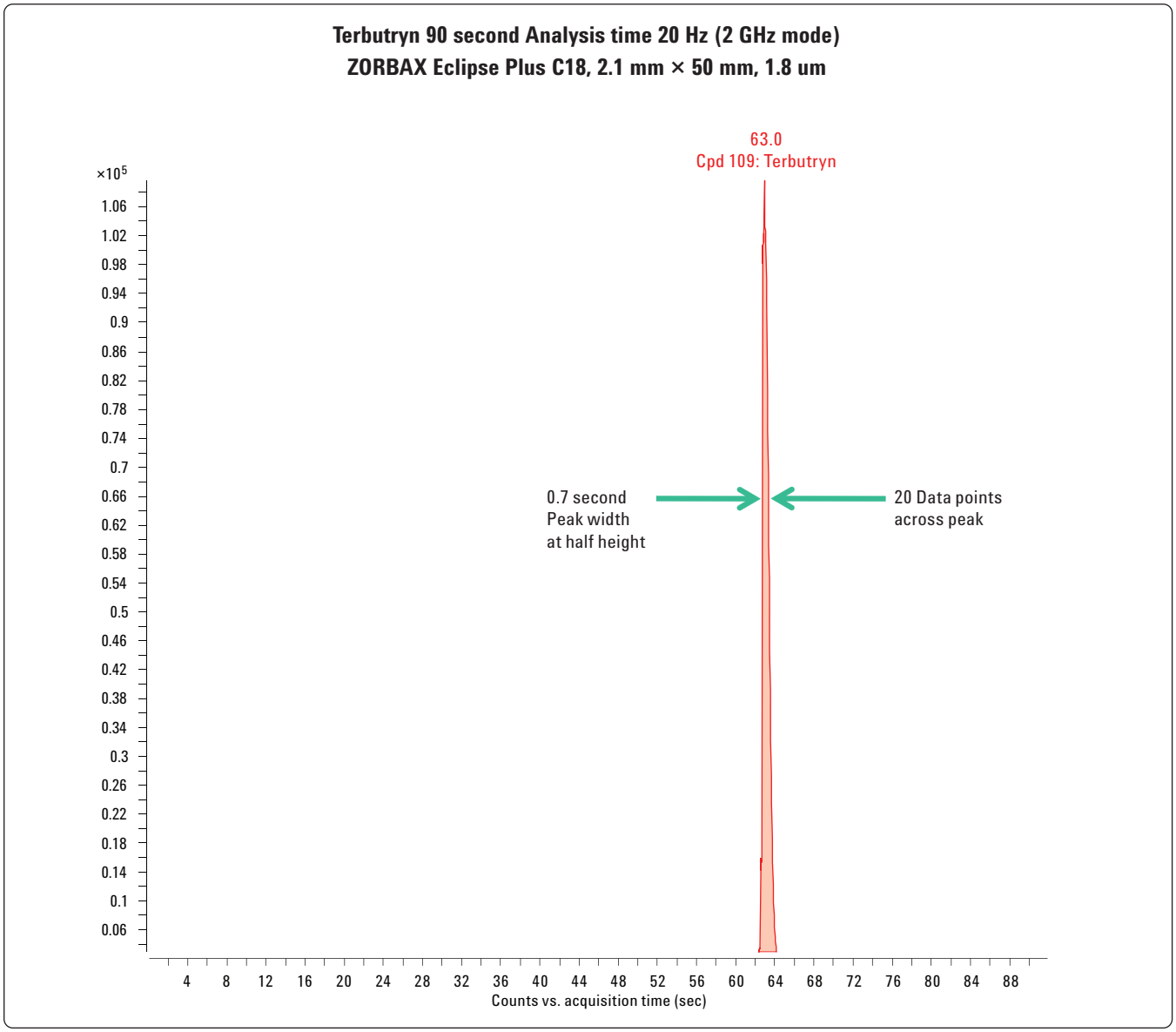

Figure 5. Peak width of 0.7 sec at half-height using the Agilent 1290 Infinity with a ZORBAX Eclipse Plus-C18, 2.1 mm × 50 mm with a flow rate of 1.5 mL/min at a pressure of 900 bar.

Part 3: Chromatographic considerations with UHPLC/MS

Optimization of chromatographic and mass spectrometry conditions for the best use of UHPLC includes the following ideas. First, it is important to have at least 20 MS data points across each peak in order to obtain the 1–2 sec peak widths of ultrafast chromatography. An illustration of the importance of data cycles per second is shown in Figure 6.

The peak broadening that appears at the lower cycle rates is caused by a smoothing routine meant to shape peaks for good integration and quantitation and is a common procedure in all chromatographic software. Therefore, when using triple quadrupole LC/MS instruments, it is recommended to use a short dwell time of 5 ms and the dynamic MRM procedures to insure that 20 cycles per peak are obtained. In the case of LC/TOF-MS and LC/Q-TOF-MS instruments it is only necessary to set the software to obtain 20 spectra per second across the mass range that is acquired.

Secondly, quantitative aspects of analysis are a consideration in good UHPLC practice. Here it is important that maximum peak sensitivity be obtained. Figure 7 shows an example where caffeine is maximized for peak intensity and peak area by adjusting the gradient and flow rate until the maximum signal is obtained. In this case, a 6-min gradient resulted in the optimum signal-to-noise (S/N) ratio of 180 and an area count of 55,000 counts at a retention time of 1.7 min. Note that the signal-to-noise drops to half at a value of 91 with the longer gradient of 30 min but a retention time increase of only 0.2 min. Thus, it is important to test various flow rates and retention times to optimize signal strength; especially of the polar and early eluting compounds in a chromatographic analysis.

A final consideration in good UHPLC chromatography is the suppression of LC/MS signal. It was mentioned earlier and must be emphasized that standards in fast and ultrafast analysis will often show little or no suppression because of the purity of the standard mixture. However, real samples may show suppression; therefore, it is important to dilute samples or to purify them in extraction procedures to limit the amount of matrix that is present. Finally, it is valuable to use deuterated or C-13 labeled standards when measuring pharmaceuticals in wastewater and other complex matrices. This is the recommended procedure of EPA Method 1694 and the readers are referred to our application note on this topic [2].

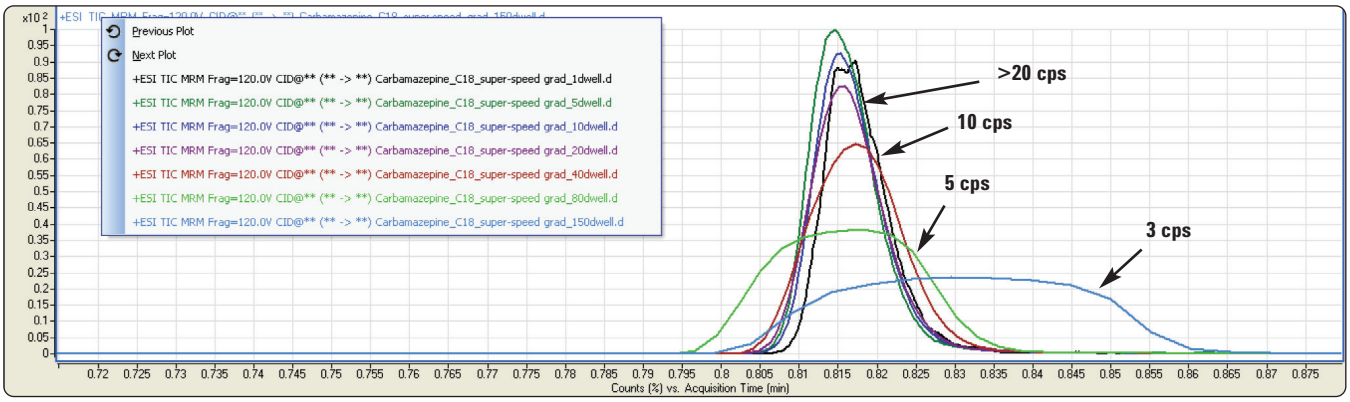

Figure 6. This figure shows the effect of dwell time and data points per peak and how it affects the peak shape for a single compound, carbamazepine, from a 1-sec peak at > 20 cycles per second (cps) to 3–4 sec peak at 3 cycles per second (cps).

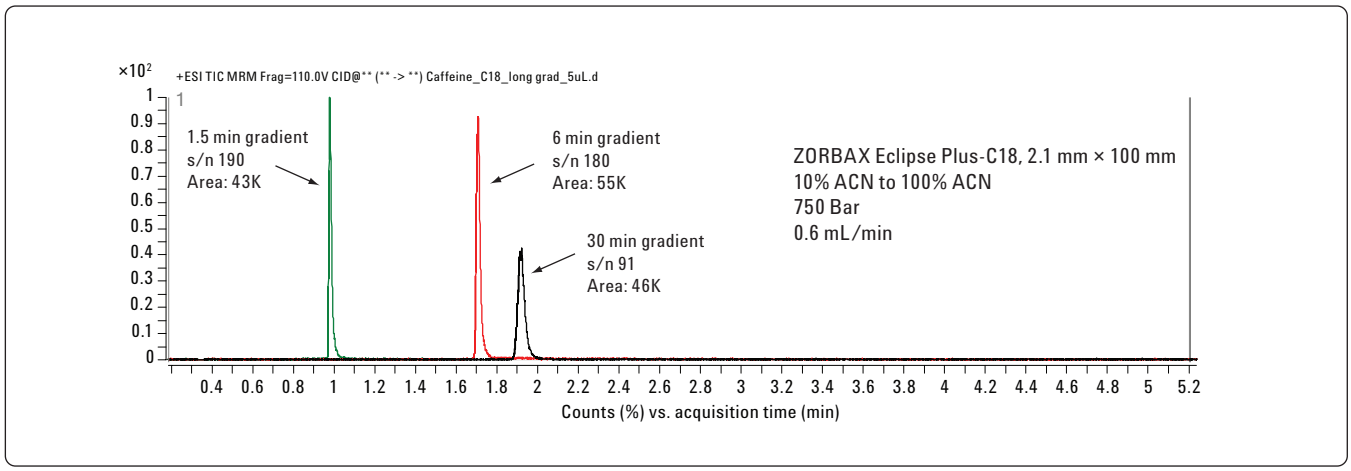

Figure 7. The effect of retention time on the signal to noise (S/N) and the area counts for caffeine in LC/MS analysis.

Conclusions

In conclusion, we recommend the ZORBAX Eclipse Plus-C18, 2.1 mm × 50 mm columns for rapid resolution and ultrafast chromatographic separations, while for maximum peak capacity the ZORBAX Eclipse Plus-C18, 2.1 mm × 100 mm is a better choice. Our results show that fast flow rates greater than 1.5 mL/min may be used and pressures greater than 1000 bar are possible with confidence and reliability. Finally, we see the new Agilent 1290 Infinity LC as the best example of the evolution of chromatography from the gravity columns of Tsweet to the UHPLC realm of ultrafast high pressure liquid chromatography "made-easy."

Acknowledgements

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2. I. Ferrer, E. M. Thurman, J. A. Zweigenbaum, "EPA Method 1694: Agilent's 6410A LC/MS/MS Solution for Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS," 2008, Application Technologies publication 5989-9665EN,
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