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ADDRESSING THE CHALLENGES OF COMPLEX MIXTURE ANALYSIS WITH SUB-2 µM PARTICLE LIQUID CHROMATOGRAPHY: LONG COLUMNS AND HIGH TEMPERATURES

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INTRODUCTION

The characterization of complex mixtures, such as pharmaceutical impurities, biological fluids, environmental samples, and peptides digests provides some of the most challenging tasks currently facing the analytical scientist.

Liquid chromatography, used with combinations of ultraviolet, fluorescence, and electrochemical detection, together with mass spectrometry, has been used extensively to address these tasks. Liquid chromatography is popular because of the compatibility of the separation system with the samples/matrix, the selectivity derived from mobile phase/stationary phase options, and the reduced need for complex derivatization-associated techniques such as GC and GC/MS.

The separation of these complex mixtures often demands lengthy analysis to generate the high resolution chromatograms required to fully characterize the sample. It is often necessary to use orthogonal separation systems (column chemistry, organic modifier, or buffer pH) to fully and confidently characterize the sample – a time-consuming and laborious process. Reducing analysis time improves productivity and makes for more efficient use of personnel and high-priced instrumentation such as mass spectrometers.

The ability to produce high-resolution separations with peak capacities in excess of 1000 reduces the need for orthogonal separations, ensuring that all the components in the sample are detected – thereby increasing confidence in the result.

Two approaches are available to produce high-resolution chromatograms: using long columns, and using small particles. The former results in longer assay times, and resolution only increases by the square-root of the column length. Thus, if the column length is doubled, resolution increases by a factor of 1.4, but the analysis time is doubled. Since resolution is inversely proportional to particle size, halving the particle size doubles the chromatographic resolution for any given column length.

However, reducing the particle size requires increasing the optimal mobile-phase flow rate, hence reducing the analysis time for a given column length. Reducing particle size, therefore, offers an attractive option for high-resolution separations.

Operating the column at elevated temperature reduces solvent viscosity and allows the use of longer columns and/or higher flow rates, again resulting in improved chromatographic performance. Combining the chromatographic benefits of sub-2 µm particles, higher temperature operation, and long columns with the low-dispersion, high-pressure capability of the Waters® ACQUITY UltraPerformance LC® (UPLC®) System facilitates the generation of extremely high-resolution chromatograms.

Figure 1. ACQUITY UPLC System with the Q-Tof Premier Mass Spectrometer.

The ability to generate these high-resolution separations permits the rapid return of critical information to aid the drug development process, the discovery of new targets, and the detection of toxicological markers.

In this application note, we show how sub-2 µm particle LC, operated at elevated temperatures, has been applied to complex mixture analysis.

EXPERIMENTAL

The separations were performed on an ACQUITY UPLC® System coupled to a Q-Tof Premier™ hybrid quadrupole time-of-flight (TOF) mass spectrometer.

Ginseng was processed by steam extraction and the resulting powder sample dissolved in a mixture of methanol/water (1:1). Representative bile sample was obtained from control Wistar rats. Representative urine was obtained from 16-week-old Zucker rats. The urine and bile samples were prepared by dilution with water 1:4 prior to analysis. The samples were injected onto an ACQUITY UPLC BEH C_{18} 2.1 x 150 mm column, or two 2.1 x 150 mm columns, connected in series and eluted with an aqueous formic acid/acetonitrile gradient. Detection was performed using a hybrid quadrupole TOF mass spectrometer.

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C_{18} Column 2.1 x 150 mm, 1.7 µm
Column temp.:	90 °C
Flow rate:	800 µL/min
Mobile phase A:	Aqueous formic acid (0.1%)
Mobile phase B:	Acetonitrile
Gradient:	Various

MS conditions

MS system:	Waters Q-Tof Premier Mass Spectrometer
Ionization mode:	ESI Positive
Capillary voltage:	3200 V
Cone voltage:	35 V
Desolvation temp.:	450 °C
Desolvation gas:	900 L/Hr
Source temp.:	130 °C
Acquisition range:	100 to 1000 m/z
Collision energies:	High 25 V, low 5 V

Chromatographic considerations

The highest performance of an HPLC column is reached at the minimum of the plate-height vs. velocity curve. This velocity can be calculated by taking the first derivative of the van Deemter equation with respect to the linear velocity *u*:

$$H = A \cdot d_p + B \cdot \frac{D_m}{u} + C \cdot \frac{d_p^2}{D_m} \cdot u \qquad (1)$$

D_m is the molecular diffusion coefficient and d_p is the particle size of the packing material.

$$u_{\min} = \frac{D_m}{d_p} \cdot \sqrt{\frac{B}{C}} \qquad (2)$$

The coefficients *B* and *C* are constants that depend on the packing material and the retention factor of the analyte.

The pressure required to reach this linear velocity can be calculated from the Kozeny-Carman equation:

$$\Delta p = f(\varepsilon_i) \cdot \frac{\varepsilon_t \cdot u_{\min} \cdot \eta \cdot L}{d_p^2} \qquad (3)$$

Where e_i and e_t are the interstitial and total porosity, respectively, *h* is the viscosity of the mobile phase, and *L* is the length of the chromatography column.

Combining equations 2 and 3 gives the pressure required to reach the minimum of the van Deemter curve, i.e. the maximum column performance:

$$\Delta p = f(\varepsilon_i) \cdot \varepsilon_t \frac{1}{d_p^3} \cdot \sqrt{\frac{B}{C}} \cdot \eta \cdot D_m \qquad (4)$$

Note that the product of the viscosity and the diffusion coefficient enters this equation. The viscosity decreases with increasing temperature, while the diffusion coefficient increases with increasing temperature. As a matter of fact, the relationship between viscosity and diffusivity can be found in the equations commonly used to estimate the diffusion coefficient of a solute in a liquid. This is shown in the following figure f figure or the Wilke-Chang (26) and the Scheibel (27) equations:

$$D_m \cdot \eta = 7.4 \cdot 10^{-8} \cdot \frac{1}{V_S^{0.6}} \cdot \sqrt{\Psi_B \cdot M_B} \cdot T \qquad (5)$$

$$D_m \cdot \eta = 8.2 \cdot 10^{-8} \cdot \frac{1}{V_S^{1/3}} \cdot \left[1 + \left(\frac{3 \cdot V_B}{V_S}\right)^{2/3}\right] \cdot T \qquad (6)$$

T is the temperature (in °K); V_S is the molar volume of the sample. Quantities with the subscript B reflect the solvent: V_B is the molar volume, M_B is the molecular weight, and Y is the association factor. In both equations:

$$D_m \cdot \eta = const \cdot T \qquad (7)$$

Consequently, the pressure required to reach the maximum column performance increases with temperature:

$$\Delta p = f(\varepsilon_i) \cdot \varepsilon_t \frac{1}{d_p^3} \cdot \sqrt{\frac{B}{C}} \cdot const \cdot T \qquad (8)$$

While this result may be counterintuitive to many, it should be pointed out that this effect is not large. For the temperature range under consideration here, it is on the order of 20 percent.

RESULTS AND DISCUSSION

Natural products: Ginseng

Natural products are currently receiving increased levels of interest as potential medicine, as herbal products – Traditional Chinese Medicines (TCM), for example – and as a source for individual compounds that form the basis for drug discovery research. It is essential for scientists to be able to separate and characterize these samples, to compare sample quality from location to location, those harvested at different times of the year, or to identify the individual components in the sample for structure activity investigations.

Ginseng is a species of the *Panax* genus: slow-growing perennial plants with fleshy roots belonging to the family *Araliaceae*. Ginseng grows in the Northern Hemisphere in East Asia (China, Korea, and Eastern Siberia). It is characterized by the presence of ginsenosides.

Both American and *Panax* (Asian) *ginseng* rhizomes are taken orally, as stimulants, and in the treatment of type 2 diabetes. Ginseng can also be found in energy drinks, usually in varieties of tea or in functional foods. Usually ginseng is present at sub-clinical doses and does not have measurable medicinal effects.

The chromatogram in Figure 2 shows the separation of Chinese *Panax ginseng* using two coupled 2.1 x 150 mm 1.7 µm ACQUITY BEH C_{18} Columns. The columns were maintained at 90 °C and eluted with a 5 to 95% aqueous formic acid/acetonitrile gradient. The resulting chromatogram produced peaks with an average peak width of 4.1 seconds at the base, delivering a peak capacity of 870 for a one-hour separation.

The inset figure highlights the resolving power of the system, with the baseline of the two peaks eluting with a retention time of 11 minutes. The high-resolution separation allows for the easy characterization and comparison of the samples.

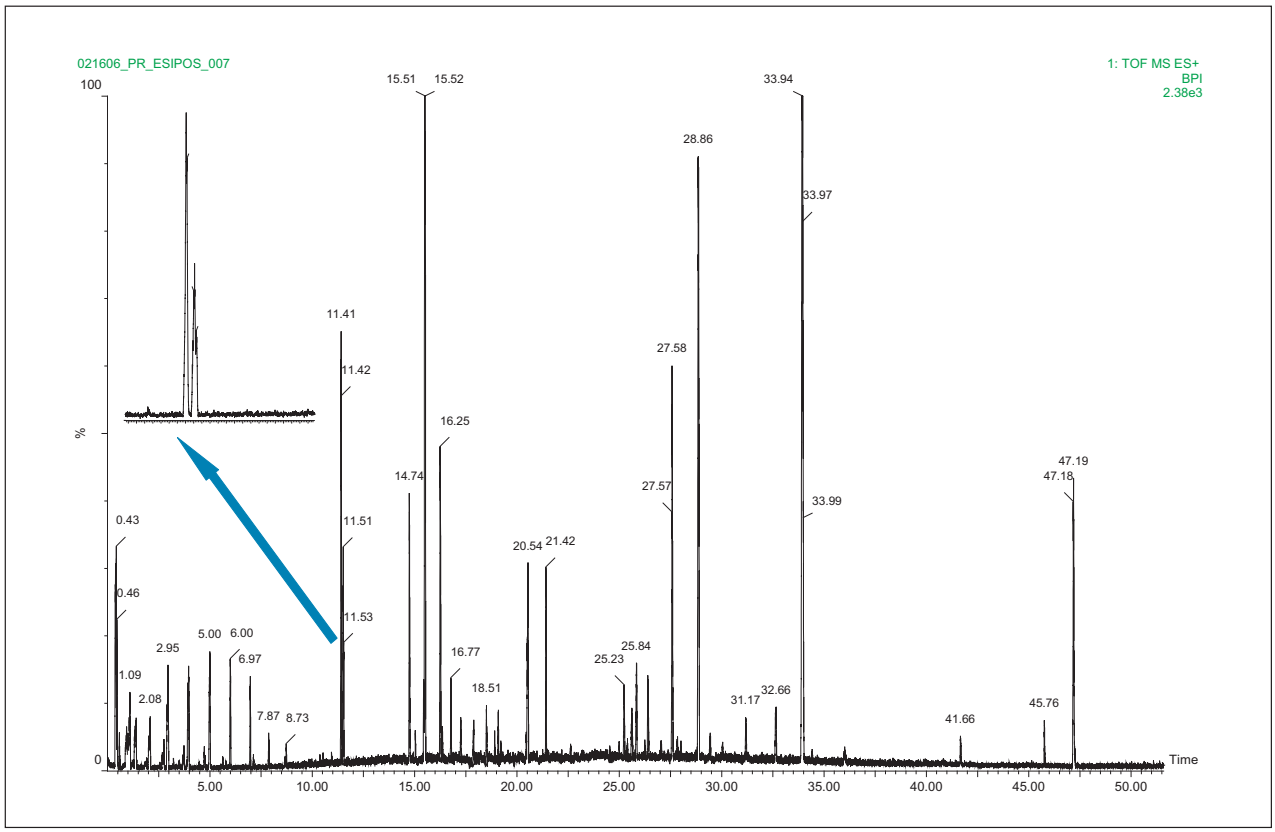

Figure 1. UPLC/MS separation of Chinese ginseng extract.

Biological sample: bile

Bile is produced by hepatocytes in the liver, draining through the many bile ducts that penetrate the liver, then into the common hepatic duct into the gall bladder. Bile acts as a detergent, helping to emulsify fats and aiding in their absorption. The most important compounds in bile are the salts of taurocholic acid and deoxycholic acid, which aid the break down of fat globules via emulsification.

Besides its digestive function, bile serves as the route of excretion for the hemoglobin breakdown product (bilirubin) created by the spleen, which gives bile its distinctive color.

Excretion into the bile is an important route of elimination for several drug and endogenous compounds, especially the larger polar molecules that are often eliminated via the feces. A major consequence of biliary excretion is that the compounds come into contact with the gut microflora, which, themselves, can metabolize the compounds – converting them into more lipid-soluble metabolites that can be reabsorbed via the intestine, portal vein, and the liver.

This enterohepatic recirculation can lead to increased half-life, toxicity, and saturated routes of metabolism. Understanding these biliary metabolites is an essential part of drug metabolism studies for regulatory submissions.

The chromatogram displayed in Figure 3 shows the separation of rat bile using a 1.7 µm 2.1 x 150 mm ACQUITY UPLC Column. The column was operated at 60 °C and eluted with a gradient of 20 to 80% acetonitrile/aqueous formic acid at 800 µL/min over 20 minutes. This separation has an average peak width of 2 to 3 seconds at the base, giving a peak capacity of 400 and allowing for the complete characterization of the sample over a short period of time.

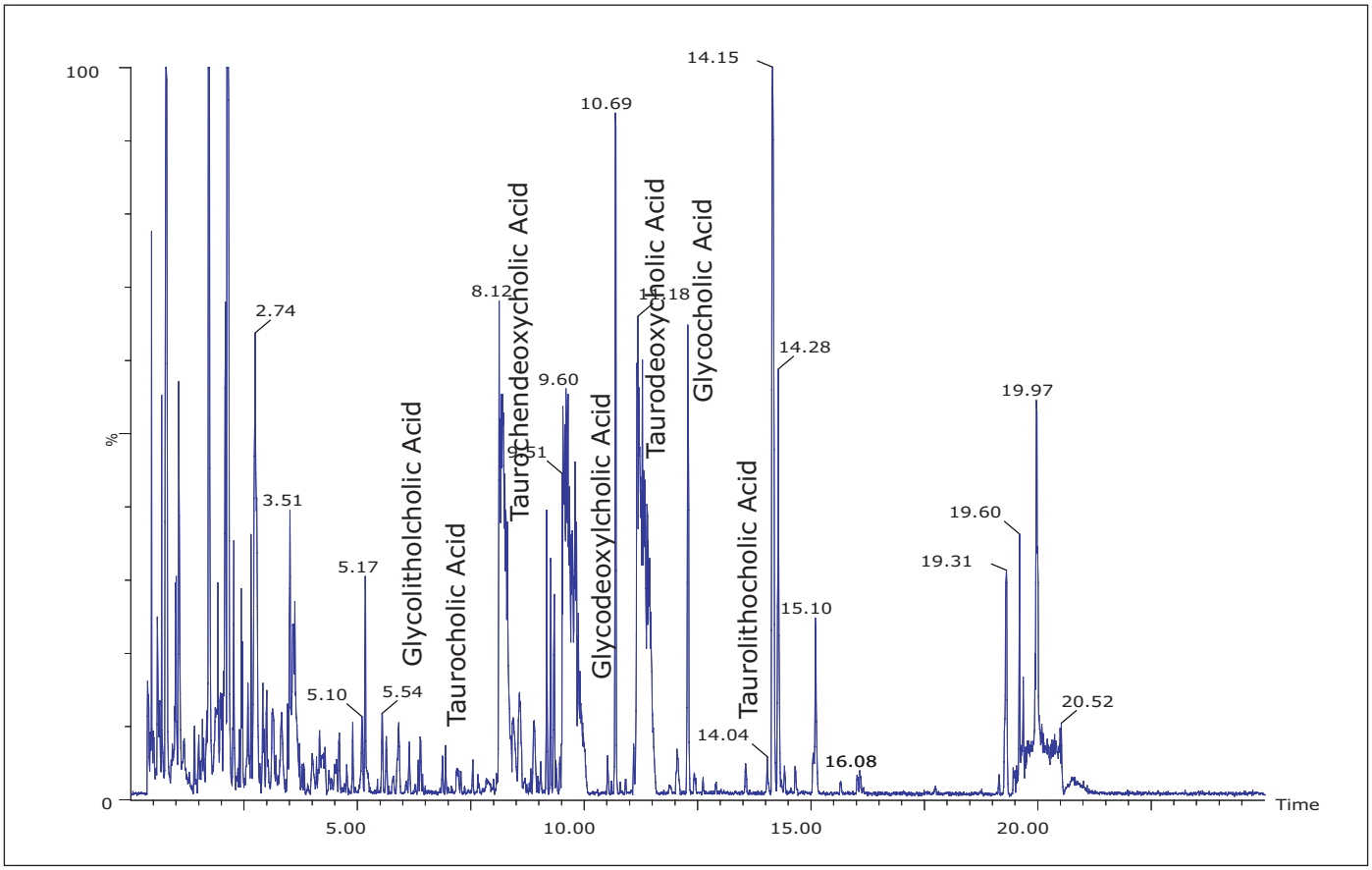

Figure 3. UPLC/MS separation of diluted rat bile sample.

Urine analysis

Urine represents the main route for elimination of drugs and endogenous metabolites from the body. The relatively small and polar compounds are eliminated from the bloodstream by the kidneys, via filtration of the blood through the glomerulus, diffusion into the tubules, and active transport into the tubular fluid. Larger molecules do not pass out the glomerulus; lipid-soluble molecules undergo reabsorption via the tubules in the kidney.

Urine contains information about the metabolic fate of exogenous compounds such as drugs. It also contains compounds that provide diagnostic information about the toxicological state of the mammalian system.

The ability to fully characterize a urine sample, therefore, allows for the identification of metabolites of a candidate pharmaceutical, detection and identification of toxicity markers, and detection of toxins in therapeutic drug monitoring. Increasing the resolving power of the chromatographic system improves detection limits and MS spectral quality, simplifies data analysis, and reveals more information about the sample.

The data shown below in Figure 4 illustrate the separation of a rat urine sample from an animal with early-onset type 2 diabetes. The urine sample was analyzed on a 2.1 x 300 mm (2 x 150 mm) column operated at 90 °C and eluted with a 5 to 95% acetonitrile gradient over 60 minutes at 800 µL/min. The average peak at the base is 3.4 seconds, providing a peak capacity of 1024 for a one-hour separation and permitting significantly more information to be extracted from the sample.

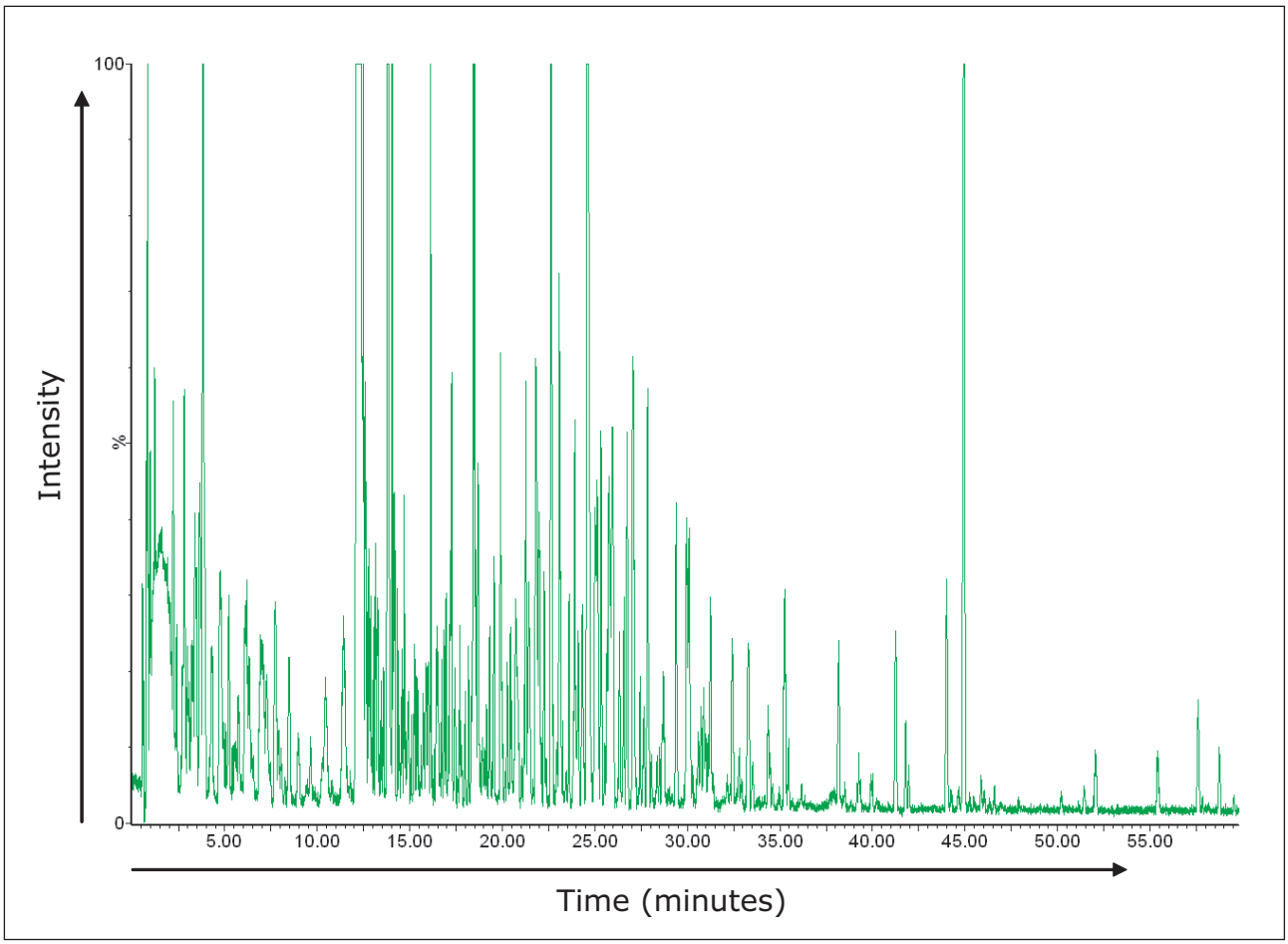

Figure 4. Separation of a rat urine sample using two coupled 2.1 x 150 mm 1.7 µm C_{18} ACQUITY UPLC BEH Columns, at an operating pressure of 13,500 psi.

CONCLUSION

The challenge of analyzing complex mixtures requires an analytical system and methodology that, together, extract the maximum amount of information from the sample in the shortest time to optimize productivity. The ACQUITY UPLC System has been specifically designed to produce chromatograms of the highest resolution, allowing scientists to fully interrogate complex samples.

The ACQUITY UPLC System has low-dispersion characteristics, precise flow delivery characteristics, and uses a sensitive detector to realize the chromatographic potential of sub-2 µm porous chromatographic material. The rugged design of the instrument facilitates the use of high operating backpressures, permitting the use of longer columns for high-resolution separations.

The use of elevated temperature in liquid chromatography does not increase the maximum obtainable performance in either isocratic or gradient mode for a given column. It does, however, allow the same performance to be obtained in less time. Increasing the column temperature allows the use of longer columns, generating gradient peak capacities in excess of 1000 in just 60 minutes.

The chromatograms produced using these sub-2 µm particles are significantly superior to those produced by 3 to 3.5 µm particles, which generate separations of peak capacities in the region of 200 to 300 per hour.

The combination of sub-2 µm particles used at elevated operating temperatures, with optimized flow rates, and in long column formats allows for complex samples, including natural products, biological fluids, and pharmaceutical compounds, to be investigated in much greater detail than before – all in a time frame of one hour or less.

This improves the ability of scientists to understand more about their samples and maximize return on large capital investments such as mass spectrometers.

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