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Improving LC–MS sensitivity through increases in chromatographic performance: Comparisons of UPLC–ES/MS/MS to HPLC–ES/MS/MS

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Abstract

Recent technological advances have made available reverse phase chromatographic media with a 1.7 μm particle size along with a liquid handling system that can operate such columns at much higher pressures. This technology, termed ultra performance liquid chromatography (UPLC), offers significant theoretical advantages in resolution, speed, and sensitivity for analytical determinations, particularly when coupled with mass spectrometers capable of high-speed acquisitions. This paper explores the differences in LC–MS performance by conducting a side-by-side comparison of UPLC for several methods previously optimized for HPLC-based separation and quantification of multiple analytes with maximum throughput. In general, UPLC produced significant improvements in method sensitivity, speed, and resolution. Sensitivity increases with UPLC, which were found to be analyte-dependent, were as large as 10-fold and improvements in method speed were as large as 5-fold under conditions of comparable peak separations. Improvements in chromatographic resolution with UPLC were apparent from generally narrower peak widths and from a separation of diastereomers not possible using HPLC. Overall, the improvements in LC–MS method sensitivity, speed, and resolution provided by UPLC show that further advances can be made in analytical methodology to add significant value to hypothesis-driven research.

Keywords: UPLC; Mass spectrometry; β-Agonists; Isoflavones; Tamoxifen; Ephedra alkaloids

1. Introduction

Progress in HPLC separation efficiency has been driven over the last several decades by improvements in manufacturing of silica-based particles. As particles decreased from the 10 μm size in the 1970s to the 3 μm range in the 1990s, the throughput and resolving power of HPLC columns has increased significantly [1]. However, the use of short columns packed with 3 μm particles does have practical limits to the combination of chromatographic speed and resolving power available because of the inability of pumping systems to perform at pressures in excess of several thousand psi [1]. The recent commercialization of porous hybrid organic–inorganic silicon-based particles with a narrow size distribution in the range of 1.7 μm has enabled a new level of performance, but only through the use of newly developed technology that permits pumping and injection of liquids at pressures in excess of 10,000 psi [1,2]. Full implementation of such highly efficient chromatographic separations (peak widths at half-height <1 s) into analytical methods is further challenged by the requirement for detectors fast enough to accept and process data on this new time scale. This need is particularly acute for LC–MS-based methodology where great effort has been invested to improve sensitivity, to increase the information content of individual analytical methods used to support hypothesis-driven research, and the relentless push to increase sample throughput. This paper

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describes the adaptation of this new chromatographic technology to address specific hypothesis-driven investigations of toxicological relevance to understand better the practical advantages in terms of chromatographic efficiency, analyte sensitivity, and method speed.

The goal of this investigation was to compare analytical performance between HPLC-based mass spectrometric methods that have been optimized in our laboratory with that obtained by adapting these methods to UPLC. These methods were specifically chosen because of the frequent need in this laboratory to develop high sensitivity methods for characterization and quantification of multiple analytes that are adequately resolved in a minimum amount of time for use in exposure and pharmacokinetic assessments. This goal were achieved by conducting a true side-by-side determination of performance using a liquid handling system capable of operation in either mode connected to a common triple quadrupole mass spectrometer.

2. Experimental

2.1. Reagents

All solvents were HPLC grade and Milli-Q water was used throughout. Sigma Chemical Co. (St. Louis, MO) supplied the formic acid, salbutamol, terbutaline, fenoterol, clenbuterol, tamoxifen, des-methyltamoxifen, 4-hydroxytamoxifen, ephedrine, pseudoephedrine, methylephedrine, phenylpropanolamine, genistein, and daidzein. Indofine Chemical Co. (Hillsborough, NJ) supplied equol. Plantech (Reading, UK) supplied dihydrodaidzein and dihydrogenistein. Cimaterol and mabuterol were obtained from Boehringer–Ingelheim (St. Joseph, MO), zilpaterol from Hoechst Roussel Vet (Clinton, NJ), and ractopamine from Eli Lilly (Indianapolis, IN). Brombuterol, ring-labeled ^{13}C6-clenbuterol, and 4-sulfoxy-tamoxifen were synthesized in-house and characterized spectroscopically (UV, NMR) and chromatographically (LC/ES/MS/MS). The deuterated internal standards used in this study were d4-genistein and d3-daidzein, obtained from Cambridge Isotope Laboratories (Andover, MA); d4-equol, was a generous gift from Dr. Kristiina Wahala, University of Helsinki; d3-ephedrine and d3-pseudoephedrine were obtained from Ceilliant Co., Round Rock, TX); d5-*N*-desmethyl tamoxifen, and d5-4-hydroxy-tamoxifen were obtained from Toronto Research Chemicals Inc. (North York, Ont., Canada); labeled tamoxifen ($^{13}C_2$, ^{15}N) was obtained from Isotec Inc. (Miamisburg, OH).

A soy supplement labeled “Genistein”, purchased from a local health food store as a convenient source containing aglycones and various substituted glucoside forms of isoflavones. Tablets were analyzed in triplicate by extraction, filtration, dilution, and analysis using LC–MS. The tablets (1.5 g) were pulverized in a mortar then extracted into methanol (20 ml) with stirring for 2 h at room temperature.

2.2. Liquid chromatography

All ultra performance liquid chromatography (UPLC) and HPLC were performed using a Waters Acquity liquid handling system (Waters Assoc., Milford, MA). All UPLC separations were achieved using a 1 mm × 50 mm Acquity reverse phase column (1.7 μm particles, Waters). All injection volumes were 15 μl, all separations of neat standard solutions or soy tablet extracts in mobile phase were performed at ambient temperature, and the flow rate was 200 μl/min. In practice, this flow rate was dictated by the target column back pressure of 10,000 psi, even though this configuration of UPLC hardware is rated for as high as 15,000 psi. Typical plate counts determined for this Acquity column were greater than 250,000 plates/m. The choice of packing material, a bridged ethylsiloxane–silica hybrid particle with C18 alkyl groups, was used for all separations because no other phases were commercially available at the time of this investigation.

UPLC separation of soy isoflavones and metabolites used isocratic elution with 40% acetonitrile and 60% 0.1% formic acid (aq); soy isoflavones plus tamoxifen metabolites used isocratic elution with 40% acetonitrile/60% 0.1% formic acid (aq) for 0.8 min followed by a linear gradient to 60% acetonitrile over 1.65 min, then held at 60% acetonitrile for 1 min (total run time 3.5 min); β-agonists used isocratic elution with 2% acetonitrile/98% 0.1% formic acid (aq) for 0.5 min followed by a linear gradient to 4% acetonitrile over 2 min, followed by a linear gradient to 30% acetonitrile over 3 min then returned to 2% acetonitrile for 0.5 min (total run time 6 min); *Ephedra* alkaloids used isocratic elution with 2% acetonitrile/98% 0.1% formic acid (aq) (total run time 4 min); soy isoflavone glucosides used isocratic elution with 5% acetonitrile/98% 0.1% formic acid (aq) for 1 min followed by a linear gradient to 32% acetonitrile over 3 min then returned to 5% acetonitrile for 1 min (total run time 5 min).

HPLC separations of soy isoflavones were achieved using a Luna C18-2 column (either 1 mm × 30 mm or 2 mm × 30 mm, 3 μm particles, Phenomenex Co., Torrance, CA) eluted at 200 μL/min with 40% acetonitrile and 60% 0.1% formic acid (aq); HPLC separation of soy isoflavones and tamoxifen metabolites was achieved using a Luna C18-2 column (2 mm × 150 mm, 3 μm particles, Phenomenex) eluted at 220 μL/min with an isocratic elution using 40% acetonitrile in 0.1% formic acid (aq) and the entire effluent was directed into the mass spectrometer (total run time 10 min); HPLC separation of β-agonist growth promoters was achieved on a Betamax Base analytical column (2 mm × 100 mm, 5 μm particles, Thermo Hypersil-Keystone, Bellefonte, PA) equipped with a C18 Security Guard cartridge (2 mm × 4 mm, 2 μm particles, Phenomenex) at a flow rate of 0.3 μL/min. The mobile phase gradient consisted of 0.1% formic acid (aq) and acetonitrile. Initial gradient conditions were set to 5% ACN and held for 2.5 min before incorporating a linear gradient increasing to 65% ACN over 6 min. At 8.6 min the gradient

was programmed to initial conditions to reequilibrate the column for 3.4 min (total run time 12 min); HPLC separation of sympathomimetic *Ephedra* alkaloids was achieved on a Luna C18-2 column (2 mm × 150 mm, 3 μm particles, Phenomenex) eluted at 200 μL/min with an isocratic elution using 4% acetonitrile in 0.1% formic acid (aq) and the entire effluent was directed into the mass spectrometer (total run time 15 min); HPLC separation of soy isoflavone glucosides was conducted as previously reported [9] using an isocratic elution at 5% acetonitrile in 0.1% formic acid (aq) for 3 min followed by a linear gradient to 50% acetonitrile over 15 min followed by an isocratic portion at 50% acetonitrile for an additional 5 min (total run time 23 min).

Mobile phases were prepared by mixing reagent acetonitrile with either Milli-Q water or filtered buffers (0.45 μm nylon) of specified pH.

2.3. Mass spectrometry

The entire effluent from either the HPLC or UPLC column was directed into the electrospray (ES) source of a Waters Quattro Premier triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK). Positive ions were acquired in the multiple reaction monitoring (MRM) or precursor ion scanning modes using a desolvation temperature of 350 °C and a source temperature of 100 °C. Cone voltages and collision energies were optimized for each MRM transition used. In all cases, dwell times for individual MRM transitions were varied to achieve approximately 15 data points across the respective HPLC or UPLC chromatographic peak. In some cases, this required dwell times, inter-channel delays, and inter-scan delays of 5 ms, the fastest scanning rate possible. All data were acquired at 16 points/scan. Full acquisition details are given in the respective figure legend.

3. Results and discussion

3.1. Soy isoflavones and metabolites

Our previous human and animal studies of internal exposures to phytoestrogens from soy consumption typically require information about levels of active parent isoflavones, genistein and daidzein, along with active metabolites, including equol, dihydrodaidzein, and dihydrogenistein [3,4]. The low levels of soy phytoestrogens often observed in humans requires the use of MS/MS using MRM mode for maximum sensitivity detection of these five analytes using a primary transition for quantification and a secondary confirmatory transition along with three deuterated internal standards for use in quantification (genistein, daidzein, equol). The requirement for analysis of large sample sets requires optimization for high throughput methods of solid phase extraction and LC separation [3]. Fig. 1 (left panel) shows an HPLC–ES/MS/MS analysis (11 MRM transitions total) of the mixture of phytoestrogens using a 1 mm × 30 mm column. No significant changes in *S*/*N* were observed for any analyte when the identical conditions were used with a 2 mm × 30 mm Luna column (data not shown). The right panel of Fig. 1 shows the analogous UPLC separation using a 1 mm × 50 mm column. This optimized isocratic HPLC method permits adequate resolution of all components in under 1 min. Although analyte retention and separation was virtually unchanged by using UPLC with the same mobile phase and flow rate, the improved resolution of UPLC reduced peak width and accordingly increased the *S*/*N* responses for all analytes in the range of 1.8–8-fold (individual *S*/*N*-fold increases: equol, 8; daidzein, 4.2; dihydrodaidzein, 5.5; genistein, 1.8; dihydrogenistein, 2.8).

This application contrasts an HPLC–MS analysis previously optimized for throughput and adequate peak separations with a UPLC analysis. In this case, the UPLC analysis produces higher sensitivity for all analytes through improvements in peak efficiency, even though no improvement in analysis time was achieved. By contrast, a recently published HPLC–UV method for analysis of total serum isoflavones (genistein, daidzein, glycitein) required 15 min run time and produced peak widths in the range of 1.5–2 min [5].

3.2. Tamoxifen metabolites and soy isoflavones

Studies designed to understand how diet can affect drug pharmacokinetics, metabolism, and pharmacodynamics require accurate measurements of circulating levels of bioactive dietary components in conjunction with levels of parent drug and its important metabolites. Tamoxifen is a successful adjuvant therapy for women with estrogen-dependent breast cancer and several chromatographic investigations of tamoxifen and bioactive metabolites have been published [6–8]. Our studies of soy isoflavone–tamoxifen interactions in animal models of post-menopausal breast cancer [6] have prompted us to seek evidence for such effects in women consuming both soy and tamoxifen. The separation of these analytes is shown in Fig. 2 (HPLC, left panel; UPLC, right panel). The same isoflavones, metabolites, and internal standards shown in Fig. 1 were included along with tamoxifen (Tam), 4-hydroxy-Tam, 4-sulfoxy-Tam, *N*-des-methyl-Tam and the four corresponding labeled internal standards. The analysis was conveniently separated into two time functions. The HPLC separation required use of a 2 mm × 150 mm column and the total run time was 10 min. By contrast, UPLC produced comparable peak separations but required a run time of only 3.5 min, approximately a three-fold improvement in speed. The sharper UPLC peak width gave increases in *S*/*N* for all but two analytes (individual *S*/*N*-fold increases: daidzein, 1.4; dihydrodaidzein, 1; dihydrogenistein, 1.2; genistein, 2.4; equol, 3.7; 4-sulfoxy-Tam, 1; 4-hydroxy-Tam, 1.3; N-des-methyl-Tam, 3.4; Tam, 9.8). In addition, some significant differences in selectivity were observed between the two columns for 4-sulfoxy-Tam and 4-hydroxy-Tam.

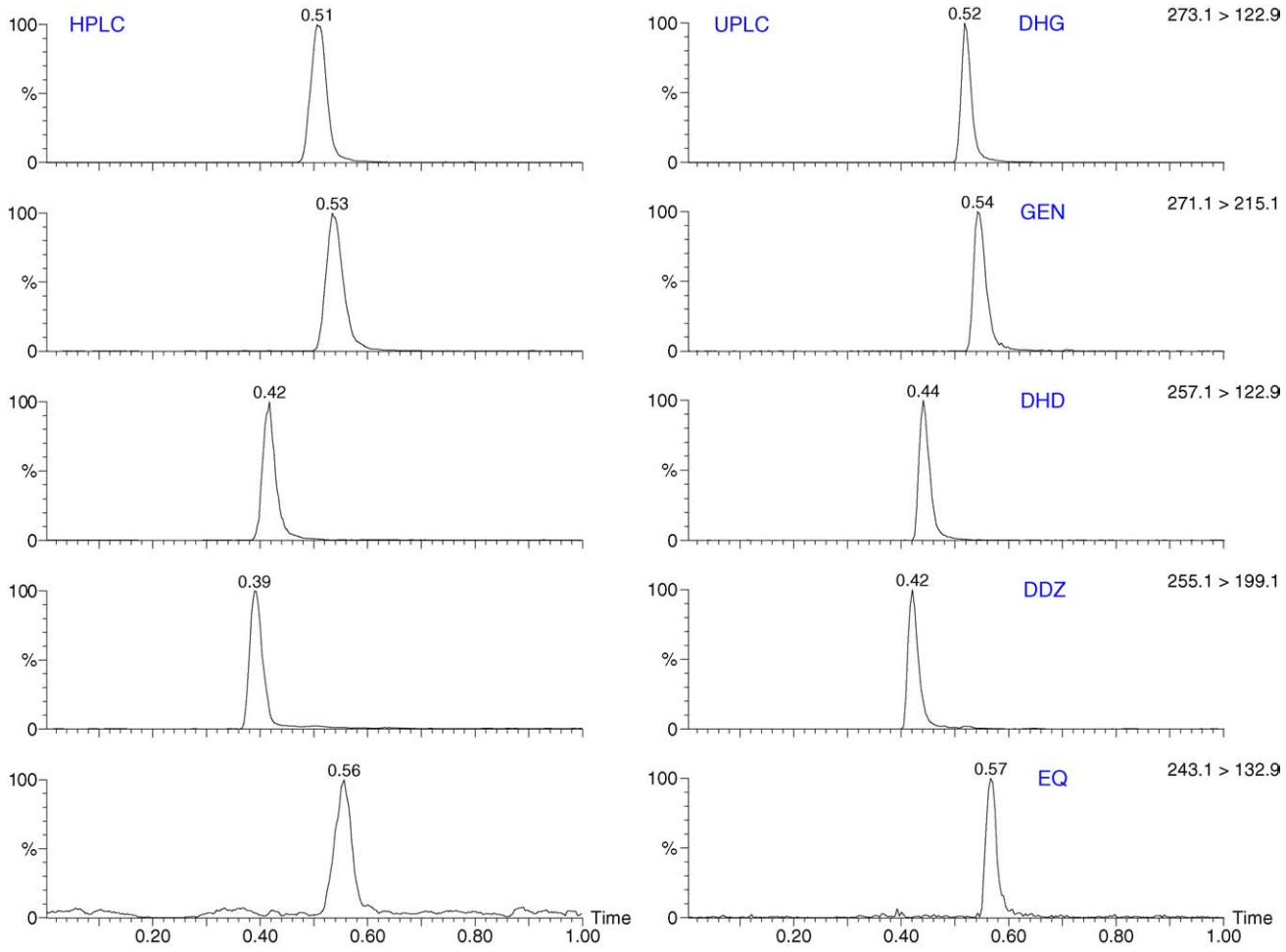

Fig. 1. Comparison of HPLC–MS/MS and UPLC–MS/MS Separations for Soy Isoflavones and Metabolites. The MS acquisition for both analyses consisted of 11 MRM transitions for equol (EQ $[M + H^+] = m/z$ 243 and its deuterated internal standard), daidzein (DDZ $[M + H^+] = m/z$ 255 and its deuterated internal standard), dihydrodaidzein (DHD $[M + H^+] = m/z$ 257), genistein (GEN $[M + H^+] = m/z$ 271 and its deuterated internal standard), and dihydrogenistein (DHG $[M + H^+] = m/z$ 273) for UPLC (right panel) and HPLC (left panel). HPLC: 10 ms dwell, 5 ms inter-channel delay, and 5 ms inter-scan times; UPLC: 15 ms dwell, 5 ms inter-channel delay, and 25 ms inter-scan times.

In this gradient elution application, significant improvement in sensitivity and analysis time were achieved by using UPLC for determining this combination of nine analytes and seven internal standards. By contrast, a recent publication of a capillary HPLC–MS method for analysis of 4-hydroxy-Tam in plasma required 15 min run time and produced a peak width of 0.5 min [7]. Similarly, a recently published HPLC-fluorescence method for Tam and four metabolites in plasma required 70 min run time and produced peak widths of 2.5–7 min [8].

3.3. β-Agonists

Growth promoting agents that act as β-adrenergic receptor agonists are widely used illegally by livestock producers for the competitive advantage given in meat production. Many chemically distinct but pharmacologically related compounds have been detected in livestock making broad screening assays specific for many analytes critical to regulatory compliance programs. We have published HPLC–ES/MS/MS methodology to detect with high sensitivity (<1 ppb) a number of β-agonists in livestock tissues (liver and retina) using MRM mode [9,10]. The chemical and chromatographic diversity of β-agonists makes the separation a critical component of the analytical method. The gradient HPLC chromatogram of 9 β-agonists using a cyano-phase column (2 mm × 150 mm) is shown in Fig. 3 (left panel and [9]) and the corresponding gradient UPLC chromatogram using the hybrid C18-silica reverse phase is shown in the right panel. Both separations employed an initial isocratic elution to resolve the polar β-agonists followed by a gradient step to resolve the non-polar compounds. At first appearance, the separations are similar; however, this finding was quite unusual because all previous attempts to separate these structurally diverse β-agonists using reverse phase HPLC had failed. This finding suggests novel selectivity from the hybrid reverse phase. For a similar degree of analyte peak separation, the total run time of

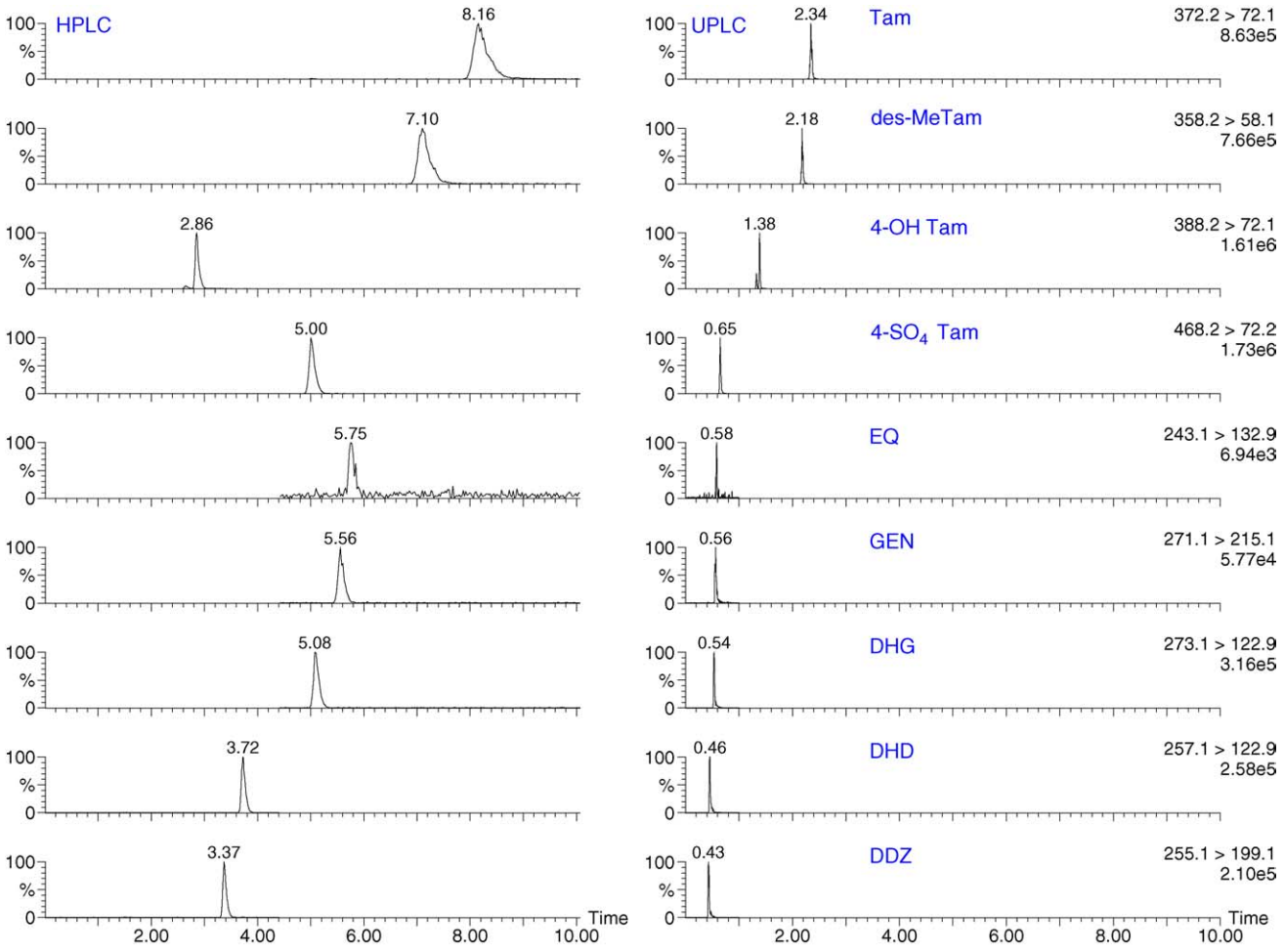

Fig. 2. Comparison of HPLC–MS/MS and UPLC–MS/MS Separations for Soy Isoflavones/Metabolites and Tamoxifen/Metabolites. The MS acquisition for both analyses consisted of 19 MRM transitions for equol (EQ $[M + H^+] = m/z$ 243 and its deuterated internal standard), daidzein (DDZ $[M + H^+] = m/z$ 255 and its deuterated internal standard), dihydrodaidzein (DHD $[M + H^+] = m/z$ 257), genistein (GEN $[M + H^+] = m/z$ 271 and its deuterated internal standard), and dihydrogenistein (DHG $M + H^+ = m/z$ *273),* 4-sulfoxy-tamoxifen ($[M + H^+] = m/z$ 468 and its deuterated internal standard), 4-hydroxy-tamoxifen ($[M + H^+] = m/z$ 388 and its ^{13}C, ^{15}N-labeled internal standard), N-des methyl-tamoxifen ($[M + H^+] = m/z$ 358 and its deuterated internal standard), tamoxifen ($[M + H^+] = m/z$ 372 and its ^{13}C, ^{15}N-labeled internal standard) for UPLC (right panel) and HPLC (left panel). HPLC: 100 ms dwell, 10 ms inter-channel delay, and 10 ms inter-scan times; UPLC: 20 ms dwell, 5 ms inter-channel delay, and 5 ms inter-scan times.

5.5 min for the UPLC separation was an improvement in speed over the 8.6 min for the HPLC method. In addition, some significant sensitivity improvements were observed, especially for the late-eluting analytes in the gradient elution (individual *S*/*N*-fold increases: cimaterol, 1.1; terbutaline, 1.3; salbutamol, 1.3; zilpaterol, 1.2; clenbuterol, 3.6; ractopamine, 1.9; fenoterol, 4.8, mabuterol, 2.0; brombuterol, 3.4).

The additional resolving capability of UPLC over HPLC is further illustrated by the partial resolution of ractopamine diastereomers (Fig. 3, 4.42 min RT, m/z 302 → 164 transition). It should be noted that no attempt was made to completely resolve these peaks although, based on our experience with resolution of other structural isomer pairs, it is likely that minor changes would probably suffice. In our previous HPLC studies, which focused on ractopamine alone, no evidence for separation of diastereomers was ever observed [9,10]. In this application, significant improvement in resolution, sensitivity, and analysis time were achieved by using UPLC for determining this combination of nine analytes and an internal standard. By contrast, a recent publication of a HPLC–MS method for analysis of 12 β-agonists in bovine retina and liver required 22 min run time and produced a peak width of 0.6–0.8 min [11].

In addition to sensitive detection of illegal drug residues, regulatory programs that monitor drug residues in livestock also require unambiguous confirmation of structure with sufficient specificity to justify legal action. Typically, this specificity is provided through the use of mass spectrometric fragmentation patterns. Our previous work described the use of intensity ratios from at least three MRM intensities for each analyte to confirm by criteria set by major regulatory agencies (US FDA, EU) the presence of β-agonists in liver and retina at residue levels relevant to detecting and

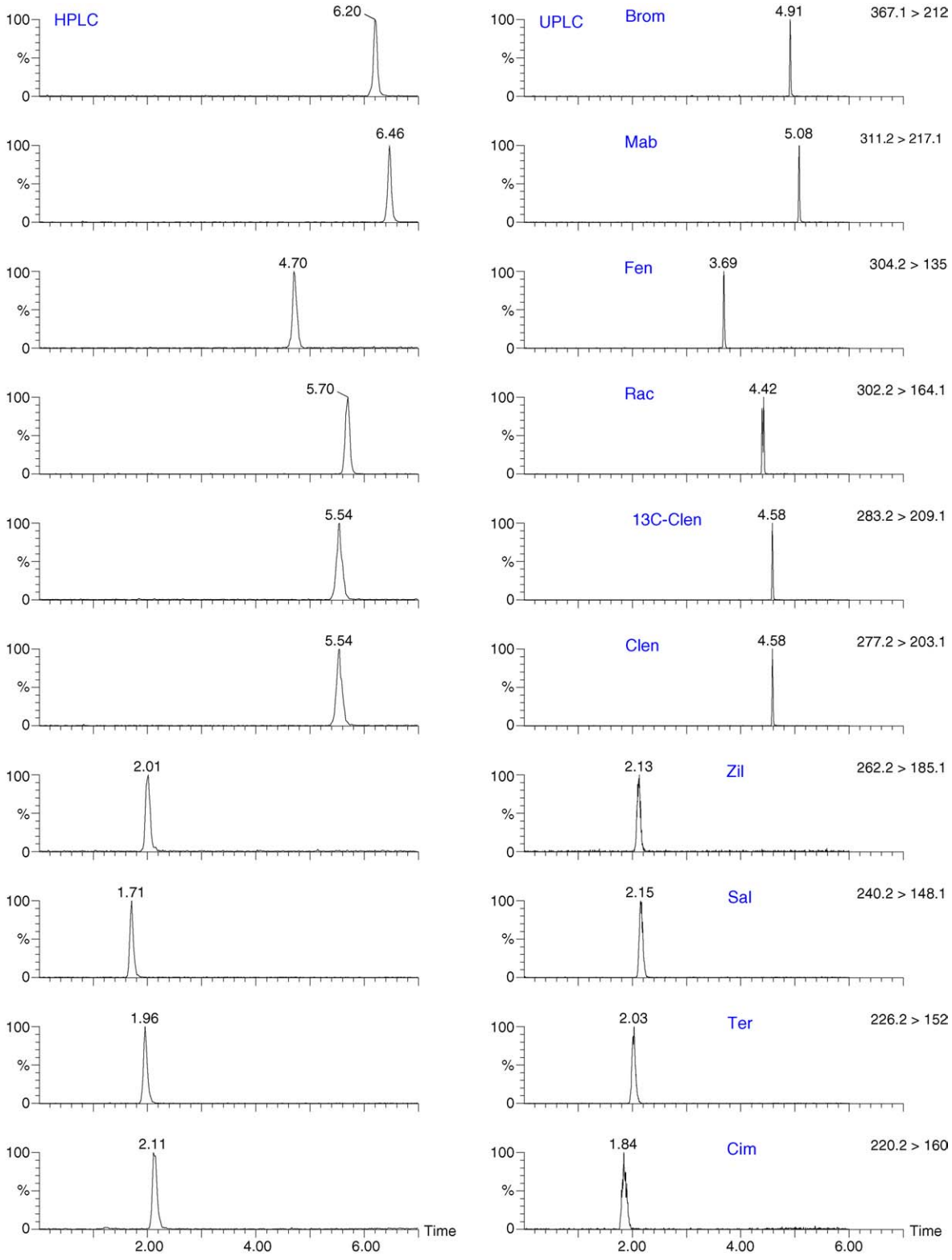

Fig. 3. Comparison of HPLC–MS/MS and UPLC–MS/MS Separations for β-agonists. The MS acquisition for both analyses consisted of 28 MRM transitions consisting of three transitions (one principal and two confirmatory) for each of nine analytes and one internal standard transition. UPLC (right panel) and HPLC (left panel) chromatograms are shown for the principal transitions only: cimaterol (Cim, $[M + H^+] = m/z$ 220), terbutaline (Ter, $[M + H^+] = m/z$ 226), salbutamol (Sal, $[M + H^+] = m/z$ 240), zilpaterol (Zil, $[M + H^+] = m/z$ 262), clenbuterol (Clen, $[M + H^+] = m/z$ 277 and its ^{13}C-labeled internal standard, $[M + H^+] = m/z$ 283), ractopamine diastereomers (Rac, $[M + H^+] = m/z$ 302), fenoterol (Fen, $[M + H^+] = m/z$ 304), mabuterol (Mab, $[M + H^+] = m/z$ 311), brombuterol (Brom, $[M + H^+] = m/z$ 367). HPLC: 100 ms dwell, 10 ms inter-channel delay, and 50 ms inter-scan times; UPLC: 25 ms dwell, 5 ms inter-channel delay, and 5 ms inter-scan times.

confirming illegal drug use (<1 ppb, [9]). The fast scanning capabilities (>5 ms) of the Premier triple quadrupole mass spectrometer made it feasible to put all 28 MRM transitions into one time function, although in this case the separation and retention time stability provided by UPLC did not require this additional constraint (i.e., two time functions were used). No change in sensitivity was observed in decreasing MRM dwell time from 75 to 25 ms, the minimum needed to produce UPLC peaks consisting of at least 15 data points. The ability to further reduce scan times on the UPLC–MS/MS system described should provide significant additional value as we increase the number of target β-agonists in our confirmatory analysis of tissue residues.

3.4. *Ephedra* alkaloids

Ma-Huang *(Ephedra sinica)* is a natural source of alkaloids that are often used in various nutritional supplements marketed as weight-loss, energy-enhancing, or body-building products. The *Ephedra* alkaloids include ephedrine, pseudoephedrine, nor-ephedrine (phenylpropanolamine), nor-pseudoephedrine, methyl-pseudoephedrine, and methyl-ephedrine [12,13]. These alkaloids all have the same physiological actions qualitatively but differ in their potencies. Our studies on cardiovascular and developmental effects of alkaloids from *Ephedra*- and the closely related *Citrus aurantium*-based nutritional supplements require analytical methodology to relate pharmacokinetic behavior to pharmacodynamic effects in different species. The separation of *Ephedra* alkaloids is shown in Fig. 4 (HPLC, left panel; UPLC, right panel). The isocratic HPLC method had adequate resolution to separate to baseline the diastereomers, ephedrine and pseudoephedrine, but this required use of a 2 mm × 150 mm column and a total run time of 12 min. By comparison, the isocratic UPLC method required a run time of 4 min to achieve a similar degree of peak separation. In addition, sensitivity improvements were observed for all components (individual *S*/*N*-fold increases: nor-ephedrine (phenylpropanolamine), 1.5; ephedrine, 4.8; pseudoephedrine, 4.7; methyl-ephedrine, 2.6).

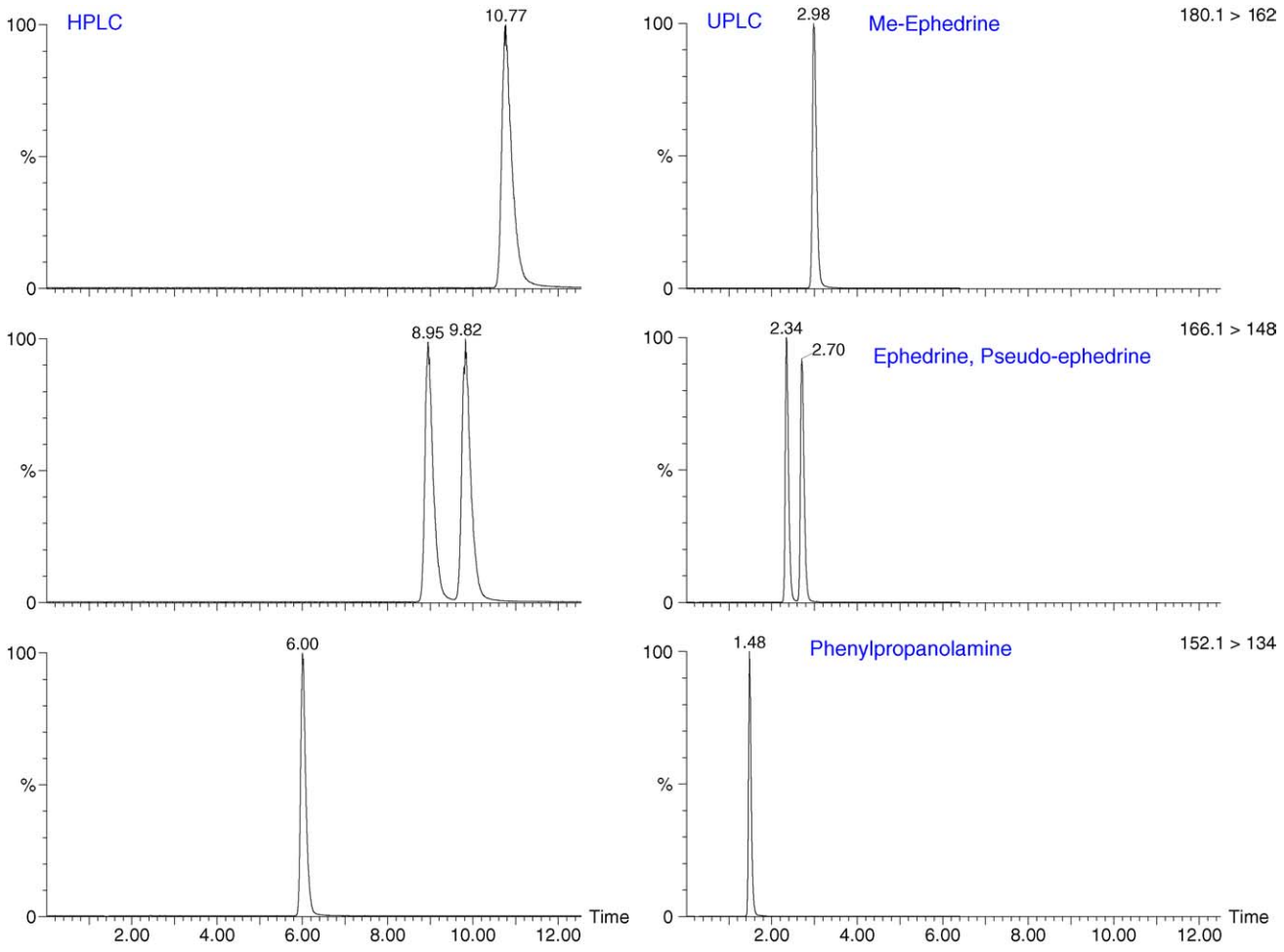

Fig. 4. Comparison of HPLC–MS/MS and UPLC–MS/MS Separations for *Ephedra* Alkaloids. The MS acquisition for both analyses consisted of 6 MRM transitions including the principal transitions for each of four analytes and two deuterated internal standards. Shown are the principal transitions for phenylpropanolamine ($[M + H^+] = m/z$ 152), ephedrine and pseudo-ephedrine ($[M + H^+] = m/z$ 166), and methyl-ephedrine ($[M + H^+] = m/z$ 180). HPLC and UPLC: 100 ms dwell, 10 ms inter-channel delay, and 10 ms inter-scan times.

In this application, significant improvement in sensitivity and analysis time were achieved by using UPLC for determining this combination of four analytes and two internal standards. By contrast, recent publication of a well-optimized HPLC–MS method for analysis of six *Ephedra* diasteromers plus caffeine in plasma required 10 min run time and produced peak widths of 0.6–0.7 min [13].

3.5. Precursor ion scanning to identify components in complex botanical mixtures

The increasing popularity of botanical products for treatment and prevention of diseases and other lifestyle issues makes identification of bioactive components of paramount concern. Separations of such complex mixtures and mass spectral identifications can be particularly challenging because often multiple botanical products are combined into single commercial products. One powerful way to reduce complexity is through the use of precursor ion scanning of analyte classes. One example we have previously investigated was the various isoflavone conjugates present in whole soy, in soy-based nutritional supplements, and in human serum [14]. Analysis of the supplement used gradient HPLC to separate glycosides, acetyl glucosides, and malonyl glucosides of daidzein, glycetein, and genistein and used precursor ion scanning function of the triple quadrupole mass spectrometer over an appropriate mass range to identify the individual components [14].

The total ion chromatogram (TIC) of the glucosides derived from each isoflavone present in a commercial nutritional supplement is shown in Fig. 5 (HPLC, center panel; UPLC, right panel). In both cases, an initial isocratic portion was used followed by gradient elution. The HPLC separation required use of a 2 mm × 150 mm column and the total run

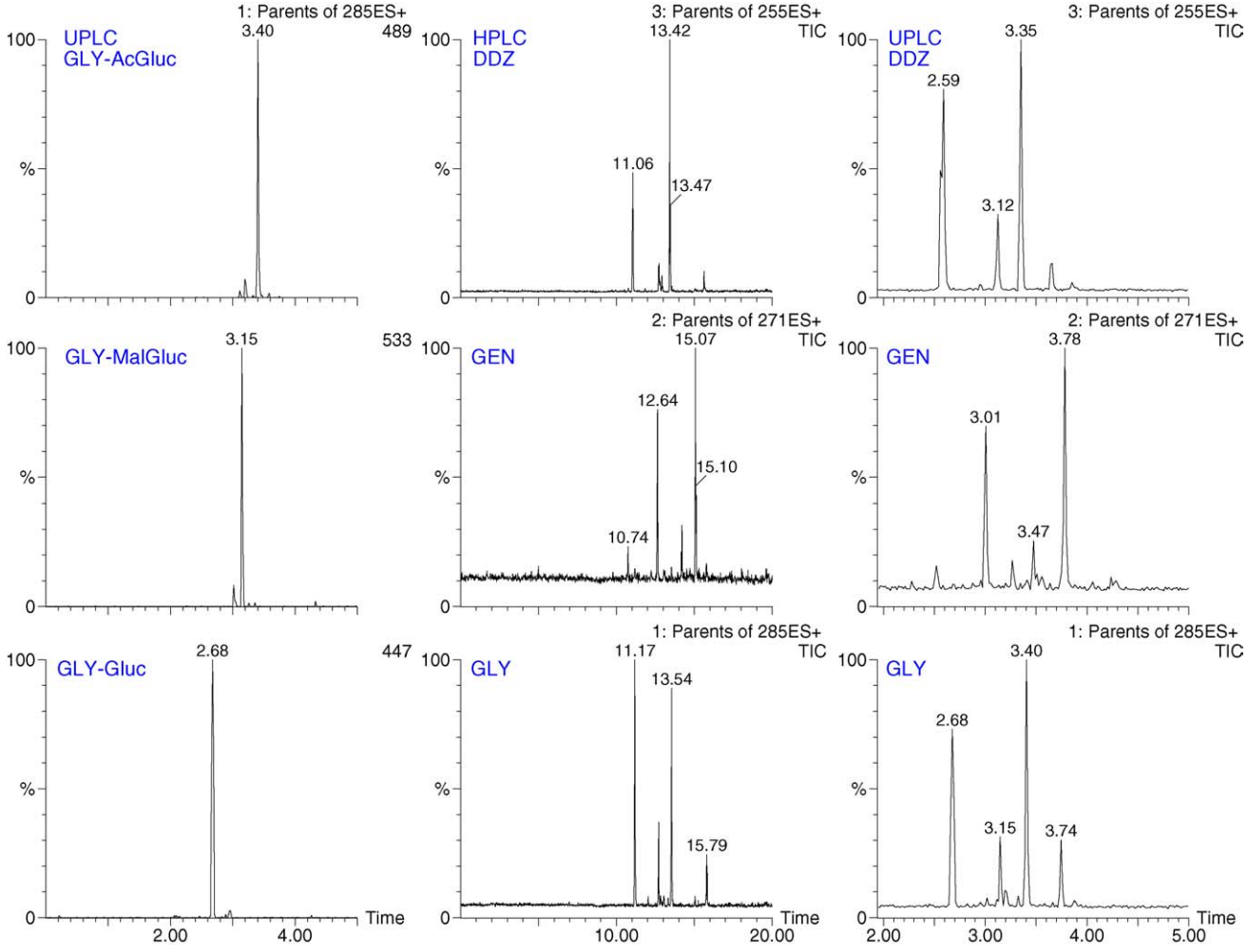

Fig. 5. Comparison of HPLC and UPLC in the Analysis of Soy Isoflavone Glucoside Conjugates in a Nutritional Supplement Using Precursor Ion Scanning. The MS acquisition for both analyses consisted of precursor ion scanning using separate functions for glycetein conjugates (parents of *m/z* 285), genistein conjugates (parents of *m/z* 271), and daidzein conjugates (parents of *m/z* 255). The center panel shows the respective total ion chromatograms for HPLC and the right panel shows the total ion chromatograms for UPLC. The left panel shows a representative deconvolution of one precursor ion scan (GLY, parents of *m/z* 285) from the UPLC separation into individual components based on the respective protonated molecule for glycetein glucoside (RT 2.68 min for $[M + H^+] = m/z$ 447), glycetein malonylglucoside (RT 3.15 min for $[M + H^+] = m/z$ 533), or glycetein acetylglucoside (RT 3.40 min for $[M + H^+] = m/z$ 489). Both acquisitions were recorded at 1000 amu/s.

time was 23 min. By contrast, UPLC produced comparable peak separation but required a run time of only 5 min, approximately a five-fold improvement in speed. Even though the UPLC peak widths observed were approximately half as wide, no significant differences in *S*/*N* were observed for the individual components.

As an example of the deconvolution provided by parent ion scanning, the individual peaks for the glucoside (*m/z* 447), malonyl-glucoside (*m/z* 533), and acetyl-glucoside (*m/z* 489) for glycetein are shown for the UPLC separation in Fig. 5 (left panel). The effect of quadrupole scanning speed was also investigated for the mass range required for this application (*m/z* 240–560). As the scanning speed was increased from 500 amu/s (the maximum scanning speed for our previous-generation triple quadrupole instruments) to 4000 amu/s, minimal effects on the *S*/*N* for each chromatographic response of individual isoflavone glucosides were observed. There were minor increases in width of the corresponding mass spectra (0.55–0.80 Th) observed as the scan speed was increased over the specified range. It should be noted that scanning speeds of 3000 amu/s were required to obtain our stated criterion of approximately 15 data points across the very narrow UPLC peaks.

In this application, significant improvement in analysis time were achieved by using UPLC (5 min versus 23 min) permitting full utilization of the rapid scanning speeds possible with this triple quadrupole MS to show the utility of this approach for analysis of complex mixtures of natural products. By contrast, a previous analysis of soy isoflavone conjugates (nine total) in soy foods required run times of 25 min and produced peak widths of 2–3 min by using selected ion monitoring of conjugates from each isoflavone in separate runs [15].

4. Conclusions

As the information content required from every sample analysis increases, as analyte numbers and complexity increase, and as the need to push limits of quantification to lower and lower levels in shorter and shorter times become more critical to the conduct of hypothesis-driven investigations, optimized performance of both chromatographic and mass spectral components of LC–MS systems becomes more and more critical. Tandem MS measurements available using a triple quadrupole spectrometer, such as multiple reaction monitoring and precursor ion scanning, are powerful tools for high sensitivity detection and for simplifying complex samples, respectively; however; the goal of increasing overall performance of these techniques can be further enhanced by reducing constraints imposed by the chromatographic separation. In the five examples presented in this paper, we have shown that use of UPLC generally produced an improvement in sensitivity and total analysis time, although not in all cases. Improvements in total analysis time and chromatographic efficiency from use of UPLC were even more evident vis a vis selected comparable analyses from the recent literature [5,7,8,11,13,15]. It was also interesting that the improvements in sensitivity observed from use of UPLC were compound-dependent and not simply arithmetical as predicted based on theoretical predictions of chromatographic peak shapes. Further work remains to understand this result. It was also noted that the hybrid particles used in UPLC often showed unique selectivity when compared to conventional HPLC packings and that extraordinary separations of geometric isomers could be achieved through UPLC without additional effort. These results were not completely unexpected based on the physical–chemical differences in hybrid particles versus pure silica. The recent development of alternate UPLC phases (polar-embedded reverse phase, phenyl, C8) also suggest that further improvements in analytical separations can be made by virtue of the different selectivities. Overall, it appears that UPLC can offer significant improvements in sensitivity, speed, and resolution that auger well for future applications in LC–MS analysis.

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References

[1] M.E. Swartz, B.J. Murphy, Ultra performance liquid chromatography: tomorrow's HPLC technology today, LabPlus Int. June (2004).
[2] C.M. Henry, Chem. Eng. News 82 (2004) 68.
[3] N.C. Twaddle, M.I. Churchwell, D.R. Doerge, J. Chromatgr. B 777 (2002) 137.
[4] A.H. Wu, M.C. Yu, C.C. Tseng, N.C. Twaddle, D.R. Doerge, Carcinogenesis 25 (2003) 77.
[5] B.F. Thomas, S.H. Zeisel, M.J. Busby, J.M. Hill, R.A. Mitchell, N.M. Scheffler, S.S. Brown, L.T. Bloeden, K.J. Dix, A.R. Jeffcoat, J. Chromatgr. B 760 (2001) 191.
[6] Y.H. Ju, D.R. Doerge, K.F. Allred, C.D. Allred, W.G. Helferich, Cancer Res. 62 (2002) 2474.
[7] R.S. Plumb, H. Warwick, D. Higton, G.J. Dear, D.N. Mallett, Rapid Commun. Mass Spectrom. 15 (2001) 297.
[8] K.-H. Lee, B.A. Ward, Z. Desta, D.A. Flockhart, D.A. Jones, J. Chromatgr. B 791 (2003) 245.
[9] L.D. Williams, M.I. Churchwell, D.R. Doerge, J. Chromatogr. B 813 (2004) 35.

[10] M.I. Churchwell, C.L. Holder, D. Little, S. Preece, D.J. Smith, D.R. Doerge, Rapid Commun. Mass Spectrom. 16 (2002) 1261.
[11] A.C.E. Fesser, L.C. Dickson, J.D. MacNeil, J.R. Patterson, S. Lee, R. Gedir, J. Assoc. Off. Anal. Chem. Int. 88 (2005) 61.
[12] B.J. Gurley, P. Wang, S.F. Gardner, J. Pharm. Sci. 87 (1998) 1547.
[13] P. Jacob, C.A. Haller, M. Duan, L. Yu, M. Peng, N.L. Benowitz, J. Anal. Toxicol. 28 (2004) 152.
[14] D.R. Doerge, H.C. Chang, C.L. Holder, M.I. Churchwell, Drug Metab. Disp. 28 (2000) 298.
[15] S. Barnes, M. Kirk, L. Coward, J. Agric. Food Chem. 42 (1994) 2466.