Clearly Better LC/MS Solutions

A compendium of product information, technical articles, and applications

How Agilent's LC and MS systems are clearly better together for high-throughput quantitation, high-resolution characterization, and fast, easy nanospray LC/MS

Agilent Technologies

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Introduction

Liquid chromatography/mass spectrometry (LC/MS) combines the physical separation capabilities of liquid chromatography (LC) or high-performance liquid chromatography (HPLC) with the mass analysis capabilities of mass spectrometry (MS). LC/MS is used for many applications that require very high sensitivity and specificity, with the goal of specific detection, potential identification, or quantitation of compounds within a complex mixture.

Agilent Technologies offers a unique combination of best-in-class LC technology, exceptional MS spectral quality, powerful data analysis tools, and optimized, single-source workflow solutions that address a full range of qualitative and quantitative applications. An industry leader in both LC separations and MS detection technology, Agilent offers you clearly better LC/MS systems for all of your analytical needs requiring robust, high-performance, ultra-competitive solutions. You'll find us right at the center of key applications such as environmental analysis, food safety analysis, metabolomics, proteomics, biologics, and pharmaceutical analysis.

Clearly better together: Agilent 1290 Infinity LC and 6000 Series LC/MS systems

Agilent's 1290 Infinity LC System delivers on both fast and optimal separations. The system provides unprecedented, best-in-class UHPLC speed, resolution, and sensitivity—and better HPLC performance, too.

The breakthrough 1290 Infinity LC System is the ideal front end for any Agilent mass spectrometer and allows you to optimize your separations to the needs of your MS analysis. Take full advantage of UHPLC speed and use the 1290 Infinity LC System's unmatched peak capacity and resolution to eliminate co-elution and peak overlap.

ZORBAX RRHD 1.8 µm columns, available in popular ZORBAX bonded phases, deliver the resolution and peak definition required for critical LC/MS analyses, as well as robust, reliable performance over the extended operating range of the 1290 Infinity LC System.

An infinitely better LC platform for LC/MS. Because of its amazingly wide operating power range (from 1200 bar at 2 mL/minute to 800 bar at 5 mL/minute), Agilent's 1290 Infinity LC System is able to deliver faster separations without loss of resolution—plus unequaled flexibility, methods compatibility, and investment protection.

UHPLC performance creates demands on the mass spectrometer. Fortunately, Agilent's MS technology breakthroughs allow the MS to keep up with faster UHPLC separations.

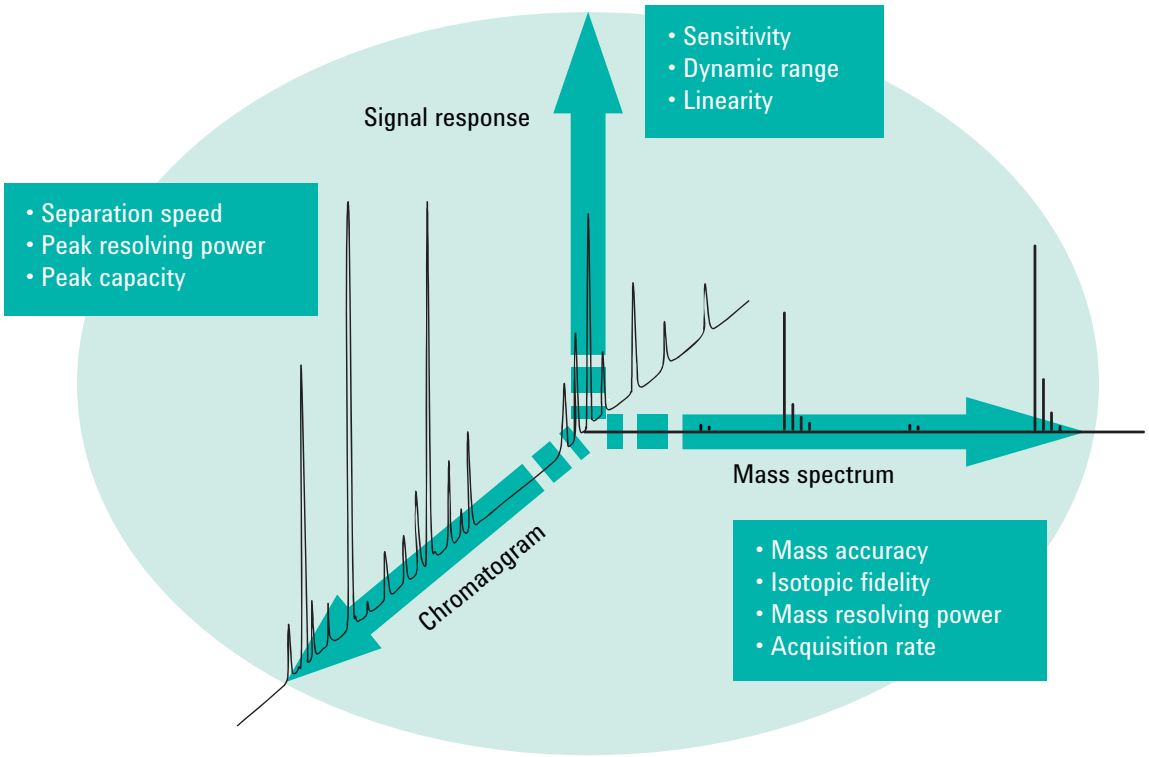
The 6400 Series Triple Quadrupole (QQQ) LC/MS systems provide:

- **Fast, sensitive MS/MS.** An innovative collision cell eliminates cross-talk and enables very fast MRMs with just 1-ms dwell times. This enables hundreds of compounds to be analyzed in a single run, which is essential for applications such as the analysis of pesticides in food.
- **Innovative, dynamic MRM acquisition mode.** Up to 4,000 compounds can be quantified without the need to manually set up time segments.
- **Faster polarity switching (30 ms).** Ensure compatibility with even narrow UHPLC peaks. A list of MRM transitions of differing polarity can be monitored with sufficient speed to achieve reproducible peak areas for even a second-wide UHPLC peak.

The 6200 Series Time-of-Flight (TOF) and 6500 Series Quadrupole Time-of-Flight (Q-TOF) LC/MS systems provide:

- **Scan rates up to 20 MS or 10 MS/MS spectra per second.** Quality data without any loss in accuracy, spectral quality, or resolution (unlike ion cyclotron instruments where resolution decreases rapidly as you increase the speed of the measurement).

Agilent QQQ, TOF, and Q-TOF LC/MS systems all benefit from tremendous sensitivity from Agilent Jet Stream technology, as well as have excellent dynamic range—typically five orders in-spectrum, which improves detection of low-abundance compounds in the presence of higher-abundance components.



Clearly better LC/MS optimizes all analytical dimensions.

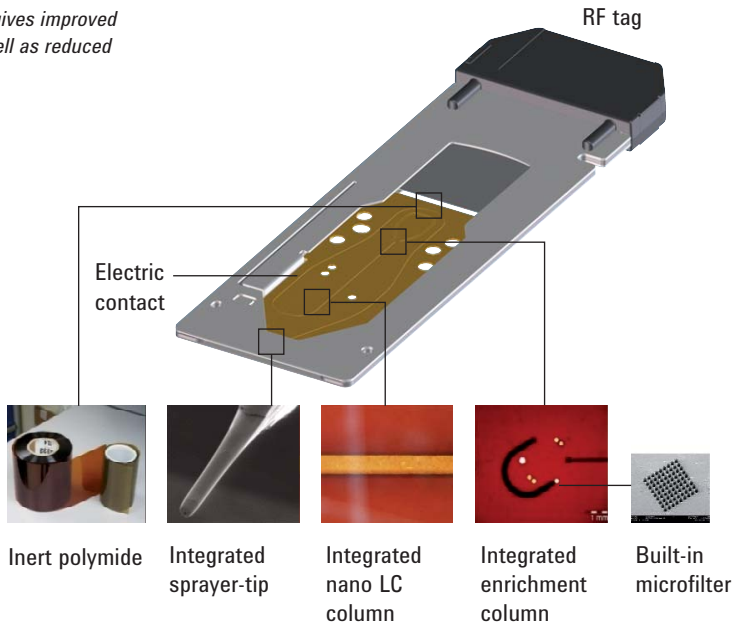
Clearly better together: Agilent 1200 HPLC-Chip and 6000 Series LC/MS systems

The Agilent 1200 Series HPLC-Chip/MS system is based on revolutionary microfluidic chip technology specifically designed for nanospray LC/MS. The system includes 1200 Series Capillary and Nanoflow Pumps, Micro-Well Plate Sampler with Thermostat, Chip Cube MS interface, and any Agilent 6000 Series mass spectrometer. System control is through either Agilent ChemStation or MassHunter software.

The second-generation HPLC-Chip II technology incorporates a carbon-ion-implanted filter, improving surface characteristics dramatically for optimal contact and sealing, as well as reducing friction between rotor and polyimide chip. These improvements double chip lifetime and lower the cost per analysis as well as improve chip-to-chip and run-to-run reproducibility. With Agilent's HPLC-Chip II, the Agilent HPLC-Chip/MS system takes you to new levels of nanospray MS reliability, robustness, sensitivity, and ease of use, allowing you to enter applications such as:

- Biomarker discovery and validation
- Intact monoclonal antibody characterization
- Small molecule analysis, such as DMPK
- Phosphopeptide analysis in post-translational modification (PTM) studies

HPLC-Chips are available for a wide range of application needs. Second-generation technology with new carbon-ion-implanted filter gives improved surface characteristics for optimal contact and sealing, as well as reduced friction between rotor and polyimide chip.



High-throughput quantitation demands fast, reliable, robust separations and streamlined analysis and data processing workflows. With the Agilent Infinity 1290 LC system, you can improve your separation quality and reduce your analysis time to process more samples each and every day. Agilent QQQ, Single Quadrupole, Q-TOF, and TOF mass spectrometers integrate seamlessly with all Agilent HPLC systems and allow walk-up introduction and processing of sample batches.

New dynamic MRM methods on the Agilent 6400 Series QQQ instruments create new capabilities to tackle large, multi-analyte assays and to accurately quantify exceedingly narrow peaks from fast Agilent 1200 Series RRLC and 1290 Infinity LC separations. Dynamic MRM methods yield equivalent, or better, quality data and results as compared to traditional time-segment-based methods—plus easier method development and modification.

Technical overview: New dynamic MRM Mode improves data quality and triple quad quantification in complex analyses

Abstract

Multiple Reaction Monitoring (MRM) mode has become the preferred method for the quantitative analysis of known or target compounds using triple quadrupole mass spectrometry. The current solution for MRM analysis uses time segmentation, where a method is divided into a series of time segments and predefined sets of MRM transitions are monitored for each segment. As sample complexity increases (*e.g.*, quantifying very low levels of hundreds of pesticide residues in a wide variety of food matrices), very real practical limitations in the time-segmentation methodology become apparent. A better solution is required.

New dynamic MRM methods on the Agilent 6400 Series Triple Quadrupole instruments create new capability to tackle large multi-analyte assays and to accurately quantify exceedingly narrow peaks from fast Agilent 1200 Series RRLC and 1290 Infinity LC separations. Examples of pesticide analysis and rapid screening of drugs of abuse are highlighted. Dynamic MRM methods yield equivalent, or better, quality data and results as compared to traditional time-segment-based methods—plus easier method development and modification.

Introduction

Utilizing multiple reaction monitoring (MRM) with a triple quadrupole tandem mass spectrometer enables extraordinary sensitivity for multi-analyte quantitative assays. The first quadrupole (Q1) selects and transmits a precursor ion with a specific *m/z*. This ion is then fragmented in the second quadrupole (Q2 collision cell), and a specific product ion with a defined *m/z* is selected and transmitted in the third quadrupole (Q3). See Figure 1. The combination of a specific precursor mass and a unique product ion is generally an unambiguous and sensitive method to selectively monitor and quantify a compound of interest. Since two stages of mass selection are utilized, MRM assays are particularly useful for the specific analysis of target compounds in complex mixtures and matrices. MRM mode has become the preferred method for the quantitative analysis of known or target compounds.

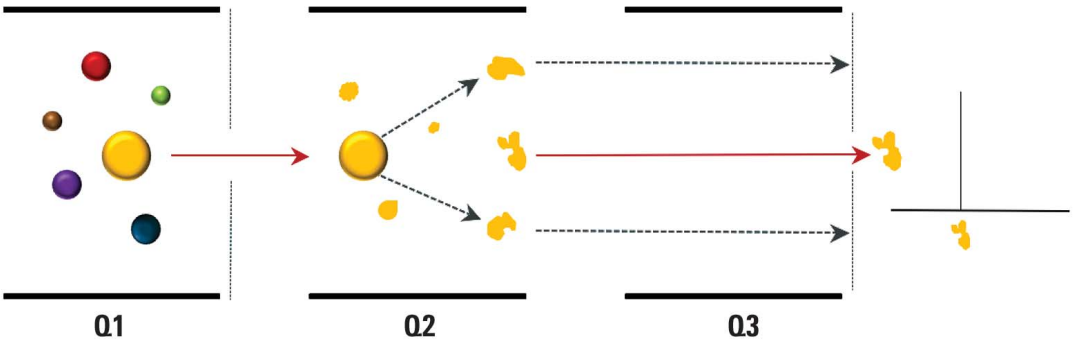


Figure 1. A schematic diagram of MRM mode on a triple quadrupole instrument. The precursor ion is selected in Q1, fragmentation occurs in Q2, and the product ion is selected by Q3. Since two stages of mass selectivity are utilized, there is very little interference from background matrix resulting in excellent sensitivity.

The limitations of time segment methods

The current solution to complex sample analysis is time segmentation. A method is developed with multiple, predefined time segments and the triple quadrupole MS is programmed to perform MRM assays for only those analytes that elute during each segment. Figure 2 shows an example of a method with four time segments. One set of MRM transitions is analyzed during segment 1, another set during segment 2, and so forth. The benefit of such a method is that, rather than performing MRM scans for all analytes during the entire method, during any given segment the triple quadrupole only monitors MRM transitions for the analytes that elute in that segment. The result is that there are fewer MRM transitions during each MS scan, allowing the mass spec method to use a longer dwell time and/or to reduce the overall cycle time for each MRM scan so that there are more data points per peak.

However, there are some limits to what can be accomplished with time segment methods. As the number of analytes in a method increases, so too will the number of concurrent MRM transitions in each segment. It will be necessary to either reduce the dwell times for these transitions or to increase the cycle time for each MS scan.

Reducing dwell times (the amount of time required for the triple quadrupole to analyze a single MRM transition) can compromise MS data integrity by introducing collision cell cross-talk (insufficient clearing of the collision cell between individual MRM experiments such that some product ions from a previous MRM may be detected in the subsequent MRM). Maintaining the same dwell time but increasing the overall MS cycle time may mean that not enough data points are collected during the elution of a very narrow LC peak to allow for reliable quantitation. Both of these factors can lead to compromises in data quality.

There is an additional challenge using time segments. In order not to compromise any data, the change from one segment to the next must occur during a time when no peaks are eluting from the LC column. In complex analyses such as pesticide analysis, where many co-eluting peaks are monitored at almost every time point during the chromatogram, this can be a formidable challenge as is highlighted in Figure 2. Furthermore, there is always the risk that adding analytes to a method may require complete redevelopment of a method to introduce these chromatographically quiet zones where segment changes can occur.

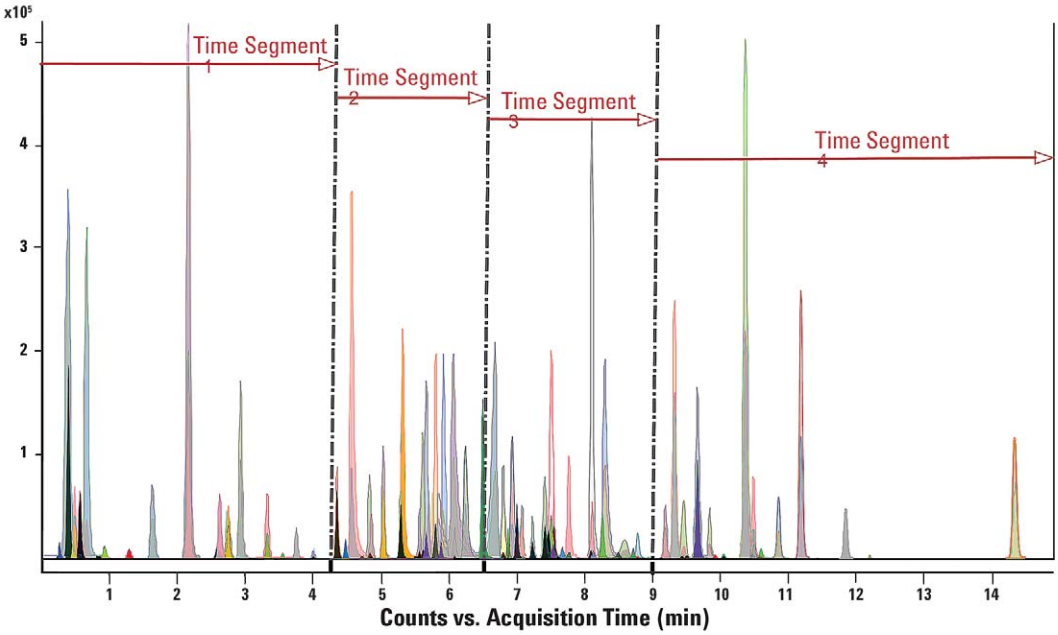


Figure 2. Dividing the chromatogram into time segments. Detection of a complex pesticide mixture demonstrates the advantages and some of the limitations of time segment based MRM quantitation.

Introducing dynamic MRM mode

Agilent's new and unique analytical method approach is now available on all 6400 Series Triple Quadrupole LC/MS systems. MassHunter acquisition software allows the user to choose conventional MRM or dynamic MRM mode. Ion transitions and a retention time window for each analyte are stored in a method. MRM transition lists are then built dynamically throughout an LC/MS run, based on the retention time window for each analyte. In this way, analytes are only monitored while they are eluting from the LC and valuable MS duty cycle is not wasted by monitoring them when they are not expected. An added benefit of this approach is that MassHunter MS Optimizer software can readily determine and store optimal transition ions for each target analyte, greatly simplifying dynamic MRM method set up.

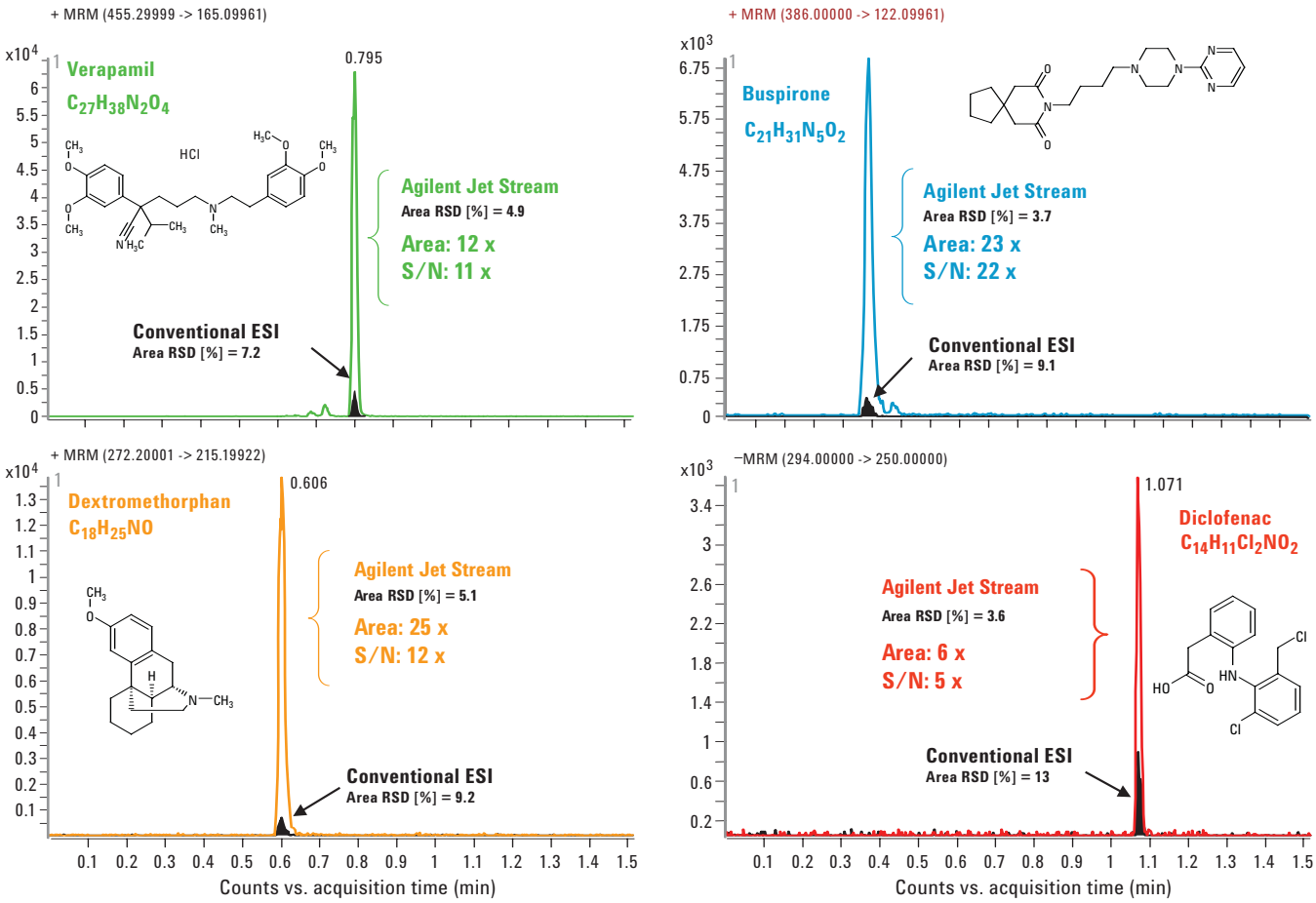
This approach addresses the limitations of the time segment methods for a large batch of compounds by replacing the group segmentation with individual time windows for every analyte transition and by dramatically reducing, on average, the number of individual MRM transitions that are monitored during each MS scan.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-3595EN.

Pharmaceutical analysis: Extended ionization capability of thermal gradient focusing ESI in high-throughput *in vitro* ADME assays

Several factors cause fast LC/MS/MS method development in the drug discovery area to be an arduous task. Combination of ESI/APCI sources offers broad response with multiple ionization modes, but optimization can be difficult and some sources limit flow rates to 1 mL/min, while others compromise chromatographic performance. The scan speed of the mass spectrometer needs to be fast enough to acquire an adequate number of data points across the narrow peaks generated using sub-2 µm columns. At typical fast LC conditions, current HPLC systems (pressure limit ~ 400 bar) would yield back pressures greater than the threshold limit. In this application example, we utilized the Agilent 1290 Infinity UHPLC system coupled with an Agilent 6460 Triple Quadrupole mass spectrometer comprising thermal gradient focusing ESI (Agilent Jet Stream technology, AJS) to streamline high-throughput bioanalytical method development using *in vitro* metabolic stability samples. Incubations of the substrates buspirone, verapamil, dextromethorphan, or diclofenac were carried out separately. An aliquot was taken at increasing time points from each incubate and then pooled together for analysis. AJS technology was compared to conventional orthogonal ESI using generic source values. The Agilent 1290 Infinity LC Triple Quadrupole MS/MS system, which allows flow rates up to 2 mL/min, pressures up to 1200 bar, dwell times as low as 1–2 ms, and polarity switching time of 30 ms, achieved an analysis time of less than 1.1 min without sacrificing quantitative data quality. Due to the high data acquisition rate provided by the Agilent 6460 Triple Quadrupole mass spectrometer, compounds ionizing in positive and negative modes were analyzed in a single run. An adequate number of data points (>10) could be collected across the extremely narrow peaks (Average full width half maximum [FWHM] <1.3 sec) generated by the Agilent 1290 Infinity LC system. AJS showed enhanced area response and signal-to-noise in comparison to conventional orthogonal ESI.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4932EN.



Overlaid MRM chromatograms obtained using AJS in comparison to conventional orthogonal ESI for the metabolic stability substrates after 35 minutes of incubation with rat liver S9 fraction.

Pharmaceutical analysis: High-throughput bioanalytical method development using UHPLC/triple quadrupole mass spectrometry

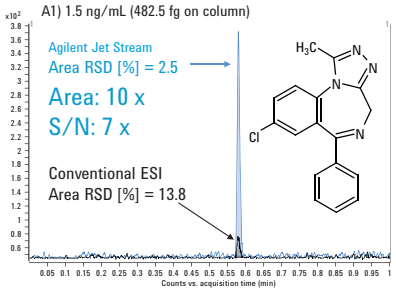
Several factors cause fast LC/MS/MS method development in the bioanalytical area to be an arduous task. In order to maintain sensitivity while speeding up analysis time, target analytes should not elute in the chromatographic region affected by ion suppression. The scan speed of the mass spectrometer must be fast enough to acquire an adequate number of data points to define the narrow peaks generated using sub-2 µm columns. In this application example, we utilized the Agilent 1290 Infinity LC system coupled to an Agilent 6460 Triple Quadrupole mass spectrometer comprising thermal gradient focusing ESI (Agilent Jet Stream technology, AJS) to streamline high-throughput bioanalytical method development using alprazolam spiked in human plasma (concentration range: 2 nM to 5000 nM, corresponding to 0.06 ng/mL to 1544 ng/mL). AJS technology was compared to conventional orthogonal ESI using generic source values.

The Agilent 1290 Infinity LC Triple Quadrupole MS/MS system, which allows flow rates up to 2 mL/min, pressures up to 1200 bar, and dwell times as low as 1–2 ms, achieved an analysis time of less than 0.5 min without sacrificing quantitative data quality. The greater column efficiency of the Agilent rapid resolution high definition (RRHD) columns resulted in narrow peaks, increased analyte peak height, excellent resolution from matrix components, and improved analyte response (sensitivity).

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4933EN.

Alprazolam ($C_{17}H_{13}ClN_4$) spiked in human plasma, RRHD ZORBAX Ecilpse Plus C18

Agilent 1290 Infinity LC
method 1: 1.0 mL/min



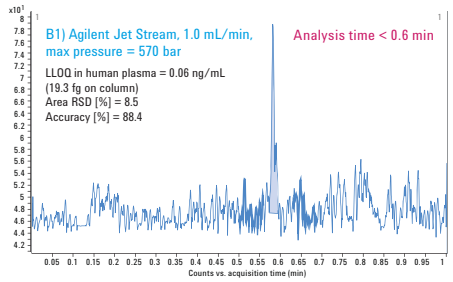
A1) Overlaid MRM chromatograms obtained using AJS in comparison to conventional orthogonal ESI for 1.5 ng/mL alprazolam spiked in human plasma.

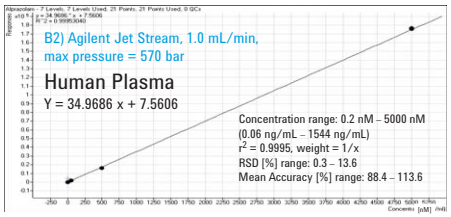
B1) MRM chromatogram at the LLOQ for alprazolam spiked in human plasma using AJS and flow rate = 1 mL/min (LLQQ = 0.06 ng/mL, 19.3 fg on column).

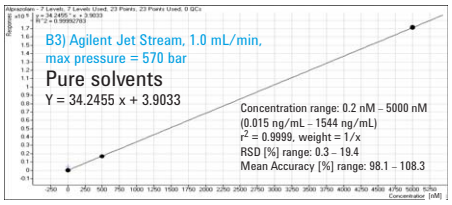
B2) Calibration curve obtained using AJS. 4.5 orders of linear dynamic range in human plasma was demonstrated.

B3) Calibration curve obtained at flow rate 1.0 mL/min for alprazolam spiked pure solvent. The amount on column for these samples corresponds to the amount on column obtained for the spiked and treated human plasma samples. The comparison between the slopes of the calibration curves obtained in spiked human plasma (slope = 34.97) and in pure solutions (slope = 34.25) shows that the slopes are practically the same, suggesting the absence of any significant matrix effect on quantification.

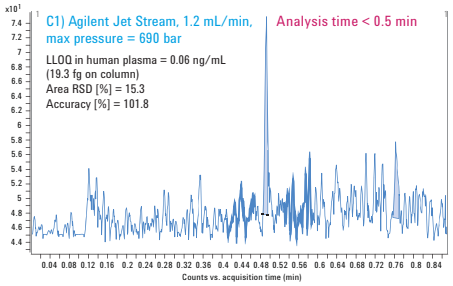
Agilent 1290 Infinity LC
method 1: 1.0 mL/min

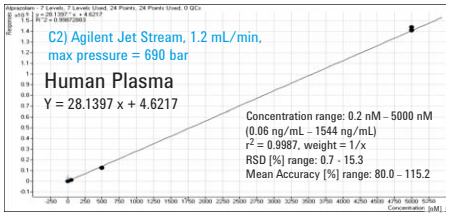






Agilent 1290 Infinity LC
method 2: 1.2 mL/min





C1) MRM chromatogram at the LLOQ for alprazolam spiked in human plasma using AJS and flow rate = 1.2 mL/min (LLQQ = 0.06 ng/mL, 19.3 fg on column). Increasing the flow rate resulted in a run time < 0.5 min without sacrificing quantitative data quality.

C2) Calibration curve obtained using AJS at flow rate = 1.2 mL/min. 4.5 orders of linear dynamic range, good accuracy and precision were demonstrated in human plasma at 1.2 mL/min.

A1) Overlaid MRM chromatograms obtained using AJS in comparison to conventional orthogonal ESI. B1) and C1) MRM chromatograms at the LLOQ (0.06 ng/mL, 19.3 fg on column) using AJS and flow rates = 1.0 and 1.2 mL/min, respectively. B2) and C2): Calibration curves obtained using AJS at 1.0 mL/min and 1.2 mL/min, respectively. B3) Calibration curve of alprazolam in pure solvents obtained using AJS at 1.0 mL/min shows practically the same slope in comparison to human plasma indicating the absence of any significant matrix effect.

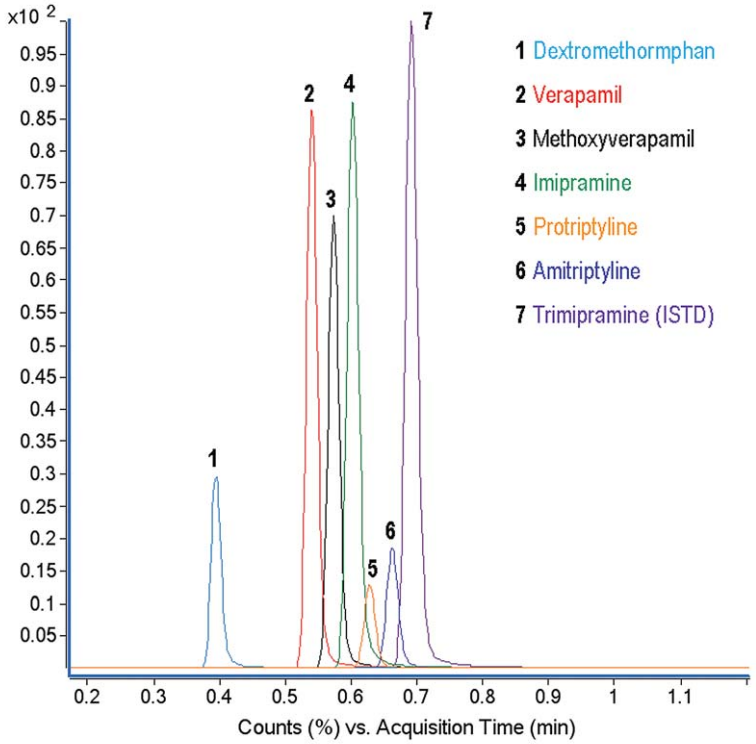
Pharmaceutical analysis: Using dried blood spots in combination with UHPLC and enhanced ion generation ESI to streamline pharmacokinetic assays

The use of LC/MS/MS for quantitative bioanalytical measurements may be considered the hallmark of pharmacokinetic (PK) screenings. Preclinical *in vivo* screens involve the administration of a lead compound to a number of animals (typically mice or rats) whose plasma is consequently monitored over the course of time for drug absorption and decay. Nowadays, PK evaluation is conducted early in drug discovery to prioritize and proceed with new chemical entities (NCE) that have the best chance to pass the later stages in drug development. However, large numbers of NCE's, combined with the pressure to quickly and cheaply fail non-drug-like compounds early, require fast sample turnaround. The term cassette dosing (a.k.a. "cocktail" or "N-in-one") refers to an approach that is widely used to accelerate PK screenings. This entails the simultaneous administration of several drugs to the single laboratory animal. The parallel testing leads to fewer rodents, less animal handling, less sample preparation, and reduced analysis. This approach complements ethical arguments and has considerable cost benefits.

This work presents the LC/MS/MS bioanalysis of a cassette containing six pharmaceuticals. Prime objectives were to demonstrate maximum productivity without sacrificing chromatographic resolution and/or sensitivity. An Agilent 1290 Infinity LC with an Agilent rapid resolution high definition (RRHD) short column (50 x 2.1 mm ID, 1.8 µm) was coupled to an Agilent 6460 Triple Quadrupole LC/MS for two reasons: high-end sensitivity and the system's capability to handle fast flow rates without the necessity to split. The high flow rates can be accommodated by the Agilent Jet Stream technology.

Dried Blood Spot (DBS) technology was used to extract the cassette from small volumes of whole blood. DBS technology offers advantages over traditional plasma sampling for preclinical and clinical assays. For example, in preclinical laboratories, the reduced volumes of blood needed to prepare DBS samples allows serial bleeding of a reduced number of rodents, which aids overcoming inter-individual variations. This could improve the quality of PK data in addition to the related ethical and cost benefits.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4705EN.



Representative MRM-chromatogram showing the quality of separation of six compounds plus the internal standard (ISTD). The chromatogram was obtained at mobile phase flow rate of 1 mL/min from a DBS extract. Concentration of drugs in the cassette prior to DBS extraction was 500 ng/mL.

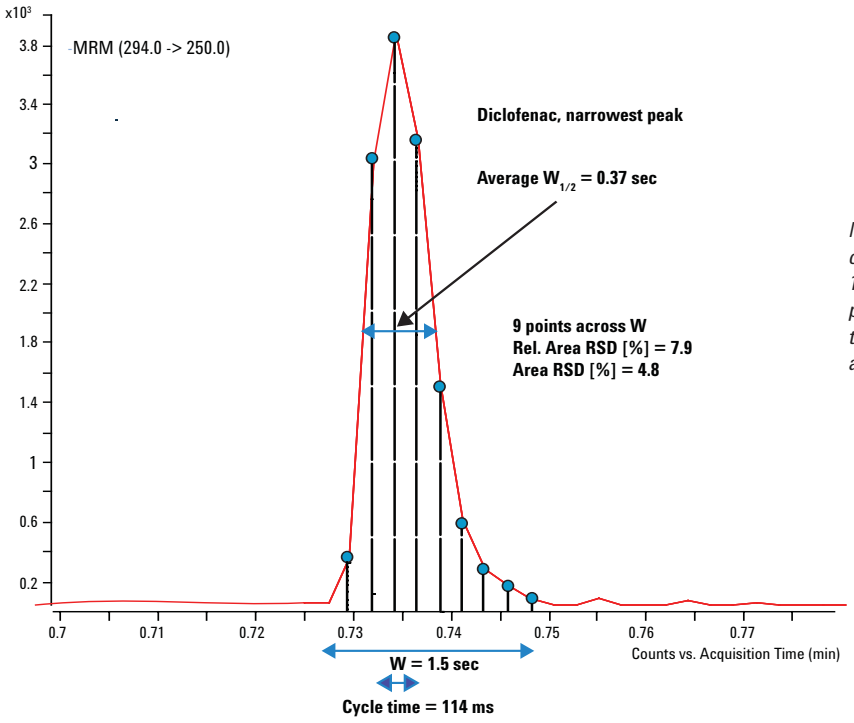
Pharmaceutical analysis: Metabolic stability study using cassette analysis and polarity switching in an ultra-high performance liquid chromatography (UHPLC)-triple quadrupole LC/MS system

In vitro ADME assays like metabolic stability play an important role in the early understanding of *in vivo* pharmacokinetic characteristics and help to discard non-drug-like compounds that would fail later stages of development. Thus, fast screening methods are needed to screen the large collection of new chemical entities that need characterization in the early stages of drug discovery. Typically, metabolic stability assessment is performed using the cassette approach and fast LC/MS/MS methods. In the cassette approach, a cocktail of substrates ionizing either in positive or in negative mode is created post-incubation in order to reduce the number of samples to analyze. Fast LC methods, which use high mobile phase flow rates and short sub-2 µm columns, allow reducing the analysis time and therefore increasing sample throughput. Detection is usually performed using a triple quadrupole mass spectrometer working in multiple-reaction-monitoring (MRM) scan mode.

Cassettes are typically designed so that substrates ionizing either in positive or negative mode are pooled together for LC/MS/MS analysis. The reason for that is because MS/MS analysis may show challenges with quantitative data quality if the MS detector is not able to acquire data fast enough in order to collect at least nine to ten data points across the extremely narrow peaks generated using fast chromatography. Nevertheless, pooling of substrates ionizing in positive and negative mode would present a pronounced flexibility advantage.

This work describes the advantage of combining not only cassette analysis and fast LC but also using fast polarity switching MS/MS analysis to increase throughput and flexibility in a metabolic stability assay, while maintaining good precision and accuracy.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4469EN.



MRM chromatogram of diclofenac obtained using a flow rate of 1.5 mL/min. The graphic shows the peak width at half height (0.37 sec) and the number of data points collected across the peak (nine data points).

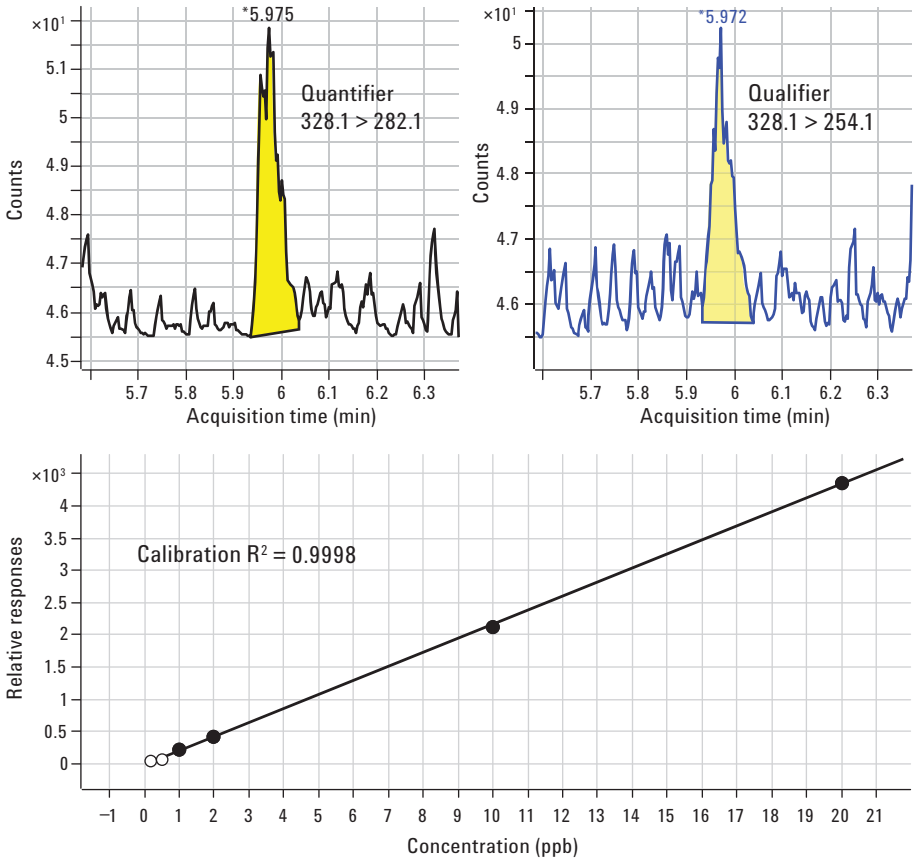
Pesticide analysis: Determination of pesticides in baby food by UHPLC/MS/MS using the Agilent 1290 Infinity LC system and the Agilent 6460 triple quadrupole LC/MS

Due to diversity of pesticides used in food protection and the globalization of the food industry, the monitoring of programs that cover a large number of pesticides is important. The application of UHPLC systems combined with the new generation triple quadrupole mass spectrometers facilitates the analysis of pesticides in challenging matrices such as food samples. As a result of the high sensitivity and the high scan rate capabilities of the Agilent 6460 triple quadrupole mass spectrometer, the simultaneous qualitative and quantitative multiresidue analysis of a large set of pesticides at trace levels can be performed.

The high sensitivity is essential for the analysis of these compounds in derived products, where the concentrations will be a fraction of the concentration in the raw material.

In this respect, baby food is a challenging matrix. This application note describes the quantitative analysis of 40 pesticides in baby food at levels below the maximum residue level (MRL) (10 µg/kg fruit or vegetable) specified in EC Regulation 396/2005, which was implemented in September 2008. A QuEChERS extraction and dispersive SPE method was applied to isolate the pesticides from the baby food matrix. An Agilent 1290 Infinity LC was used to perform the separation on a rapid resolution high definition (RRHD) ZORBAX Eclipse Plus column. The total analysis time was 10 minutes (including 1.5 minutes of re-equilibration) and detection limits ranged from 10 to 500 ng/kg using dynamic MRM and two transitions (quantifier and qualifier) per compound. Three different baby food compositions were analyzed. Extraction performance criteria such as repeatability, recovery (accuracy), and sensitivity were investigated.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-5028EN.

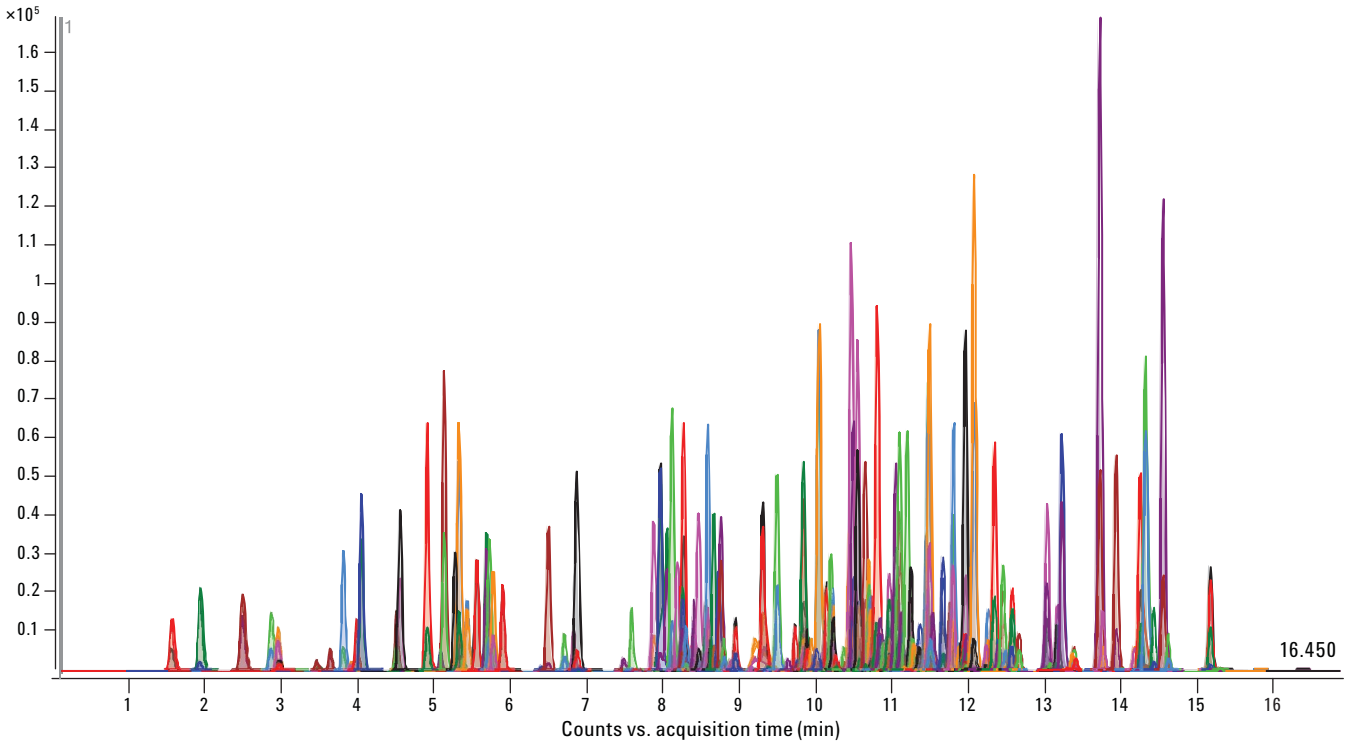


Ion traces for two transitions at the limit of detection (0.5 ppb standard solution) and calibration curve for the pesticide fluazifop.

Pesticide analysis: Multi-residue pesticide analysis with dynamic multiple reaction monitoring and triple quadrupole LC/MS/MS

The analysis of pesticide residues in food and environmental samples is challenging due to the low concentrations and large number of analytes that need to be monitored and quantified. In addition, method development for liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) with a triple quadrupole instrument is laborious and time consuming because of the compound-dependent parameters that need to be optimized. This application note describes how pesticide residue LC/MS/MS methods can be set up quickly and efficiently using the Agilent Pesticides Application Kit. This application kit contains a pesticide test mix, a 600-compound pesticide MRM database, a quick-start guide, and several dynamic multiple reaction monitoring (MRM) methods, which can easily be incorporated into a specific method for pesticide residue analysis. The Pesticides Dynamic MRM database contains compounds commonly monitored around the world and provides fast, customized method development of the analysts' list of pesticides. Results from a 100- and 300-compound mixture are demonstrated with an Agilent 1200 SL Series Rapid Resolution LC and the Agilent 6460 Series Triple Quadrupole LC/MS System with Agilent Jet Stream technology. The 300-compound mixture was also analyzed using an Agilent 1290 Infinity Ultra High Pressure Liquid Chromatograph (UHPLC) and a 6460 LC/MS. With the higher pressure capabilities of the Agilent 1290 Infinity UHPLC, rapid separations with higher peak capacity and less peak overlap than the Agilent 1200 Series RRLC were produced. Using a spinach matrix spiked with 16 pesticides, the performance of a complete method with the SampliQ extraction and dispersive SPE kits and the Agilent LC/MS/MS triple quadrupole on a typical food matrix was demonstrated.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4253EN.



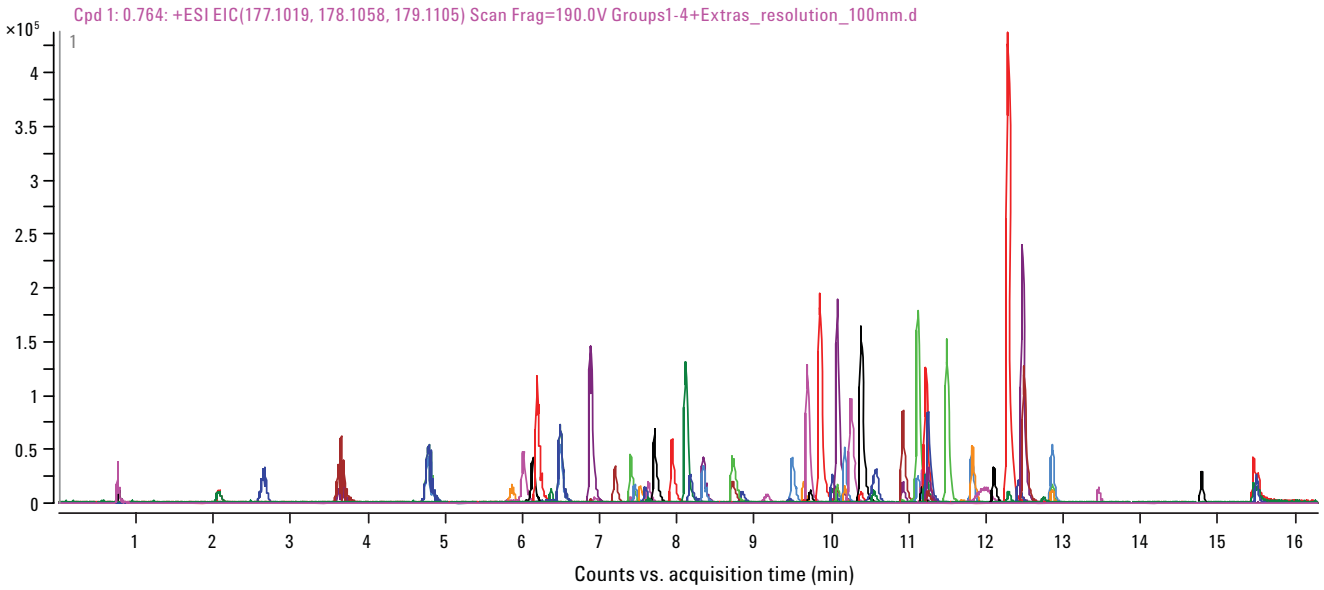
EIC of 224 pesticides using the Agilent 1200 Series SL LC and the Agilent 6460 Triple Quadrupole LC/MS systems.

Environmental analysis: Environmental applications of the Agilent 1290 Infinity UHPLC—the evolution of chromatography

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 ultra-high performance liquid chromatography (UHPLC).

Another 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.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4409EN.



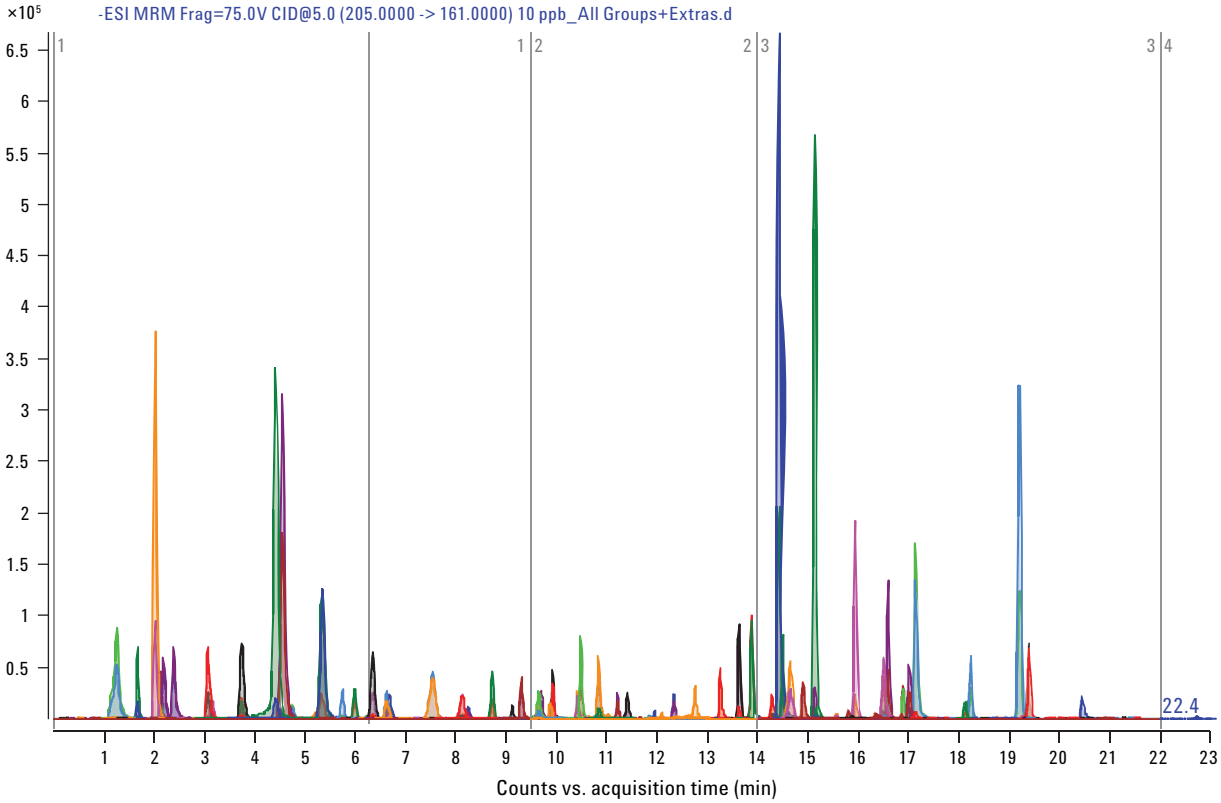
Increased peak capacity showing the separation of the entire list of EPA Method 1694 pharmaceuticals plus 15 new compounds for a total of 90 pharmaceuticals in less than 20 minutes by using a ZORBAX Eclipse Plus-C18, 2.1 mm × 100 mm, 1.8-µm packing material with UHPLC using the Agilent 1290 Infinity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

Environmental analysis: Ultrasensitive EPA Method 1694 with the Agilent 6460 LC/MS/MS with Jet Stream technology for pharmaceuticals and personal care products in water

The analytical challenge of measuring emerging contaminants in the environment has been a major research focus of scientists for the last 20 years. Pharmaceuticals are an important group of contaminants that have been targeted, especially in the last decade. In the area of pharmaceuticals and personal care products (PPCPs), there is one EPA method (although not yet promulgated) addressing the analysis of these analytes, which is EPA Method 1694 published in December 2007. In this method, the standard EPA protocol uses solid-phase extraction (SPE) for water samples followed by analysis with LC/MS using a tandem mass spectrometer with a single transition for each compound.

This application note describes the latest Agilent solution to this EPA method, which is demonstrated with the Agilent 6460 LC/MS Triple Quadrupole with Agilent Jet Stream technology. The number of compounds in the method has been increased by 14, which includes not only the standard analytes in EPA Method 1694 (70 analytes of their 74—four were not available to us) but also 14 commonly found pharmaceuticals and 23 labeled internal standards for a total of 107 compounds. The chromatography has been shortened by reducing the analysis from four groups to two groups of analytes, in spite of the increased number of compounds. Furthermore, the analysis times have been reduced from a total of 90 minutes to less than 30 minutes, while achieving a 10- to 100-times increase in sensitivity, depending upon the analyte detected. The result is a robust analytical method for PPCPs in water that may be analyzed rapidly and sensitively while maintaining the highest analytical standards for correct analysis.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4605EN.



New Group I analytes in positive ion electrospray. This group includes the original Group 1, 2, and 4 of EPA Method 1694 plus 14 commonly found pharmaceuticals for a total of 85 compounds, with internal standards.

High-resolution characterization requires excellent separations, exceedingly accurate-mass measurement, and spectral interpretation tools to identify compounds and modifications. Proprietary HPLC-Chip technology provides robust, high-resolution separation of small, complex samples. The 1290 Infinity LC offers similar performance for larger samples. Using Ultra-High Definition Q-TOF technology, Agilent 6538 and 6540 Accurate-Mass Q-TOF mass spectrometers deliver sub-ppm mass accuracy and resolution in excess of 40,000, with exemplary speed, sensitivity, and linear dynamic range.

Pharmaceutical analysis: Software-assisted, high-throughput identification of main metabolites of pharmaceutical drugs

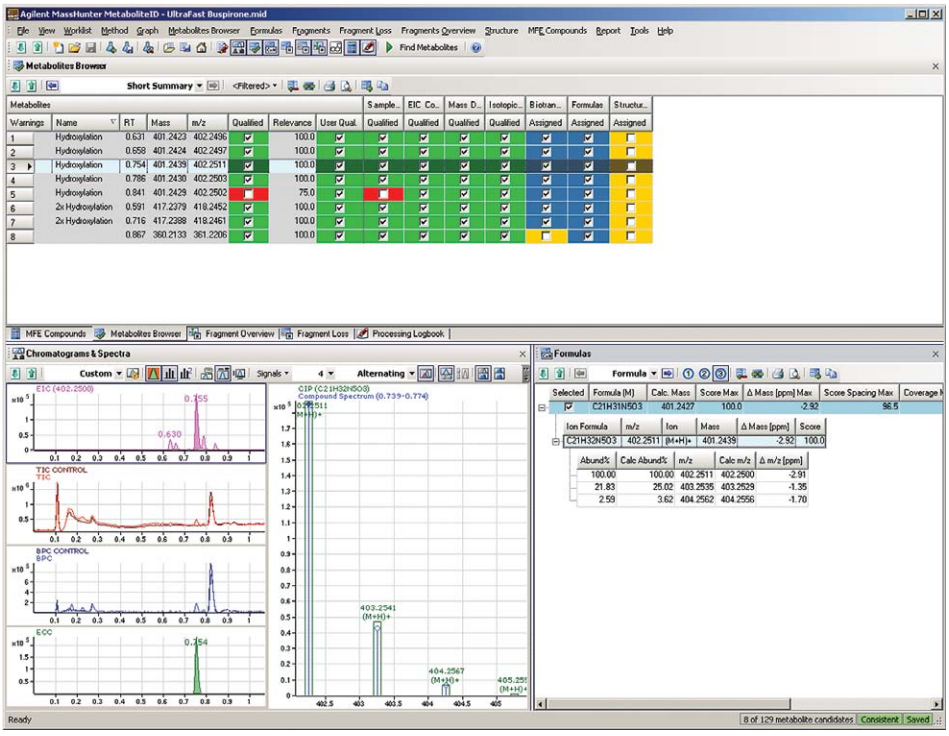
In modern pharmaceutical drug development it is of crucial importance to analyze the adsorption, distribution, metabolism, and excretion (ADME) properties of possible new drug candidates as quickly as possible in order to make decisions about further investments in the development of a special compound. To find compounds with the correct properties it is essential to screen a large number of compounds for their ADME properties, which requires work in an high-throughput environment. This application note describes the use of the Agilent 1290 Infinity LC system, the Agilent 6530 Q-TOF MS system, and the MassHunter Metabolite Identification software for fast, high-throughput identification of main metabolites of new pharmaceutical drug candidate compounds.

Data analysis method in the MetID software

The first step in the analysis comprised a comparison between the data file that contained the metabolite compounds (metabolite sample) and the data file that contained only the parent drug (control sample). All detectable mass signals were extracted from the MS-level data using the Molecular Feature Extraction (MFE) algorithm. Related compound isotope masses and adduct masses were grouped together into discrete molecular features, and chemical noise was removed. The compounds lists of the metabolized sample and the control were then compared.

All new compounds or those that increased two-fold in the metabolized sample were considered potential metabolites and were subjected to further analysis by different algorithms. The algorithms can identify and qualify new metabolites, or just qualify metabolites found by another algorithm. In this high-throughput experiment all algorithms' results were weighted equally and combined into a final identification relevance score. Metabolites were qualified when their final score was above the stringently defined relevance threshold. The results from all algorithms were collated in a results table, which could be inspected at-a-glance and reported.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5989-9924EN.



Result table showing an at-a-glance summary of buspirone metabolite analysis with overall identified metabolites, extracted ion chromatograms (EIC), extracted compound chromatograms (ECC), isotopic pattern analysis, and calculated formulas.

Pesticide analysis: An application kit for multi-residue screening of pesticides using LC/TOF or Q-TOF with a Pesticide Personal Compound Database

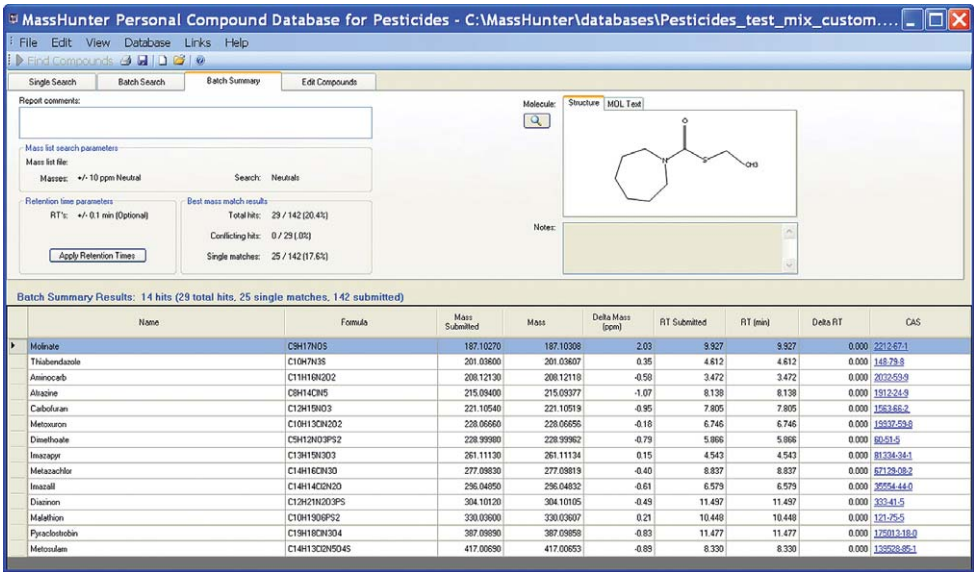
Because over 1000 pesticides have been in use over the last century and new pesticides are being developed, there is a great need to perform both targeted and non-targeted screening in food and the environment. The Agilent time-of-flight (TOF) mass spectrometers provide both high mass resolution and mass accuracy that allow comparison of the measured mass to the exact mass of an ionized compound. In addition, the tandem hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer provides the capability of both screening and confirming compounds in one instrument. Both liquid chromatography (LC) combined with TOF MS and Q-TOF MS provide a robust and sensitive means to perform this type of screening at levels required by the international community. Because TOF is a pulsing instrument the resulting data is always full spectra, which allows the screening of compounds that are sought (targeted) and those that may not be expected (non-targeted). In contrast LC/MS/MS with a triple quadrupole in its most sensitive mode, multi-reaction monitoring (MRM), provides targeted screening and confirmation only.

Recently Agilent has introduced the Pesticide Personal Compound Database (PCD) consisting of 1600 compounds and pesticides. With PCD the analyst can use the pesticide database as is for non-targeted screening or create custom databases from the read-only supplied database. The custom database can be edited by changing entries, adding, and deleting entries. In addition, a powerful feature of updating retention times allows the users' custom database to be modified with retention times from the users' chromatographic conditions. The analyst can create as many custom databases with LC-dependent retention times as needed. This allows easy targeted (compounds verified with standards run with specific conditions) and non-target analysis (compounds in the database that have not been verified). The ability to detect and identify compounds not being sought in food and environmental samples can be very important.

Customized databases with user added retention times

One of the powerful benefits of the supplied database is that it can be saved to a user-customized database. To create a read-write customizable database, the user selects the "File" menu item and the "New Database." The software then allows selection of an existing database and then the naming of a new database. A description can also be given. When "Create" is selected the database with the new name contains all the entries of the selected database. In this way multiple custom databases can be created. The user can run standards with unique chromatographic conditions and easily update retention times in their custom database.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4251EN.



Pesticide Personal Compound Database (now with Personal Compound Database Library, PCDL, not shown) customized with retention times from a positive ion test mix.

Fast, easy nanospray LC/MS is a reality with the proprietary Agilent HPLC-Chip. The HPLC-Chip/MS system is the ideal platform for routine, high-sensitivity analysis of low-volume samples. The second generation HPLC-Chip II technology doubles the lifetime of the chip, lowers the cost per analysis, and improves chip-to-chip and run-to-run reproducibility. With this technology, the Agilent HPLC-Chip/MS system takes you to new levels of nanospray MS reliability, robustness, sensitivity, and ease-of-use, empowering you to enter new application frontiers such as:

- Protein characterization
- Post-translational modification (PTM) studies
- Biomarker discovery and validation
- Small molecule analysis, such as DMPK

Protein characterization: Primary characterization of a monoclonal antibody using Agilent HPLC-Chip accurate-mass LC/MS technology

Recombinant monoclonal antibodies (mAbs) represent an important class of biopharmaceutical products with a wide range of diagnostic and clinical applications. The worldwide market for monoclonal antibodies is projected to reach $26 billion by 2010–2011. These therapeutic glycoproteins are usually produced from mammalian cell lines (hybridomas), then purified, concentrated, and exchanged into an appropriate formulation buffer prior to being sold. Although mAbs are relatively stable biomolecules, a number of chemical modifications and degradation reactions can occur during manufacturing, formulation, and storage, thereby necessitating reliable and sensitive methods for the characterization of protein purity and structural integrity. Accurate-mass measurements of intact proteins, whole subunits, or domains are useful for the rapid verification of sequence composition and identification of posttranslational modifications and sample handling artifacts.

LC/MS technology is a powerful and sensitive technique for the characterization and identification of proteins. Nanoflow LC/MS enables faster and more sensitive protein identification while minimizing sample and solvent consumption. Agilent's microfluidic-based HPLC-Chip integrates sample preparation, chromatographic separation, and nanoelectrospray formation for efficient, high-sensitivity nanospray LC/MS. Agilent Accurate-Mass LC/MS TOF and Q-TOF systems deliver exceptional mass resolution, mass accuracy, sensitivity, and data processing capabilities for optimal MS characterization of proteins. In this note, we report the rapid LC/MS characterization of low nanogram levels of an intact mAb and its fragments using the Agilent HPLC-Chip system coupled to an Accurate-Mass Q-TOF.

Analysis of intact antibody

Figure 1 shows the averaged mass spectrum of the intact antibody after elution from the HPLC-Chip. A very well distributed series of peaks is seen corresponding to the *m/z* of the many different charge state species of the intact antibody. Small satellite peaks within each charge state are also observed (see inset), corresponding to either adducts or modifications of the mAb. To gain more insight into the possible modification/adduct, deconvolution of the intact mass spectrum was performed using a maximum entropy algorithm (Figure 2). The deconvoluted spectrum shows three major mass peaks at 148812.81 Da (calculated mass from sequence is 148811.95 Da; measured mass accuracy of 5.7 ppm), 147367.94 Da, and 145922.00 Da. The mass differences between the peaks are marked in Figure 2. The observed mass difference of 1444.87 Da (calculated mass=1445.35 Da) is attributed to one unit of the glycan (G0F) attached to the mAb. The other observed mass difference of 2890.81 Da (calculated mass=2890.7 Da) corresponds to two such units of G0F glycan, indicating that the mAb sample has three species: intact mAb with a pair of G0F glycans (148812.81 Da), mAb with one unit of G0F glycan (147367.94 Da), and mAb devoid of any N-inked glycan part (145922.00 Da). Another peak at 148974.97 Da was assigned to the addition of a hexose unit to the G0F glycan part of the antibody (observed mass increase of 162.16 Da; calculated mass is 162.14 Da), denoted as G1F in Figure 2.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-3445EN.

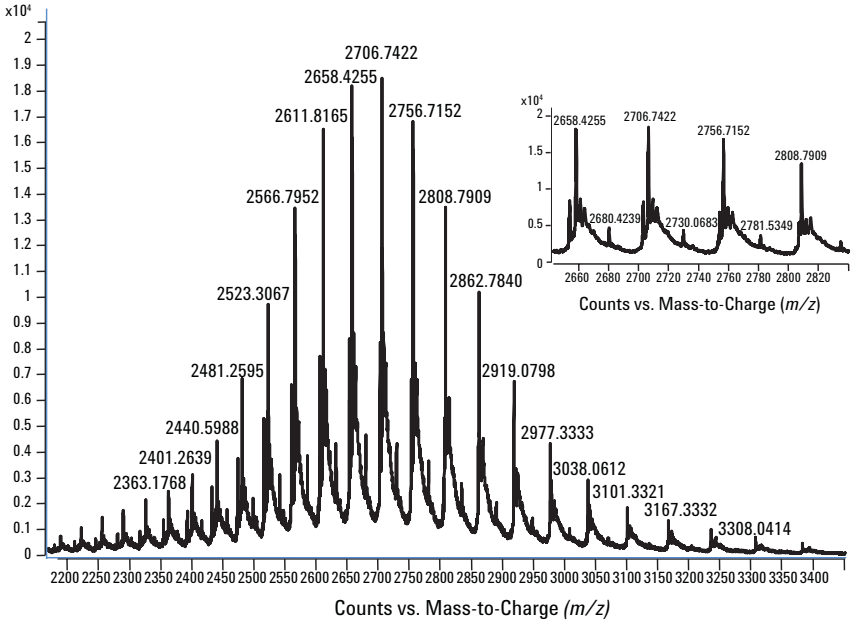


Figure 1. Mass spectrum of intact antibody with inset showing the expanded view of charge states of the antibody.

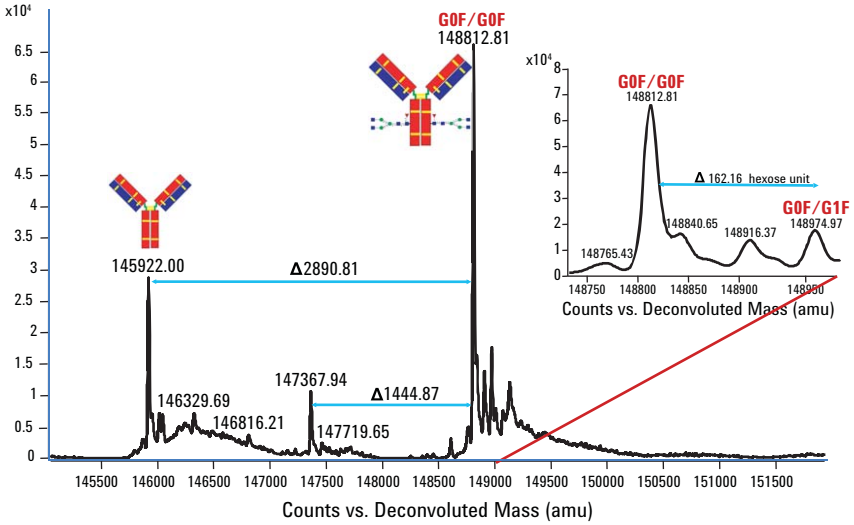


Figure 2. Deconvoluted spectrum of intact antibody with inset showing the expanded view of a small amount of G1F modification (addition of hexose unit to G0F) of the mAb.

Protein characterization: Peptide mapping of a monoclonal antibody using a microfluidic-based HPLC-Chip coupled to an Agilent Accurate-Mass Q-TOF LC/MS

The sequence confirmation of therapeutic monoclonal antibodies is of prime importance before product release. Peptide mapping using LC/MS is an established analytical tool for the confirmation of amino acid sequences of monoclonal antibodies. In this application note, a purified monoclonal antibody (mAb) was subjected to proteolytic digestion followed by peptide separation and mass determination on a HPLC-Chip coupled to an Accurate-Mass Q-TOF LC/MS. The data obtained from LC/MS were analyzed using the powerful features of the MassHunter Qualitative Analysis software package. The MS results of the trypsin digest yielded 95% sequence coverage of heavy chains and 85% of light chains, and in combination with different proteases, 100% coverage of both chains. The matched peptides were searched at a mass accuracy of 5 ppm. Furthermore, the MS/MS results obtained for peptides confirm the peptide sequence of mAb. This method of using HPLC-Chip/MS technology in combination with True High-Definition TOF technology for antibody characterization is a valuable method in the biopharmaceutical industry for QC studies. The results of this work continues our studies toward complete characterization of monoclonal antibodies using advanced Agilent platforms ideal for the biopharma market.

Results and discussion

Peptide mapping is routinely used for the assessment of antibody quality. Figure 1A shows the base peak chromatogram (BPC) of trypsin-digested mAb obtained using the HPLC–Chip/MS system for nanospray LC/MS. Inspection of the chromatogram reveals only peptide peaks with no undigested protein product. The extracted ion chromatograms (EICs) of some of the peptides for both light and heavy chains are shown in Figure 1B. These results show the high performance of HPLC-Chip separation, which is important in peptide mapping.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4587EN.

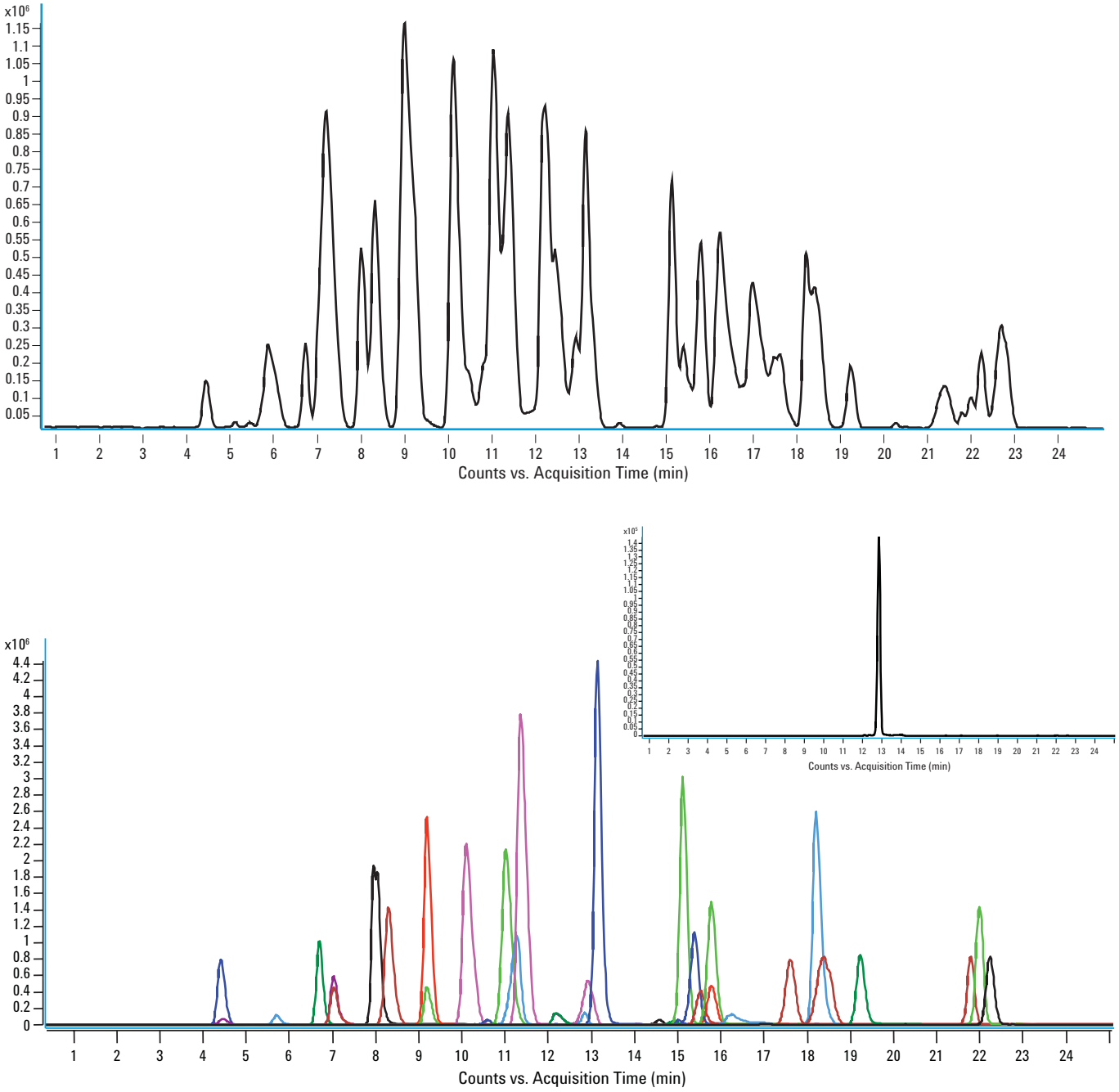


Figure 1. A. BPC of trypsin-digested mAb on a C18 HPLC-Chip.
B. EIC of some peptides from mAb light chains and heavy chains. The inset shows the expanded view of the peak with a narrow width of approximately six seconds.

PTM studies

Moving beyond protein identification, more and more researchers are focusing on the protein phosphorylation mechanism, an important post-translational modification process, to understand signal transduction in the cell. To investigate the phosphoproteome in more detail, phosphorylated proteins and peptides of interest need to be enriched prior to LC/MS/MS analysis due to the low abundance of phosphorylated proteins in complex mixtures. The Agilent Phosphochip, developed specifically for phosphopeptide analysis, integrates an enrichment column, analytical column, associated connection capillaries, and a nanospray emitter directly on a single, small, reusable microfluidic chip. It provides an easy to use, robust, and reliable nanospray LC/MS platform when compared to conventional nanocolumn LC/MS technology. Integration of enrichment columns with different stationary phase materials on the chip can be used to selectively capture phosphopeptides during the sample loading process.

PTM studies: Facilitating phosphopeptide analysis using the Agilent HPLC Phosphochip

Since biological samples are extremely complex in composition and can contain a large variety of different phosphopeptides, prefractionation of the peptides is required before the samples can be efficiently analyzed by LC/MS/MS. One common approach for prefractionation is strong cation exchange (SCX), which can separate peptides based on their net charge. Tryptic peptides most often have two basic sites, the N-terminus of the peptide and the C-terminal lysine or arginine residue required for tryptic cleavage, resulting in a 2+ net charge. Phosphopeptides, in contrast, will mainly have a single net charge as the phosphorylated residue is negatively charged and compensates for one positive charge.

Although this strategy enriches phosphopeptides into a few SCX fractions, the same fractions can also contain other peptides with a net single charge. This includes acetylated peptides and peptides derived from protein C-termini, as well as the many other cellular compounds that can carry a single charge, all of which often are much more abundant than the phosphorylated peptides of interest. Protein derived from a human osteosarcoma cell line (U2OS) was digested with trypsin to show the enrichment improvement that can be achieved on such samples using the Phosphochip. The resulting peptides were separated by SCX. A single SCX fraction expected to contain the majority of peptides with a net single charge was analyzed both with a regular HPLC-Chip and with the Phosphochip (200 µg each). As can be seen in Figure 1, the vast majority of the sample was retained in the flowthrough of the Phosphochip. It is noteworthy that a large amount of the total sample (over 90% of the total combined signal) is observed in the flowthrough analysis on the Phosphochip. The subsequent analysis of the LC/MS/MS experiment using the Spectrum Mill search engine on the human IPI database allowed the identification of 109 unique phosphopeptides in the elution fraction of the Phosphochip with a Spectrum Mill score of at least 9. In contrast, only 11 phosphopeptides were identified when the same sample was analyzed with a regular C18 HPLC-Chip. As expected, the major non-phosphopeptide components hindered phosphopeptide identification for the sample without the enrichment step.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4098EN.

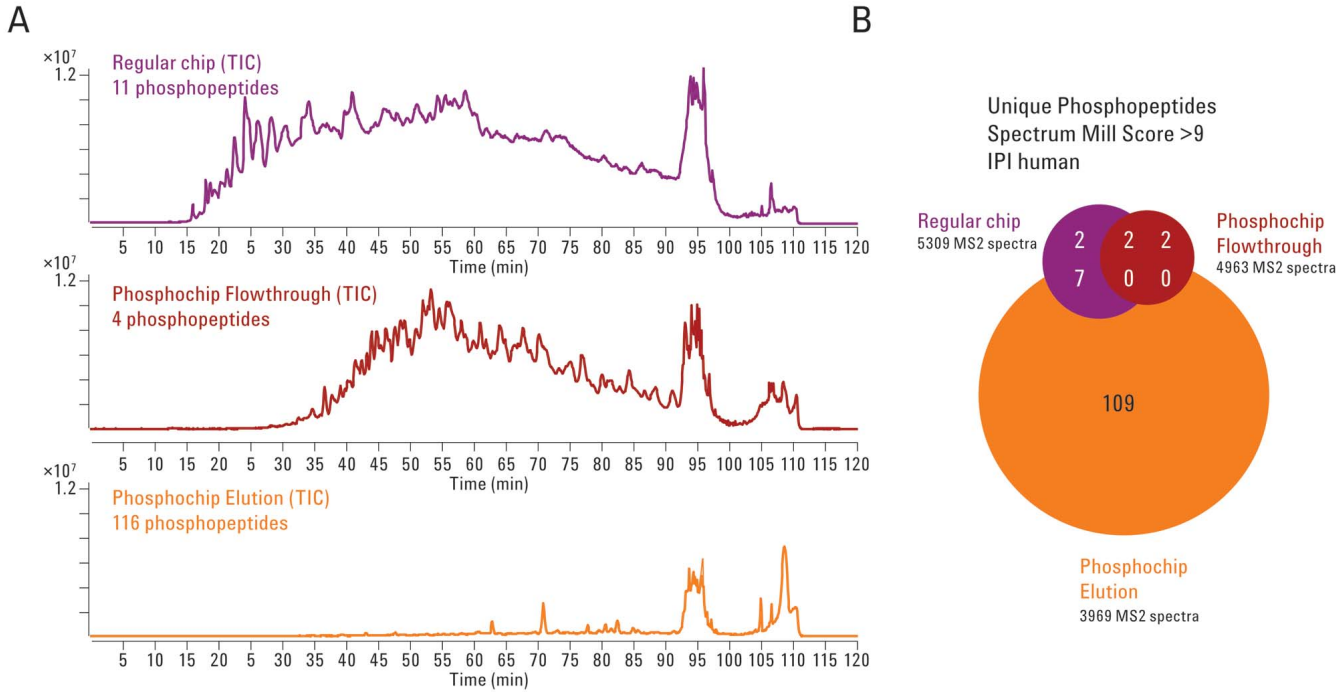


Figure 1. Comparison of phosphopeptide analysis on a regular HPLC-Chip and a Phosphochip. (A) Total ion current chromatograms of the U2OS singly charged peptide SCX fraction analyzed on a regular HPLC-Chip (top, purple) and on a Phosphochip (flowthrough in red, elution in orange). (B) The number of unique phosphopeptides identified with Spectrum Mill in the LC/MS/MS runs on the regular HPLC-Chip and in both the flowthrough and elution of the Phosphochip, as shown in panel A. Indicated are the total number of MS2 spectra searched and the scoring and database used.

PTM studies: Custom HPLC-Chip enables new research in glycan expression

Glycan expression is another PTM mechanism under intense investigation. Structurally diverse glycans are found on mammalian cell surfaces and in extracellular matrices. They mediate cell-cell recognition and cell-cell and cell-matrix interactions, but the exact details are largely a mystery.

Professor Joseph Zaia and his team at the Mass Spectrometry Resource within the Boston University School of Medicine develop LC/MS methods to study the range of glycans that are expressed under various cell conditions. An innovative Agilent HPLC-Chip has now solved many of the difficulties that were inherent with LC/MS characterization of glycans.

The custom HPLC-Chip has given Zaia's lab a research tool that enables new biomedical discoveries. Agilent collaborators provided an experimental makeup flow (MUF) HPLC-Chip, which allows Zaia's team to add organic solvent postcolumn. They achieve a consistent spray over the entire gradient, which extends the range of glycans they can measure. In addition, they no longer need to adjust source voltages during the run, so they can now perform overnight, unattended analyses.

Professor Zaia spoke about the large-scale studies he can now conduct to examine differences in the temporal and spatial expression of glycosaminoglycans (GAGs). For this critical research, the LC/MS system must provide consistent, reliable data for triplicate runs of dozens of samples. "The system has to be stable for days to get through the set of samples we need to handle. Over the past year and half, we've been able to use the system for these large sample sets. Previously, we would not have had the instrument stability to address these research questions."

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-5155EN.

Biomarker discovery and validation

Biomarker discovery and validation is the first step in the new paradigm of drug development involving biotechnology. In clinical research or pharmaceutical development, differential protein expression analysis is employed in putative protein biomarker determination. Featuring HPLC-Chip/MS, Agilent offers a label-free identification and relative quantification workflow for rapid screening of potential biomarkers, and subsequent targeted validation of biomarker candidacy.

Biomarker discovery: A highly accurate mass profiling approach to protein biomarker discovery using HPLC-Chip/MS-enabled ESI-TOF MS

Biological samples are frequently very complex, and protein levels can vary greatly. To address this complexity, we employed a combination of high chromatographic performance and extreme sensitivity by using an HPLC-Chip/6210 TOF MS system to resolve and detect proteins. Data from one sample showed *E. coli* lysate spiked with BSA and serotransferrin generated a total ion chromatogram (TIC) that demonstrated a highly complex sample (Figure 1).

Further analysis was performed on a single peptide (m/z = 504.2506) by generating an extracted ion chromatogram (EIC) using a narrow mass window of ± 1.9 ppm. The EIC showed multiple peaks, further indication of the complexity of the sample (Figure 2). A single peak at 9.2 min was identified from the EIC and the full mass spectrum was obtained for this time point (Figure 3).

The spectrum showed that the peptide at m/z = 504.2506 is relatively low in abundance compared to other ions. These data illustrate a situation that can occur when searching for biomarkers. The combination of the HPLC-Chip with the 6210 TOF LC/MS system was able to detect a low-level peptide within a highly complex sample. More importantly, with typical data-dependent acquisition this particular low-abundance peptide would likely not be selected for MS/MS analysis because of the presence of more abundant ions.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5989-5083EN.

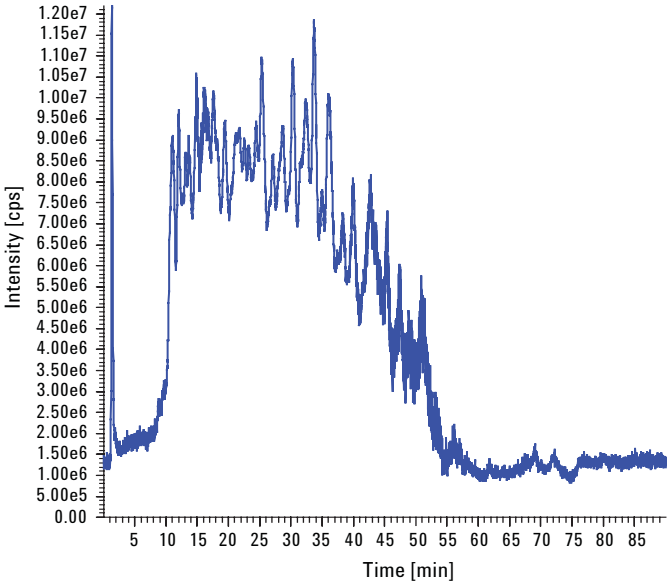


Figure 1. Total ion chromatogram (TIC) for Sample B.

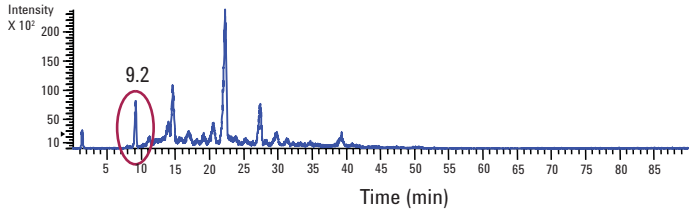


Figure 2. Extracted ion chromatogram (EIC) for m/z 504.2507 +/– 1.9 ppm.

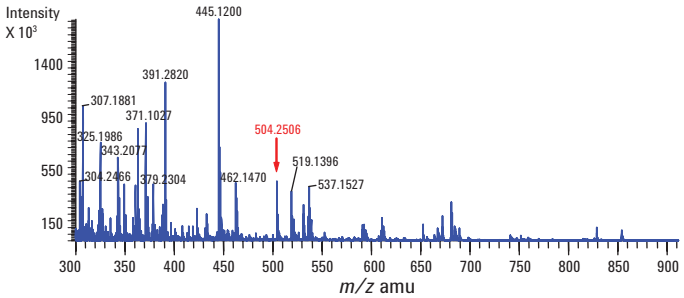


Figure 3. Full MS at 9.2 minutes. Peptide with m/z *504.25 would most likely have not been chosen for MS/MS using regular data-dependent strategies because more intense peaks are present at this time point.*

Biomarker discovery: Profiling approach for biomarker discovery using an Agilent HPLC-Chip coupled with an Accurate-Mass Q-TOF LC/MS

Human plasma is extensively used for clinical diagnosis and early disease detection. Plasma is composed of classical blood proteins as well as tissue leakage proteins present in a wide dynamic range of concentrations. Highly sensitive, accurate, and reproducible techniques are required to identify biomarkers in such complex biological samples.

A profile-directed biomarker discovery approach involves initial differential expression analysis followed by targeted identification of differentially expressed proteins. In this study, a very small amount (100 femtomole and 10 femtomole) of horseradish peroxidase was spiked into human plasma and a profile-directed approach was used for the identification of the spiked protein.

Plasma digest samples were analyzed in four replicates using an Agilent 6520 Accurate-Mass Q-TOF LC/MS configured with a 1200 Series HPLC-Chip/MS. Total ion chromatograms of four replicate LC/MS analyses of one sample are shown in Figure 1 demonstrating the excellent reproducibility achieved in the LC/MS runs.

LC/MS data were extracted and evaluated using a specialized molecular feature extractor (MFE) algorithm in MassHunter Qualitative Analysis software. MFE takes raw data as the input and outputs a list of molecular features. A molecular feature represents a chemical entity such as a compound or a peptide. MFE reports a feature combining the abundance information of all its isotope clusters, different ion species such as multiple charge states, as well as dimers and adducts. Nearly 25,000 molecular features were observed in the LC/MS runs of plasma digest demonstrating the complexity of the sample (Figure 2). Retention times, masses, and abundances of all features from each LC/MS run were compiled by MFE into feature lists.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4404EN.

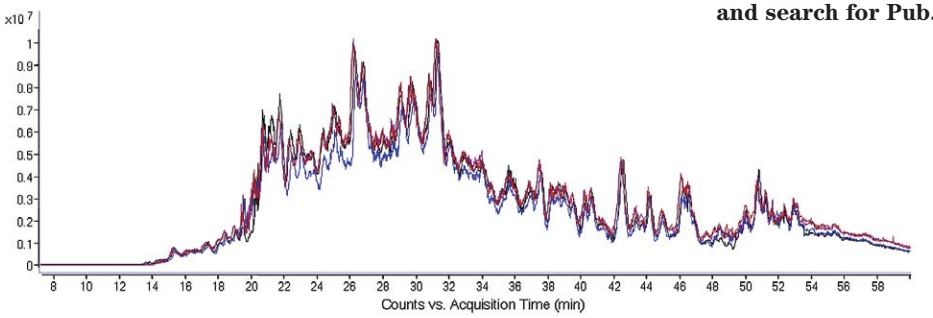


Figure 1. Total ion chromatogram of four replicate LC/MS runs of plasma digest.

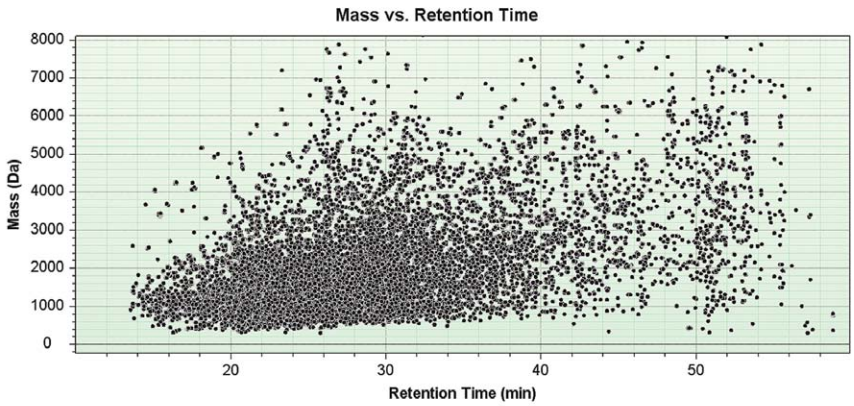


Figure 2. Mass versus retention time plot of the molecular features observed in LC/MS runs of plasma digest.

Biomarker discovery: The Agilent HPLC-Chip/6210 TOF LC/MS enables highly accurate profiling of peptide maps for differential expression studies

Shotgun proteomic approaches utilize data-dependent MS/MS acquisition, which identifies only a subset of the actual proteins present in the entire sample; these proteins usually represent the higher abundance proteins that are not necessarily of biological significance in disease. As a result, extensive fractionation of complex samples may be necessary to identify meaningful protein biomarkers associated with disease, thus dramatically increasing the number of analyses required per sample.

Dr. Pierre Thibault, a principal investigator at the Institute for Research in Immunology and Cancer (IRIC) and the director of the Proteomics Core Facility, stresses the difficulty of biomarker discovery. "When using a non-targeted shotgun proteomics approach, an overwhelming amount of MS/MS data can be acquired; out of that data, only a small subset will represent proteins of interest. This significant challenge [is like searching for] the proverbial differentially expressed needle in a widely diverse proteomic haystack. Indeed, proteins of interest might only represent 5% of the overall population, and appropriate strategies are required to successfully identify these candidates."

Dr. Thibault's research program consists of developing a reproducible LC/MS proteomics platform for applications in cancer and immunology, and is heavily focused on characterizing low-level amounts of proteins and potential biomarkers in complex cell extracts.

Dr. Thibault favors the Agilent HPLC-Chip/6210 TOF LC/MS for his research. "The novel microfluidics approach with the HPLC-Chip/TOF system eliminates all those uncertain issues with traditional nano-separation and nanoelectrospray. Dead volumes, dispersion issues, and sample losses are minimized because the pre-columns and columns are integrated into a chip format, allowing us to mine complex proteomics samples reliably and efficiently. This approach has also proven to be very reproducible and we have a more consistent platform day-in, day-out. We have lost the fear of wondering if we will get the same performance when we run our samples; there is now a level of reliability in our comparative sample studies."

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5989-5084EN.

Biomarker validation: Data showing the feasibility of confirming putative biomarkers using multiple reaction monitoring on the Agilent 6410 Triple Quadrupole LC/MS/MS system

Confirmation of putative protein biomarkers in complex biological samples requires an instrumental method that is fast, highly selective, and sensitive. Multiple reaction monitoring (MRM) on a triple quadrupole (QQQ) mass spectrometer (MS) provides superior sensitivity and selectivity for targeted compounds in complex samples. MRM also offers high precision and rapid MS cycle time, which makes it an ideal technology for validating biomarkers in a high-throughput fashion.

In this study, we tested the MRM methodology by spiking standard protein digests into human plasma digest. To enable detection of low-level proteins, the most abundant proteins were removed by immunodepletion using the Agilent Multiple Affinity Removal System. We spiked various concentrations that represented the wide dynamic range that would be observed in that biological fluid.

In order to quantitate protein biomarkers, the MRM mode of the Agilent 6410 QQQ LC/MS/MS system was used to detect peptides. The MassHunter Quantitative Analysis software was then used to generate calibration curves. Various amounts of luciferase and peroxidase were spiked into aliquots of immunodepleted human plasma; the resulting samples were then analyzed. In an MRM experiment with a QQQ MS, Q1 (the first quadrupole) is set to pass a desired precursor ion, Q2 is used as a collision cell to fragment that precursor ion, and Q3 is set to monitor a specific fragment ion.

For this study, the Peptide Selector module within the Agilent Spectrum Mill software was used to help choose MRM transitions that would produce sensitive analyses. Peptide Selector first performs an *in silico* digestion of the proteins, based on sequences that are manually copied into the software or are retrieved by an accession number from a database. The simulated digestion provides a list of component peptides. The Peptide Selector then filters the peptides based on user input such as the number of missed cleavage sites, the mass range, and the specific amino acid composition. The software lists precursor and product ions for these peptides. It also highlights the best product ions for MRM experiments, based on the cleavages that are expected to produce intense signals (*i.e.*, N-terminal side of Pro and C-terminal side of Asp and Glu). The software can also search any user-selected database to determine the uniqueness of any peptide transitions that are being considered.

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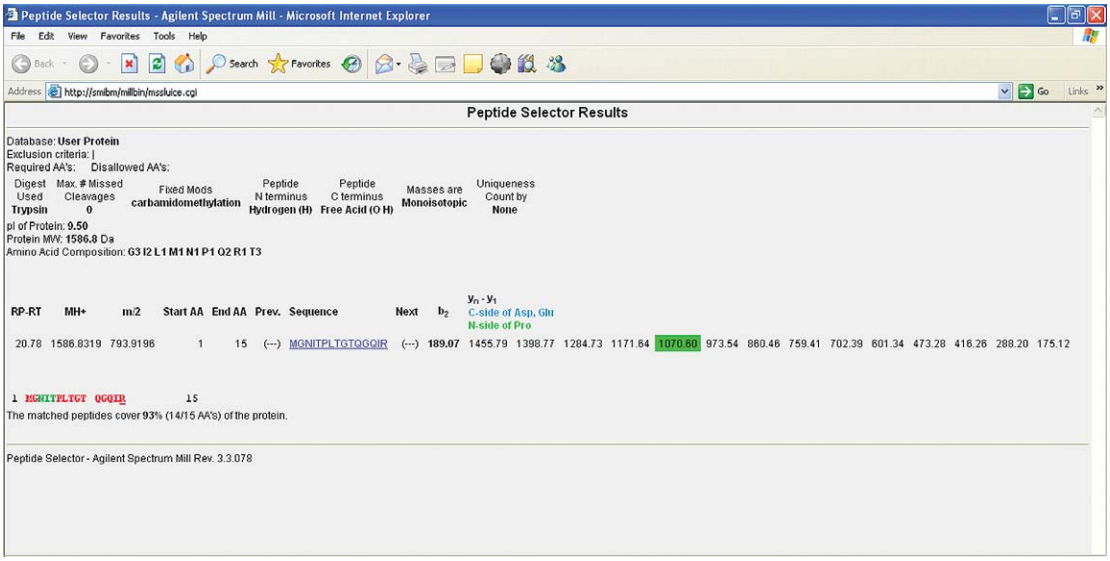

The Peptide Selector module in Agilent Spectrum Mill software assisted in the selection of MRM transitions that would produce the lowest detection limits.

Biomarker validation: High-throughput protein quantitation using multiple reaction monitoring

Peptide quantitation using multiple reaction monitoring (MRM) has been established as an important methodology for biomarker validation. Quantitative proteomics can require high throughput, as often hundreds of target peptides need to be monitored in each sample and thousands of biological samples may need to be analyzed. The dynamic MRM algorithm allows the system to acquire transition ion data only during the retention window when each peptide is eluting. This reduces the number of concurrent ion transitions and therefore improves quantitation and sensitivity. In this study, peroxidase was spiked at different concentrations into human plasma to demonstrate the entire workflow from biomarker discovery to validation. Reproducibility of peak abundances and retention time at nanoflow range were studied with 443, 2000, and 3293 ion transitions using a dynamic MRM method on a nanoflow LC/MS system. For this work, the HPLC-Chip was interfaced to an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) and an Agilent 6410 Triple Quadrupole (QQQ) LC/MS.

The Q-TOF data was searched against the SwissProt database using Agilent Spectrum Mill MS Proteomics Workbench. A new tool within Spectrum Mill, MRM Selector, was used to directly generate the dynamic MRM methods based on results from the Q-TOF database search results. After LC/MS analysis, the results were analyzed using MassHunter Quantitative Analysis software. From this, quantitative batch report XML files were imported into Mass Profiler Professional software, a chemometrics software package designed specifically for mass spectrometry data. Spiked-in peptide features were analyzed in the context of human serum peptides via principal components analysis (PCA). Additionally, a naïve hierarchical clustering analysis was performed.

The biomarker validation workflow is illustrated in Figure 1. As a first step, the samples were run on HPLC-Chip/Q-TOF in data-dependent MS/MS mode. The HPLC-Chip provided excellent reproducibility as shown by the overlaid base peak chromatograms (BPC) of five replicate injections (Figure 2).

Q-TOF data was searched using Spectrum Mill software. A dynamic MRM list was generated using MRM Selector based on the validated peptide hits. MRM Selector is a utility tool in Spectrum Mill workbench that allows the user to select the MRM transitions from the experimental MS/MS data. The user can input several parameters to filter the ion transitions to be monitored on QQQ. The MRM Selector results contain protein accession number and peptide sequence, ion transition values, retention time (RT), peak width, collision energy, and fragmentor values. The saved list can be pasted directly into the QQQ acquisition software.

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5990-4276EN.

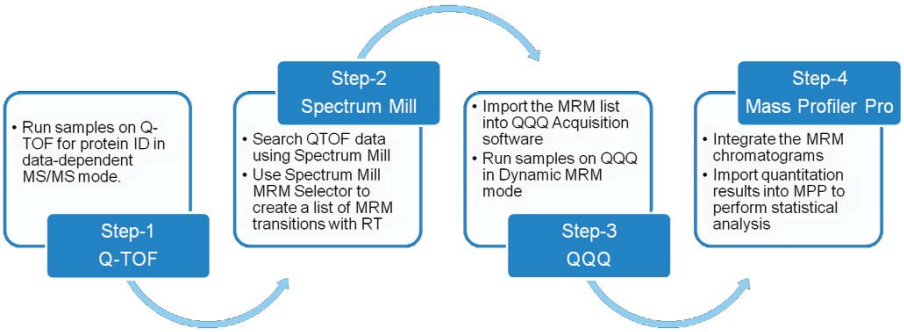

Figure 1. Biomarker validation workflow.

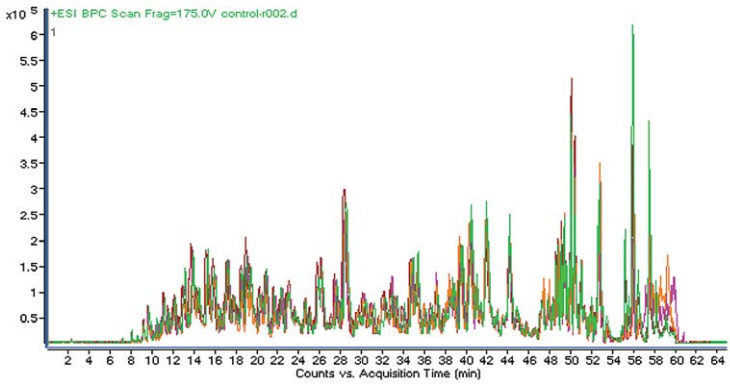

Figure 2. Overlaid BPCs from five replicate runs of depleted human plasma tryptic digest on HPLC-Chip/Q-TOF. The samples were analyzed in data-dependent MS/MS mode for protein identification.

Small molecule analysis: HPLC-Chip/Triple-Quadrupole MS for quantification of pharmaceuticals in diminishing small volumes of blood

Using HPLC-Chips coupled to the Agilent 6410 Triple Quadrupole LC/MS allows for substantial cost savings in the drug metabolism and pharmacokinetic (DMPK) laboratory. Based on the system's outstanding detection sensitivity, the volumes of blood drawn in animal PK studies can be reduced considerably, which would be commensurate with ethical arguments and could also be envisaged for pediatric studies. This permits the reduction of animal size and a concomitant cutback of the amount of the active pharmaceutical entity per PK study. Furthermore, it can increase PK data quality due to the ability to serially sample blood from the same small-size rodent without jeopardizing its physiological welfare and without increasing recovery periods.

We developed a method to specifically quantify a test drug, nortriptyline, out of 10 µL of whole blood utilizing the Agilent 1200 Series HPLC-Chip and the Agilent 6410 QQQ system.

Major steps in the optimized extraction procedure were spotting the blood onto FTA Elute MicroCards, punching disks from the dried spot, extraction of nortriptyline using 75% ACN solutions and the dilution step to obtain sample solutions with 10% ACN content. 5 µL of these extracts were quantified employing the Agilent ultra-high capacity chip (UHC-Chip), which was specially designed for such small molecule applications.

Recovery obtained from our optimized extraction process was 101%. Intraday precision ranged from 6% to 16%, respectively, at higher and the lowest concentration quantified, which was well in accordance with recommendations made by the US FDA.

In addition we showed that HPLC-Chip systems show great robustness even when used for the analysis of extremely complex biological matrices such as those contained in blood. More than 500 blood-extracts were analyzed without significant change in pressure drop over the entire period of analysis while retention and chromatographic performance was stable (Figure 1).

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5989-9896EN.

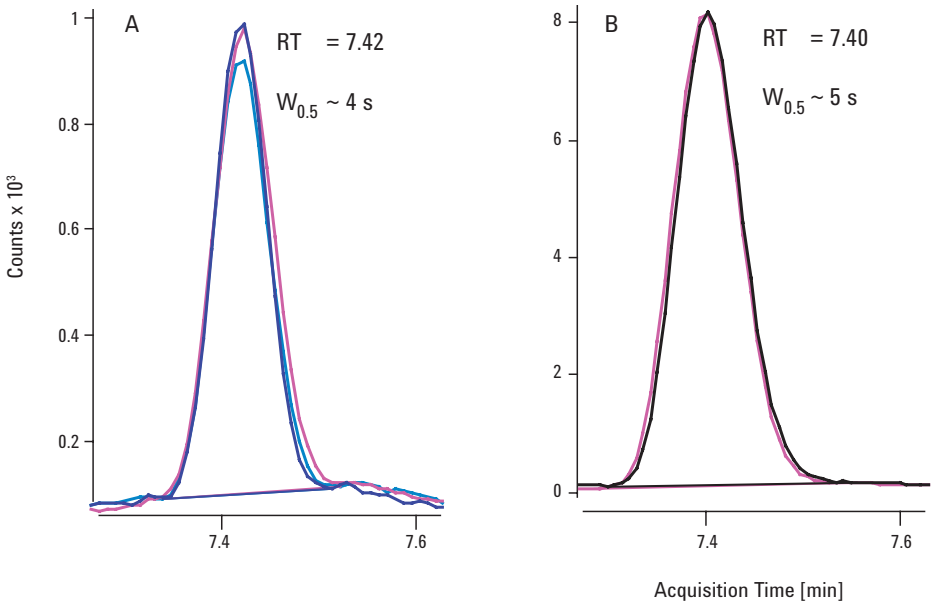

Figure 1. Superimposed quantifier-chromatograms obtained from blood extract analyses number 350–352 (A) and 511–512 (B).

Small molecule analysis: Ultra-high capacity small molecule chips for the quantification of pharmaceuticals using triple-quadrupole mass spectrometry

The creation of a new pharmaceutical drug consumes vast amounts of capital. Cost savings may become essential, even for leading pharmaceutical companies. Substantial savings can be achieved by the miniaturization of laboratory processes, provided the quality of the results is uncompromised. A prime example for this situation exists in the drug metabolism and pharmacokinetics (DMPK) laboratory where PK studies are carried out in the preclinical development phase.

During PK studies the potential lead compound is administered to animals from which blood is taken over the course of time to monitor drug absorption and decay using quantitative LC/MS analysis. Here, the cost per study may be reduced markedly by using smaller animals. However, the animal's size sets a limit to the blood volume that can be drawn per unit time and serial bleeding of few or even only one animal, which reduces the variability in the TK profile, results in even lower volumes per bleed. Consequently, studies with small animals set extreme demands on the analytical system with respect to both the capability to handle small sample volumes and detection sensitivity.

The Agilent HPLC-Chip/MS system meets these strong demands. The HPLC-Chip incorporates an enrichment column and a nanoanalytical column from which a very short and narrow channel leads to the chip's nanospray tip. Miniaturization of the analytical column dimensions and the resulting low flow rates of a few hundredths of nanoliters result in less sample dilution and improved overall analyte ionization efficiency. This ultimately leads to the pronounced sensitivity advantage of the chip compared to conventional LC-ESI-MS. The challenge with pharmaceuticals lies in the wide variety of polarities present. It has to be ensured that none of the molecules are lost during the on-chip enrichment process. In this study, we illustrate the design of an ultra-high capacity small molecule HPLC-Chip (UHC-Chip), which features a 500-nL enrichment column and a 150 mm x 75 µm analytical column. We present data of the technical evaluation for quantification of small molecules in combination with the Agilent 6410 Triple Quadrupole MS. The test mix contained four pharmaceutical compounds, covering a wide range of polarities (logP 0 to 4.2).

Pre-concentration of wide polarity and concentration ranges

Pharmaceutical compounds covering a wide polarity range (logP values ranging from 0 to 4.2) were chosen to evaluate the performance of the UHC-chip. Figure 1 shows extracted ion chromatograms of seven drugs with atenolol (logP 0) as the first and imipramine (logP 4.2) as the last eluting peak. Chromatogram A shows the results for the standard small molecule chip with a 40 nL enrichment column. Analysis of this mixture on a small volume enrichment column revealed a marked correlation between analyte hydrophobicity and detected signal intensity. These results were in agreement with the preconcentration process on a reversed phase (RP) column—after loading the sample onto the enrichment column, mobile phase was pumped through in order to remove contaminants potentially interfering with MS analysis (for example, salts). The volume of mobile phase was user-defined by the injection flush volume (IFV) in the Agilent MassHunter software. Thus, the enrichment process was simply an isocratic elution at very low % B.

Since compound migration speed through the enrichment column depended on the hydrophilicity of the molecule, hydrophobic compounds were trapped, whereas very polar compounds came through because the enrichment column volume was very small compared with the volumes of mobile phase flushed through it.

The 500-nL enrichment column in the new UHC-chip has more than 12 times higher capacity than the standard 40-nL enrichment column small molecule chip making it much more suitable to retain hydrophilic compounds. Figure 1B shows the analysis of the same sample mix as in Figure 1A but using the UHC-Chip. Responses of all peaks were similar, which indicated retention of all compounds over the entire polarity range (logP 0 to 4.2).

For more information on this topic, go to www.agilent.com/chem, click the Literature Library link, and search for Pub. No. 5989-7967EN.

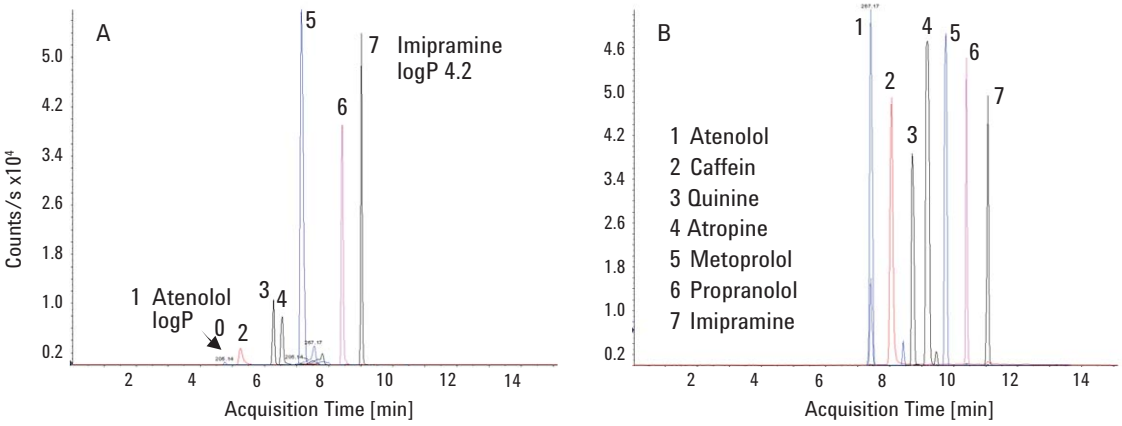

Figure 1. Analysis of a seven-compound mixture with a hydrophobicity range from logP 0 to 4.2. Loading mobile phase: ACN/water, 2:98 v/v (0.05%TFA). Panel A shows the EIC for the 40-nL standard enrichment column small molecule chip and panel B for the 500-nL enrichment column on the UHC small molecule chip.

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