

The Waters Acquity Ultra-Performance Liquid Chromatograph and the Micromass Quattro Premier Triple Quadrupole Mass Spectrometer

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Section I

Introduction

An LC/MS/MS is a liquid chromatograph (LC) system coupled with a quadrupole mass spectrometer (MS). MS quadrupole is the most common mass analyzer, which allows low scan time (<100 ms) and is ideal for LC or GC inlets. When analyzing with LC/MS/MS, the LC separates compounds by conventional chromatography on a column. After the compounds enter the quadrupole mass detector, the solvent is removed and the compounds are ionized. Ions are filtered on the basis of their mass-to-charge ratio (m/z). Ions below and above a certain m/z value will be filtered out depending on the ratio of the direct current (DC) and alternating current (AC) voltages. By ramping the voltages on each set of poles, a complete range of masses can be passed to the detector.

Liquid chromatography can separate metabolites that are not volatile and have not been derivatized. As a result, LC/MS can analyze a much wider range of chemical species than GC/MS. Samples commonly analyzed by LC/MS include amino acids (18 out of 20 amino acids can be derivatized, but the remaining two cannot) and sugars larger than trimers. The LC/MS/MS setup has higher compatibility detecting polar compounds such as organic acids, organic amines, nucleosides, ionic species, nucleotides, and polyamines compared to a GC/MS/MS. LC/MS is better suited for a discovery based approach when researching unknown metabolites, or when many of the targeted metabolites GC/MS incompatible due to volatility issues.

Advantages of LC/MS/MS:

- MS provides exceptionally clean product (fragment) ion chromatograms for quantification;
- Useful for the rapid screening of complex samples where analytes of interest are known;
- Compound identity confirmation can be achieved with MS/MS using the product ion scan mode;
- By detecting a specific product ion (precursor ion mode) or charged fragments resulting from a neutral loss (neutral loss mode), a compound of interest can be classified.

LC/MS/MS Components

The LC/MS/MS we used is a Waters [ACQUITY UPLC separation module](#) coupled with a Waters Micromass [Quattro Premier XE](#) Mass Spectrometer.

(1) UPLC

The ACQUITY UPLC Console contains four major parts: binary solvent manager, sample manager, column manager options, and detector options (Figure 1).



Fig. 1 System overview of ACQUITY UPLC

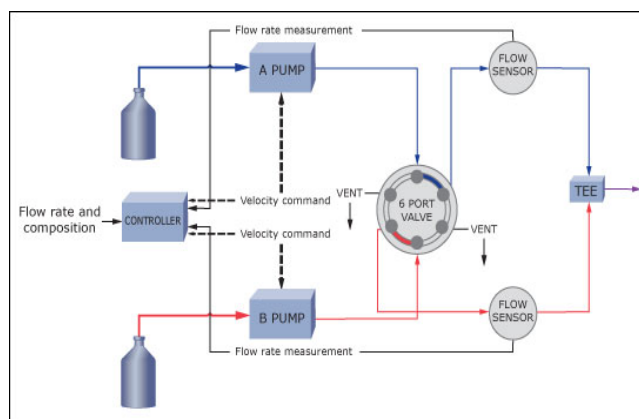


Fig. 2 Schematic of Binary solvent manager

- Binary solvent manager: Each of the solvent manager's two independent pump systems, A (left) and B (right), contains two linear-drive actuators. Each actuator pair comprises a single reciprocating 'serial' pump that delivers precise flow of a single solvent. The two pump systems combine their two solvents at a filter/tee mixer. From there, the solvent mixture flows to the sample manager (Figure 2). A gradient elution program is commonly used so that the eluent composition (and strength) is steadily changed during the analysis. This increases separation efficiency, decreases the retention time and improves peak shape by minimizing tailing.

The chromatography software controls the two solvents' mixing ratio by varying the flow of pump A relative to that of pump B. A pressure transducer in each pump head relays pressure to the solvent manager, whose firmware measures pump head pressures during the pumping cycle. Thus the solvent manager independently pre-compresses the solvents in both the A and B portions to ensure consistent solvent delivery and minimize pump-induced detector baseline disturbances.

- Sample Manager: Plates are transferred in a programmable order from the Sample Organizer to the ACQUITY UPLC System Sample; Manager for processing plate exchange shuttle replaces a plate in

the Sample Manager within 15 seconds to support high sample-throughput processing; It can accept any combination of ANSI-compliant well plates and vial holders. Temperature control is of 4 to 40 °C in 1 °C increments. The ACQUITY system also supports a sample organizer which increases the level of automation and sample processing throughput by extending the capacity of the system, by more than tenfold for samples in standard ANSI-compliant 96 or 384 well plates; or more than threefold for samples in conventional 2 mL vials.

- UPLC Columns: Columns can be divided into two types: Normal phase column and Reverse phase column. Normal phase column: Stationary phase: higher polar rigid silica, or silica-based compositions. Mobile phases: relatively nonpolar solvent. More polar solvent has higher eluent strength and the least polar component is eluted first.

Reverse phase column: Stationary phases: nonpolar hydrocarbons, waxy liquids, or bonded hydrocarbons (such as C18, C8, etc.). Mobile phase: polar solvents or mixtures such as methanol-water or acetonitrile-water. Less polar solvent has higher eluent strength and the most polar component is eluted first. Reverse phase columns are more commonly used in UPLC.

The ACQUITY UPLC system also supports a column manager for high-productivity sample processing (Figure 3). It provides automated switching for up to four columns with dimensions up to 2.1 mm in internal diameter (I.D.) and 150 mm in length, as well as a bypass channel for flow injections.

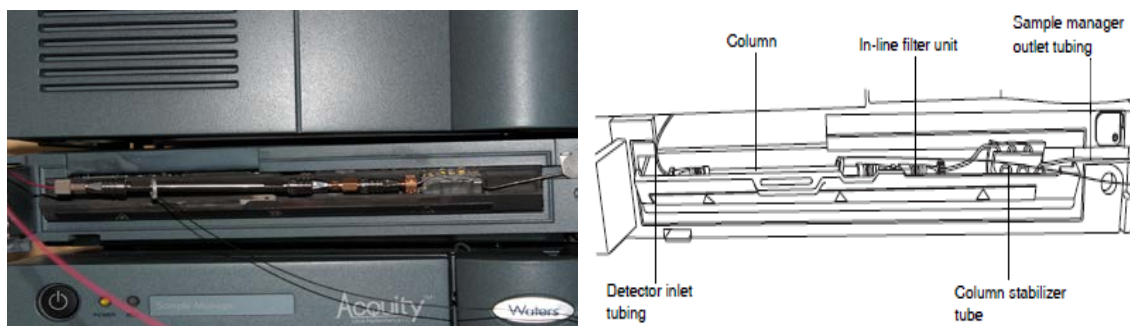


Fig. 3 An ACQUITY BEH C18 (1.7 μm , 2.1 X 50 m) UPLC reversed phase column and the schematic

- Detectors: The system can be configured with in-house optical detectors (e.g. TUV, PDA, or ELS) or connected to a separate Mass Spectrometer. Here MS/MS detector is used and explained in details as follows.

(2) MS/MS

The [Quattro Premier](#) is a high performance tandem quadrupole mass spectrometer designed for routine LC-MS-MS operation. The instrument may be coupled to the following liquid introduction systems: LC system, to provide molecular weight information from an LC run or to perform target analysis and quantification; Syringe pump, for analysis of precious, low-concentration compounds. The Quattro Premier XE Schematic is shown in Figure 4.

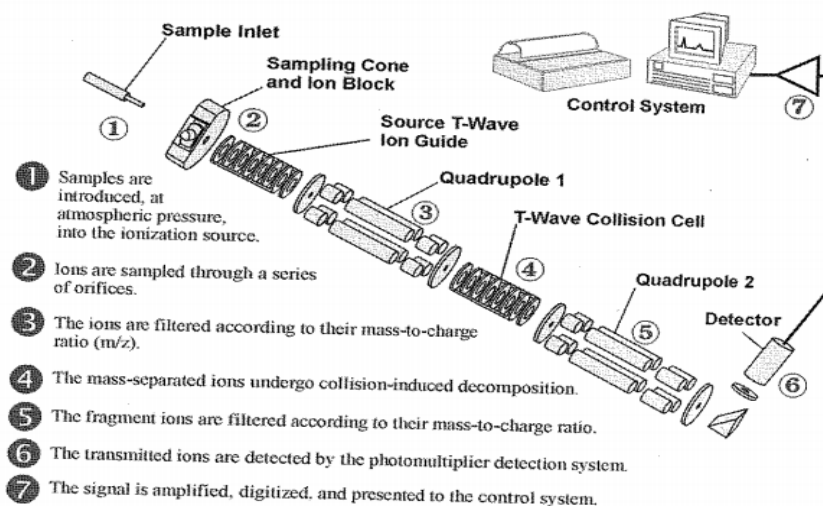


Fig. 4 Quattro Premier XE Schematic

After a sample is introduced, at atmospheric pressure, into the ionization source ① and ionized, ions are sampled through a series of orifices ②, then the ions are filtered according to their mass-to-charge ratio (m/z) at the first quadrupole ③, and the mass-separated ions undergo collision-induced decomposition at the T-Wave Collision Cell ④, then the fragment ions are filtered according to their mass-to-charge ratio at the second quadrupole ⑤, finally the transmitted ions are detected by the photomultiplier detection system ⑥ and the signal is amplified, digitized, and presented to the control system ⑦.

MS/MS operating modes are shown in Table 1. The MS1 mode in which MS1 is used as the mass filter is directly analogous to a single quadrupole MS. The MS2 mode of operation is used with collision gas and when switching rapidly between MS and MS/MS operation; it can be useful for instrument tuning and calibration before MS/MS analysis or error checking. The Selected Ion Recording (SIR) mode of operation can also be used as a quantification mode when no suitable fragment ion can be found to perform a more specific analysis.

Table 1 MS/MS operating modes

	MS1	Collision Cell	MS2
Daughter (product) Ion Spectrum	Static (Parent mass selection)	RF only (pass all masses)	Scanning
Parent (Precursor) Ion Spectrum	Scanning		Static (Daughter mass selection)
Multiple Reaction Monitoring	Static (Parent mass selection)		Static (Daughter mass selection)
Constant Neutral Loss Spectrum	Scanning (synchronised with MS2)		Scanning (synchronised with MS1)

(3) Other components

- Sample Inlet:

An HPLC system or an infusion pump delivers sample to either an electrospray ionization (ESI) probe or atmospheric pressure chemical ionization (APCI) probe and serves as the sample inlet.

Electrospray ionization (ESI) is an ionization technique where a sample solution is sprayed into a strong electric field in the presence of nitrogen to assist desolvation. The droplets formed evaporate in a region maintained at a vacuum causing the charge to increase on the droplets. The multiply charged ions then enter the analyzer. The most obvious feature of an ESI spectrum is that the ions carry multiple charges, which reduces their mass-to-charge ratio compared to a singly charged species. This allows mass spectra to be obtained for large molecules.

Atmospheric pressure chemical ionization (APCI) generally produces protonated or deprotonated molecular ions from the sample via a proton transfer (+ve) or abstraction (-ve). The sample is vaporized in a heated nebulizer before emerging into a cloud of solvent ions formed within the atmospheric source by a corona discharge. Proton transfer or abstraction takes place between the solvent ions and the sample.

- **Vacuum System:**
An external rotary pump and an internal split-flow turbomolecular pump combine to create a vacuum. The turbomolecular pump evacuates the analyzer and ion transfer region.
- **Data System:**
The data system collects information from the mass analyzer. The data system consists of: An embedded PC; an external workstation, and the Masslynx software.
- **MassLynx Software:**
MassLynx software, a Windows based application, enables the following operation:
Configuring Quattro Premier XE; Creating inlet and MS methods that define operation parameters for a run; Tuning and calibrating Quattro Premier XE; Running samples; Monitoring the run; Acquiring data.

Analysis with LC/MS/MS

Analysis with LC/MS/MS can be explained from two respects: (1) Method set-up (containing Mass Spectrometer Method set-up and LC Method Set-up) and (2) Sample analysis.

(1) Method set-up

Set-up Mass Spectrometer method:

- **Ion source selection:**
Based on the chemical properties of target compounds, select proper ion source (e.g., ESI+ve, ESI-ve, APCI+ve, and APCI-ve).
- **Syringe pump injection:**
Fill the syringe with standard solution of the target compound, using syringe pump for injection.
- **Optimize MS/MS parameters:**
Steps:
 - (1) ES/APCI Source: Optimize the voltages for ESI/APCI probe, Sample Cone, Extraction Cone and Hexapole; Source temperature; Desolvation and Cone Gas.
 - (2) Analyzer: Resolution, Ion energy; entrance; collision; exit.
 - (3) Diagnostics:
 - (4) Determine the parent and daughter ions for qualification and quantification.
 - (5) Save as MS File.

Set-up LC method:

Optimize the conditions for LC, determine: (1) LC column; (2) Gradient program of solvents. (3) Retention time of the analytes (Set up the functions that the mass spectrometer uses to scan the instrument during an acquisition). (4) Set solvent delay. Save as the Inlet File.

(2) Sample Analysis.

- Edit Sample List: (1) Sample name; (2) Import MS File; (3) Import Inlet (LC) File; (4) Import Tune File; (5) Edit sample type: Recovery, calibration, analyte.
- Prepare Mobile Phase: Solvent A, Solvent B, weak wash, strong wash etc.
- Prime: 50% Solvent A, 50% Solvent B, 2.7 min.
- Condition: Load LC method, wait for Delta (the difference between the maximum and minimum pressure of the column) decreases to 10.
- Run.

NOTE:

To analyze samples, standards should be run first to check the retention time; standard solutions with a series of concentrations should be run to establish calibration curves.

Section II

Procedure for PPCP analysis with LC/MS/MS.

(1) Filtrate samples with GF/F to remove particles.

(2) Perform SPE to remove impurities and concentrate target PPCPs:

Use HLB Oasis® 200 mg columns to extract PPCPs. These cartridges contain the Oasis HLB sorbent, which is a universal polymeric reversed-phase sorbent (30 µm) that can be used for the extraction of a wide range of acidic, basic, and neutral compounds from various matrices. The columns were sequentially preconditioned with 3 mL Dichloromethane (DCM), 5 mL Methanol (MeOH) and 7 mL Milli-Q water (H₂O), and the samples were then loaded onto the cartridges at 10 mL/min after which the cartridges were rinsed with 3 mL H₂O and 5 mL of 5% MeOH. The cartridges were dried for 40 minutes under vacuum and then the analytes were eluted with 6 mL MeOH followed by 4 mL MeOH/DCM (70:30). The eluate was then dried under a gentle stream of nitrogen at 35 °C to a volume of 0.5 mL and then volumed up to 1 mL with MeOH.

(3) Analysis with LC/MS/MS:

The PPCPs were measured according to Method 18CD (Vanderford et al 2012). Separation and detection of analytes were achieved using Ultra Performance Liquid Chromatography followed by Tandem Mass Spectrometry (UPLC-MS-MS). A Waters ACQUITY UPLC separation module was used for liquid chromatography and a Quattro Premier XE Micromass was used for mass spectrometry.

Analytes were separated using an ACQUITY BEH C18 (1.7 μ m, 2.1 X 50 m) UPLC column. These bridged, ethylsiloxane, hybrid particles can mechanically endure high-pressure conditions upto 15000 psi. The column dimensions allow optimal MS-compatible flow rates, and matched outlet tubing minimizes the effect of extra-column volume.

Both ESI (+) and ESI (-) modes were used for detection. The mobile phases for ESI (+) were Solvent A: 0.05% aqueous formic acid with 1 g ammonium formate and Solvent B: Methanol/ Acetonitrile (1:1) with 0.1% formic acid at a flow rate of 0.35 mL/min. The gradient program started with 3% solvent B, increased linearly to 95% solvent B in 10 minutes, and stayed at 95% solvent B for 6 minutes after which it was brought back to 3% solvent B in 1 minute and kept at 3% solvent B for 8 minutes. The total run time was 25 minutes. The mobile phases for ESI (-) were Solvent A: 40 mg/L aqueous ammonium acetate and Solvent B: 100% methanol at a flow rate of 0.4 mL/min. The gradient program started with 3% solvent B, increased linearly to 25% solvent B in 1 minute, then increased linearly to 99% solvent B in 5 minutes, stayed at 99% solvent B for 5 minutes, then decreased back to 3% solvent B in 3 minutes and was kept at that composition for 7 minutes. The total run time was 20 minutes. Both internal and external standard calibration was used. Internal standard calibration was used for compounds for which internal standards were available (Table 2 and Table 3).

Table 2: ESI- Compounds and Corresponding Transitions

Name	Transition (Parent>Daughter)	Use
Estradiol	270.96>144.83	Hormone
Estrone	268.81>144.94	Hormone
Furosemide	328.68>204.82	Loop Diuretic
Gemfibrozil	248.88>120.88	Anti-cholesterol Drug
Ibuprofen	204.93>160.98	Anti-inflammatory Drug
Naproxen	228.82>169.88	Analgesic
Naproxen d3	231.84>172.79	Internal Standard
Sucralose	394.78>354.78	Artificial Sweetener
Warfarin	306.86>160.79	Blood Thinner

Table 3: ESI+ Compounds and Corresponding Transitions

Name	Transition (Parent > Daughter)	Use
Acetaminophen	151.87>109.91	Analgesic
Atenolol	267.02>145.09	Beta-blocker
Atenolol d7	274.15>145.05	Internal Standard
Atorvastatin	559.1>440.2	Anti-cholesterol Drug
Atorvastatin d5	564.08>445.12	Internal Standard
Caffeine	194.97>137.98	Caffeinated Drinks
Ciprofloxacin	332.01>314.13	Antibiotic
Cotinine	176.97>80.08	Nicotine Metabolite
DEET	192.09>119.04	Insect Repellant
Diclofenac	295.83>213.91	Anti-inflammatory
Diltiazem	414.83>177.83	Calcium Channel Blocker
Diphenhydramine	255.98>166.89	Antihistamine
Miconazole	416.72>160.83	Antifungal drug
Primidone	218.93>161.95	Anti-epileptic
Propranolol	260.05>182.95	Beta-blocker
Propranolol d7	267.19>182.96	Internal Standard
Ranitidine	315.07>176.04	Treat Ulcers & Heart Burn
Ranitidine d6	321.08>176.04	Internal Standard
Salbutamol	239.97>147.87	Asthma Treatment
Sulfamethoxazole	253.94>155.9	Antibiotic
Sulfamethoxazole d4	258.13>160.01	Internal Standard
TCEP	286.84>225.01	Fire Retardant
Trimethoprim	291.05>230.09	Antibiotic
Trimethoprim d9	300.12>234.15	Internal Standard

(4) LC/MS/MS software operation procedure.

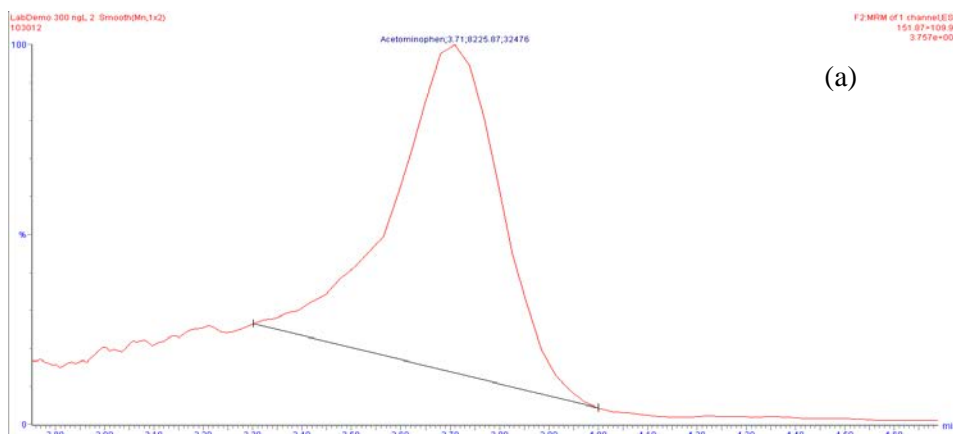
- Edit sample list:
Under Masslynx :

Enter : File name; File text (e.g., date); Bottle number; Inject volume. Import methods (Double click), choose: MS File(e.g., PPCP 2012_consensus_ESI pos. exp); Inlet File(e.g. PPCP 2012_3); MS Tune File: PPCP2012. Ipr.

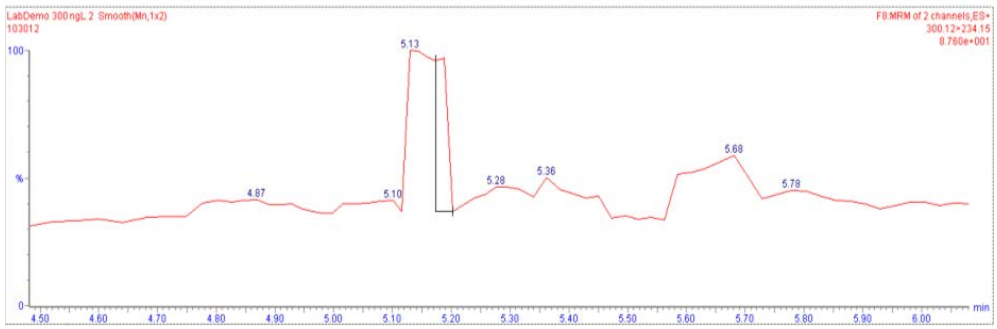
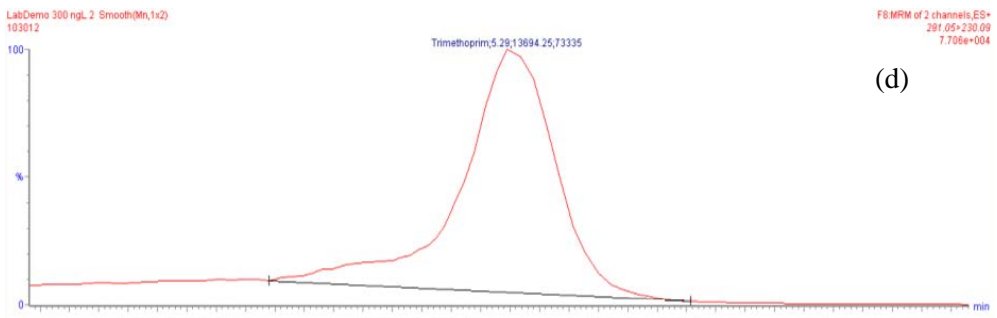
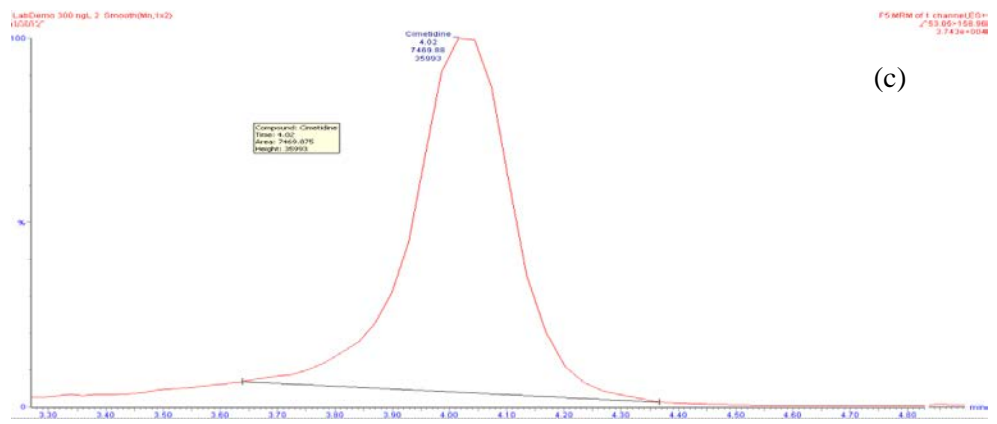
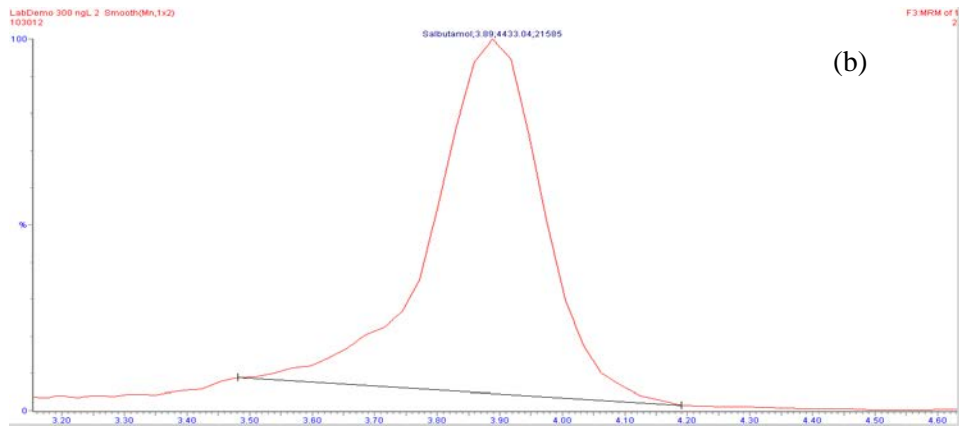
- Turn on Gas for MS:
Under Quattro Premier:
Turn on: API GAS;
COL GAS;
Click: Press for operate.
- Prime the lines:
Under ACQUITY UPLC Console:
Binary Solvent Manager→Control→Prime A/B Solvents (for 2.7 min)
- Condition UPLC column:
Inlet method→Load method →turn on the lamp
Wait for the delta of the column pressure decreases to 10.
- After checking that all the buttons have turned green, run the sample list.

(5) Results

For Lab Demo, we prepared 300 ng/L PPCPs standard solution which was analyzed by LC/MS/MS. Some selected chromatograph peaks are shown in Figure 5. These chromatograms monitor reactions where a precursor ion isolated by one mass filter, passes into a collision cell in which it breaks into products. Product ion is selected by the second mass filter for passage to the detector, hence we can see that this process is extremely selective for just one analyte and vastly increases the signal-to-noise ratio for the analyte.



(a)



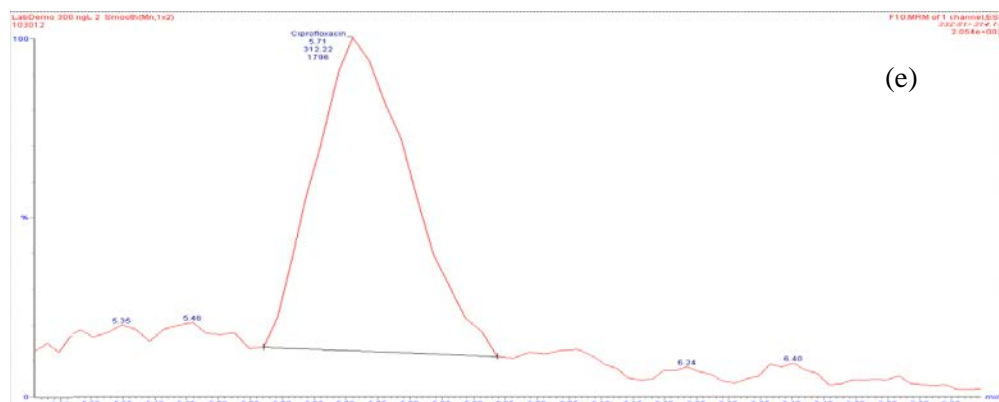


Fig. 5 Chromatograph peaks of (a) Acetaminophen, (b) Salbutamol, (c) Cimetidine, (d) Trimethoprim, (e) Ciprofloxacin. (C = 300 ng/L)

References:

- [1] Harris, Daniel C. 2007. Quantitative Chemical Analysis. W. H. Freeman and Company.
- [2] Waters Corporation. 2006. Acquity UPLC System: Operator's Guide. 71500082502/Revision C.
- [3] Waters Corporation. 2007-2009. Acquity UPLC Photodiode Array eλ Detector. 71500160703/Revision B.
- [4] <http://www.ecs.umass.edu/cee/reckhow/courses/772/>
- [5] Waters Corporation. Micromass Quattro micro API Mass Spectrometer: Operator's Guide. 71500058602 Revision A
- [6] Vanderford, B.J., Drewes, J.E., Hoppe-Jones, C., Eaton, A., Haghani, A., Guo, Y.C., Snyder, S., Ternes, T., Schleusener, M. and Wood, C., J. (2012). Evaluation of Analytical Methods for EDCs and PPCPs via Interlaboratory Comparison, Water Research Foundation, Denver, CO.