High Throughput LC/MS Quantitation for Drug Mixture

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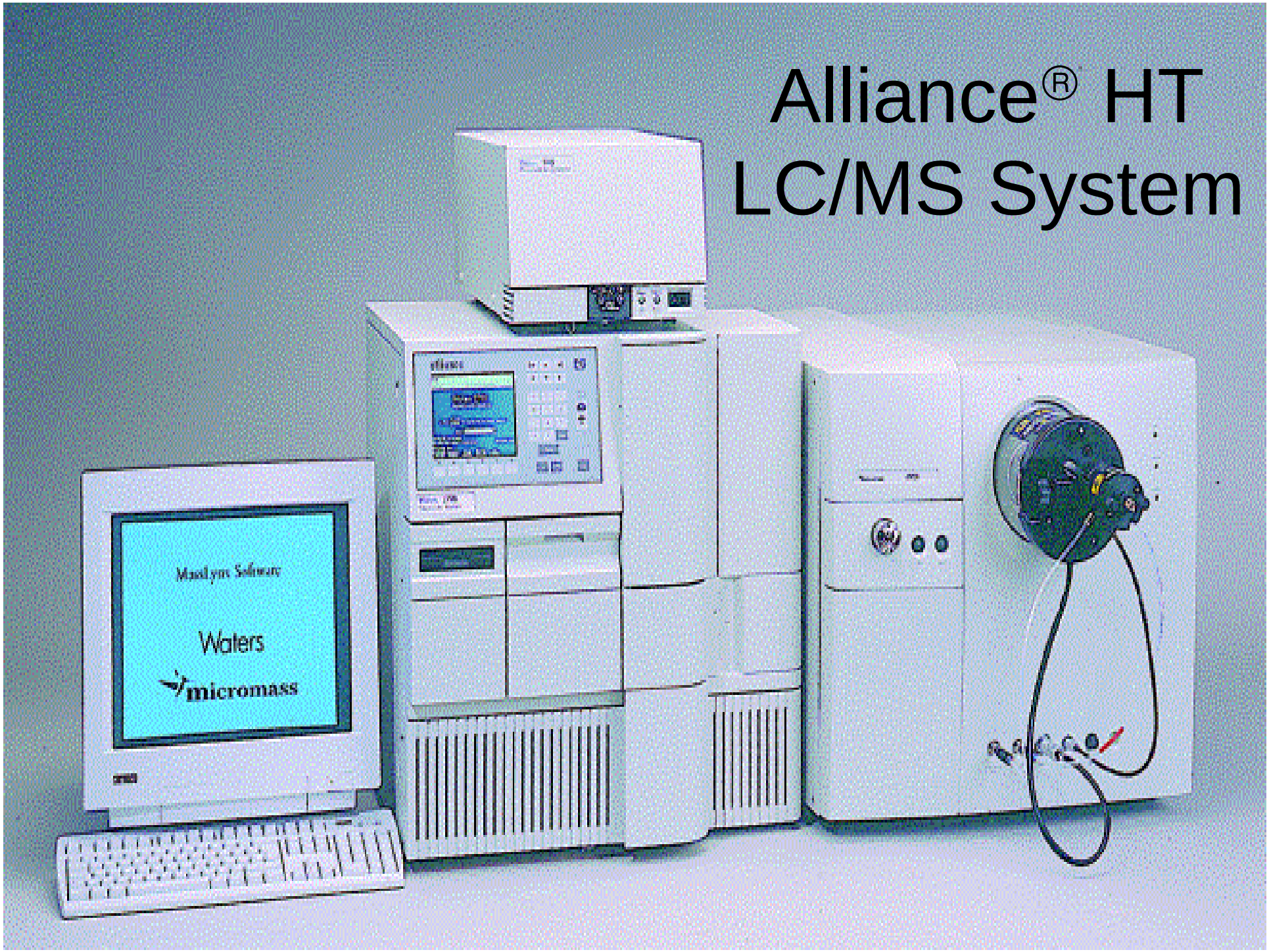
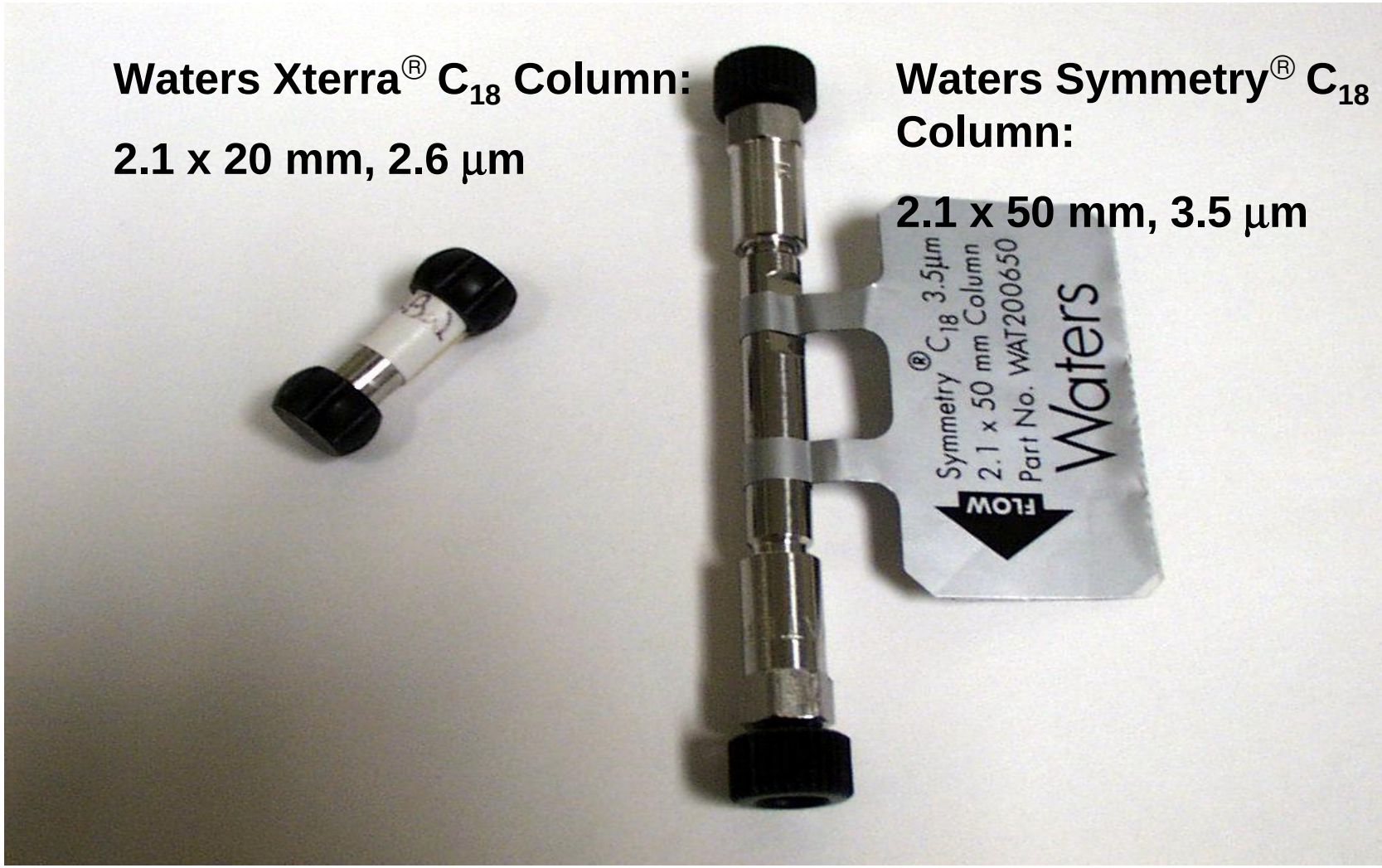

Alliance® HT
LC/MS System
MassLynx Software
Waters
micromass

Waters Xterra® and Symmetry® Columns



Introduction

In recent years, substantial effort have been made to increase the number of lead compounds produced in drug discovery. Because of the need of rapid turn around for discovery projects, the time allowed for method development is sharply decreased. And there is an ever increasing need to analyze more samples faster and cheaper.

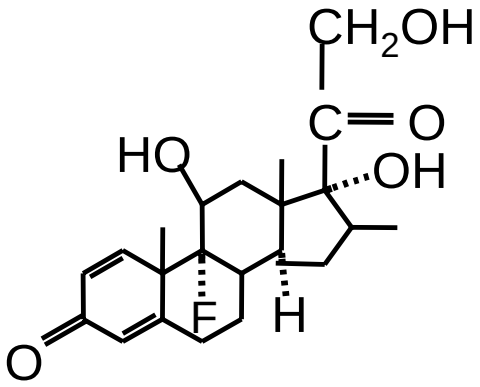
Among the diverse analytical techniques available today, mass spectrometry seems to be the most versatile tool to cope with the demands of high throughput analysis. And LC/MS is the preferred method of choice in most cases.

In this paper, we described a a fast LC/MS method applied to a six drug compound model. The fast LC method was converted from a conventional LC method. By applying fundamental concepts of fast LC, we were able to reduce the cycle time from more than 20 minutes to 2.5 minutes. The analytes were detected by a single quadruple MS detector in electrospray mode by flow splitting. By applying"on-the-fly" positive and negative polarity switching, all six analytes in the mixture were analyzed within one injection. No carryover was observed. The quantitation limits of this "real life" assay model was studied so that the LOD, LOQ, linearity, precision and accuracy for each analyte were defined. The LC/MS quantitation procedure (including MS optimization, data acquisition, quantification and data reporting) can be automated by applying the QuanLynx™ (Part of MassLynx™ software).

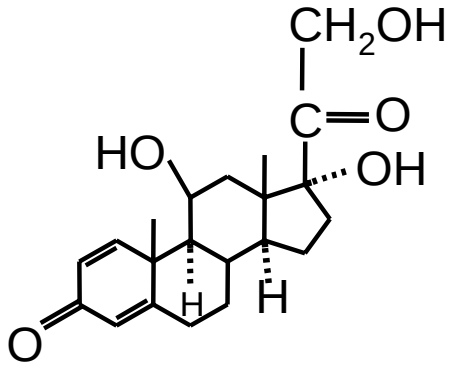
Major Features

- **Convert a conventional LC method to a fast LC method**
  - apply fast LC fundamentals
  - utilize Waters Alliance® HT system - an integrated solvent delivery manager designed for high throughput applications in LC and LC/MS.
  - Reduce the cycle time for analysis to 2.5 minutes from more than 20 minutes
- **Analysis carried on shorter Waters Xterra® C_{18} columns (packed with 2.6 μm particles)**
- **Electrospray MS detection on Waters ZMD™ 2000 MS Detector:**
  - "on-the-fly" positive/negative polarity switching allowed all compounds to be analyzed within one injection
  - LC flow split to accommodate low flow requirement of electrospray to maintain or increase sensitivity
- **Quantitation performance evaluated:**
  - for each analyte, the LOD, LOQ, linearity, inter- and intra-assay precision and accuracy were defined
  - No carryovers
  - Automation by QuanLynx™

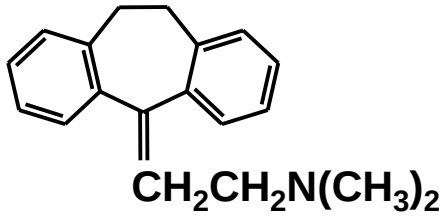
Model Analyte



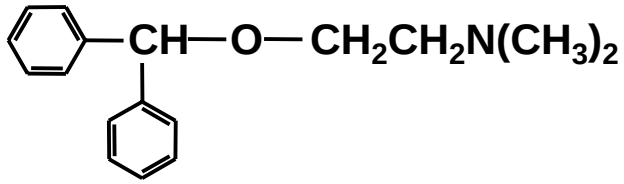
Bethamethasone MW 392
[378-44-9]



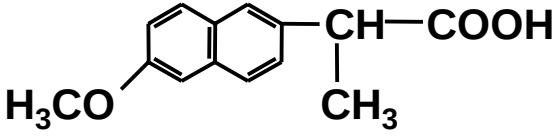
Prednisolone MW 360
[50-24-8]



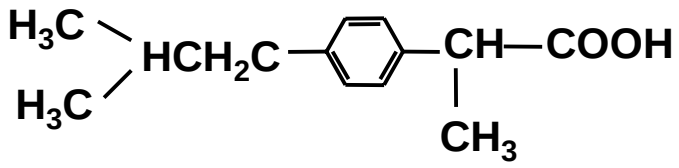
Amitriptyline MW 277
[549-18-8]



Diphenhydramine MW 255
[147-24-0]



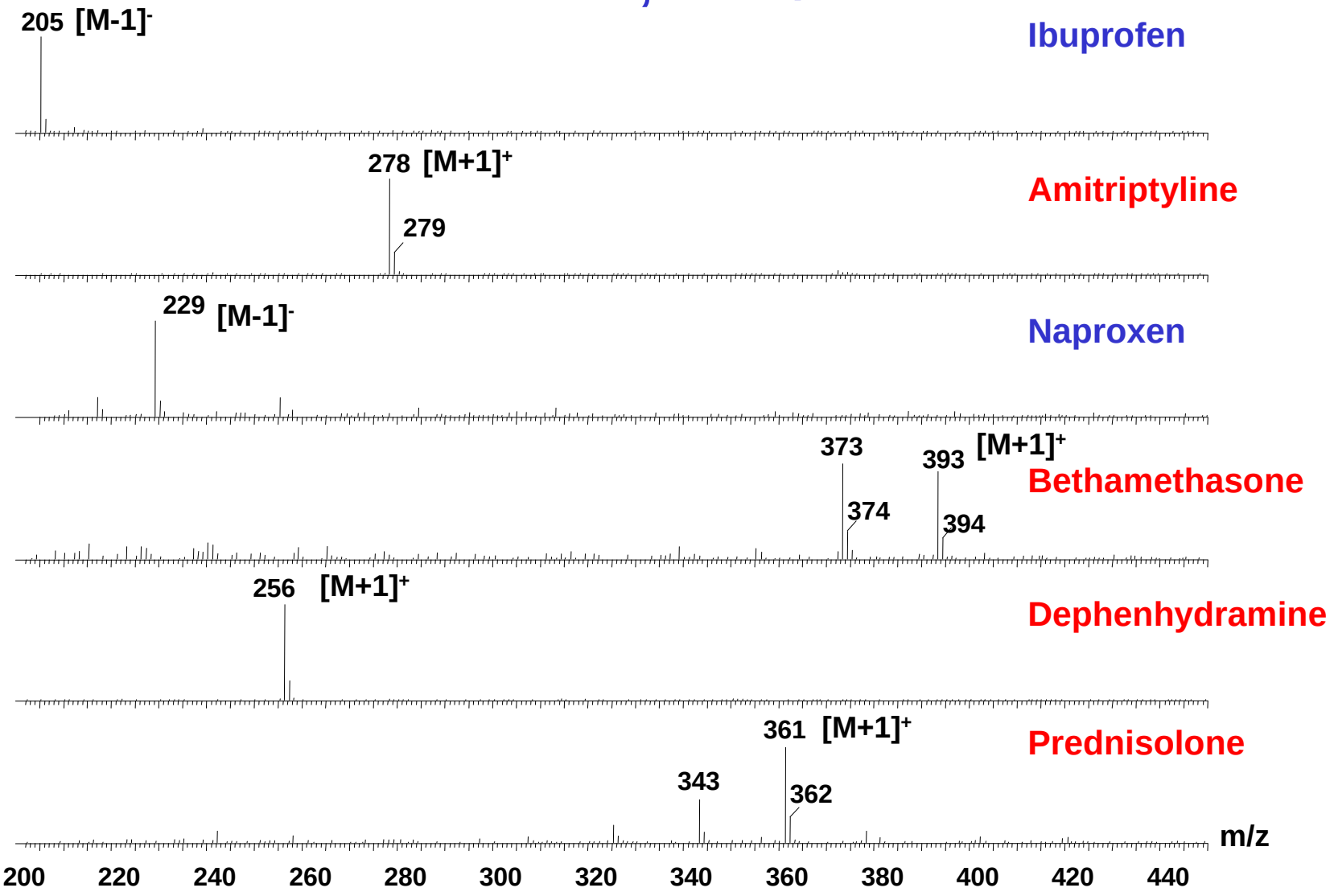
Naproxen MW 230
[22204-53-1]



Ibuprofen MW 206
[15687-27-1]

MS Spectra of the Analytes

Full Scan, ESI+ /ESI-



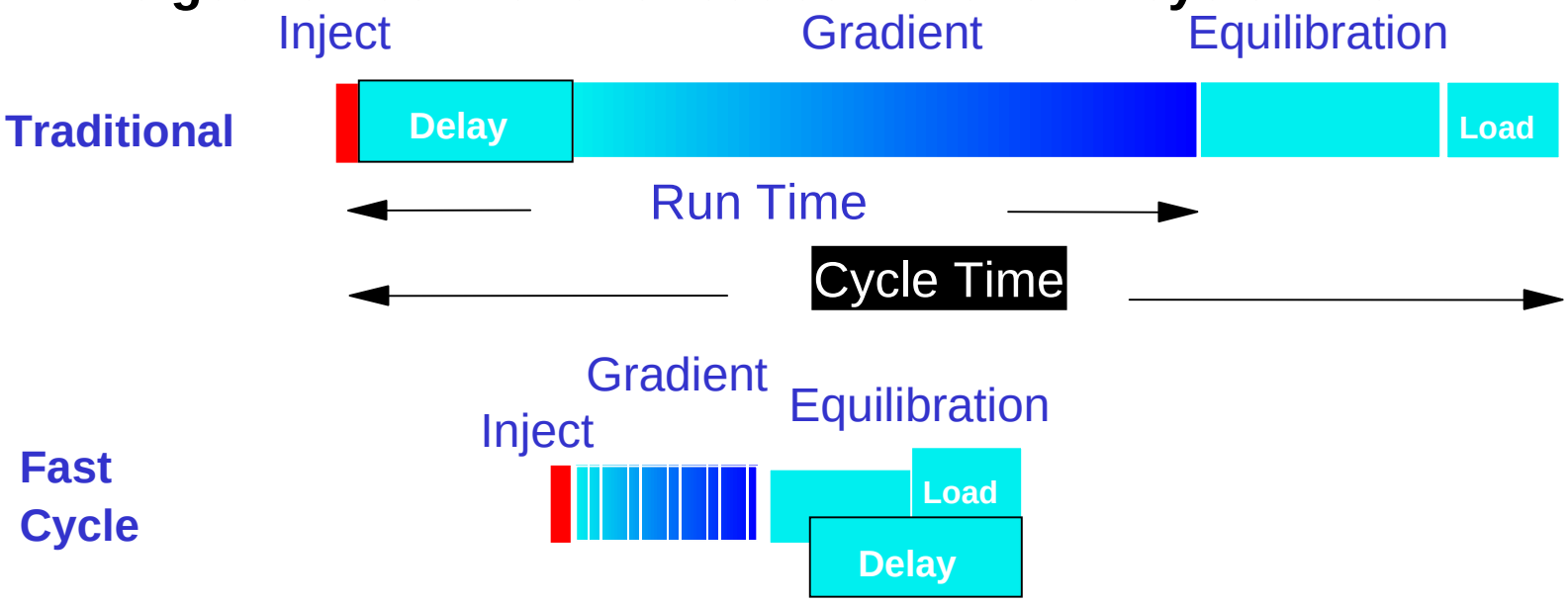
How To Reduce Analysis Cycle Time

- In HPLC analysis, the time required for analyzing a sample is the cycle time

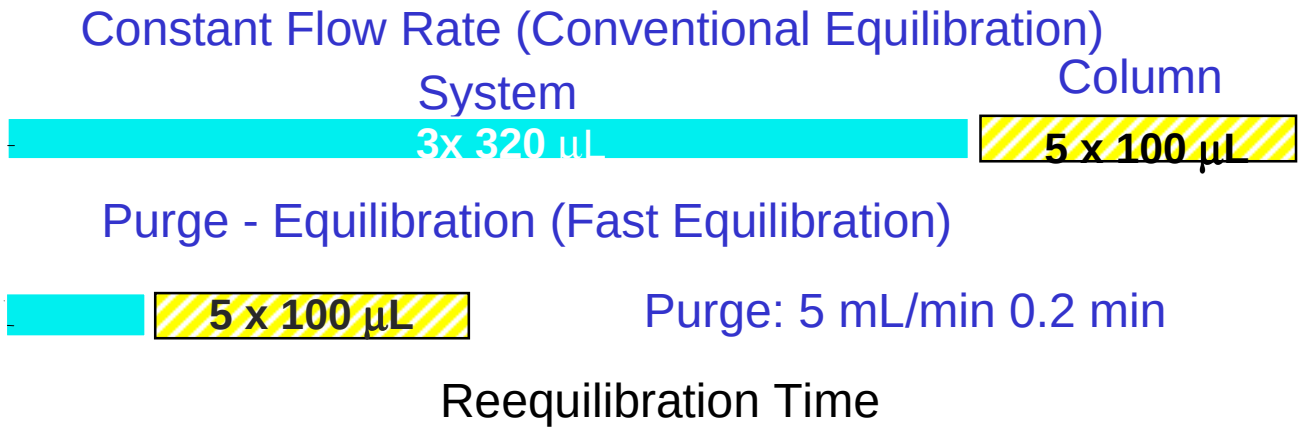
  Cycle time is the time that required from injection to injection.

  – sample withdraw, loading and injection time
  – gradient time
  – delay time
  – equilibration time

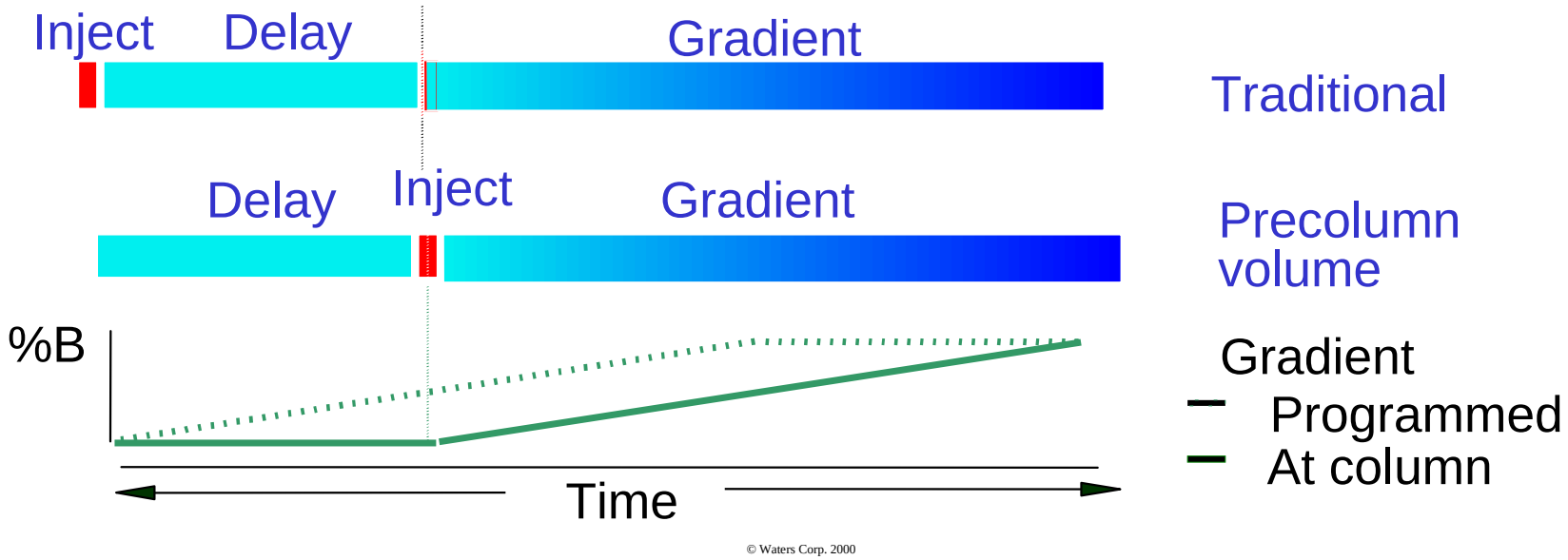
  The goal of fast LC is to reduce the overall cycle time



- Shorten LC run time
  - Use shorter columns with smaller I.d. and smaller particle size
  - Increase flow rate
  - Apply higher column temperature
- Reduce Re-equilibration Time
  - Time needed for system equilibration: 3 x system volume + 5 x column volume
  - System equilibration can be accomplished in 0.2 minutes by switching the column off-line and apply high flow rate

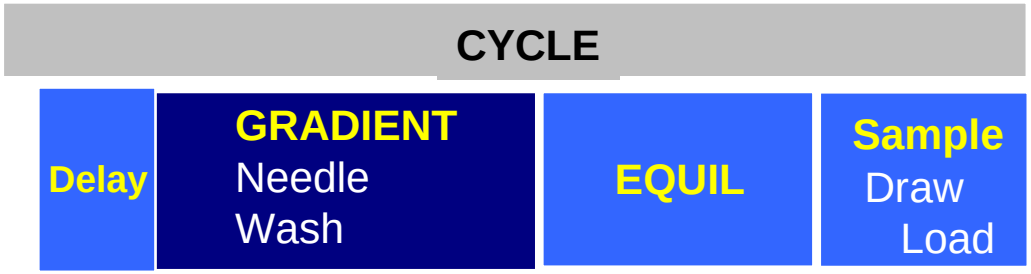


- Apply pre-column volume concept
  - pre-column volume is a programmable software parameter in both Alliance® and Alliance® HT systems
  - it allows the injector to be loaded and gradient to be started, but sample is only injected onto to the column until the gradient reaches the column
  - the effect of pre-column volume is that the delay volume is at the end of the cycle, instead of beginning
  - the effect of pre-column volume is to compress the beginning of the chromatogram



Make processes parallel

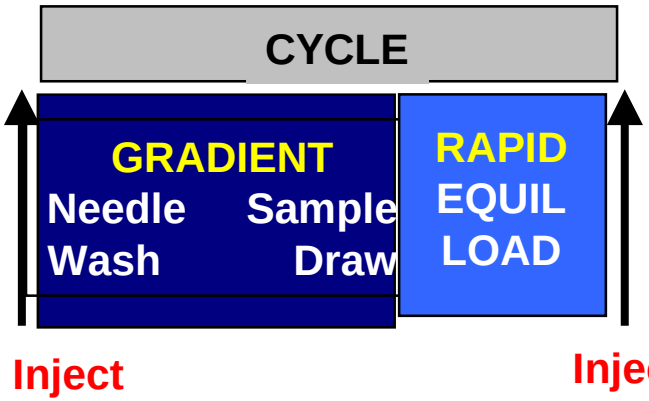
This can be accomplished easily with Waters Alliance HT solvent delivery system



Sequential Process

Initial Conditions

Inject **Inject**



Inject **Inject**

Parallel Process

Experimental Conditions

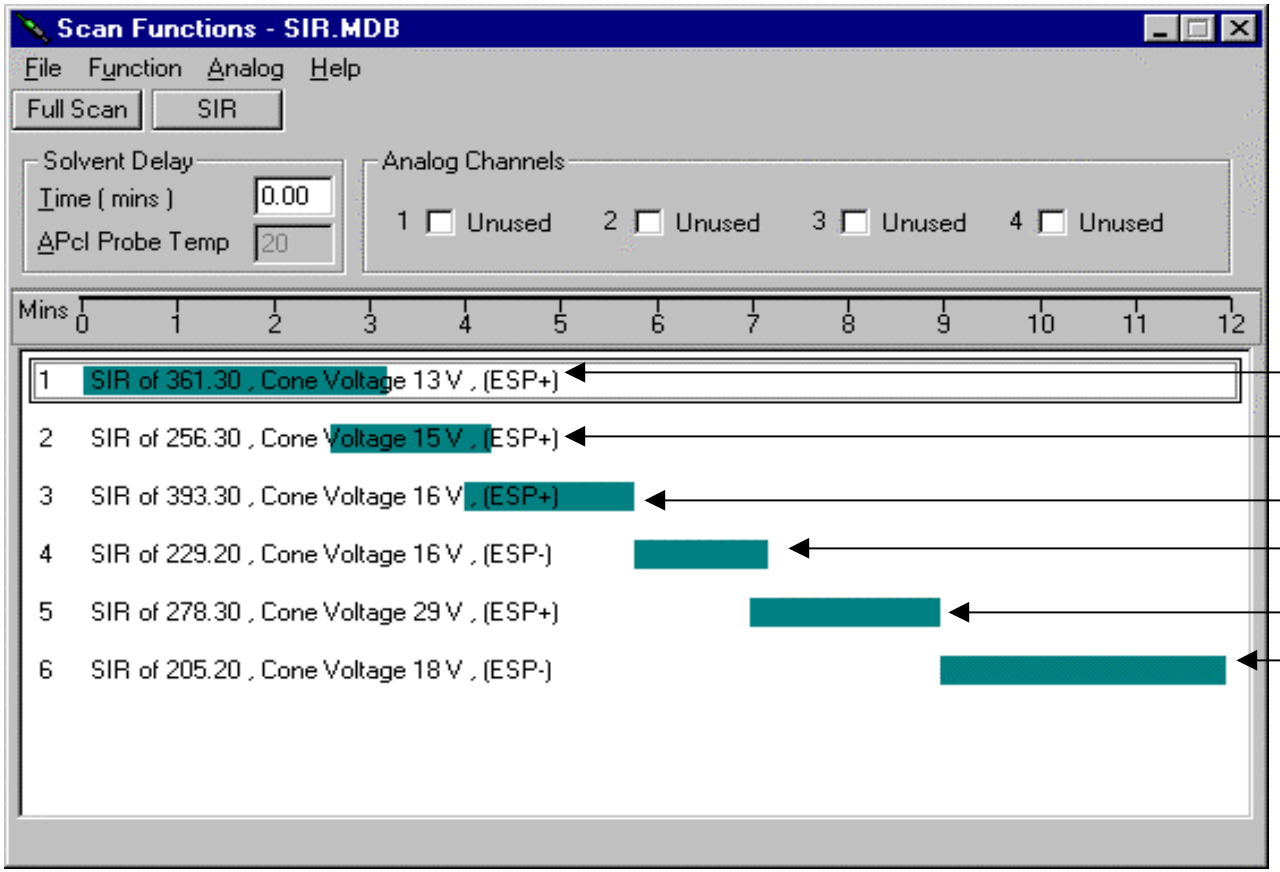
Conventional LC

- **HPLC: Waters Alliance® 2690**
  - Column: Waters Symmetry® C_{18} 2.1 x 50 mm 3.5 µm
  - Column T: 30°C
  - Mobile Phase: A: 10 mM NH4OAc in Water pH 5.0
    B: 10 mM NH4OAc in AcN
  - Flow Rate: 0.3 mL/min
  - Injection Volume: 10 µL
  - Gradient:

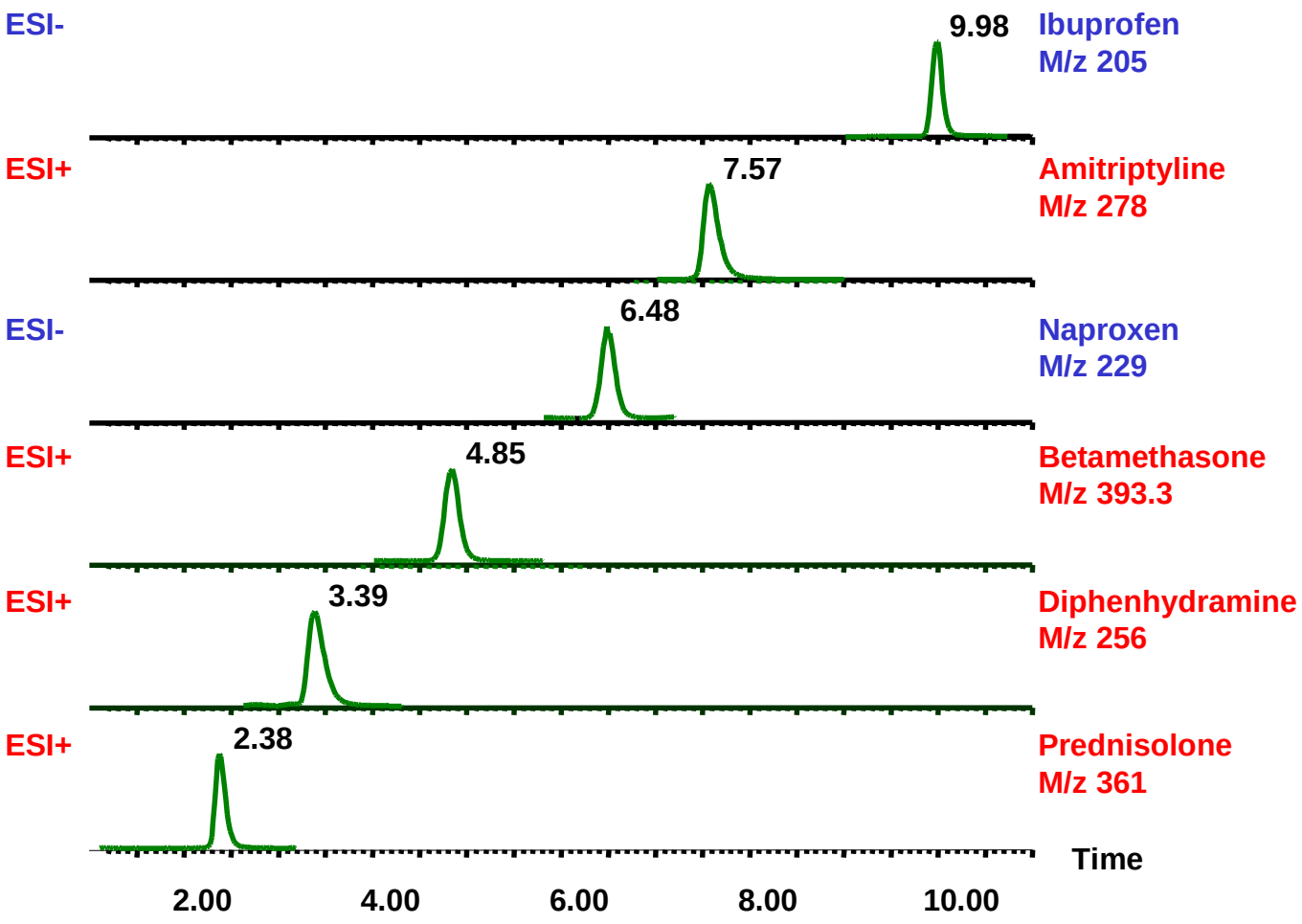
Time	A%	B%	Curve
0 min	75	25	--
8 min	25	75	8
12 min	0	100	1
20 min	75	25	1

MS: Waters ZMD® 2000 MS Detector

Ionization:	ESI+ or ESI-
Source T:	150°C
Desolvation T:	300°C
Capillary Voltage:	2.85 kv
Data Acquiring:	SIR (Single Ion Monitoring)



Extracted Mass Chromatograms



Fast LC

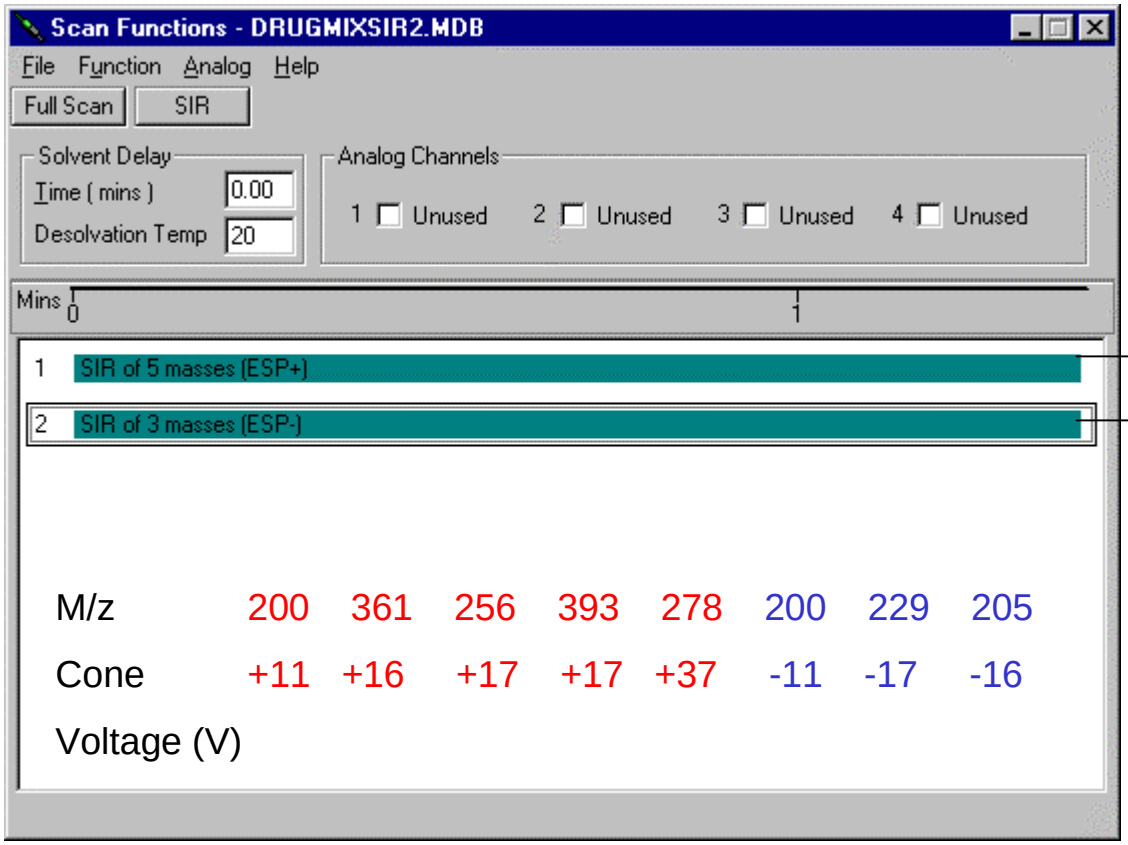
- **HPLC: Waters Alliance® HT 2790**
  - Column: Waters Xterra® 2.1 x 20 mm 2.6 µm
  - Column T: 40ºC
  - Mobile Phase: A: 10 mM NH4OAc in Water pH 5.0
    B: 10 mM NH4OAc in AcN
  - Gradient:

Time	A%	B%	Curve
0 min	75	25	--
0.7 min	55	45	6
1.4 min	0	100	1

  - Pre-Column Vol: 100µL
  - Flow Rate: 1.0 mL/min (Split flow, 150uL/min into MS)
  - Injection Volume: 5 µL Full loop (4 times over fill)
  - Fast Equilibration: System 5.0 ml/min 0.20 min
    Column 1.0 ml/min 0.35 min
  - Wash Parameters: Injector Port: 10 s
    Needle Exterior: 10 s

MS: ZMD® 2000 MS Detector:

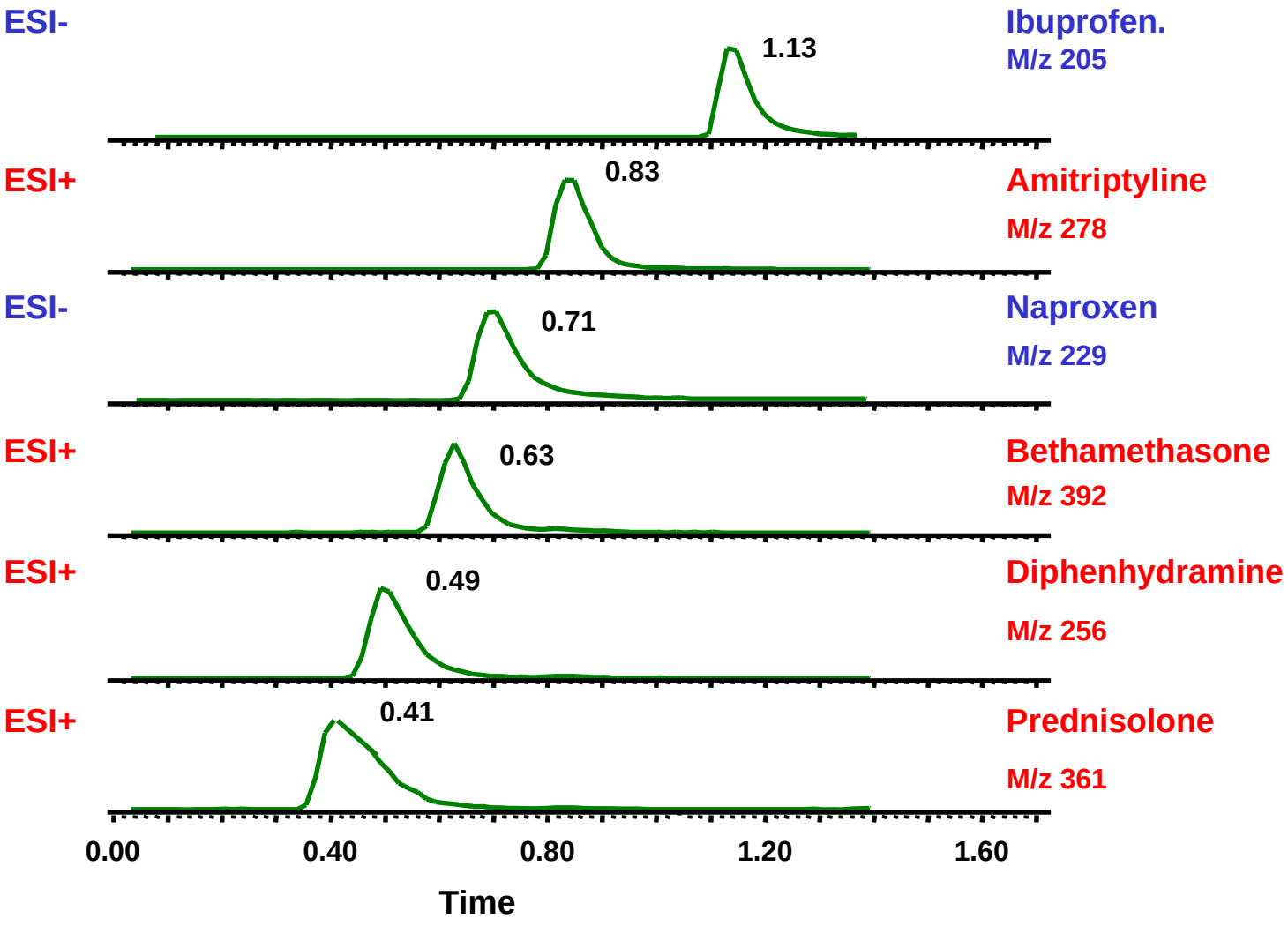
Ionization:	ESI+ or ESI-
Source T:	130°C
Desolvation T:	250°C
Capillary Voltage:	2.85 kv



M/z 200, 361, 256, 393, 278

M/z 200, 229, 205

Extracted Mass Chromatograms



How Fast Should You Go

- How fast you should go will depend on the purpose of the analysis. Shorter run time does not necessarily results into shorter cycle time.
- In reality, there is a practical limit on how fast you can go. Faster is not necessarily better for your specific analysis. This is especially true for quantitative work.
- For quantitation, appropriate wash cycle is necessary to ensure that carryover is minimal from injector needles, injector valves and the LC columns. This will prolong the cycle time of the analysis.
- It also takes time for samples to be drawn and loaded. Water Alliance® HT allow this procedure to be hidden behind the gradient in parallel mode provided the gradient time is long enough.

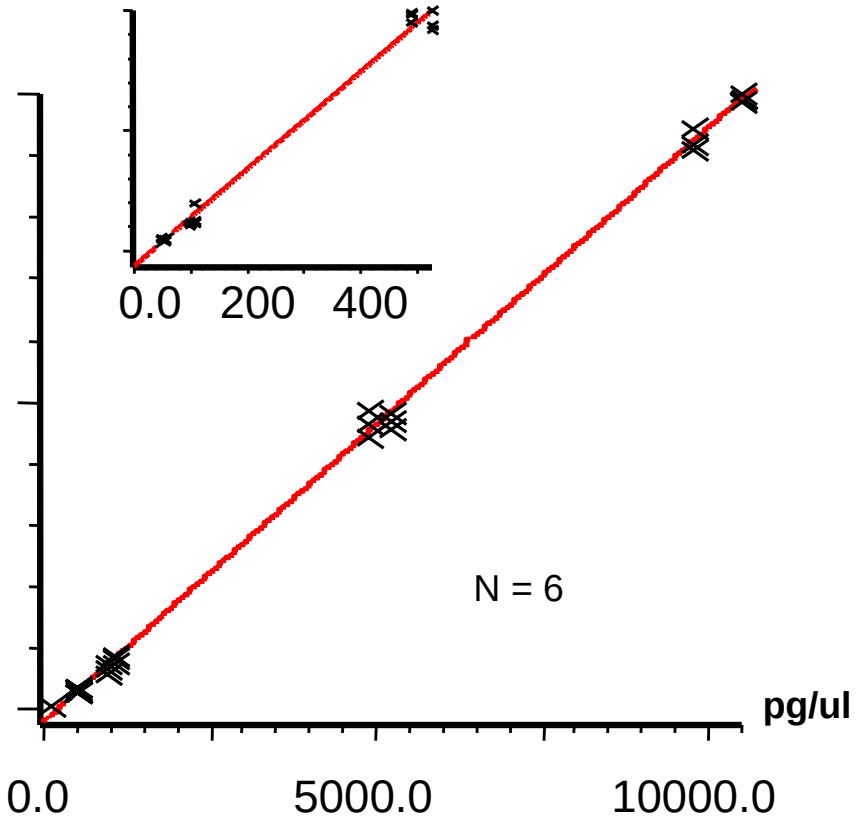
Quantitation with Fast LC

- The calibration curves shown below were constructed in the following way:
  - They are the results of 6 injections in 3 consecutive days.
  - Each day, two injections were made. Each injection was made from a separate vial.
  - Two sets of stock solutions were prepared and they were alternated between points.
- The limit of detection (LOD) and the limit of quantitation (LOQ) of each analyte was determined by measuring the signal to noise ratio (S/N).
  - LOD: S/N = 3; LOQ: S/N = 10
- The QC samples were injected 6 times each day and repeated in 3 consecutive days.
  - Intra-assay CV (%): within day precision, average of 3 days results
  - Iner-assay CV (%) between day precision, take all 18 injections as one group of data
  - Iner-assay Error (%) between day accuracy, take all 18 injections as one group of data

Calibration Curves

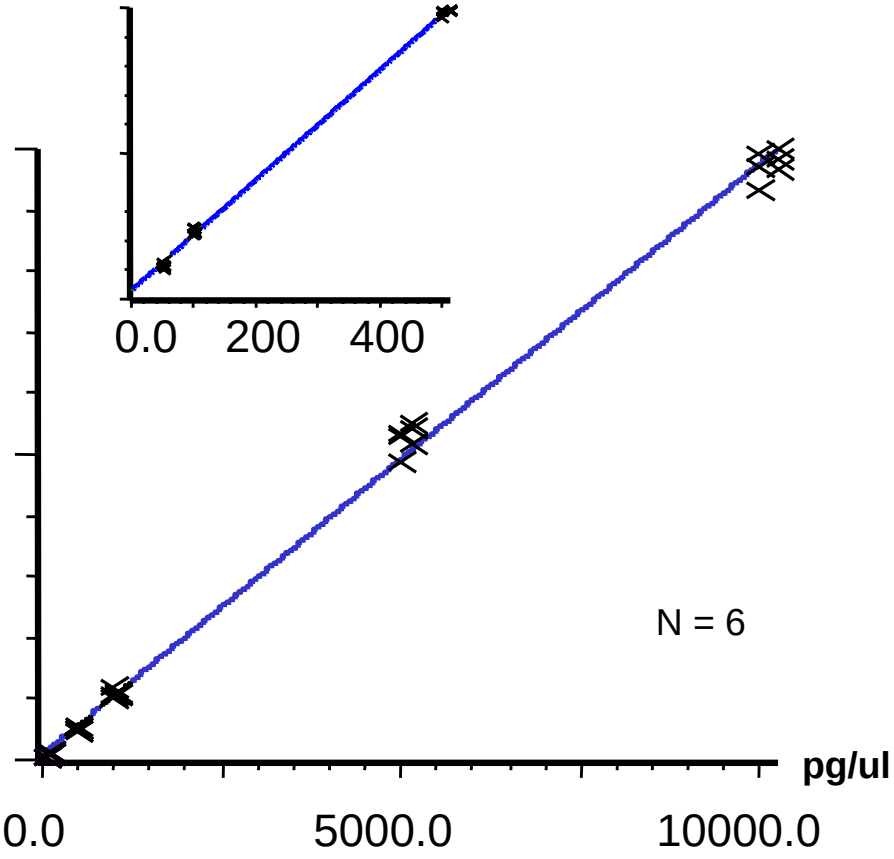
Amitriptyline, ESI+

Correlation coefficient: r^2 = 0.998737
Cal. curve: 508.205 * x + -104334
Response type: External Std, Area
Curve type: Linear, Origin: Exclude,
Weighting: Null, Axis trans: None



Naproxen, ESI-

Correlation coefficient: r^2 = 0.997367
Cal. curve: 22.9011 * x + 1656.93
Response type: External Std, Area
Curve type: Linear, Origin: Exclude,
Weighting: Null, Axis trans: None



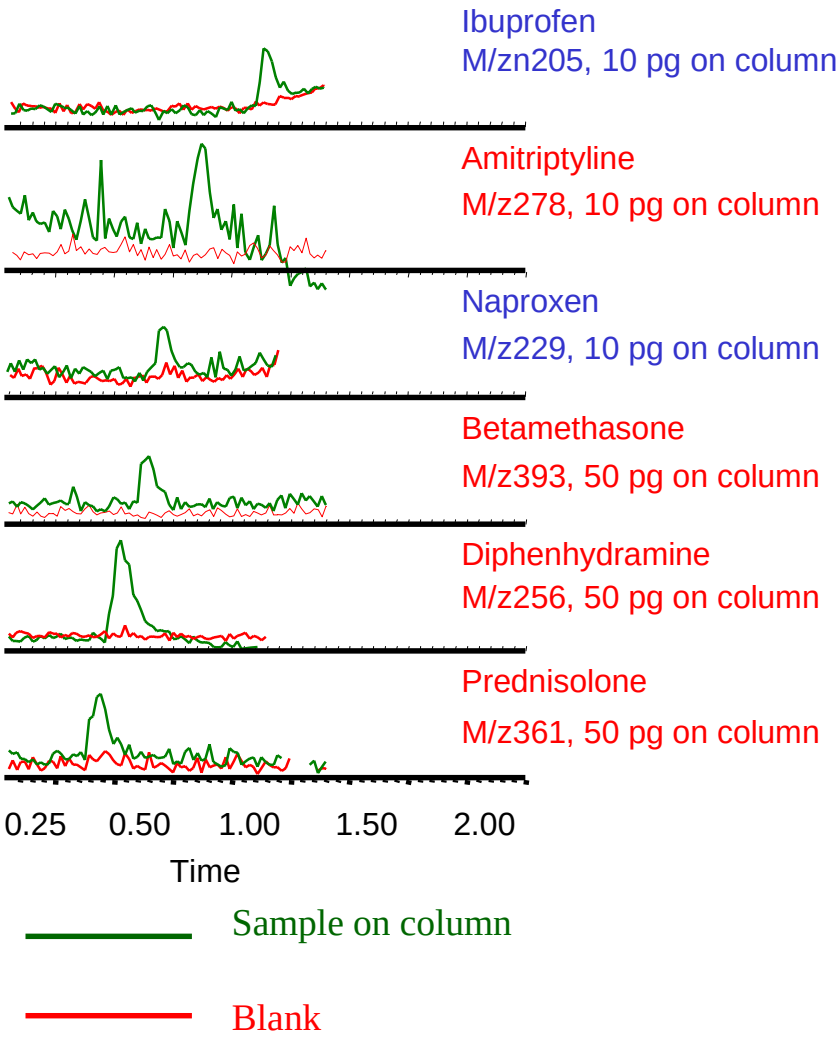
Quantitation Results

	LOD (pg)	LOQ (pg)	R^2	Intra-Assay CV (%) N=6	Inter-assay CV (%) N=18	Inter-assay Error (%) N=18
Pred.	**55.2**	**184**	**0.992**	**3.77**	**4.40**	**1.62**
Diphen.	**13.4**	**46.0**	**0.998**	**1.27**	**4.57**	**-8.00**
Betha.	**62.5**	**208**	**0.993**	**3.30**	**4.48**	**-0.00**
Amitrip.	**8.42**	**28.1**	**0.999**	**2.06**	**2.63**	**-7.29**
Napro.	**46.1**	**154**	**0.997**	**2.72**	**4.60**	**14.5**
Ibu.	**6.43**	**21.4**	**0.996**	**1.56**	**4.63**	**-0.66**

All precision and accuracy data shown here were obtained at low QC level (3 x LOQ).

The LOD and LOQ was calculated based on total injection amount to the column. The LC flow was split about 1:7 ratio before the MS.

The Effect of Carryover



No carryover was observed with the system and the method used for the project. Shown here are the example of one set of experiments.

Samples were running in the following sequence: Blank, Series of standards from low concentration to high concentration (1ng/ml to 10 ug/ml), and Blank.

Shown here are the comparison of the lowest signal detectable for each analyte with the blank injection (injected after the highest concentration of sample).

Quantitation Automated by QuanLynx™

- The LC/MS quantitation procedure can be automated by utilizing QuanLynx™ software from the MassLynx™ package
- QuanLynx™ allows MS condition optimization, data acquisition, data processing to be performed in one step

Conclusions

- Utilizing the Waters Alliance® HT 2790 solvent delivery system, by applying and coordinating the fast LC principles, the cycle time for the drug mixture was reduced to 2.5 minutes from more than 20 minutes.
- Quantitation results were obtained with no carryover.
- At low QC level, the intra-assay precision was less than 3.8%, the inter-assay precision was less than 4.7% and the inter-assay error was less than 14.5% for the whole methods.
- The LC/MS quantitation process (optimization, data collection, and calculation) can be automated with QuanLynx™.

Acknowledgment

- Bonnie Alden from Waters Corporation for providing the Xterra columns