

LC World Talk

SHIMADZU'S NEWSLETTER FOR THE HPLC GLOBAL COMMUNITY

Shimadzu Introduces the LCMS-8030, the World's Fastest Triple Quadrupole Mass Spectrometer

- LCMS-8030 Ultra Fast Tandem Quadrupole Mass Spectrometer, The Next Generation of Mass Spectrometer
- NEXERA, the World's Only No Compromise UHPLC
- Long-term Automated HPLC Analysis of Microdialysis Samples from Multiple Freely Moving Animals
- Impurities Analysis in Pharmaceuticals: FDA Regulations on Genotoxic Impurities in Pharmaceuticals
- Melamine Analysis Using the LCMS-8030





LCMS-8030 Ultra Fast Tandem Quadrupole Mass Spectrometer

The Next Generation of Mass Spectrometer

Triple quadrupole mass spectrometry is the method of choice for accurate quantification and confirmation of trace-level analytes in complex matrices. From the detection of drugs and metabolites in biological specimens to environmental contaminants and pesticides in food, analysts the world over are challenged with detecting an increasing number of target analytes with greater sensitivity and in more samples than ever before.

Shimadzu understands these requirements. That is why we have combined our experience from different fields to build a mass spectrometer that can keep pace with the chromatographic resolving power of our world-leading UHPLC systems without any speed limitation. The LCMS-8030 couples the power of a Triple Quadrupole Mass Spectrometer with unparalleled speed to provide the ideal complement to both Nexera UHPLC and Prominence HPLC systems.

With ultra fast MRM transitions and ultra fast polarity switching, LCMS-8030 is truly a universal detector for UHPLC or HPLC.



FEATURES

■ Ultra-high Speed

Fusion of Ultra Fast MRM and Ultra Fast Polarity Switching

The world's fastest polarity switching speed (15 msec) and minimized MRM transition time (1 msec) realize high-throughput MRM analysis.

Ultra Fast Scan Speed of 15,000 u/sec

The LCMS-8030 adopts the ultra-high-speed scanning technology cultivated in the development of the GCMS-QP2010 Ultra and LCMS-2020.

UFSweeper™ Technology

UFSweeper collision cell technology accelerates product ions out of the collision cell, minimizing sensitivity losses and cross talk.

■ High Reliability

Long-term Stable MS/MS Analysis

The proven ionization source and optimized desolvation line minimize instrument downtime, providing consistent performance between maintenance intervals.

Dramatically Minimized Cross Talk

UFSweeper technology minimizes cross talk that is associated with shorter dwell times and which leads to poor quantitative results in MRM.

Excellent Linearity with a Wide Dynamic Range

Ultra-fast analysis coupled with a wider dynamic range has been achieved with an ultra-fast pulse-counting detector and conversion dynode system.

Ultimate in Front End HPLC Performance

Nexera UHPLC integrates seamlessly with the LCMS-8030 to deliver high-speed, high-quality LC/MS/MS analysis.

■ User-Friendly

Minimize Instrument Downtime with Easy Maintenance

The simplified maintenance and robust instrument design increases work efficiency.

Single-vendor Solution Provides Seamless Operation

LabSolutions software offers a unified platform for LCMS-8030 and Nexera control, easy operation and efficient data browsing.

Ultra-fast MRM Acquisition

With high-resolution, high peak capacity separations, mass spectrometry detection needs to be ultra fast in order to acquire sufficient data points across a peak to deliver accurate and reliable quantitation. Coupled with Nexera UHPLC, the LCMS-8030 not only delivers the fastest MRM acquisition times available today, with dwell times of 1 msec, it also acquires data with a polarity switching time of just 15 msec. Figure 1 illustrates a 2-minute elution of 226 pesticides using the LCMS-8030 with the Nexera UHPLC system. Ultra fast polarity switching and ultra fast MRM analysis times deliver reliable and accurate quantitation.

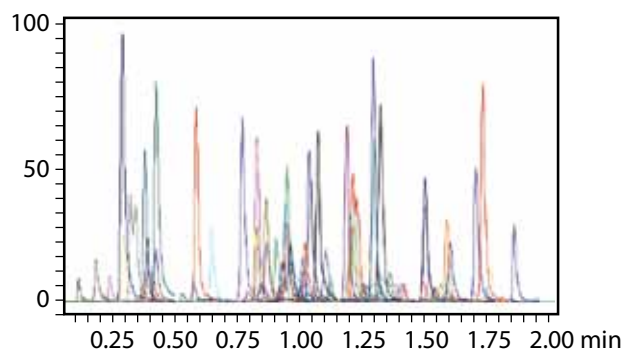


Figure 1: MRM analysis of 226 pesticides in two minutes; concentration of each pesticide: 10 ppb, peak width: less than 2 sec

Ultra Fast Scan Speed of 15,000 u/sec

In product ion scan measurement (automatic MS/MS, or synchronized survey scan) using MRM as the trigger, mass spectra of a product ion can be obtained during an MRM transition. Compound structure can be simultaneously confirmed by the resulting product ion spectra due to the high selectivity achieved during MRM with no interference from co-eluting substances.

Figure 2 illustrates a 1.5-minute elution of 29 pesticides using the LCMS-8030 with the Nexera UHPLC system. Ultra fast polarity switching and ultra fast MRM transitions deliver reliable and accurate quantitative results. Furthermore, synchronized survey scan technology, utilizing a high-speed scanning rate of 15,000 u/sec, allows full spectrum scans within a series of MRM measurements, providing confirmation of target compounds with information-rich product ion spectra.

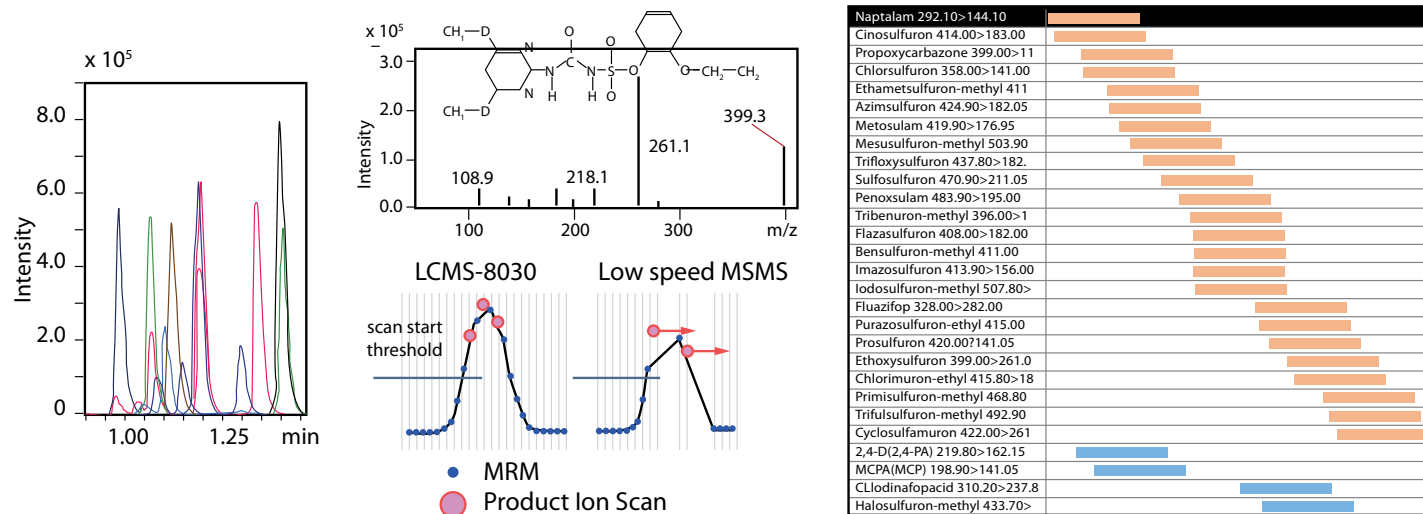


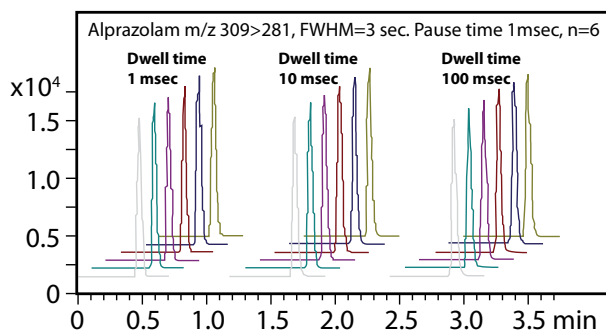
Figure 2: MRM transition and product ion scan measurement triggered by the MRM transition

UFsweeper Technology

UFsweeper is a unique technology created by Shimadzu that delivers unparalleled efficiency and speed. It accelerates ions out of the collision cell by forming a pseudo-potential surface. The result is higher CID efficiency and ultra fast ion transport, which prevent the sensitivity losses and cross talk that are observed on other systems.

In the UFsweeper collision cell, there is no ion loss even at a 1 msec dwell time as ions are accelerated from the pressurized collision cell without losing momentum. In addition, higher RF power capability minimizes the pause time between each MRM transition. Figure 3 shows reproducibility data in MRM analysis of alprazolam. Even at a 1 msec dwell time and a 1 msec pause time, the LCMS-8030 provided good reproducibility without signal loss. 500 MRM transitions in one second are now possible!

UFsweeper technology dramatically reduces cross talk, a common problem associated with shorter dwell times. In multiple reaction monitoring (MRM), cross talk leads to poor quantitative results. In the LCMS-8030, UFsweeper technology efficiently accelerates residual ions out of the collision cell. Figure 4 demonstrates the dramatic reduction of cross talk during ultra fast MRM transitions.



Dwell Time	%RSD
100 msec	0.48
10 msec	0.79
1 msec	1.92

Figure 3: Reproducibility of alprazolam; dwell times: 1 msec, 10 msec and 100 msec; pause time between each MRM transition: 1 msec

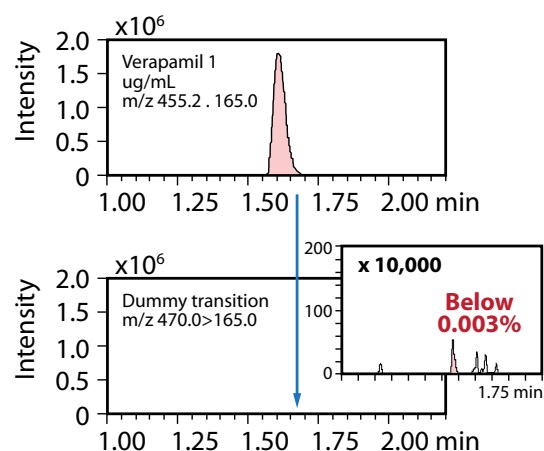


Figure 4: Cross talk evaluation of verapamil in ultra fast MRM analysis; dwell time: 3 msec; pause time between each MRM transition: 3 msec

Reliable LC/MS/MS Analysis

The ion optics in the LCMS-8030 have been fully optimized for ion transport to the detector using a series of RF and DC ion guides before Q1. This design has a significant impact on challenging matrices. For example, in the case of 10 ppb pesticides mixture spiked into tomato extract, 1400 individual 1 μ l aliquots were injected over a period of 2 days. The result is an outstanding 2.19% reproducibility as shown in Figure 5.

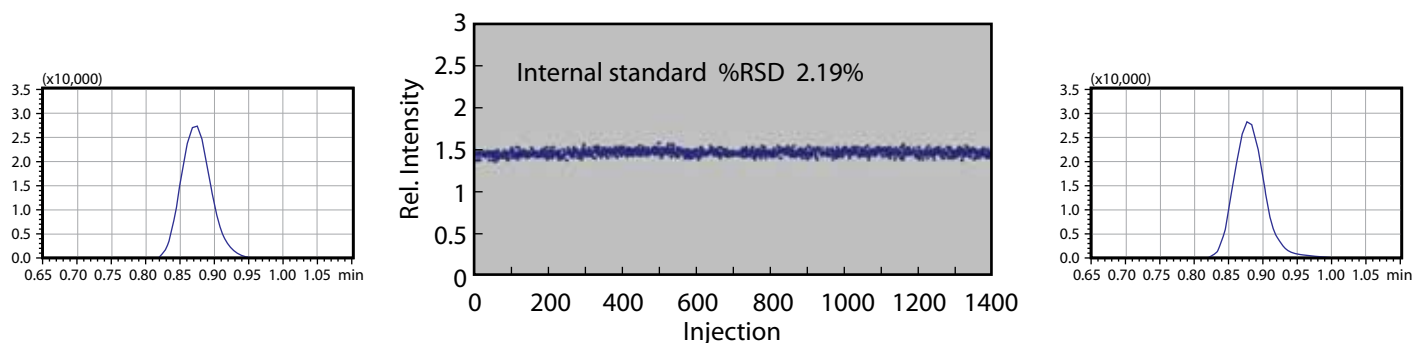


Figure 5: Results of long-term stability test; 1400 analyses (47 hours) of chromafenizide-spiked tomato extract

Ultra fast analysis coupled with a wider dynamic range has been achieved with an ultra fast pulse-counting detector and conversion dynode system as shown in Figure 6. The development of a unique semi-floating high-voltage power supply realizes ultra high-speed polarity switching (15msec).

Std. Conc. ng/mL	Conc. ng/mL	Accuracy %	Area % RSD (n=6)
0.005	0.0058	116.35	7.75
0.01	0.0108	109.37	3.36
0.05	0.0477	95.38	3.11
0.1	0.0907	90.55	1.43
0.5	0.4708	94.15	0.79
1	0.9702	97.02	1.29
5	4.9995	98.98	0.31
10	9.6907	96.92	0.70
50	49.9108	99.83	0.58
100	100.4682	100.48	0.40

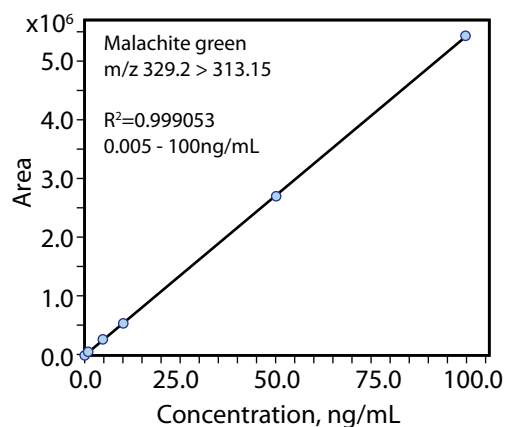


Figure 6: 10-point calibration curve of malachite green from 5 ppt to 100 ppb; the calibration curve shows a linear range of 4.5 orders of magnitude

Minimize Instrument Downtime with Easy Maintenance

With the LCMS-8030, maintenance has never been simpler or more accessible. The robust design of the LCMS-8030 allows maximum uptime and results in a system that can handle the most complex matrices. Easy maintenance of the desolvation line without breaking vacuum minimizes instrument downtime (Figure 7).

Figure 7: Simple assembling and disassembling of DL (Desolvation Line)



Single-vendor Solution Provides Seamless Operation

The combination of Shimadzu's LCMS-8030 and Nexera UHPLC brings together the latest hardware on a single platform for the next generation of ultra fast technology. The unified platform provides unmatched qualitative and quantitative analysis, increased productivity, and accelerated workflows for high-throughput data analysis (Figure 8). Also, all software operations are handled seamlessly, reducing PC conflicts and the need for user intervention.

The Quantitation Browser in LabSolutions LCMS software provides intuitive functionality that contributes to more efficient data processing. With the Quantitation Browser, peak information, quantitative results, and statistical calculations of a series of data can be rapidly viewed in a single window as shown in Figure 9.

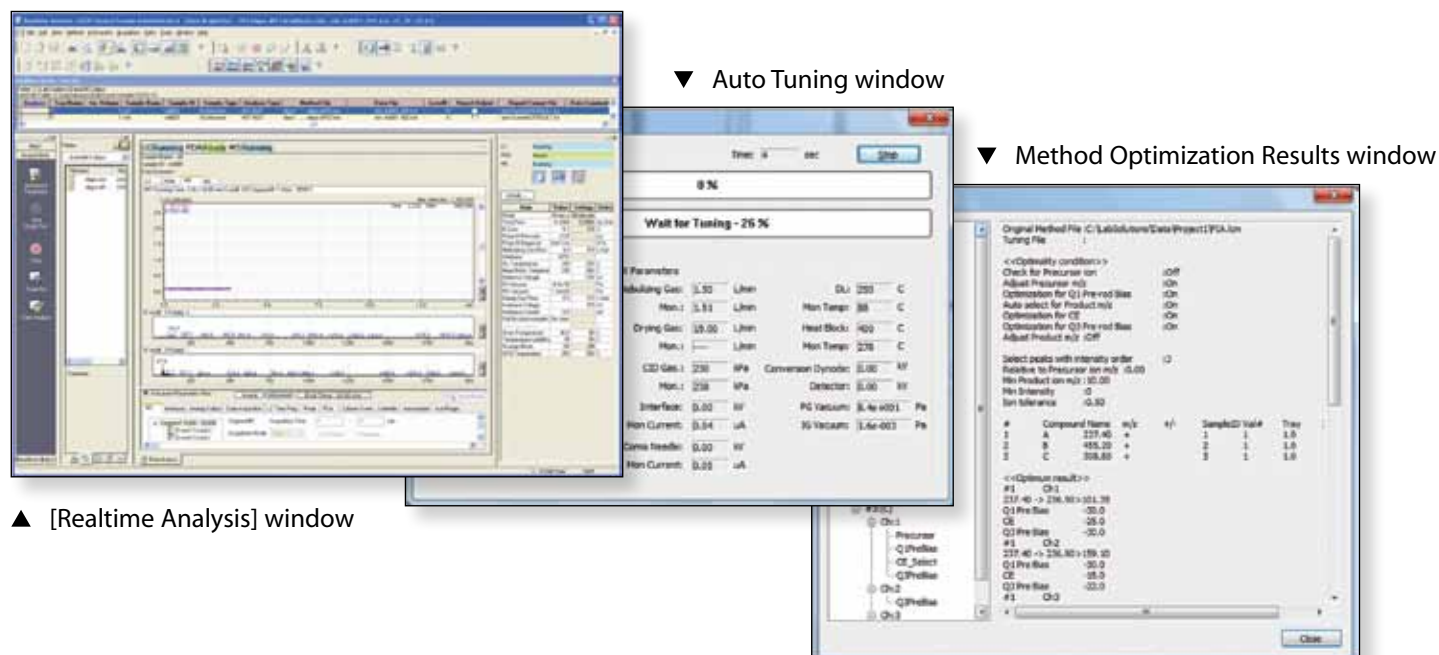


Figure 8: Operation windows of LabSolutions

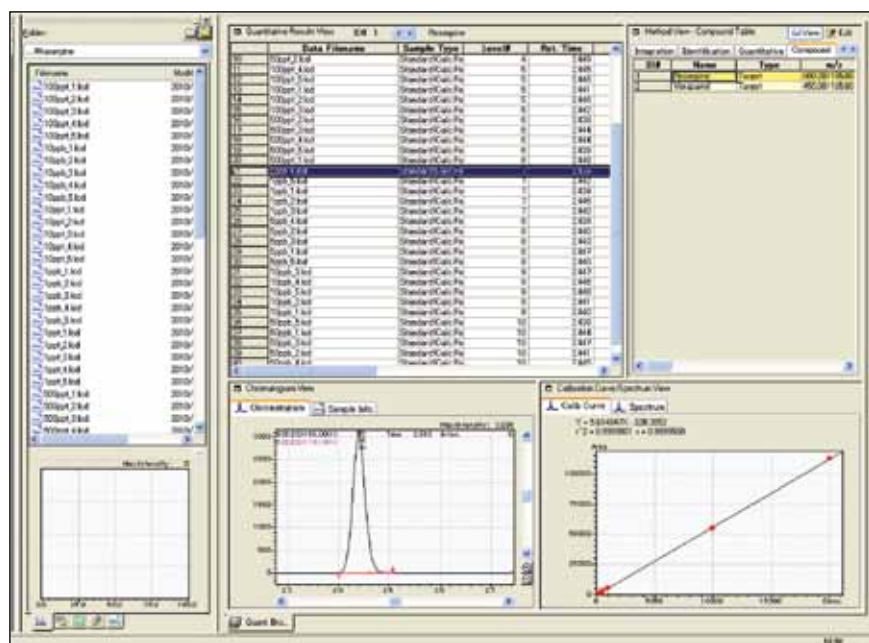


Figure 9: Quantitation Browser window

Nexera



the World's Only No Compromise UHPLC

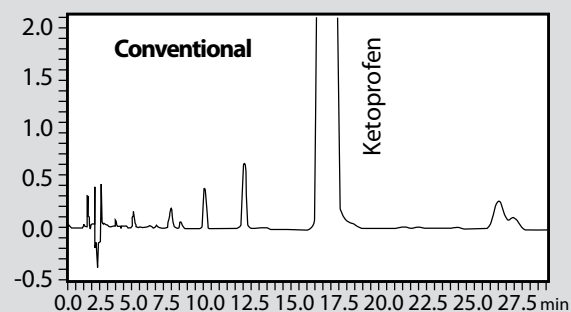
Nexera UHPLC is the most powerful tool to save cost and time per sample, not only offering speed but also superior data quality, versatility, ruggedness, and performance for all applications. The maximized pressure range and unparalleled system quality guarantee maximum performance in all fields.

Nexera addresses all analysis requirements, including conventional/ultra-high speed/ultra-high resolution/high temperature, with no compromise in data integrity. The pressure range of up to 130 MPa allows Nexera to maximize performance of sub-2-micron particle columns. The combination of this maximized pressure range and high-resolution columns realizes genuine ultra-high speed and ultra-high resolution analysis.

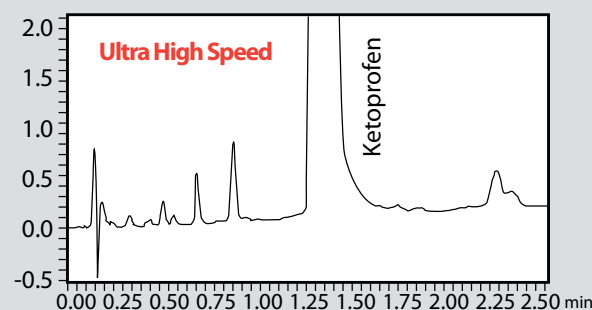
The following are examples of analytical data obtained using Nexera UHPLC. The data demonstrates the ultra-high speed and ultra-high resolution that can be obtained with this system. ☒

Features

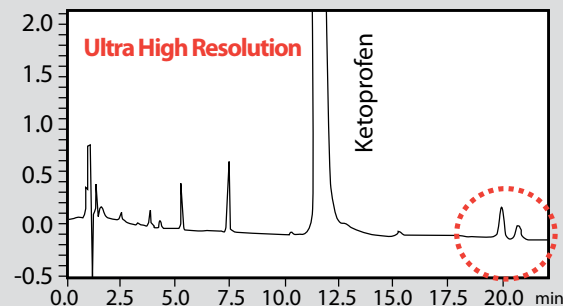
- The stable UHPLC solvent delivery up to 130 MPa (19,000 psi) offers the widest analysis range, enabling the use of sub-2- μ m and fused-core columns as well as conventional columns.
- The micro plunger-driven precision solvent delivery control and high-efficiency low-volume mixer (20 μ L) guarantee precise gradient delivery even at the rapid concentration shift in fast gradient programming.
- Nexera's SIL-30AC autosampler features the world's fastest sample injection; in addition, it now includes auto-pretreatment and overlapping functions as standard as well as an optional loop-injection method configuration to minimize delay volume.
- The SIL-30AC also ensures a new level of low carryover. Reduction of the needle contact area, special coatings, surface treatments, a new needle seal and thorough rinsing of the sample path with multiple rinse solvents provides strong support for high-sensitivity analyses, such as LC/MS/MS analysis.
- The Rack Changer II accommodates up to 12 sample plates to allow up to 4608 samples, incorporating a 4 to 40°C cooling function.
- Nexera achieves excellent injection-volume reproducibility, even with a 0.1 μ L injection step, and excellent retention time reproducibility to ensure UHPLC data quality.
- The CTO-30A column oven provides precise temperature control up to 150°C with a newly designed small-volume pre-heater, a proprietary Intelligent Heat Balancer and optional column cooling to minimize temperature distribution.
- Nexera has been built around modular flexibility, allowing multiple system configurations to address a variety of applications, including multidimensional LC, online SPE, low-pressure gradient configuration and method development.
- Nexera is easily compatible with Shimadzu LCMS and third-party mass spectrometers.



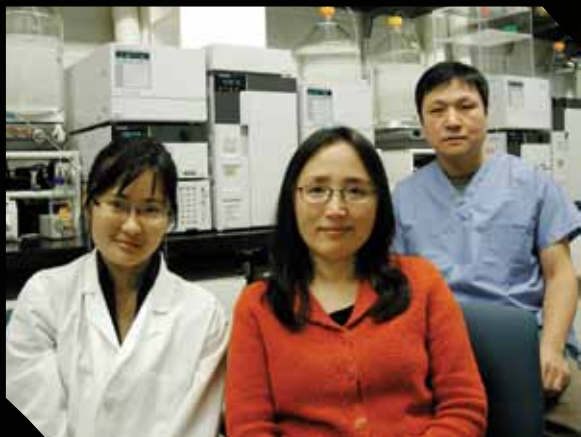
Column : ODS (150 mmL x 4.6 mm I.D., 4.6 μ m)
Mobile Phase : 0.1% Formic acid in Water/Acetonitrile = 65/35
Flow Rate : 1.0 mL/min
Column Temp : 40°C
Detection : UV 254 nm
Pressure : 6.5 MPa



Column : ODS (50 mmL x 2.1 mm I.D., 1.8 μ m)
Mobile Phase : 0.1% Formic acid in Water/Acetonitrile = 65/35
Flow Rate : 0.8 mL/min
Column Temp : 40°C
Detection : UV 254 nm
Pressure : 50 MPa



Column : ODS (250 mmL x 2.1 mm I.D., 1.8 μ m)
Mobile Phase : 0.1% Formic acid in Water/Acetonitrile = 65/35
Flow Rate : 0.5 mL/min
Column Temp : 40°C
Detection : UV 254 nm
Pressure : 118 MPa



Authors: Liang Samantha Zhang, left; Jimo Borjigin, middle; Tiecheng Liu, right.



Long-term Automated HPLC Analysis of Microdialysis Samples from Multiple Freely Moving Animals

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Circadian rhythms are present in almost all organisms and biological processes. These daily rhythms control homeostasis and influence various aspects of life, including health, behavior and cognitive functions. The best marker for studying circadian rhythms is the hormone melatonin produced in the pineal gland of mammals. Unlike activity, heart rate or body temperature, melatonin production rhythm is very precise and is less influenced by hunger, sickness or stress.

To fully take advantage of melatonin as a circadian marker, the Borjigin Laboratory developed a method to automatically analyze consecutive samples of pineal dialysates from multiple rats. With the current system configuration, each rat is sampled every 20 minutes in experiments that can last up to two months.

The ability to frequently measure an individual's pineal secretion allows for the precise definition of melatonin onset and offset, the identification of individual differences, as well as detection of change in circadian phase under various experimental conditions. The automation and high throughput of this technique bypass the labor-intensive and error-prone manual handling of dialysates, and provide an accurate profile of daily melatonin production rhythm.

METHOD

Overview

The HPLC system consists of one Shimadzu SCL-10A VP controller, two Shimadzu LC-20AD isocratic pumps, a CTO-20AC column oven containing 2 Supelco C18 reversed phase columns, two RF-10AXL detectors, two VICI Cheminert® sample injectors (2-position/10-port actuator), and a VICI digital sequence programmer. Each system is designed to analyze pineal dialysates from four rats, with two rats to each detector.

As shown in Figure 1, detectors A and B analyze dialysates from rats A1 and B1 simultaneously for 10 minutes, then switch to rats A2 and B2. Each rat is thus analyzed every 20 minutes, and consecutive samples can be measured for up to two months.

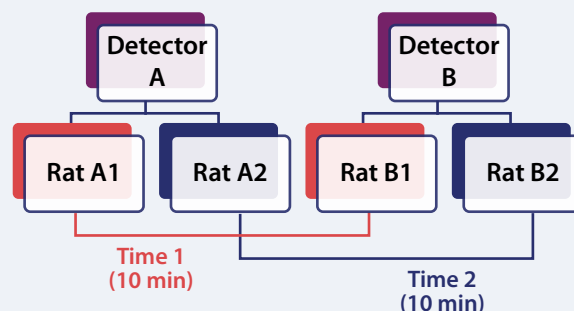


Figure 1: VICI digital sequence programmer controls the 2-position/10-port actuator. Detector A analyzes dialysate from rat A1 while detector B simultaneously analyzes rat B1. Dialysates from rats A2 and B2 are then subsequently analyzed. The analysis switches back and forth between rat 1 and rat 2 every 10 minutes.

Sample Acquisition

Rats are implanted with microdialysis probes through their pineal gland. For a detailed description of probe construction and surgery, see (Borjigin and Liu 2008). Artificial cerebral spinal fluid is delivered to each implant via Instech peristaltic pumps, with two rats per pump at 2 μ l per minute. Each rat is linked to the peristaltic pump through a series of PEEK tubing connected through an Instech dual-channel swivel. The swivel is mounted on a counterbalance arm providing both vertical and horizontal mobility.

Freely moving rats tethered to the swivel are housed individually in cages situated in light controlled chambers. Dialysates are collected and delivered to the HPLC system through the sample injector (Figure 2). Two rats are connected to each sample injector. Staggered sample collection and analysis doubles the output of this system; while the dialysate from one rat is analyzed, the dialysate of the other rat is collected in the 20 μ L loop of the sample injector, with the excess running off into the waste. Every 10 minutes, the sequence programmer gives a signal to the fast microelectric actuator, and the previously collected dialysate is injected from the 2-position/10-port valve.

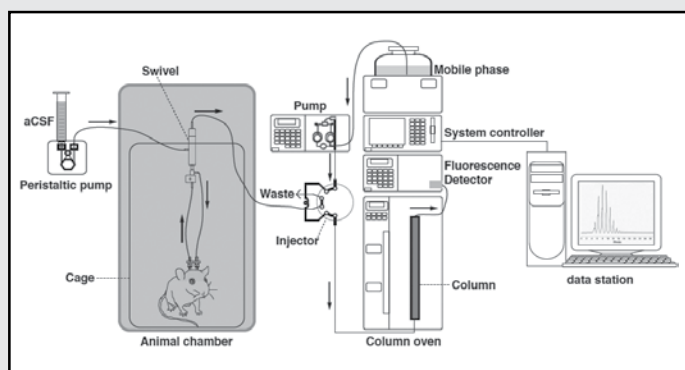


Figure 2: The general outline of the HPLC system. Components and connections for analysis of one rat are shown. Artificial cerebral spinal fluid is pumped through the pineal microdialysis implants. The dialysate from the pineal gland is collected in the sample injector, which is then injected into the HPLC column. For simplicity, a 1-position/6-port valve is shown instead of the 2-position/10-port valve used in the actual sample injector. Excess dialysate and mobile phase are delivered into waste lines.

Data Analysis

For the purpose of circadian rhythm studies, the peaks of interest on a chromatograph are melatonin (MT) and its precursors, serotonin (5-HT) and N-acetylserotonin (NAS). These indoles are naturally fluorescent (Chin 1990). Separation of each sample is conducted by reversed phase C18 column, maintained at 45°C. The mobile phase is pumped at 1.5mL per minute, and consists of 34% methanol with about 10 mM sodium acetate. Due to slight differences in each system, the exact concentration of sodium acetate must be adjusted for each detector so that the NAS, 5-HT and MT peaks are present and distinct during each run.

The final adjusted retention times of the three peaks from nighttime pineal dialysates are shown in Figure 3. Note that dialysates are directly analyzed without any purification process, and it is of importance to ensure additional peaks do not interfere with the peaks of interest.

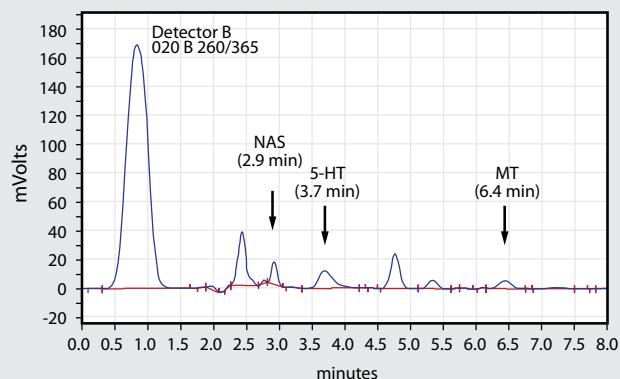


Figure 3: A typical nighttime trace of a single pineal dialysate sample shows the peaks for N-acetylserotonin (NAS), serotonin (5-HT) and melatonin (MT) at their ideal retention times. The acquisition time window is 8 minutes.

Data collection and sequence processing is performed on CLASS-VP firmware from Shimadzu. The sequence consists of two alternating methods set up for two detectors. Method 1 pertains to rat A1 and B1 and method 2 pertains to rat A2 and B2. The acquisition time window for each run is 8 minutes followed by 2 minutes of system equilibration, for a combined time of 10 minutes for each method.

The sequence is processed daily, and the resulting report is pasted into a preformatted Microsoft Excel worksheet. The baselines of certain MT peaks are checked and adjusted manually if necessary – these include the daily maximum value, as well as peaks during the rising and falling phases of MT production.



Experiments

Rats are maintained in 24-hour cycles of light and darkness. Data obtained every 20 minutes for each rat form a profile of pineal NAS, 5-HT and MT concentrations through time. Figure 4 shows two consecutive days of data for a rat housed in cycles of 12 hours of light (6am to 6pm) and 12 hours of darkness (6pm to 6am). The concentration of 5-HT remains relatively constant, spiking only right before onset of NAS and MT.

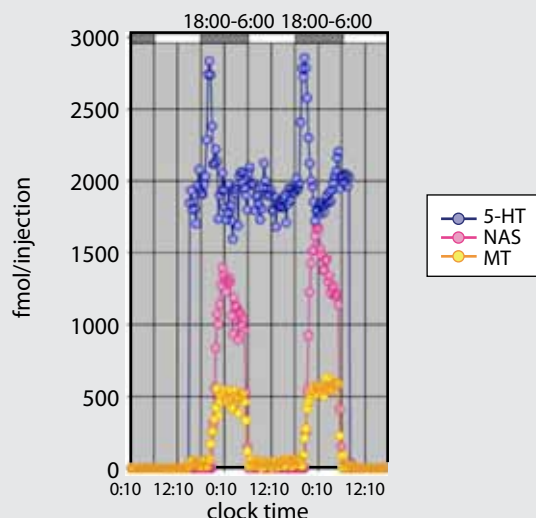


Figure 4: Two consecutive days of data from a single rat are displayed, showing the concentrations of NAS, 5-HT and MT produced in the pineal gland over time. The shaded bars above the graph indicate periods of darkness.

Both NAS and MT are detectable only at night; their concentrations rise dramatically several hours into the night, and fall to basal levels before the light comes on each day. These rhythms are so consistent that the daily MT profile looks almost identical when the rat is entrained to constant conditions (Figure 5). The precision and frequency in which the data are obtained through the automated HPLC system allows for an unambiguous determination of MT onset and offset, as defined by when the MT concentration rises above and falls below 20% of the nightly maximum value (represented by the dotted line in Figure 5).

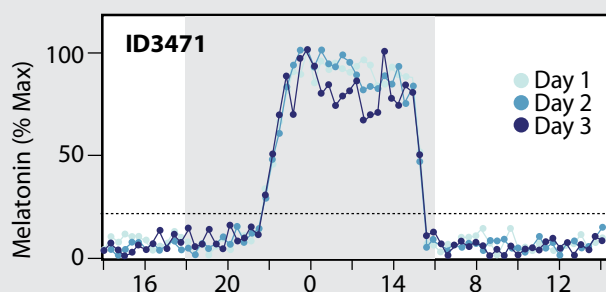



Figure 5: The daily MT profiles of a single rat are shown for three consecutive days. The gray shaded area from 18:00h to 6:00h represents time of darkness. The y-axis displays a percentage of the daily maximum value. The dotted line represents 20% of the daily maximum value, defining MT onset and offset timing.

Conclusions

As other circadian markers such as activity, heart rate and body temperature could only give a rough estimate of daily rhythmic onset, the ability to distinctly and clearly identify both onset and offset of the melatonin rhythm for long periods of time within the same individuals offers many new insights into the circadian system.

Through this technique, individual circadian chronotypes can be identified (Liu and Borjigin 2006). Also, the duration in which an individual adjusts to a new schedule (commonly known as jetlag) can be clearly determined by noting the number of days it takes for both the MT onset and offset to re-stabilize after a change in the lighting schedule (Liu and Borjigin 2005). Individual differences in re-adjusting to jetlag can also be determined through this technique.

Furthermore, the dynamics in which the MT onset and offset independently shift during jetlag is interesting in and of itself, and may inform us of the molecular dynamics of the central circadian clock. Various types of drugs, such as isoproterenol, can be infused directly to the pineal gland through the microdialysis probes or delivered to animals via systemic injection, and their effects on the circadian system can be easily monitored across multiple cycles via the automated HPLC system.

Online microdialysis integrated with real-time HPLC analysis is a powerful tool for providing information on the circadian pacemaker, especially in combination with molecular and pharmacological tools. Other applications of this system are certainly possible for the long-term in-vivo analysis of various biological substances in freely moving animals. 

Citations

Borjigin J and Liu T (2008) Application of long-term microdialysis in circadian rhythm research. *Pharmacol Biochem Behav* 90:148-155.

Chin JR (1990) Determination of six indolic compounds, including melatonin, in rat pineal using high-performance liquid chromatography with serial fluorimetric-electrochemical detection. *J Chromatogr* 528:111-121.

Liu T and Borjigin J (2005) Reentrainment of the circadian pacemaker through three distinct stages. *J Biol Rhythms* 20:441-450.

Liu T and Borjigin J (2006) Relationship between nocturnal serotonin surge and melatonin onset in rodent pineal gland. *Journal of Circadian Rhythms*.

Impurities Analysis in Pharmaceuticals:



Genotoxicity is the property of a compound known to have irreversible effects on the structure and functionality of the DNA in cells and cause DNA loss, DNA replication errors, mutations, and chromosomal abnormalities. Knowledge of genotoxicity information is vitally important from a safety perspective when developing new pharmaceuticals.

At the end of 2008, the FDA issues a draft guidance, “Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches.” This guidance established and applied a Threshold of Toxicological Concern (TTC) to genotoxic impurities in pharmaceutical ingredients.

The TTC indicates the acceptable daily intake that presents no genetic risk to humans below this threshold. It is effectively an estimated safely value such that the risk of developing cancer throughout the person’s life does not exceed one in a million. It defines the acceptable daily intake as 1.5 ug max., assuming that the person takes the drug over 12 months (Table 1). For example, for a daily dose of 30 mg, the acceptable daily intake concentration of the impurity is given by:

$$\text{Concentration Limit (ppm)} = \text{TTC (mg/day)} / \text{Dose (g/day)} = 1.5 \text{ (mg/day)} / 0.03 \text{ (g/day)} = 50 \times 10^{-6} (= 50\text{ppm})$$

Compared to the impurity value in the previous ICH guidelines, this demands higher detection sensitivity for the analysis of ultra-trace impurities.

	CLINICAL TRIAL PERIOD AND EXPOSURE PERMITTED VALUE					
Dosing period	≤ 14 Days	14 days - 1 month	1-3 months	3-6 months	6-12 months	≥ 12 months
Threshold values (µg)	120	60	20	10	5	1.5

Table 1: Permitted values for genotoxic and mutagenic impurities under the FDA draft guidelines

FDA Regulations on Genotoxic Impurities in Pharmaceuticals



Structural Analysis of Ultra-trace Impurities Using 2D-LC/LCMS-IT-TOF

The FDA draft guidance was issued to cover the verification of the genotoxicity and carcinogenicity of ultra-trace impurities in new pharmaceuticals, investigational new drugs, and generic pharmaceuticals by structural analysis of ultra-trace impurities. This demands structural analysis of ultra-trace impurities in a 200 mg daily drug dose at approximately 150 times lower levels than under the ICH guidelines. LCMS-IT-TOF analysis, which provides precise mass measurement and MSⁿ spectra, is an effective method for the structural analysis of ultra-trace impurities.



Involatile mobile phase such as phosphate buffer is commonly used for HPLC methods in quality control. Changing the mobile phase conditions to make them suitable for atmospheric ionization when performing LC/MS analysis is required. The modification of mobile phase sometimes causes problems in the identification of impurities due to changes in the elution pattern and requires additional labor to optimize method conditions. This modification process has become a bottleneck, causing a delay in the feedback required by production departments.

This 2D-LC design, which enables on-line desalting and separation of ion-pair reagents, allows for structural analysis of impurities by mass spectrometry without relying on the LC separation conditions. By using LCMS-IT-TOF for detection, it provides MSⁿ capability by the QIT and high-resolution/precise mass measurement capability by TOF.

The precise mass measurement in MSⁿ is not possible by typical LC/MS/MS instruments. Shimadzu also offers a versatile software solution to support impurity analysis workflow covering detection of ultra-trace impurities, composition prediction and structural prediction. MetID Solution Software for metabolite identification is capable of automated extraction of structurally-relevant peaks using MSⁿ spectral similarity. Formula Predictor Software is capable of formula prediction not only using mass accuracy but also combining isotopic pattern scoring, chemical rule and MSⁿ filtering.

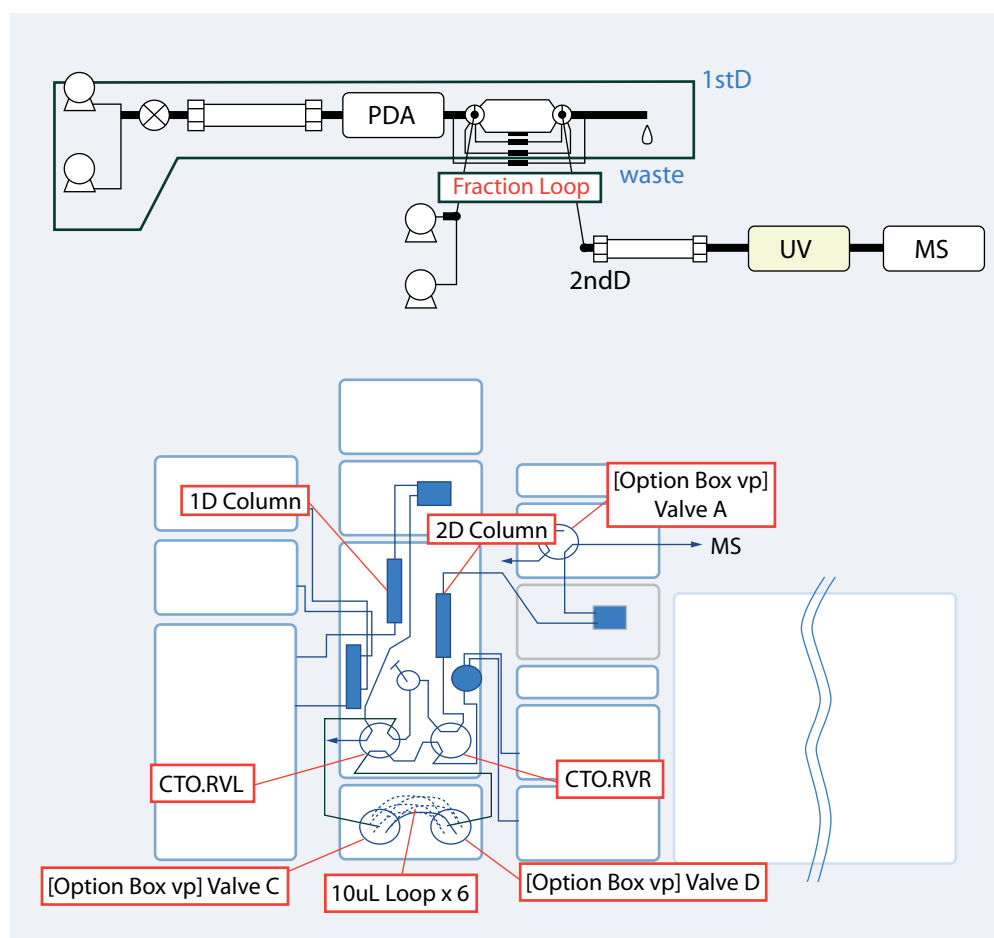


Figure 1: System configuration of 2D LC/LCMS-IT-TOF system

System Workflow

The 2D-LC/LCMS-IT-TOF consist of three parts: 1st dimensional part for impurities separation, target impurities fractionation part, and 2nd dimension analyzer (LCMS) as shown in Figure 1. Target impurities are fractionated in the loops between two valves and then delivered to the 2nd dimension for MS detection. Phosphate fractionated with impurities in the loops can be separated at the second dimension and eliminated from the divert valve between the second dimension column and the LCMS-IT-TOF mass spectrometer. This configuration enables efficient mass analysis of the target impurities without modification of analytical methods that use nonvolatile buffer conditions.

Sulfadimethoxine Impurities Analysis

Sulfadimethoxine-based sulfa drug impurities with four similar structures were prepared as model samples. The impurities were mixed with the main compound at 0.1 % each relative to the main compound. Concentration of the main compound was adjusted to be 500 ug/mL. The structures of the main compound and impurities are shown in Figure 2.

Figure 3 shows method conditions and the PDA chromatogram at the 1st dimension. Phosphate buffer for mobile phase additive was used. The system pressure trace (blue-colored) ensures that each impurity was successfully fractionated in the loops. The trapped impurities were detected by LCMS-IT-TOF using mobile phase conditions without phosphate buffer as shown in Figures 4 and 5. The accurate mass spectrum provided sufficient information to identify each impurity.

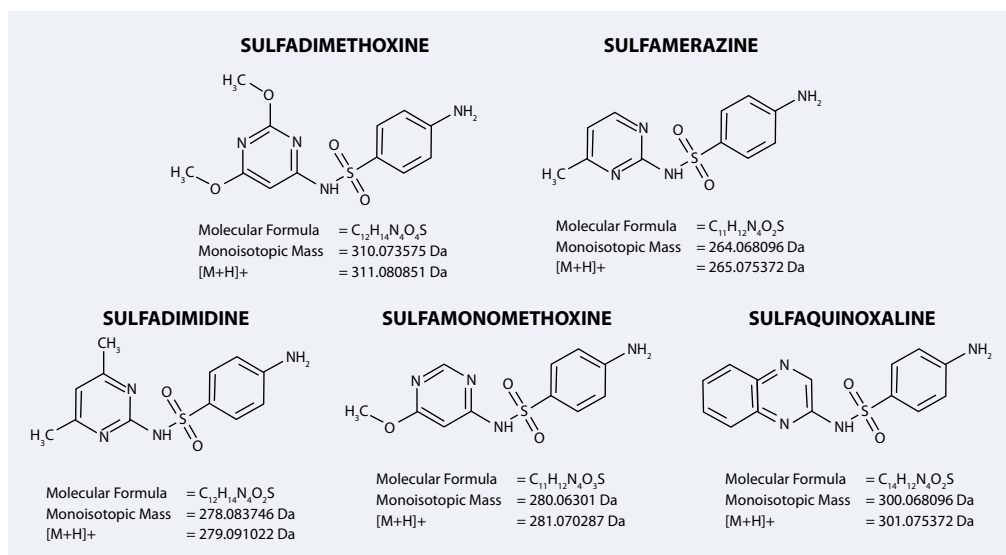


Figure 2: Structure of main compound and impurities



Analytical Conditions <LC 1stD>

Column : Shim-pack VP-ODS, 4.6mm i.d.x150mm, 5um
 Mobile phase : 0.01 mol/L Phosphate buffer (pH2.6)
 Methanol mixture
 Mobile phase flow rate : 1 mL/min
 Column temperature : 40 degrees C
 Sample injection volume : 10uL
 PDA detection wavelength : 200-350 nm (monitoring 270nm)

Analytical Conditions <LC 2ndD>

Column : Shim-pack XR-ODS 2.0mm i.d.x75mm, 2.2um
 Mobile phase A : 0.1% Formic acid solution
 Mobile phase B : Methanol
 Mobile phase ratio : 10%B(0min)-50%B(10min)-10%B(10.01-20min)
 Mobile phase flow rate : 0.3 mL/min
 Column temperature : 40 degrees C
 Sample injection volume : 10uL (Loop capacity)
 UV detection wavelength : 270nm

<MS>

Ionization mode : ESI+
 Nebulizer gas flow rate : 1.5 L/min
 Drying gas pressure : 0.15 MPa
 Applied voltage : 4.5 kV
 CDL temperature : 200 degrees C
 BH temperature : 200 degrees C
 Scan range : m/z 100-1000

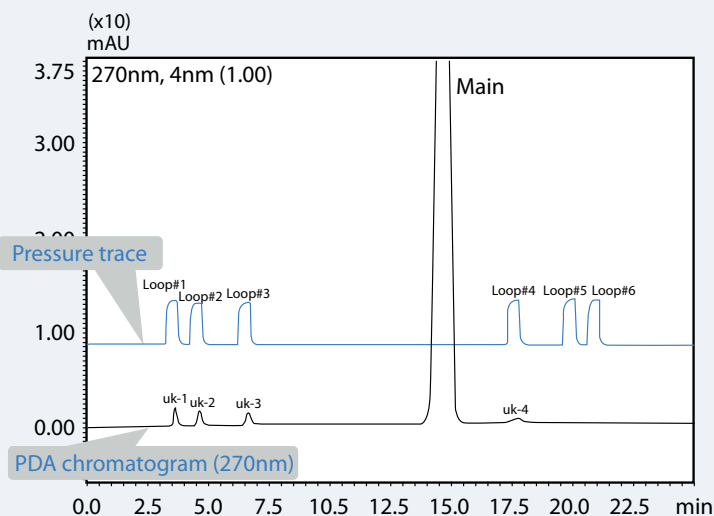


Figure 3: Method conditions and 1st-dimension chromatogram; the blue-colored trace displays the system pressure, and the black-colored trace shows the UV 270 nm signal

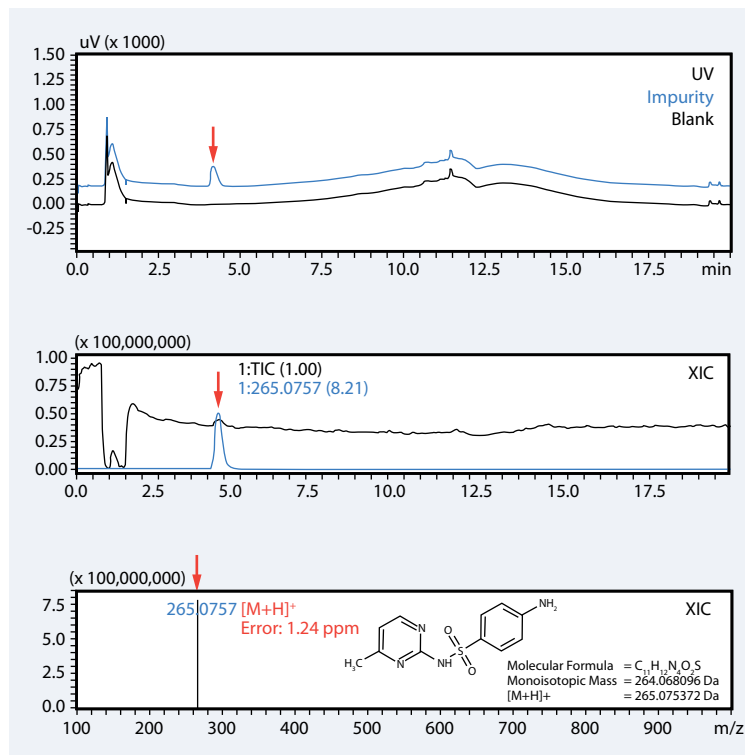


Figure 4: UV chromatogram, MS chromatogram and MS spectrum of Unk-1; blue trace: Sample, black trace: blank, red arrow: impurity

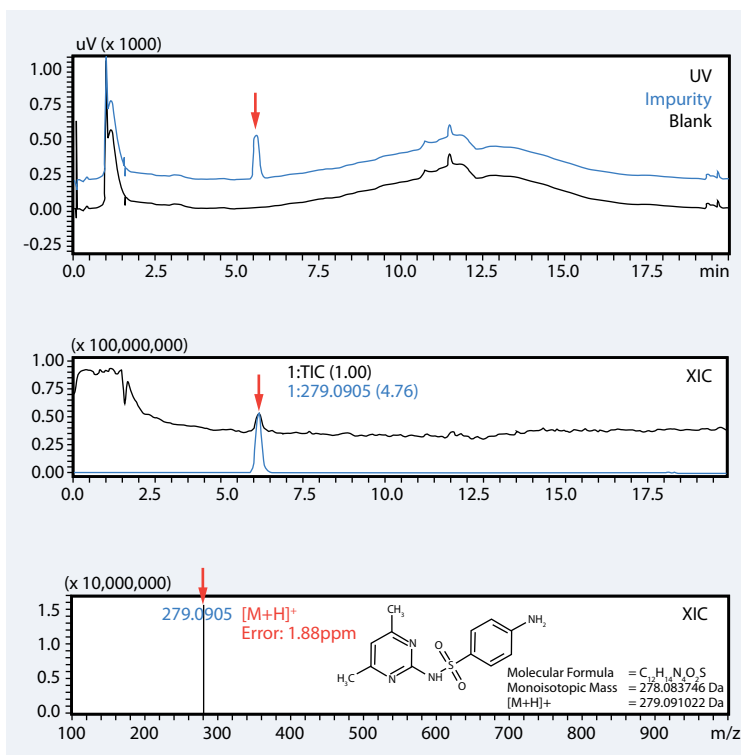


Figure 5: UV chromatogram, MS chromatogram and MS spectrum of Unk-2; blue trace: Sample, black trace: blank, red arrow: impurity



Quantitation of Ultra-trace Impurities Using the Co-Sense for Impurities System

While it's possible to use an LC/MS instrument for high-sensitivity quantitation of impurities, it's also desirable to use a highly sensitive quantitation method using a conventional detector, such as a UV detector, that provides stable analysis and low running cost. The Co-Sense for Impurities system automatically traps and concentrates target impurities eluted from a 1st dimension column. The system achieves an approximately 10 to 20-fold increase in sensitivity through the trap process and 2nd dimension separation. Dedicated software (Figure 6) adopts a graphic user interface that offers visual instrument monitoring and easy setting of method parameters.

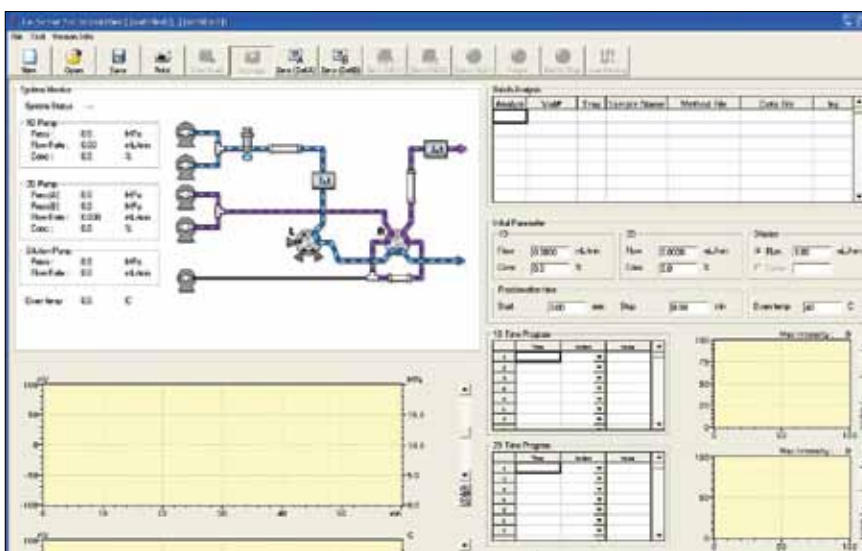


Figure 6: Co-Sense for Impurities software window

System Workflow

The flow diagram is shown in Figure 7. Target impurities are separated from main compounds and other impurities at the 1st dimension semi-preparative scale column. The target impurities eluted from the 1st dimension column are trapped on the trap column. On-line dilution by the additional pump assists in the effective trapping of impurities. The target impurities are introduced into the 2nd dimension analytical scale column, which not only provides further separation but also additional concentration of the target impurities by a difference of cross section between the 1st dimension and 2nd dimension columns.

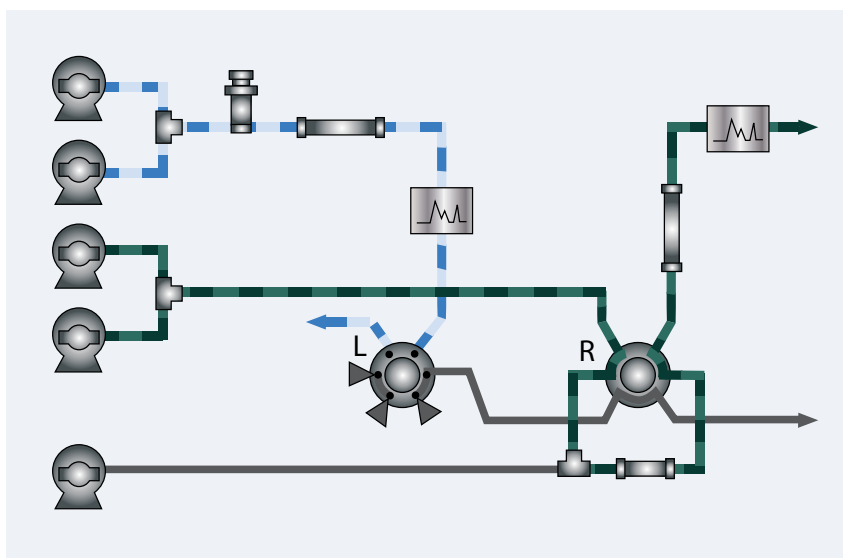


Figure 7: Flow diagram of Co-Sense for Impurities system



Comparison of Signal Intensity Using Test Sample

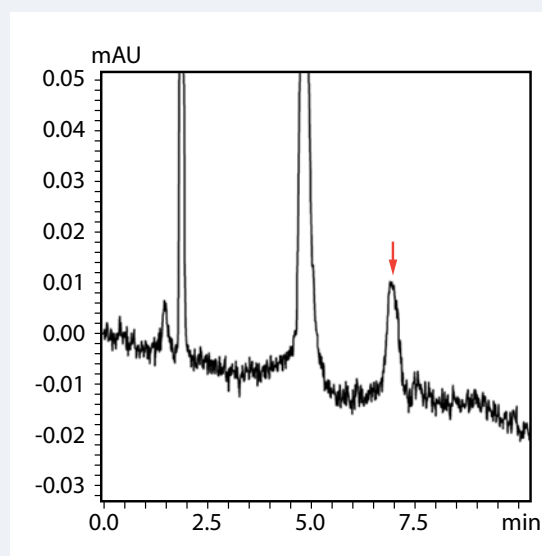
Signal intensity of an impurities peak was compared between the 1st dimension and 2nd dimension elution peaks using a methyl paraben solution in methanol (1ng/mL, 200 uL injected volume) as the test sample. As shown in Figure 8, the methyl paraben was effectively concentrated through the trapping process and the 2nd dimension separation process.

Analytical Conditions (First Dimension)

Column : Shim-pack VP-ODS 5 μ m
(10.0 mm i.d. x 150 mm L.)
Mobile Phase : A: 10 mmol/L Phosphate buffer
B: methanol, B. conc 40%
Isocratic
Flow Rate : 4.0 mL/min
Temperature : 40°C
Wavelength : 254 nm

Concentration/Trap Conditions

Column : Shim-pack PRC-ODS 5 μ m
(8.0 mm i.d. x 15 mm L.)
Dilution liquid : 10 mmol/L Phosphate buffer
Flow rate : 8 mL/min
Trap interval : 6.41 min \rightarrow 7.4 min



Analytical Conditions (Second Dimension)

Column : Shim-pack VP-ODS 5 μ m
(2.0 mm i.d. x 150 mm L.)
Mobile Phase : A: 10 mmol/L Phosphate buffer
B: acetonitrile
Gradient : 20% (8.01-13 min)
Flow Rate : 0.3 mL/min
Temperature : 40°C
Wavelength : 254 nm

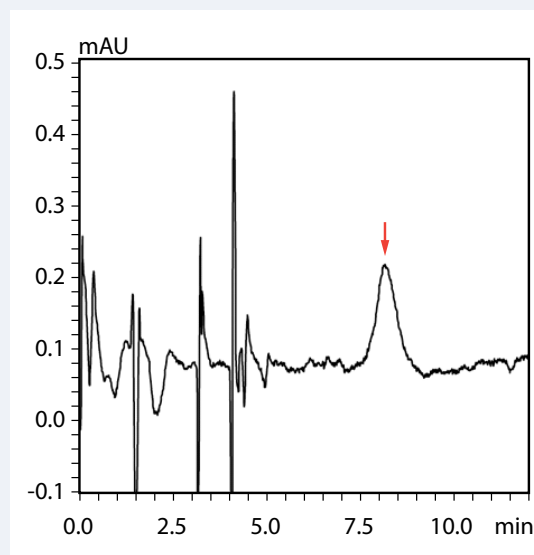


Figure 8: Comparison of signal intensity of methyl paraben between 1st dimension and 2nd dimension

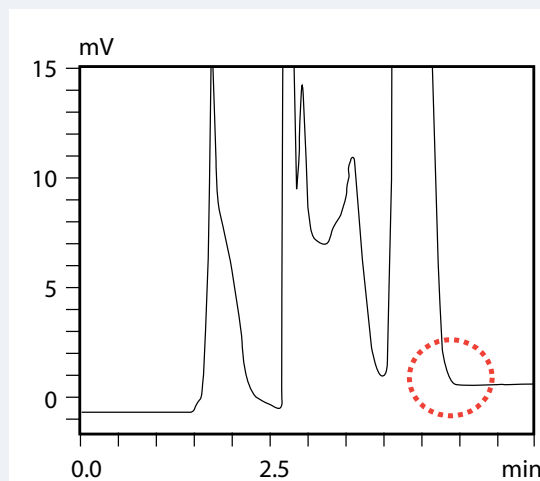


Analysis of Impurities in a Pharmaceutical

Caffeine as an impurity was spiked into a pharmaceutical (caffeine content: 0.0008%) to evaluate system performance. As shown in the 1st dimension chromatogram (Figure 9), other impurities co-eluted with caffeine. Effective concentration of the target impurity (caffeine) and optimization of separation conditions at the 2nd dimension enabled the detection of caffeine with high sensitivity and high resolution as shown in Figure 9 (pharmaceutical concentration: 0.5 mg/mL, injection volume: 1.5 mL).

Analytical Conditions (First Dimension)

Column	: Shim-pack VP-ODS 5 μ m (10.0 mm i.d. x 150 mm L.)
Mobile Phase	: A: 20 mmol/L phosphate buffer (pH 2.5) B: acetonitrile, B. conc. 15% Isocratic
Flow Rate	: 4.7 mL/min
Temperature	: 40°C
Wavelength	: 272 nm
Concentration/Trap Conditions	
Column	: Shim-pack PRC-ODS 5 μ m (8.0 mm i.d. x 15 mm L.)
Dilution Liquid	: 100mmol/L aqueous solution of ammonia acetate
Flow Rate	: 100mmol/L
Concentration	: 12mL/min
Interval	: 4.21 min \rightarrow 4.86 min



Analytical Conditions (Second Dimension)

Column	: Phenomenex Synergi 2.5 μ m Hydro-RP (3.0 mm i.d. x 100 mm L.)
Mobile Phase	: A: 100 mmol/L aqueous solution of ammonium acetate
Gradient	: B: methanol (gradient elution)
Flow Rate	: 0.4 mL/min
Temperature	: 40°C
Wavelength	: UV 272 nm

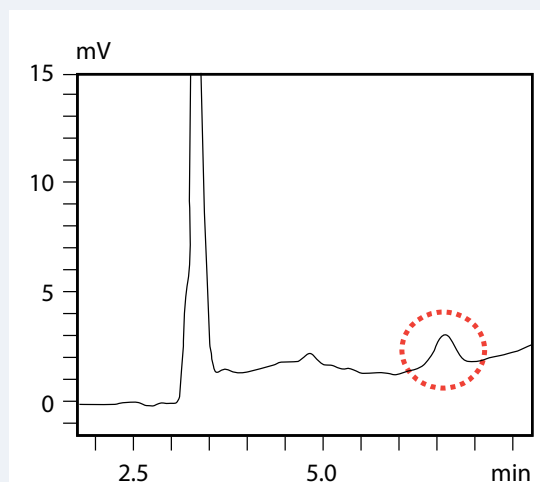


Figure 9: Analysis of impurities in a pharmaceutical by Co-Sense for Impurities system

MELAMINE ANALYSIS

Using the **LCMS-8030**



Melamine is an organic base that has been used to adulterate food products. Melamine's high nitrogen content causes spiked foods to appear to have higher protein content, and therefore higher quality, than they actually do. At low doses, melamine (Figure 1) is not particularly toxic in animals; however, at higher levels and when combined with its analogues such as cyanuric acid, melamine may crystallize in the kidneys and cause a variety of kidney-related problems.

A series of melamine scandals involving adulterated milk and pet food in recent years has harmed thousands of people and animals, and has resulted in the deaths of several dozen infants.



Regulatory limits have been imposed in many countries on the levels of melamine and related compounds in human and animal food. Given the large quantity of food products that require testing globally, extremely fast and robust methods for melamine and cyanuric acid analysis are in high demand.

A rapid LC/MS/MS analysis of melamine and cyanuric acid in food products was developed on a Shimadzu Nexera UHPLC and LCMS-8030 triple quadrupole mass spectrometer, with a run time of only 60 seconds. This method was used to measure levels of these substances in commercial pet food.

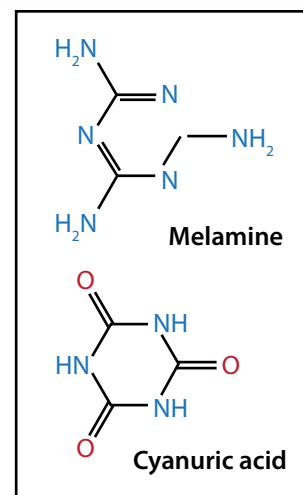


Figure 1: Structures of melamine and cyanuric acid

Method

Authentic standards for melamine and cyanuric acid were obtained and diluted for LC/MS/MS optimization. Standards were diluted to prepare a calibration curve ranging from 50 ppb to 10 ppm. A Phenomenex Kinetex HILIC column (2.1 x 50 mm, 2.6 μ m) was used at ambient temperature with an isocratic mobile phase of 90% acetonitrile in 100 mM ammonium acetate at 1 mL/min. Cyanuric acid and melamine eluted at 0.2 and 0.3 min respectively, and the total run time was 60 seconds.

Electrospray ionization was used with polarity switching. The multiple reaction monitoring (MRM) transitions of m/z 127->85 in positive mode (melamine) and m/z 128->85 in negative mode (cyanuric acid) were used. The collision energies and other MS parameters were optimized and are shown in Table 1. The ESI probe position and spray needle protrusion were adjusted to maximize the signal-to-noise ratio of the analytes. The nebulizing gas was 3 L/min, the drying gas was 20 L/min, the DL temperature was 300 $^{\circ}$ C, and the heat block temperature was 450 $^{\circ}$ C.

Dog food was obtained from a local grocery store, and crushed into fine powder. Melamine and cyanuric acid standards were each spiked into 200 mg pet food. Each sample was mixed with 10 mL of 10% acetonitrile in water, followed by vortexing and sonication for 15 min. Samples were centrifuged for 10 min at 3000 x g and 100 μ L of the supernatant was diluted ten-fold with mobile phase for LC/MS/MS analysis. Recovery was measured by comparing peak areas for an extracted standard against a spiked standard representing 100% recovery.

Type	Event#	+/-	Compound Name (m/z)	Dwell Time (msec.)	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)	Measurement Time
MRM	1	+	Melamine+H 127>85	10	-14	-20	-15	0-1
MRM	2	-	Cyanuroc acid-H 128>85	10	13	12	20	0-1

Table 1: MRM parameters

Results and Discussion

An extremely fast and reliable LC/MS/MS method for quantitation of melamine and cyanuric acid was developed. This method is over five times faster than conventional LC/MS methods for these compounds and does not require column equilibration between runs. The mass chromatograms from standards are shown in Figure 2 and those from a pet food sample extract are shown in Figure 3.

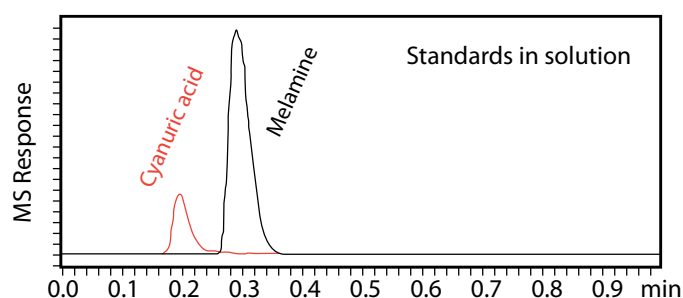


Figure 2: Chromatogram of cyanuric acid and melamine

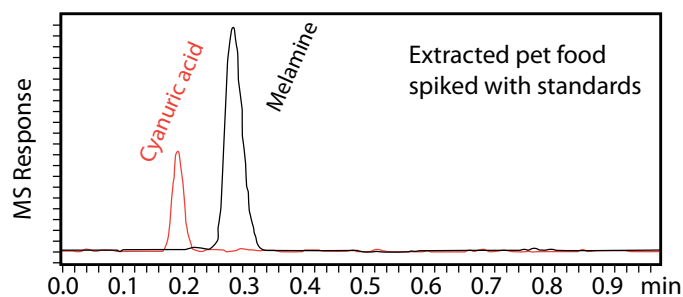


Figure 3: Chromatogram of extracted pet food spiked with standards

The use of a triple quadrupole mass spectrometer increases the selectivity of the method dramatically when compared with LC-UV or LC/MS methods using a single quadrupole mass spectrometer in selected ion monitoring (SIM) mode. For comparison, a SIM chromatogram of cyanuric acid is shown in Figure 4. At this concentration, background ions in SIM mode interfere with the detection of the analyte. By using the more selective MRM mode available on the LCMS-8030, the background was reduced and the analyte peak unambiguously detected.

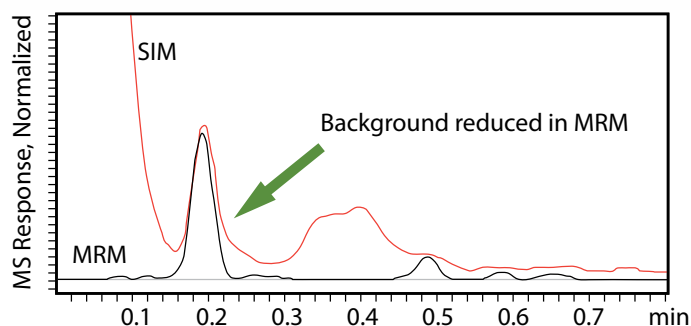


Figure 4: SIM and MRM chromatograms of cyanuric acid; MRM decreases the background, effectively increasing sensitivity

The calibration curve was linear in the tested range with an r^2 of 0.99 as shown in Figure 5. The limit of detection and quantitation were both less than 50 ppb, which is substantially lower than regulatory limits. The recovery was >90% and the reproducibility for three replicate samples was 7.03% RSD. Therefore this method is both sensitive enough to meet regulatory requirements as well as fast enough to process large numbers of samples in short timeframes.

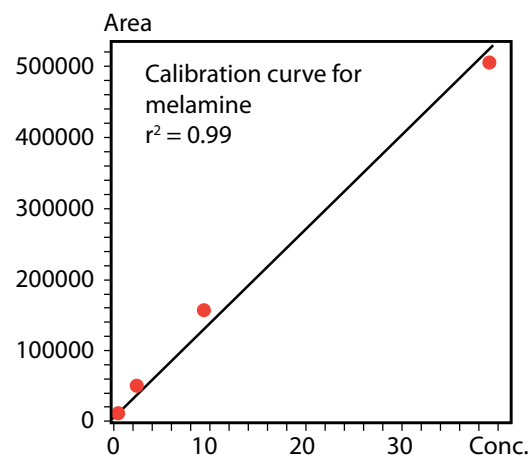


Figure 5: Calibration curve

Conclusion

This fast and reliable LC/MS/MS method for melamine and cyanuric acid is over five times faster than conventional methods for these compounds, with a run time under 60 seconds. The simple sample preparation involves only dilution and a one-step solvent extraction and can be automated for high-throughput sample analysis. The sensitivity is more than adequate to meet regulatory requirements while the speed and reliability enable rapid analysis of large numbers of samples. ☒

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