

MultiQuant™ Software Version 3.0 for Accurate Quantification of Clinical Research and Forensic Samples

Fast and Efficient Data Review, with Automatic Flagging of Outlier Results

Adrian M. Taylor and Michael Jarvis
AB SCIEX, Concord, Ontario, Canada

Introduction

Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) is a widely used analytical tool for the quantitation and identification of compounds in forensic and clinical research samples. Triple quadrupole based mass analyzers operated in Multiple Reaction Monitoring (MRM) mode (Figure 1A) deliver highly selective and sensitive quantitative results. Advancements in TripleTOF® technology enable targeted quantitation with triple quadrupole-like performance on a hybrid quadrupole / time-of-flight system (Figure 1B), with enhanced confidence in compound identifications based on accurate mass MS and MS/MS information. Quantitative information may be extracted from either the full-scan, high-resolution MS or MS/MS data, making accurate mass LC-MS/MS an interesting alternative for modern Clinical Research and Forensic labs.

Laboratories are increasingly adopting strategies to increase throughput, for example by using simplified and automated sample preparation techniques, and by multiplexing compound analyses by simultaneously screening for hundreds of target compounds in a single analysis using the *Scheduled MRM™* Pro algorithm and full-scan accurate mass techniques. These trends in increased laboratory throughput have placed greater demand on faster data processing and efficient data review, which has traditionally remained a significant bottleneck. The re-integration of peaks, the review of quantitative measurements, and the reporting of results are time consuming and labor intensive tasks.

To address the needs of modern Clinical Research and Forensic Toxicology laboratories, MultiQuant™ software version 3.0 has been designed with the goal of improving data processing efficiency, thereby accelerating the process of data review. Integrated within Analyst® software, MultiQuant™ 3.0 software enables the identification and quantification of chemicals of interest in complex samples from data files generated on AB SCIEX triple quadrupole, QTRAP®, and TripleTOF® systems.

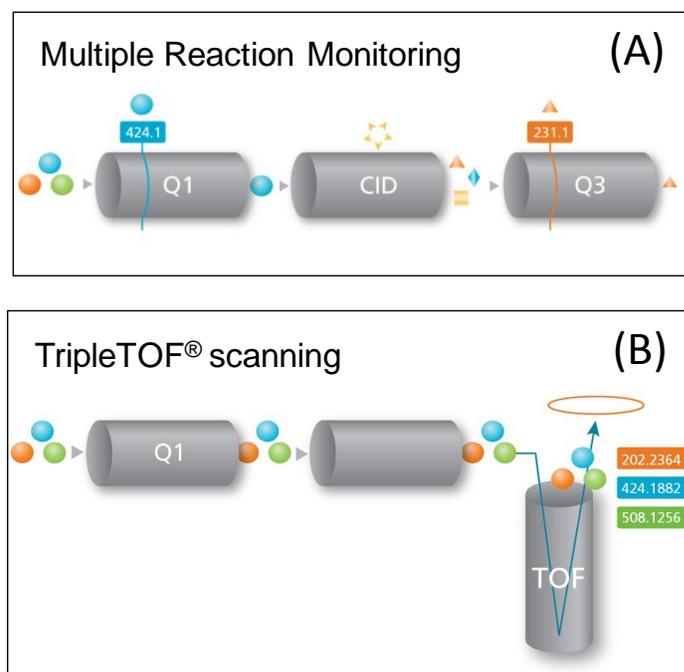


Figure 1. (A) Highly selective and sensitive quantitation using Multiple Reaction Monitoring (MRM) with triple quadrupole systems. **(B)** Highly selective and sensitive quantitation using narrow extracted chromatograms (XIC) of accurate mass TOF-MS ions generated using TripleTOF® systems

Key Features of MultiQuant™ Software Version 3.0

- Next generation data processing software from AB SCIEX with full support of Windows XP, Windows 7 (32 and 64 bit) operating systems
- Processes multiple data types from AB SCIEX triple quadrupoles, QTRAP®s and TripleTOF® systems, including data generated using the *Scheduled* MRM™ Pro algorithm and *Scheduled* MRM^{HR} as well as processing MRM³ data.
- Intuitive workspace for data processing
- Very powerful for handling multiple analytes
- Advanced Query and metric plot functionality to speed up data review
- Advanced SignalFinder™ algorithm delivers reliable integration the first time and extends dynamic range
- Processing of UV, DAD and ACD data

Recent improvements have been made in the review of the quantified data in the form of outlier flagging and ion ratio acceptance visualization features. This technical note highlights these innovative new features in MultiQuant™ software version 3.0 which significantly improve the data analysis workflow for quantitation and identification of compounds of interest in forensic and clinical research laboratories. These features include the following:

- Built-in queries for the automatic calculation and flagging of:
 - Outliers in accuracy
 - Analytes below or above a target concentration
 - Ion ratios and ion ratio tolerances
- Easy result review using the visual display of ion ratios
- Side-by-side peak review to quickly compare the response of samples
- Peak review magnifier for easy review and adjustment of (manual) peak integrations
- Additional result table columns to assess peak quality parameters, including Asymmetry Factor, Tailing Factor, Slope of Baseline, Peak Width, Points Across Peak

Experimental

Drugs of Abuse from Serum

Drug compounds were quantified and identified in serum samples after automated On-line Solid Phase Extraction (SPE) cleanup using a Spark Holland PICO System. On-line sample

cleanup was achieved using HySphere Resin GP (general purpose) 10; polydivinyl-benzene cartridges. The Spark Holland PICO was coupled to the AB SCIEX QTRAP® 4500 system for the complete On-Line SPE LC-MS/MS set up. The AB SCIEX QTRAP® 4500 system was used with Turbo V™ source and Electrospray Ionization (ESI) probe. The *Scheduled* MRM™ Pro algorithm was used to achieve best data quality while monitoring over 100 drugs using two MRM transitions per analyte to allow simultaneous quantitation and identification based on the ion ratio.

Drugs of abuse from Urine with Disposable Pipette Extraction (DPX) cleanup

130 drugs were monitored for after automated DPX sample cleanup using a Tecan EVO® 150 Liquid Handling System. A small amount of urine is pre-mixed with acetonitrile and aspirated into the DPX-WAX-S tip for mixing and sample clean up. Data was obtained using an AB SCIEX QTRAP® 4500 system in *Scheduled* MRM™ mode; coupled with a Shimadzu Prominence LC system.

High throughput screening; detecting and quantifying drugs in urine

The combination of the MPX™-2 High Throughput System and TripleTOF® 4600 LC/MS/MS System was used to investigate a high throughput screening approach with less than 50 seconds injection to injection. The AB SCIEX TripleTOF® 4600 system was operated with DuoSpray™ source and Electrospray (ESI) probe. The method consisted of a TOF-MS full scan and several looped TOF-MS/MS product ion scans for the 41 target compounds with an optimized Collision Energy (CE) used for each compound. 50 msec TOF-MS and 10 msec TOF-MS/MS accumulation time were used. Drugs in urine samples were quantified from the high throughput screening approach using MRM^{HR}. The different product ions detected in high resolution mode were used by MultiQuant™ Software Version 3.0 to automatically calculate ion ratios.

25-OH-Vitamin D3 and D2 in Serum

25-OH-Vitamin D3 and 25-OH-Vitamin D2 were extracted from commercially available serum calibration standards, using liquid-liquid extraction in 90:10 hexane/ethyl acetate. The extract was evaporated to dryness and reconstituted in LC mobile phase, and analyzed by LC/MS/MS, in positive APCI mode on the QTRAP® 4500 system. The total run-time was 3.7 minutes, and two MRM transitions were monitored for each analyte.

Flagging Outlier Results to Accelerate “Exception-Based” Data Review

MultiQuant™ 3.0 software features built-in queries, or quality control rules, that enable the user to specify compound-specific criteria for flagging outlier results. The user may choose to automatically flag results that exhibit potential problems with data quality – for example, discrepant qualifier/quantifier ion peak area ratios, or unacceptable accuracies for Standards and QC samples – or to highlight quantitative results that are above or below user-specified thresholds.

By automatically flagging results, the software enables “exception-based” data review. This accelerates the time to final result by quickly and easily calling the user’s attention only to those results that require additional review. The outlier settings are specified in the corresponding tab of the quantitation method editor, as shown in Figure 2, below.

Components | Integration & Regression | **Outlier Settings**

Accuracy for Standards Accuracy for QCs

Max. Accuracy Tolerance for LLOQ (lowest Std): % Max. Accuracy Tolerance for QC: %

Max. Accuracy Tolerance for Stds except LLOQ: %

Ion Ratio Calculated Concentration

Component	IS	Group	Ion Ratio Tolerance (%)	Lower Limit of Calculated Concentration	Upper Limit of Calculated Concentration
Acetaminophen 1	<input type="checkbox"/>	Acetaminophen		5	1000
Acetaminophen 2	<input type="checkbox"/>	Acetaminophen	20	5	1000
AKB48 1	<input type="checkbox"/>	AKB48		5	500
AKB48 2	<input type="checkbox"/>	AKB48	20	5	500
Alprenolol 1	<input type="checkbox"/>	Alprenolol		0.5	2000
Alprenolol 2	<input type="checkbox"/>	Alprenolol	20	0.5	2000
Amilofide 1	<input type="checkbox"/>	Amilofide		10	2000
Amilofide 2	<input type="checkbox"/>	Amilofide	20	10	2000
Amlodipine 1	<input type="checkbox"/>	Amlodipine		50	2000
Amlodipine 2	<input type="checkbox"/>	Amlodipine	20	50	2000
Avipiprazole 1	<input type="checkbox"/>	Avipiprazole		1	2000
Avipiprazole 2	<input type="checkbox"/>	Avipiprazole	20	1	2000
Atropine 1	<input type="checkbox"/>	Atropine		1	2000
Atropine 2	<input type="checkbox"/>	Atropine	20	1	2000
Beberine 1	<input type="checkbox"/>	Beberine		1	2000
Beberine 2	<input type="checkbox"/>	Beberine	20	1	2000
Betamethazone 1	<input type="checkbox"/>	Betamethazone		5	2000

Set criteria for flagging outliers.

Accuracy for Standards Accuracy for QCs

Max. Accuracy Tolerance for LLOQ (lowest Std): % Max. Accuracy Tolerance for QC: %

Max. Accuracy Tolerance for Stds except LLOQ: %

Ion Ratio Calculated Concentration

Component	IS	Group	Ion Ratio Tolerance (%)	Lower Limit of Calculated Concn...	Upper Limit of Calculated Concn...
Vit-D3 1	<input type="checkbox"/>	Vit-D3		30	70
Vit-D3 2	<input type="checkbox"/>	Vit-D3	20		
Vit-D2 1	<input type="checkbox"/>	Vit-D2		30	70
Vit-D2 2	<input type="checkbox"/>	Vit-D2	20		
Vit-D3-d6	<input checked="" type="checkbox"/>				

Figure 2. Built-in queries in MultiQuant™ software version 3.0 enable users to set compound-specific criteria for flagging outlier results that require further review. Flagging criteria include: accuracies for Standards and QC samples, ion ratios (ratio of qualifier ion/quantifier ion), and lower/upper calculated concentration limits for unknown samples. **Top:** Typical outlier settings for the analysis of drugs of abuse for a forensic toxicology drug screening method, in urine. **Bottom:** Example settings for a clinical research method used to analyze 25-OH-Vitamin D3 in serum.

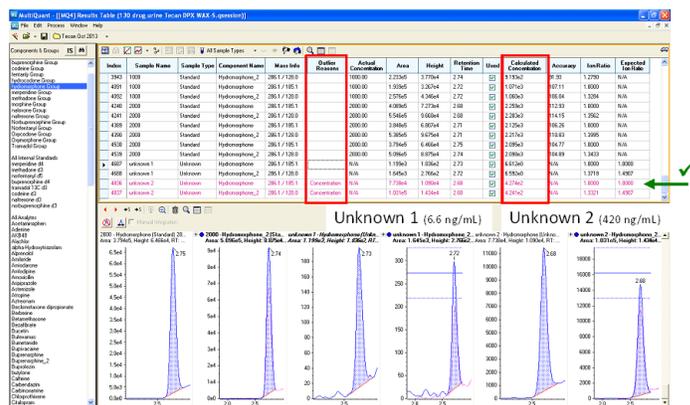


Figure 3a. Hydromorphone quantified in different urine samples. One sample was flagged (highlighted in red) because the measured concentration exceeded the user-specified cut-off level of 50 ng/mL, and the analyte was positively confirmed by the MRM ion ratio.

Index	Sample Name	Sample Type	IS	Component Name	IS Name	Component Group Name	Outlier Reasons	Actual Concentration	Calculated Concentration	Accuracy
106	25OH-D3_Std_73	Standard		Vit-D3 1	Vit-D3-d6	Vit-D3		73.00	72.22	98.94
111	25OH-D3_Std_73	Standard		Vit-D3 1	Vit-D3-d6	Vit-D3		73.00	75.24	103.07
116	25OH-D3_Std_73	Standard		Vit-D3 1	Vit-D3-d6	Vit-D3		73.00	71.09	97.39
121	Blank	Blank		Vit-D3 1	Vit-D3-d6	Vit-D3		N/A	N/A	N/A
126	Sample 001	Unknown		Vit-D3 1	Vit-D3-d6	Vit-D3		N/A	33.45	N/A
131	Sample 002	Unknown		Vit-D3 1	Vit-D3-d6	Vit-D3	Concentration	N/A	12.40	N/A
136	Sample 003	Unknown		Vit-D3 1	Vit-D3-d6	Vit-D3		N/A	30.02	N/A
141	Sample 004	Unknown		Vit-D3 1	Vit-D3-d6	Vit-D3	Concentration	N/A	76.70	N/A
146	Sample 005	Unknown		Vit-D3 1	Vit-D3-d6	Vit-D3		N/A	43.80	N/A
151	Sample 006	Unknown		Vit-D3 1	Vit-D3-d6	Vit-D3		N/A	51.45	N/A

Figure 3b. 25-OH-Vitamin D3 was measured in several unknown samples, with user-defined lower and upper concentration limits of 30 and 70 ng/mL, respectively. Two samples were automatically flagged by the software (highlighted in red) based on the measured concentrations.

Automatic Flagging of Analyte Concentrations, using Upper and Lower Limits

In forensic analyses, it is often useful to flag measured analyte concentrations above specific “cut-off” levels. In clinical research analyses, it is often desirable to highlight measured analyte concentrations that fall outside of an expected concentration range. The built-in flagging capabilities in MultiQuant™ 3.0 software allow the user to set both upper and lower concentration limits for every analyte in a method. The lower limit and upper limit of the calculated concentration can be defined directly in the quantitation method editor (Figure 2). Unknown samples having measured concentrations outside of the specified concentration limits are automatically highlighted in the results table. Figure 3 provides several examples of automatically flagged concentration measurements.

In Figure 3a, for the measurement of hydromorphone in urine, one sample is flagged (highlighted in red) because the concentration exceeds the user-specified cut-off level of 50 ng/mL. The qualifier/quantifier ion ratio matches the expected ion ratio, and therefore confirms the identity of the compound. A second unknown sample in Figure 3a also contains hydromorphone, as confirmed by the ion ratio, however the

calculated concentration does not exceed the user-defined cut-off level of 50 ng/mL, therefore the sample is not highlighted by the software.

In Figure 3b, 25-OH-Vitamin D3 was measured in several unknown clinical research samples, and the lower and upper concentration limits for automatic flagging were set to 30 and 70 ng/mL, respectively. In this example, two samples were automatically flagged by the software because the measured concentrations did not fall in the expected range. In one case the measured value of 12 ng/mL was below the lower concentration limit. In a second case the measured value of 76 ng/mL was above the upper concentration limit.

Automatic Flagging of Accuracies for Quality Control (QC) Samples and Standards

Quality Control (QC) samples provide feedback on the performance of the analytical method and assess the integrity and validity of the unknown samples analyzed within the run. Most laboratories establish method-specific acceptance criteria for the measured accuracy (%) of QC samples. Results that are outside the limits can indicate potential defects with the set-up of the assay, from the initial sample preparation step, to the data analysis step. By quickly identifying aberrant QC results, the potential of a defect can be investigated, corrected, and the test can be repeated if necessary.

Assessment of accuracy (%) for calibration standards is typically performed in order to determine the upper and lower limits of quantification for a method. Data points that do not pass the acceptance criteria are usually excluded from the calibration curve. As such, it is a great benefit to the reviewer to have automatic notification of samples that fail the user-defined acceptance criteria.

Set criteria for flagging outliers.

Accuracy for Standards

Max. Accuracy Tolerance for LLOQ (lowest Std): %

Max. Accuracy Tolerance for Stds except LLOQ: %

Accuracy for QCs

Max. Accuracy Tolerance for QC: %

Figure 4. MultiQuant™ 3.0 software allows users to specify Accuracy (%) tolerances for Standards and Quality Control (QC) samples.

MultiQuant™ 3.0 software provides the capability to automatically flag results for QC samples and standards where the accuracy (%) does not meet acceptance criteria. The “maximum accuracy % tolerance for QC”, and the “maximum accuracy % tolerance for standards”, are defined in the quantitation method, as shown in Figure 4. The user may choose to enter a global acceptance

criterion (e.g. 15%), and a separate acceptance criterion for the lowest calibrator (e.g. 20%), if desired.

Figure 5 displays several examples where results that do not pass the acceptance criteria for Accuracy (%) have been automatically flagged in the results table. This enables users to quickly identify problem areas, and to take the appropriate corrective actions.

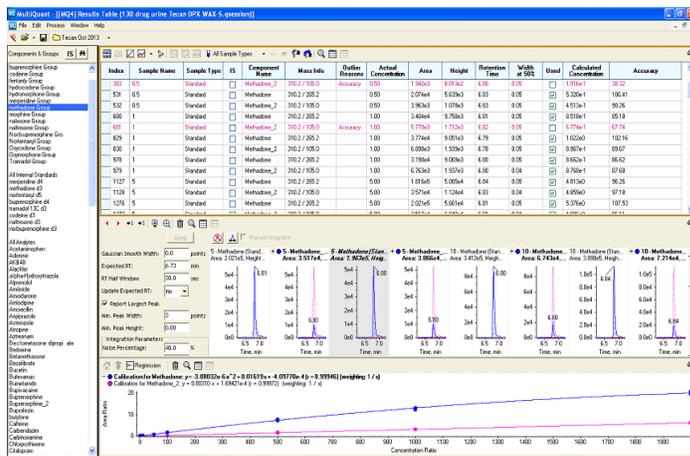


Figure 5a. Methadone detected in a urine screening method using a QTRAP® 4500 system with flagged outliers for the qualifier MRM transition at 0.5 and 1 ng/mL; highlighted in red. (calibration line of 0.5 to 2000 ng/mL with $r^2 = 0.999$ and %CV = 8.32% at 0.5 ng/mL, n = 3)

Index	Sample Name	Sample Type	IS	Component Name	IS Name	Component Group Name	Outline Floures	Actual Concentration	Calculated Concentration	Accuracy
26	25OHd3_Std_10	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		10.00	1.0971	109.66
31	25OHd3_Std_10	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3	Accuracy	10.00	1.2114	121.12
36	25OHd3_Std_10	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		10.00	8.9150	89.19
41	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	3.1031	103.45
46	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	3.1161	103.86
51	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	3.0451	101.52
56	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	2.9161	97.20
61	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	2.8351	94.43
66	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	2.9971	99.89
71	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	3.2491	108.29
76	25OHd3_Std_30	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		30.00	3.1761	105.86
81	25OHd3_Std_73	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		73.00	7.1561	98.01
86	25OHd3_Std_73	Standard	<input type="checkbox"/>	Vit-D3 1	Vit-D3-46	Vit-D3		73.00	7.4261	101.72

Figure 5b. Quality control rules have been applied to automatically flag standards with accuracies in excess of ±15% (±20% for the lowest standard) for 25-OH-Vitamin D3 measured in spiked serum standards. The analysis was performed using a QTRAP® 4500 system.

Automatic Calculation and Flagging of Analyte Ion Ratios for Compound Identification

Despite the high selectivity of MS/MS based measurements, there is always a risk of false positive findings due to interfering matrix signals. Accordingly, it is preferable to confirm any quantitative results using additional qualitative criteria. Often, a second “qualifier” fragment ion is monitored per analyte, and the ratio of the chromatographic peak areas for the “quantifier” and “qualifier” signals is calculated in each sample. The measured qualifier/quantifier ion ratio for unknown samples is compared to the expected ion ratio, which has been pre-determined from injections of unadulterated standards containing the analytes of interest.

MultiQuant™ 3.0 software automatically calculates, and compares, the measured and the expected ion ratios for every analyte. Based on user-defined “ion ratio tolerances” (see Figure 2), the software has the ability to automatically flag outliers – analyte peaks for which the measured ion ratio does not correspond to the expected ion ratio, within the specified % tolerance.

The ion ratios are updated in the results tables, in real-time, to reflect any changes that are made to the peak integrations. To provide a visual representation of the ion ratio, the software displays overlaid chromatographic traces for the quantifier and qualifier ions in the Peak Review pane. Horizontal lines in the graph area display the expected ion ratio for the qualifier ion, and the tolerance error bars. In Figure 6a and 6b, Fentanyl and 25-OH-Vitamin D3 have been quantified in serum samples, and confirmed using ion ratios. The ion ratios are tabulated, and flagged where appropriate, in the results tables.

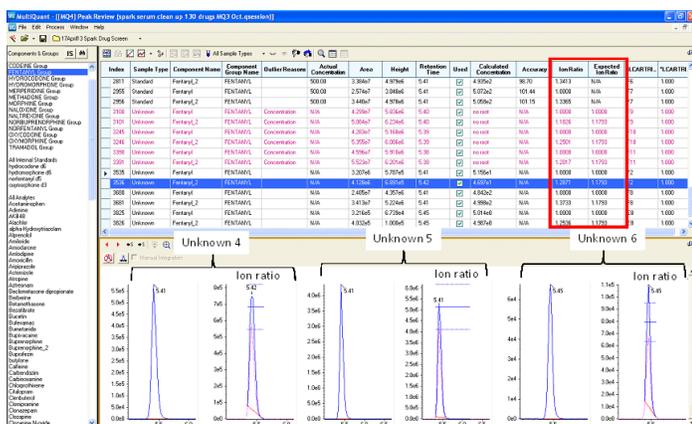


Figure 6a. Fentanyl was identified in various serum samples, using the observed ion ratio as a data quality check. The ion ratio is also visualized in the graph area using tolerance error bars.



Figure 6b. Ion ratios were calculated to confirm the identity of 25-OH-Vitamin D3, measured in serum. The calculated ion ratio values are tabulated in the Results Table, and flagged if the measured ion ratio does not match the expected ion ratio, within the set tolerance of ±20%.

Side-by-Side Peak Review

To facilitate the comparison of samples with one another, or with a reference sample, MultiQuant™ 3.0 software now allows a side-by-side peak review of chromatograms, for up to 6 samples at a time. This allows the user to quickly compare the response for a selected compound at a glance, across multiple samples.

Figure 7 shows examples of detection of Norfentanyl in different urine samples. The side-by-side review with linked intensity axis of standard and samples allowed quick identification of compounds above the target concentration of 50 ng/mL in the urine extract.

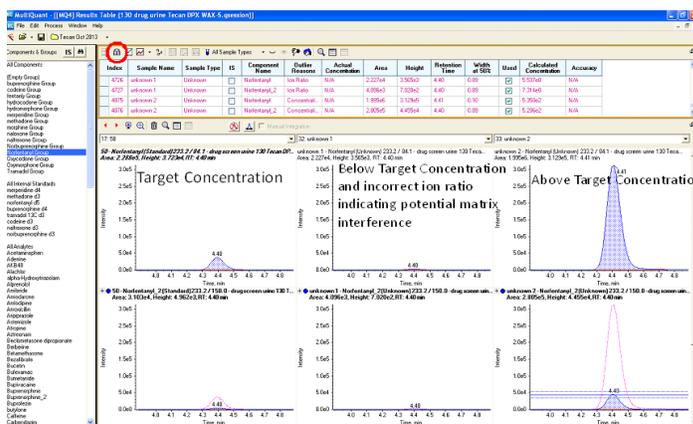


Figure 7. Fentanyl detected in urine samples. The side-by-side peak review allows comparison of the response for a selected compound at a glance, across multiple samples.

Easy Peak Review and Adjustments of Peak Integration

MultiQuant™ software offers a number of features to allow easy and efficient peak review. To facilitate efficient review and re-integration of chromatographic peaks, the software now includes a new ‘Peak Magnifier’ and ‘Peak Demagnifier’ feature.



The ‘Peak Magnifier’ allows the user to increase the size of the peak review pane for a selected chromatogram. This is particularly useful when simultaneously reviewing many chromatograms as it allows the user to quickly verify and adjust integration parameters, and perform manual peak integration. The example in Figure 8 demonstrates the magnification of a chromatographic peak for Norfentanyl measured in a urine sample.

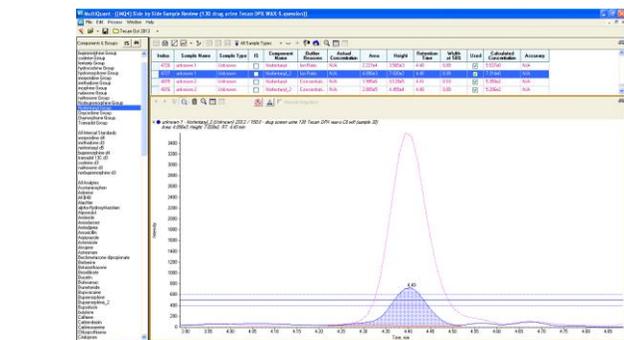
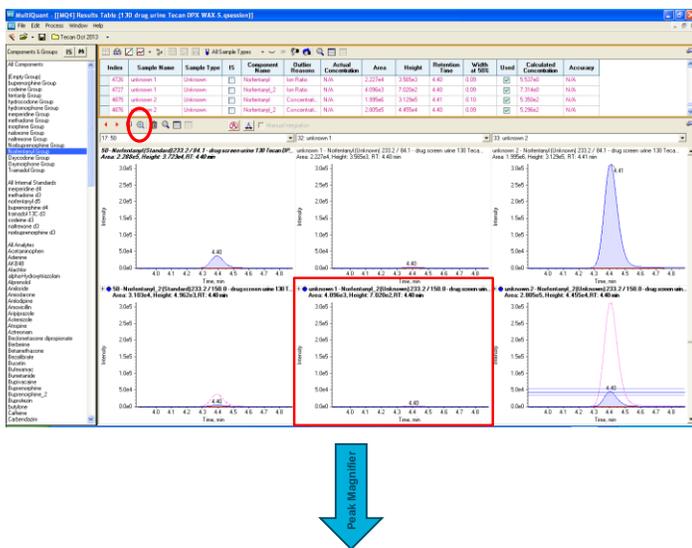


Figure 8. The 'Peak Magnifier' feature allows the user to easily transition from simultaneous review of many chromatograms to a zoomed-in view of a selected chromatogram, to facilitate review and re-integration of peaks as necessary.

Processing of TripleTOF® TOF-MS and MRM^{HR} Data

MultiQuant™ software can be used to process data generated on any AB SCIEX LC/MS/MS systems, including the high-resolution TripleTOF® systems, which have gained popularity for quantitative applications because they provide triple quadrupole-like performance as well as the ability to perform retrospective data analysis.

Figure 10 shows examples of quantification using the MRM^{HR} approach on the TripleTOF 5600+ system. On the top, meperidine is quantified in a urine sample using a high throughput screening method. On the bottom, more than 6 MRM^{HR} transitions for Norpropoxyphene are concurrently

For Research Use Only. Not for use in diagnostic procedures.

© 2013 AB SCIEX. The trademarks mentioned herein are the property of AB Sciex Pte. Ltd. or their respective owners. AB SCIEX™ is being used under license.

Document number: RUO-MKT-02-1053-A

monitored and quantitated in urine. The different product ions for each analyte are used to calculate ion ratios in the MultiQuant™ 3.0 software.

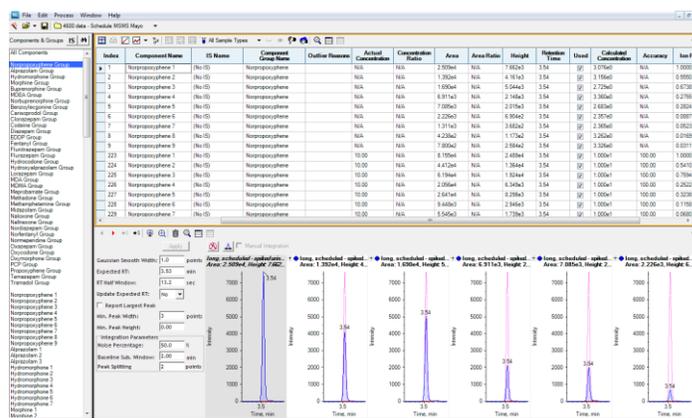
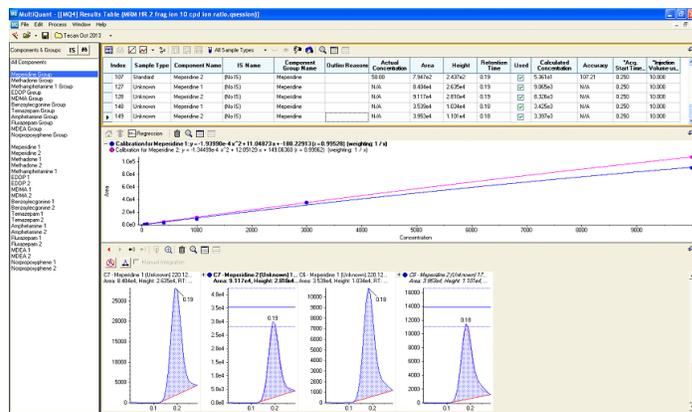


Figure 10. Top: Meperidine in an unknown urine sample was quantified using MRM^{HR} and identified based on ion ratio calculation. Bottom: More than 6 MRM^{HR} transitions are monitored for Norpropoxyphene.

Summary

New features in MultiQuant™ software version 3.0 significantly improve the data analysis workflow for data acquired from AB SCIEX triple quadrupole, QTRAP® and TripleTOF® instruments. These features include built-in queries or "quality-control rules", automatic ion ratio calculation, and side-by-side peak review with peak magnifier. These features aid in the fast and reliable quantitation and identification of compounds in forensic and clinical research samples.