



Quantitation of Cystine and Identification of Related Metabolites in White Blood Cells Using a High Resolution Accurate Mass LC/MS Approach

Application Note

Clinical Research

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Abstract

High resolution accurate mass (HRAM) LC/MS approach was demonstrated for quantitation and profiling of small molecule metabolites in complex biological samples. Excellent analytical performance was achieved in the quantitation of cystine in white blood cells (WBCs) using the ultra-high resolving power and mass accuracy of an Agilent 6530 Accurate-Mass Q-TOF LC/MS System. Further, related metabolites were successfully identified and quantitatively profiled. The HRAM LC/MS data acquired in this study can be retrospectively analyzed to search for more metabolites and biomarkers without sample re-injection.



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Introduction

Cystine levels in WBCs are commonly measured by multiple reaction monitoring (MRM) using triple quadrupole mass spectrometry.¹ Recently, Q-TOF HRAM mass spectrometry has become a promising approach in clinical research analysis in that it allows rapid method development and provides full scan accurate mass data for further metabolite and biomarker identification.²⁻⁵

In this application note, an HRAM method with great selectivity and mass accuracy is presented for quantitation of cystine in WBCs using an Agilent 6530 Accurate-Mass Q-TOF LC/MS System connected to an Agilent 1290 Infinity LC System. The quantitation performance of the Q-TOF HRAM method was evaluated and compared to that of a previously developed MRM method. Excellent sensitivity, linearity, dynamic range, precision, accuracy, and reproducibility were demonstrated in the HRAM method, which is comparable to that of the MRM method. Both methods were used to measure the WBC cystine concentrations for 23 previously analyzed samples (10 controls and 13 cystinotic unknowns) and consistent quantitation results were observed. Cystine quantitation and 44 related metabolites (Table 1) were simultaneously investigated based on the accurate mass information.

A targeted workflow is described for metabolite identification and profiling using Agilent MassHunter Qualitative Analysis and Personal Compound Database and Library (PCDL) software tools.

Table 1. Cystine, d4-cystine, and 44 related metabolites in WBCs.

Compound	Formula
L-Cystine	C₆H₁₂N₂O₄S₂
d4-Cystine	C₆H₈D₄N₂O₄S₂
Cysteamine	C ₂ H ₇ NS
beta-Alanine	C ₃ H ₇ NO ₂
Serine	C ₃ H ₇ NO ₃
Hypotaurine	C ₂ H ₇ NO ₂ S
Cysteine	C ₃ H ₇ NO ₂ S
Taurine	C ₂ H ₇ NO ₃ S
5-Oxoproline	C ₅ H ₇ NO ₃
Homocysteine	C ₄ H ₉ NO ₂ S
L-Lysine	C ₆ H ₁₄ N ₂ O ₂
O-Acetylserine	C ₅ H ₉ NO ₄
Pantoic acid	C ₆ H ₁₂ O ₄
Methionine	C ₅ H ₁₁ NO ₂ S
Cystamine	C ₄ H ₁₂ N ₂ S ₂
Thiocystine	C ₃ H ₇ NO ₂ S ₂
Carnitine	C ₇ H ₁₆ NO ₃
N-Acetylcysteine	C ₅ H ₉ NO ₃ S
Cysteic acid	C ₃ H ₇ NO ₅ S
Cysteinylglycine	C ₅ H ₁₀ N ₂ O ₃ S
Cysteine-cysteamine	C ₅ H ₁₂ N ₂ O ₂ S ₂
S-Sulfocysteine	C ₃ H ₇ NO ₅ S ₂
Acetylcarnitine	C ₉ H ₁₇ NO ₄
Pantothenol	C ₉ H ₁₉ NO ₄
Homocysteine-cysteamine	C ₆ H ₁₄ N ₂ O ₂ S ₂
Pantothenate	C ₉ H ₁₇ NO ₅
Cystathionine	C ₇ H ₁₄ N ₂ O ₄ S
g-glutamylcysteine	C ₈ H ₁₄ N ₂ O ₅ S
Cysteine-homocysteine	C ₇ H ₁₄ N ₂ O ₄ S ₂
N(epsilon)-g-glutamyllysine	C ₁₁ H ₂₁ N ₃ O ₅
Pantetheine	C ₁₁ H ₂₂ N ₂ O ₄ S
Glutathione (reduced)	C ₁₀ H ₁₇ N ₃ O ₆ S
N-[(R)-pantothenoyl]-L-cysteine	C ₁₂ H ₂₂ N ₂ O ₆ S
4'-Phosphopantetheine	C ₁₁ H ₂₃ N ₂ O ₇ PS
Glutathione-cysteamine	C ₁₂ H ₂₂ N ₄ O ₆ S ₂
S-Adenosylhomocysteine	C ₁₄ H ₂₀ N ₆ O ₅ S
4'-Phosphopantothenoyl-L-cysteine	C ₁₂ H ₂₀ N ₂ O ₉ PS
S-Adenosylmethionine	C ₁₅ H ₂₃ N ₆ O ₅ S
Glutathione-cysteine	C ₁₃ H ₂₂ N ₄ O ₈ S ₂
Adenosine-3',5'-bisphosphate	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂
Glutathione (oxidized)	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂
Dephospho-CoA	C ₂₁ H ₃₅ N ₇ O ₁₃ P ₂ S
Coenzyme A	C ₂₁ H ₃₆ N ₇ O ₁₆ P ₃ S
Acetyl-coenzyme A	C ₂₃ H ₃₈ N ₇ O ₁₇ P ₃ S
Ubiquinone-10	C ₅₉ H ₉₀ O ₄
Ubiquinol-10	C ₅₉ H ₉₂ O ₄

Experimental

Sample preparation

Calibration standards (0.02–4.0 μM) and low, medium, and high level quality control (QC) solutions were prepared by spiking cystine at varied concentrations in WBC lysates (Table 2). WBC lysate samples from 23 previously analyzed samples, calibration standards, and QC solutions were spiked with d4-cystine as internal standard at 2 μM and extracted with ice-cold acetonitrile before LC/MS analysis. WBC lysate was used as double blank and WBC lysate with 2 μM d4-cystine was used as blank.

Table 2. Calibration standard and QC solutions of cystine in WBCs.

Type	Level	Injection vol. (μL)	Concentration (μM)	fmol on-column	d4-cystine concentration (μM)
Calibration	1	2	0.02	40	2
Calibration	2	2	0.04	80	2
Calibration	3	2	0.1	200	2
Calibration	4	2	0.2	400	2
Calibration	5	2	0.4	800	2
Calibration	6	2	1	2,000	2
Calibration	7	2	2	4,000	2
Calibration	8	2	4	8,000	2
QC	Low	2	0.015	30	2
QC	High	2	0.9	1,800	2
Blank		2	0	0	2
Double blank		2	0	0	2

Instrumentation

Liquid chromatography was performed using an Agilent 1290 Infinity LC System consisting of a binary pump, vacuum degasser, high performance thermostatted autosampler, and a thermostatted column compartment. Full acquisition MS was performed on an Agilent 6530 Q-TOF mass spectrometer equipped with Agilent Jet Stream source in positive ionization mode using a mass resolving power of 10K. Liquid chromatography, ion source conditions and MS acquisition method parameters were optimized for cystine in WBC lysate, as listed in Table 3.

Data acquisition and analysis

MassHunter Workstation Software (B.03.01) was used for data acquisition. MassHunter Quantitative (Quan) Analysis Software (version B.04.00) was used for generation of calibration curves and quantitation of cystine in WBCs. Extracted ion chromatograms (EICs) of m/z 241.0311 for cystine and m/z 245.0562 for d4-cystine were employed for quantitation. The mass extraction window was 10 ppm. The quantitative capability of this HRAM method was evaluated by comparing results to that of an MRM method. Additionally, MassHunter Qualitative (Qual) Analysis Software (version B.03.01) was used for profiling and identification of cystine and the other 44 related metabolites (Table 1) from WBCs. In MassHunter Qual, the data files were processed by targeted data mining and compound identification approaches, Find by Formula (FbF) with Molecular Formula Generation (MFG) scores, and database search.

Table 3. Liquid chromatography and Q-TOF MS conditions.

LC conditions	
Column	Teicoplanin chiral column (2.1 × 250 mm, 5 μm)
Column temperature	40 °C
Injection volume	2 μL
Autosampler temperature	6 °C
Needle wash	10 seconds in wash port
Mobile phase	A = 0.025 % formic acid in water B = 0.025 % formic acid in acetonitrile
Flow rate	0.5 mL/min
Gradient	Isocratic 50:50 A:B
Stop time	4.5 min
Q-TOF MS source conditions	
Ion mode	Positive
Drying gas temperature	300 °C
Drying gas flow	7 L/min
Sheath gas temperature	400 °C
Sheath gas flow	11 L/min
Nebulizer pressure	35 psi
Capillary voltage	3,750 V
Nozzle voltage	0 V
Fragmentor voltage	200 V
Reference delivery	Agilent 1200 Isocratic pump with 100:1 splitter (p/n: G1607-60000)
Reference pump flow	0.5 mL/min for 5 μL/min to nebulizer
Reference ions	121.050873 and 922.009798
Instrument mass range	1.700 Da
Instrument mode	Extended dynamic range
Data storage	Centroid and profile
Q-TOF MS acquisition method parameters	
Mass range	100–1,000 m/z
Acquisition rate	2 Hz, 500 ms/scan

Results and Discussion

Cystine quantitation

Cystine and d4-cystine were eluted at retention time (RT) of 2.65 minutes and their EICs of $[M+H]^+$ were employed for quantitation (Figure 1). The high mass resolving power and narrow mass extraction window employed in the HRAM LC/MS method greatly decreased the endogenous interference from WBC lysate, thus significantly improved the selectivity, sensitivity, and other performance parameters (for example, linearity, range, precision, and accuracy) of the quantitative detection.

Sensitivity

In this application note, limit of quantitation (LOQ) is defined as the lowest concentration or amount of the analyte on-column that generates a signal significantly different from the blank, has a signal to noise (S/N) ratio $> 5:1$, and gives an acceptable accuracy (80–120 %), retention time reproducibility (% RSD < 20) and quantitative precision (% RSD < 20). The LOQ of cystine in WBCs is $0.02 \mu\text{M}$, or 40 fmol on-column, with an S/N ratio $> 5:1$ (Figure 2).

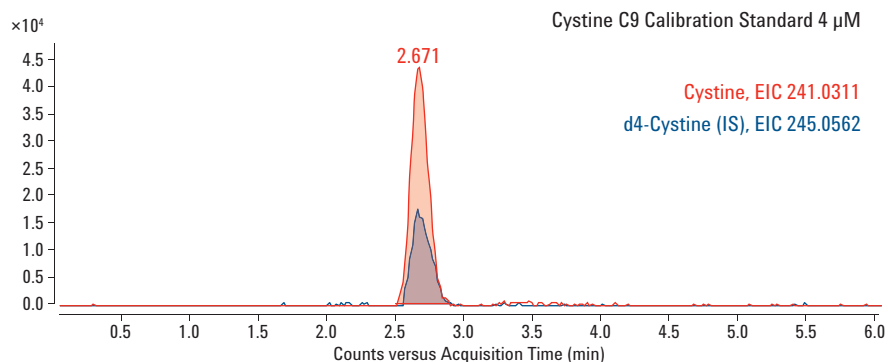


Figure 1. EICs of cystine and d4-cystine internal standard.

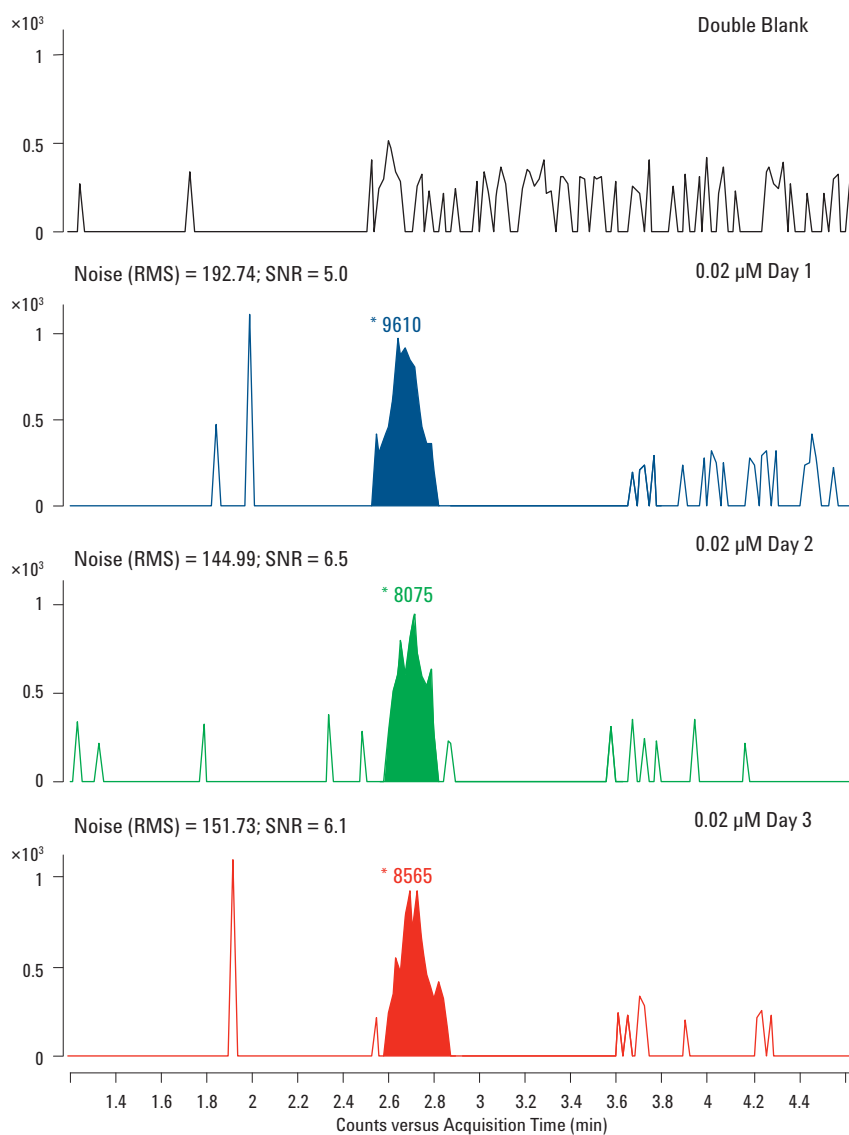


Figure 2. EICs of cystine m/z 241.0311 at LOQ level, $0.02 \mu\text{M}$ (40 fmol on-column).

As illustrated by Figure 2 and Table 4, excellent accuracy (105.3 %) and reproducibility of retention time and peak area response (% RSD = 4.5 from triplicate analysis) were obtained at the LOQ level.

Calibration curve linearity and range

Cystine calibration standard solutions were analyzed in triplicate over a three-day time period and the average calibration curve (n = 3) is demonstrated in Figure 3. The cystine calibration curve in WBCs shows excellent linearity with an average R² > 0.9999 (Day 1 = 0.99976, Day 2 = 0.99997, and Day 3 = 0.99996) in the dynamic range of 0.02 – 4 μM. As summarized in Table 4, great detection accuracy (95.2–105.3 %) and precision (% RSD = 2.0–6.2) were observed at all calibration levels. In addition, consistent detection response factors (RF) were obtained over the calibration range, with a % RSD (n = 8) of 3.4 from the eight calibration levels.

Precision and accuracy

The method inter-day and intra-day precision was evaluated from QC solutions at low and high levels over the three-day period. The results are summarized in Table 5. The average intra-day precision of Day 1 (n = 6), Day 2 (n = 6), and Day 3 (n = 12) was determined to be 5.2 % at low QC level and 4.1 % at high QC level. The inter-day precision obtained from a total of 24 replicates over the three-day analysis period was 5.0 % at low QC level and 3.8 % at high QC level. The average detection accuracy from the 24 replicates was 103.2 % at low QC level and 97.3 % at high QC level.

Table 4. Accuracy, reproducibility, and response factors at the eight calibration levels. These results were generated from triplicate analysis in a three day time period.

Cystine concentration (μM)	0.02	0.04	0.1	0.2	0.4	1	2	4
Average accuracy (% , n = 3)	105.3	95.2	95.5	98	97.8	100.6	101.2	99.7
Precision (% RSD, n = 3)	4.5	2.3	1.4	6.2	5.6	3.3	2.0	1.4
Response Factor (RF)	0.62	0.56	0.56	0.58	0.58	0.59	0.60	0.59

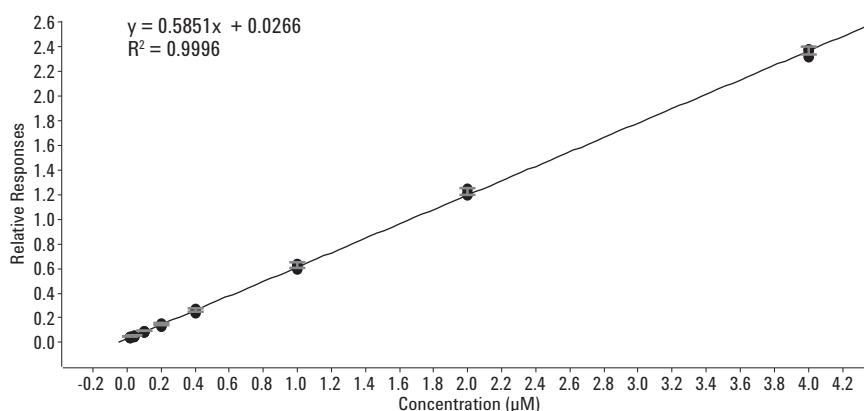


Figure 3. Calibration curve of cystine (0.02 – 4 μM) in WBC lysate.

Table 5. Accuracy, intra- and inter-day precision results determined from low and high level QC solutions.

QC levels	Intra-day precision (% RSD)			Inter-day precision (% RSD)	Accuracy (%)
	Day 1 (n = 6)	Day 2 (n = 6)	Day 3 (n = 12)	Day 1 – Day 3 (n = 24)	
Low	5.5	5.9	4.3	5.0	90.0 – 113.9
High	3.4	5.6	3.4	3.8	89.3 – 105.6

Quantitation of cystine in WBCs

The WBC cystine concentrations from 23 incurred samples were determined in triplicate using the HRAM method and the results range from 0.14 to 9.44 μM (Table 6). Great precision (% RSD < 5) was observed in the quantitative measurements. These Q-TOF HRAM quantitation results were compared to those obtained using the MRM method. The relative bias values were calculated and listed in Table 6 and the correlation plot is illustrated in Figure 4. The excellent bias of 0–19 % with an average of 5.2 % from the 23 incurred samples and the correlation coefficient (R^2) of 0.998 demonstrate the consistency of the two methods and, more importantly, the comparable quantitation capability of Q-TOF HRAM methods to more conventionally used MRM methods in complex biological matrices.

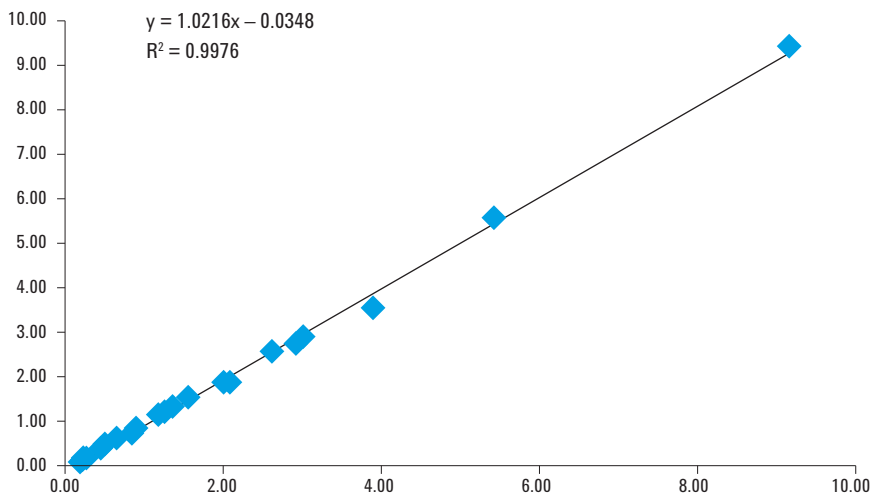


Figure 4. Correlation plot of WBC cystine levels measured for 23 incurred, retested samples using the Q-TOF HRAM method and the MRM method.

Table 6. Cystine concentrations measured in WBCs of 23 incurred samples using the Q-TOF HRAM method and the MRM method.

Sample number	Cystine concentration (μM)		
	QTOF HRAM	QQQ MRM	% Bias
1	0.16	0.17	-7
2	0.14	0.16	-8
3	0.19	0.19	-2
4	0.16	0.19	-19
5	0.22	0.20	8
6	0.52	0.46	13
7	0.43	0.42	2
8	0.68	0.62	9
9	0.78	0.82	-4
10	0.91	0.87	5
11	1.26	1.25	1
12	1.37	1.33	3
13	1.20	1.20	4
14	1.59	1.59	3
15	1.91	1.91	-8
16	1.93	1.93	-2
17	2.61	2.61	1
18	2.80	2.80	-4
19	2.61	2.61	0
20	3.58	3.58	-8
21	2.94	2.94	-2
22	5.62	5.62	4
23	9.46	9.46	3
Average			5.2

Comparison of Q-TOF HRAM and MRM methods

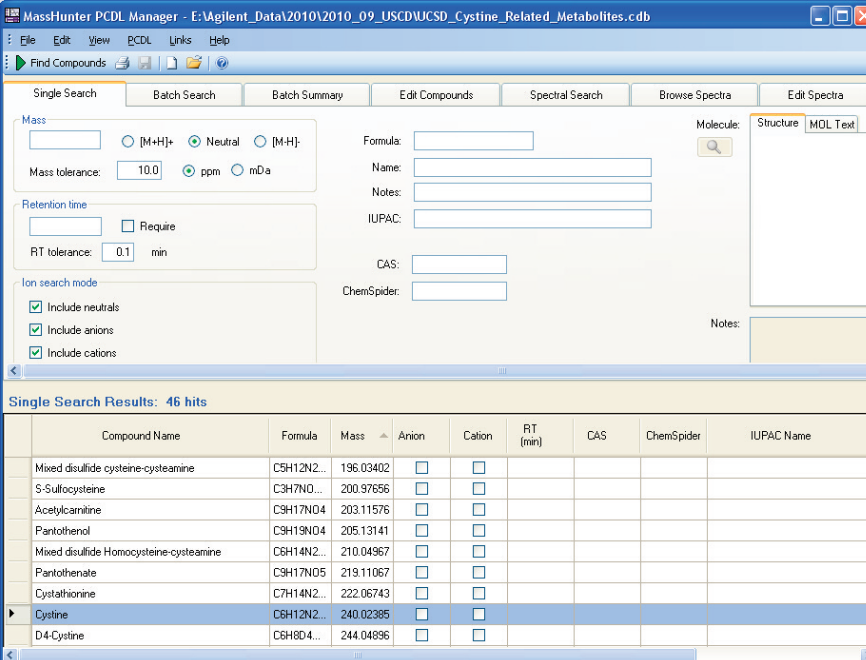
Table 7 summarizes the comparison of performance parameters achieved using the Q-TOF HRAM method and the MRM method. The HRAM method described in this application note and the MRM method give very comparable linearity, accuracy, and precision. Notably, the LOQ level of the HRAM method (40 fmol on-column) in WBC lysate is 2.5 times lower than that of the MRM method (100 fmol on-column), which demonstrates the great potential of utilizing HRAM to achieve quantitative bioanalysis with high-degree sensitivity and selectivity. Q-TOF HRAM methods are advantageous to MRM methods in that data are acquired in full scan MS mode so that fragment ion selection and collision energy (CE) optimization steps are not necessary during method development. In addition, excellent mass accuracy (< 2 ppm) was obtained for cystine at the LOQ level and in the incurred samples, which added to the confidence of cystine quantitation using HRAMS.

Table 7. Quantitation performance comparison: Q-TOF HRAM method versus QQQ MRM method.

Quantitation performance parameters		QTOF HRAM method	QQQ MRM method
LOQ (fmol on-column)		40	100
Linearity (0.02 – 4 µM)		0.9999	0.9998
Accuracy (%)		89 – 114	88 - 109
Precision (%RSD)	Intra-day	4.7	4.7
	Inter-day	4.4	4.6

Metabolite identification and profiling

Identification and quantitative profiling of the 44 related metabolites (Table 1) were performed in selected samples. A targeted data mining algorithm, FbF was utilized to search the full scan MS data against a personal compound database library that contains the formula and accurate mass information of cystine and the 44 related metabolites (Figure 5). MFG was used to yield match scores for the list of compounds found in FbF using accurate mass and isotope patterns. The triple criteria MFG scores were based on accurate mass of the monoisotopic peak, isotope spacing of the monoisotopic peak and isotope peaks, and the isotope abundance pattern.



MassHunter PCDL Manager - E:\Agilent_Data\2010\2010_09_USCD\UCSD_Cystine_Related_Metabolites.cdb

Single Search Results: 46 hits

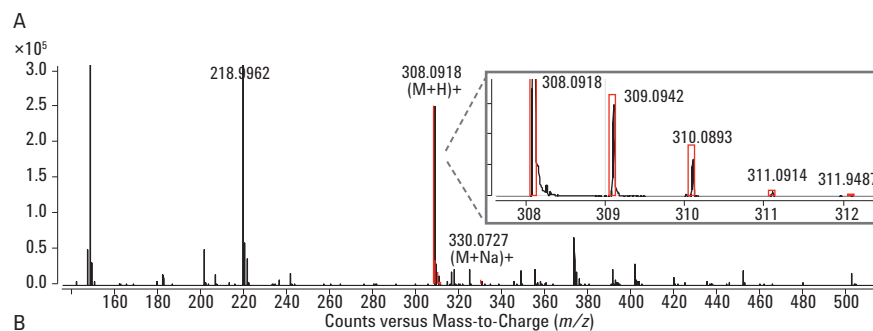
Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name
Mixed disulfide cysteine-cysteamine	C5H12N2...	196.03402	<input type="checkbox"/>	<input type="checkbox"/>				
S-Sullocysteine	C3H7NO...	200.97696	<input type="checkbox"/>	<input type="checkbox"/>				
Acetyl-carnitine	C9H17NO4	203.11576	<input type="checkbox"/>	<input type="checkbox"/>				
Pantothenol	C9H19NO4	205.13141	<input type="checkbox"/>	<input type="checkbox"/>				
Mixed disulfide Homocysteine-cysteamine	C6H14N2...	210.04967	<input type="checkbox"/>	<input type="checkbox"/>				
Pantothenate	C9H17NO5	219.11067	<input type="checkbox"/>	<input type="checkbox"/>				
Cystathionine	C7H14N2...	222.06743	<input type="checkbox"/>	<input type="checkbox"/>				
Cystine	C6H12N2...	240.02385	<input type="checkbox"/>	<input type="checkbox"/>				
D4-Cystine	C6H8O4...	244.04896	<input type="checkbox"/>	<input type="checkbox"/>				

Figure 5. Personal compound database library (PCDL) established for cystine and 44 related metabolites in WBC lysate.

As summarized by Table 8, cystine and 11 related metabolites were found and identified in the WBC lysate of incurred sample # 21 using FbF with average mass errors (MS) < 1 ppm and MFG scores > 90. Figure 6 and Figure 7 illustrate the MS spectra, isotope patterns, and MFG results for selected metabolites, for example, glutathione (reduced), and acetylcarnitine. Notably, excellent mass accuracy with average mass errors < 2 ppm was observed for the isotopes (M+1, M+2, and M+3) of glutathione (reduced) and acetylcarnitine, demonstrating the high sensitivity and in-spectrum dynamic range of the Agilent 6530 Accurate-Mass Q-TOF LC/MS System.

Table 8. Cystine and 11 related metabolites identified in the WBCs of incurred sample #21 using FbF in MassHunter Qualitative Analysis software.

Name	RT	Mass	Mass (Tgt)	Diff (Tgt, ppm)	Formula	Score	Height	Area	Algorithm
Serine	2.202	105.0427	105.0426	1.34	C3H7NO3	97.77	61504	406046	Find By Formula
Hypotaurine	2.073	109.02	109.0198	2.25	C2H7NO2S	99.22	670588	3910317	Find By Formula
Taurine	1.796	125.0147	125.0147	0.34	C2H7NO3S	97.88	573044	3474239	Find By Formula
5-Oxoproline	1.87	129.0426	129.0426	0.2	C5H7NO3	99.88	475547	2093274	Find By Formula
L-Lysine	4.53	146.1053	146.1055	-1.85	C6H14N2O2	99.46	26997	256411	Find By Formula
D-Acetylserine	2.184	147.0533	147.0532	0.8	C5H9NO4	99.46	1628564	8347389	Find By Formula
Methionine	2.332	149.051	149.0511	-0.63	C5H11NO2S	78.78	54696	236942	Find By Formula
Cysteinyglycine	2.165	178.0411	178.0412	-0.52	C5H10N2O3S	77.22	9768	61794	Find By Formula
Acetylcarnitine	3.551	203.1158	203.1158	0.38	C9H17NO4	97.6	72447	128949	Find By Formula
Cystathionine	2.646	222.0673	222.0674	-0.36	C7H14N2O4S	90.52	14704	169673	Find By Formula
Cystine	2.64	240.0241	240.0239	0.96	C6H12N2O4S2	92.77	37428	329261	Find By Formula
D4-Cystine	2.677	244.049	244.049	0.04	C6H8D4N2O4S2	97.09	8623	67312	Find By Formula
Glutathione (reduced)	2.165	307.0845	307.0838	2.2	C10H17N3O6S	97.54	496473	2913303	Find By Formula



Best	Name	RT	Mass	Mass (MFG)	Diff (ppm)	Formula	Score (MFG)	ID Source	
	Glutathione (re...	2.165	307.0845	307.0838	-2.21	C10 H17 N3 O6 S	97.43	FbF-MFG	
Species	Ion	Formula	Height	m/z	Score (MFG)	Score (MFG, MS)	Score (MFG, mass)	Score (MFG, abund)	Score (MFG, iso, spacing)
	[M+H] ⁺	C10 H18 N3 O6	268453	308.0911	97.43	97.43	96.76	99.63	
Height	Height (Calc)	Height	m/z	m/z (Calc)	Diff (ppm)	Diff (mDa)			
263467	263200.6	100	308.0918	308.0911	-2.21	-0.7			
32043	34576.3	11.9	309.0942	309.0937	-1.54	-0.5			
13632	17124.8	5.1	310.0899	310.0896	-0.75	-0.2			
1733.4	1957.7	0.6	311.092	311.0919	-0.19	-0.1			
282.7	300	0.1	312.0881	312.0924	13.81	4.3			

Figure 6. Reduced glutathione MS spectrum (A), isotope patterns (inset), and MFG results (B) from incurred sample # 21.

Conclusions

This application note describes a high resolution accurate mass Q-TOF LC/MS method with excellent sensitivity and mass accuracy for simultaneous quantitative and qualitative analysis of small molecular metabolites in complex biological samples.

- The Q-TOF HRAM method demonstrates excellent sensitivity with LOQ of 0.02 μM and 40 fmol on-column for cystine in WBCs.
- Calibration curves in WBCs show excellent linearity ($R^2 > 0.9999$) over the dynamic range of 0.02–4 μM .
- Statistics for accuracy (88–114 %), intra-day precision (% RSD < 5.9 %), and inter-day precision (% RSD < 5.0 %) were well within accepted limits.
- The concentrations of cystine were measured in WBCs with good precision (% RSD < 5.0 % in triplicate).
- Comparable quantitation capability in WBCs was demonstrated and consistent quantitation results for incurred samples were obtained using the Q-TOF HRAM method and the MRM method.
- Eighteen related metabolites were identified and quantitatively profiled in WBCs with high scores.
- Accurate mass results were obtained with average mass errors of < 1 ppm and match scores > 90.
- Powerful software processing tools (MassHunter Qual) with sophisticated data mining and feature identification algorithms (FbF and MFG) greatly facilitate metabolite identification and profiling.

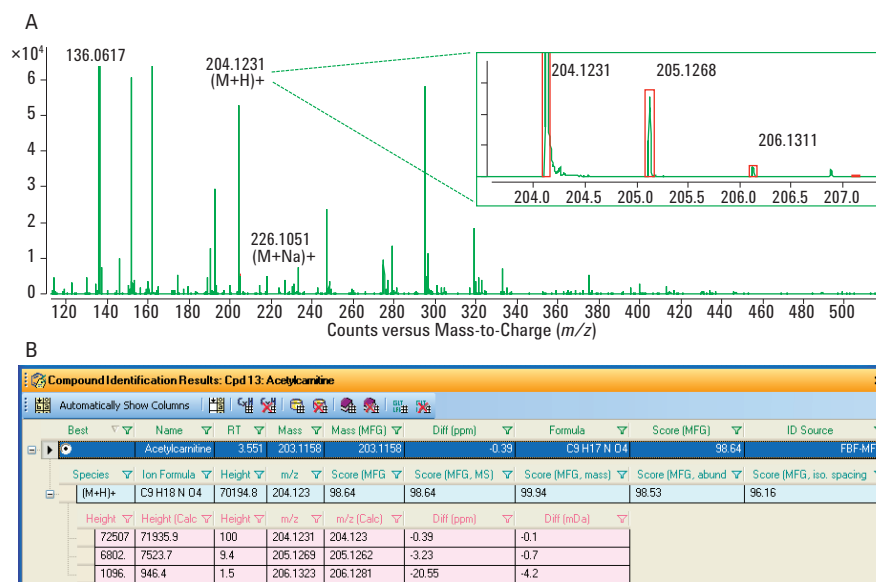


Figure 7. Acetylcarnitine MS spectrum (A), isotope patterns (inset), and MFG results (B) from incurred sample #21.

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