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Targeted Metabolomics Using the UPLC/MS-based Absolute*IDQ* p180 Kit

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APPLICATION BENEFITS

Waters® ACQUITY UPLC® System with Xevo® TQ and Xevo TQ-S mass spectrometers combines with the commercially available Absolute*IDQ* p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) to allow for the rapid identification and highly sensitive quantitative analyses of more than 180 endogenous metabolites from six different biochemical classes (biogenic amines, amino acids, glycerophospholipids, sphingolipids, sugars, and acylcarnitines). The assay is performed using MS-based flow injection and liquid chromatography analyses, which were validated on Waters' tandem quadrupole instruments.

WATERS SOLUTIONS

ACQUITY UPLC System

ACQUITY UPLC BEH Columns

Xevo TQ Mass Spectrometer

Xevo TQ-S Mass Spectrometer

TargetLynx™ Application Manager

KEY WORDS

Absolute*IDQ* p180 Kit, flow injection analysis (shotgun), targeted metabolomics, targeted lipidomics, MetIDQ software

INTRODUCTION

Global metabolic profiling (untargeted metabolomics) is used for the identification of metabolic pathways that are altered following perturbations of biological systems, as shown in Figure 1. The analysis, however, encompasses significant statistical processing that leads to a low rate of successful identification of biomarkers. Additionally, a tedious marker validation process using pure standards is often required for the identification of a particular metabolite, unless an in-house database has been previously generated. Furthermore, the sample preparation required for the extraction of metabolites is a multi-step process that, without a standardization of the operating procedures, likely contributes to the intra- and inter-laboratory variations in the measurements.

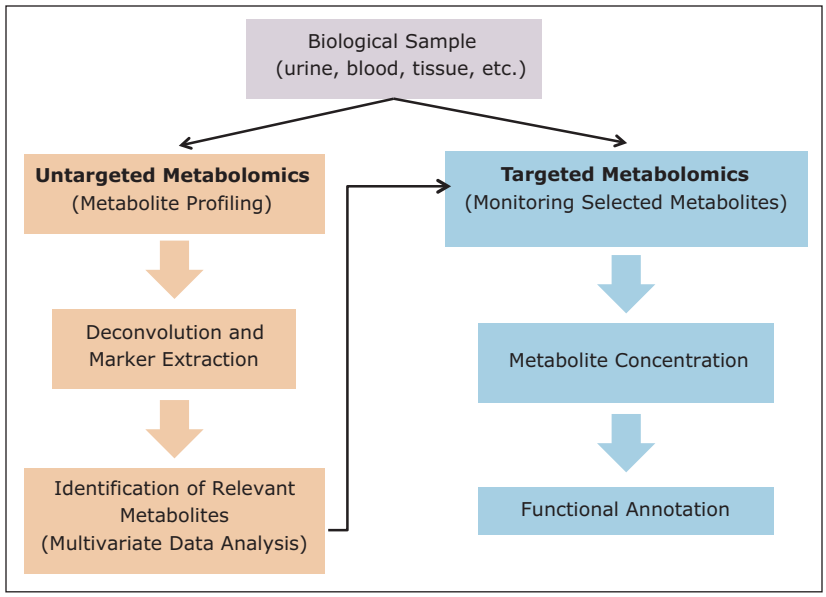

Figure 1. Workflows illustrating both untargeted and targeted metabolomics approaches.

To alleviate many of these limiting issues, another approach involves the application of targeted metabolomics assay, seen in Figure 1. The Absolute*IDQ* p180 (BIOCRATES Life Sciences AG) Kit is an MS-based assay for targeted metabolomics allowing the simultaneous identification and quantification of over 180 endogenous metabolites in biological samples.[1-2] MS-based flow injection analysis (FIA) for acylcarnitines, hexoses, glycerophospholipids, and sphingolipids as well as an MS-based LC method for amino acids and biogenic amines are used to provide a robust, high-throughput identification of preselected metabolites, as shown in Figure 2. Here, we applied this targeted metabolomics strategy to identify biochemical alterations and potential biomarkers in serum from mice exposed to 8 Gy of gamma radiation. Significant differences allowed for the identification of metabolites that could be used to develop a signature of radiation exposure in mice.

Metabolite group	No. of metabolites	FIA-MS/MS	LC-MS/MS
Amino acids and biogenic amines	40		X
Acylcarnitines	40	X	
Lyso-phosphatidylcholines	14	X	
Phosphatidylcholines	74	X	
Sphingomyelins	14	X	
Hexose	1	X	
Total	183		

Figure 2. List of metabolite classes and total metabolites covered by the kit.

EXPERIMENTAL

Mouse irradiation and sample collection

Male C57Bl/6 mice (8 to 10 weeks old) were irradiated at Georgetown University with 8 Gy of gamma rays (^{137}Cs source, 1.67 Gy/min). Blood was obtained by cardiac puncture 24 h post-irradiation, and serum was collected with serum separators (BD Biosciences, CA). All experimental conditions and animal handling were in accordance with animal protocols approved by the Georgetown University Animal Care and Use Committee (GUACUC).

Sample preparation and data analysis

Metabolites were extracted from mouse sera using a specific 96-well plate system for protein-removal, internal standard normalization and derivatization (Absolute*IDQ* p180 Kit). The preparation was performed according to the Kit User Manual. Briefly, 10 samples (n=5 sham irradiated group and n=5 irradiated group) were added to the center of the filter on the upper 96-well plate kit at 10 µL per well, and dried using a nitrogen evaporator. Subsequently, 50 µL of a 5% solution of phenylisothiocyanate was added for derivatization of the amino acids and biogenic amines. After incubation, the filter spots were dried again using a nitrogen evaporator. The metabolites were extracted using 300 µL of a 5-mM ammonium acetate solution in methanol, and transferred by centrifugation into the lower 96-deep well plate. The extracts were diluted with 600 µL of the MS running solvent for further MS analysis using Waters tandem quadrupole mass spectrometers. One blank sample (no internal standards and no sample added), three water-based zero samples (phosphate buffered saline), and three quality control samples were also added to the Kit plate. The quality controls were comprised of human plasma samples containing metabolites, at several concentration

levels, used to verify the performance of the assay and mass spectrometer. A seven-points serial dilution of calibrators was added to the kit's 96-well plate to generate calibration curves for the quantification of biogenic amines and amino acids. The kit included a mixture of internal standards for the quantification of the natural metabolites as follows: chemical homologous internal standards were used for the quantification of glycerophospholipid and sphingomyelin species; whereas, stable isotopes-labeled internal standards were used to quantify the other compound classes. The amount of internal standards was identical in each well, and the internal standard intensities of zero sample and sample wells were compared to allow conclusions on ion suppression effects.

Acylcarnitines, glycerophospholipids, and sphingolipids were analyzed using the Waters tandem quadrupole mass spectrometers (Xevo TQ and Xevo TQ-S MS) by flow injection analysis (FIA) in positive mode, as shown in Figure 3. Hexose was analyzed using a subsequent FIA acquisition in negative mode. Amino acids and biogenic amines were analyzed using an ACQUITY UPLC System connected to the Xevo tandem quadrupole and Xevo TQ-S mass spectrometers in positive mode, as shown in Figure 4.

Identification and quantification of the metabolites was achieved using internal standards and multiple reaction monitoring (MRM) detection. Data analysis and calculation of the metabolite concentrations analyzed by FIA (acylcarnitines, glycerophospholipids, sphingolipids, and hexoses) is automated using Met*IDQ* software (BIOCRATES Life Sciences AG), an integral part of the kit that imports Waters' raw data files. Analysis of peaks obtained by HPLC/UPLC® (amino acids and biogenic amines) was performed using TargetLynx Application Manager, and the results were imported into Met*IDQ* software for further processing and statistical analysis.

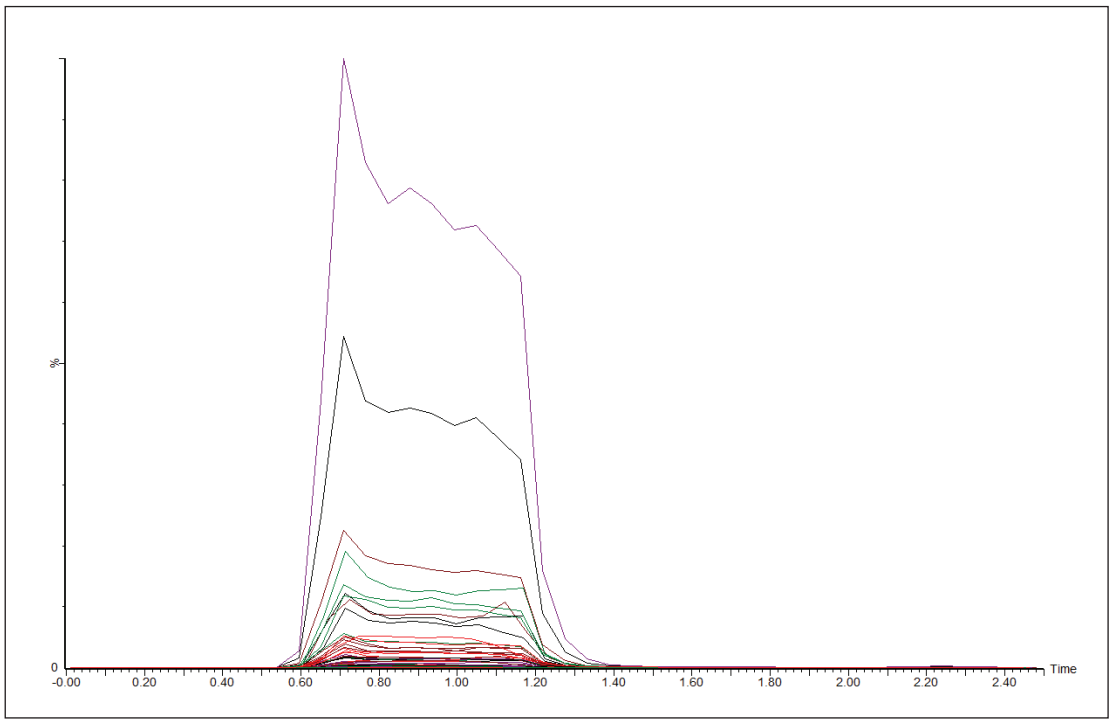

Figure 3. Representative FIA chromatogram.

LC pump settings

Mobile phase A: water and 0.2% formic acid

Mobile phase B: ACN and 0.2% formic acid

HPLC column

Column: Agilent Zorbax Eclipse XDB C_{18}, 3.0 x 100 mm, 3.5 µm

Pre-Column: SecurityGuard, Phenomenex, C_{18}, 4 x 3 mm

Step	Time (min)	Flow (mL/min)	% A	% B	Curve
0	0.00	0.5	100.0	0.0	Initial
1	0.50	0.5	100.0	0.0	6
2	4.00	0.5	30.0	70.0	6
3	5.30	0.5	30.0	70.0	6
4	5.40	0.5	100.0	0.0	6
5	7.30	0.5	100.0	0.0	6

UPLC column

Column: Waters ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm

Pre-Column: Waters ACQUITY UPLC BEH C_{18} VanGuard,™ 1.7 µm

Step	Time (min)	Flow (mL/min)	% A	% B	Curve
0	Initial	0.9	100.0	0.0	Initial
1	0.25	0.9	100.0	0.0	6
2	3.75	0.9	40.0	60.0	6
3	3.95	0.9	40.0	60.0	6
4	4.25	0.9	100.0	0.0	6
5	4.35	0.9	100.0	0.0	6

Flow injection analysis (FIA) pump settings

Step	Time (min)	Flow (µL/min)	% A	% B
0	Initial	30	0.0	100.0
1	1.60	30	0.0	100.0
2	2.40	200	0.0	100.0
3	2.80	200	0.0	100.0
4	3.00	30	0.0	100.0

Other systems settings

Instrument	Parameter	Method		
		HPLC	UPLC	FIA
Autosampler	Injection volume	10	5	20
Column Oven	Temp.	50 °C	50 °C	No column
MS	Capillary voltage	3.2	3.2	3.9
	Cone voltage	27	27	22
	Source temp.	150 °C	150 °C	150 °C
	Desolvation temp.	600 °C	600 °C	350 °C
	Cone gas	50	250	0
	Desolvation gas	720	1000	650
	Collision gas	0.15	0.15	0.15
	Collision	2	2	2

RESULTS AND DISCUSSION

The extraction of metabolites from biological samples is a key delicate step for an accurate MS analysis. A multi-step sample preparation procedure could contribute to the variation and errors in the measurements of the natural metabolites. In order to minimize these issues, step-by-step operating procedures were followed as described in the Kit User Manual and detailed in the Experimental section of this application note.

The Absolute*IDQ* p180 Kit was tested with both HPLC (Agilent Zorbax Eclipse XDB C_{18}, 3.0 x 100 mm, 3.5 µm) and UPLC (Waters ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm) columns coupled with Xevo TQ and Xevo TQ-S mass spectrometers, as shown in Figure 4. The UPLC-based assay at a flow rate of 0.9 mL/min allowed for a high-throughput separation of the selected metabolites in less than 5 min, which was considerably shorter than the HPLC-based assay at a flow rate of 0.5 mL/min, as shown in Figure 4.

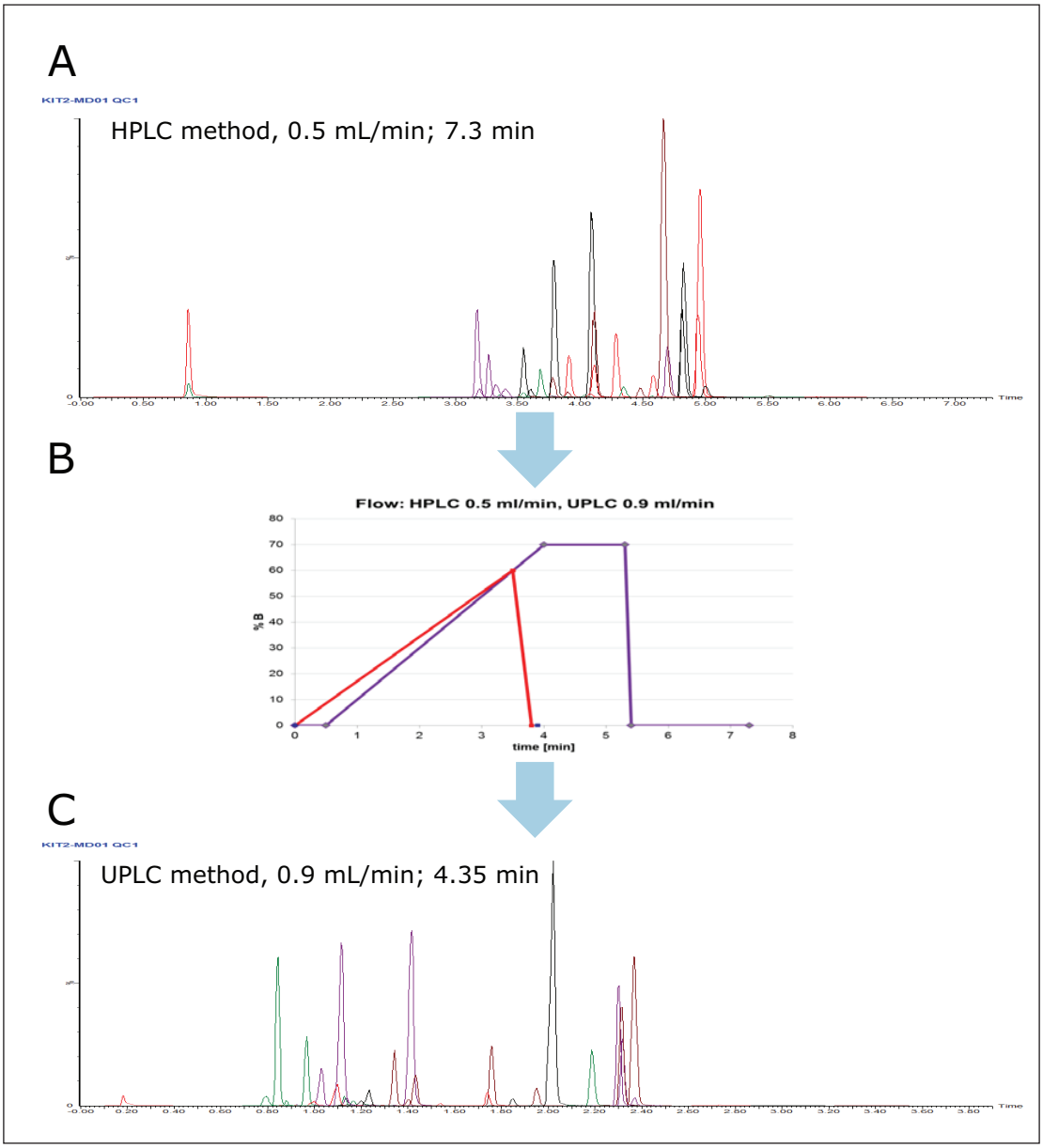

Figure 4. A.) Representative HPLC/MS chromatogram illustrating the total run time of 7.3 min. B.) Optimization of the chromatographic gradient from HPLC-based method (violet) to UPLC-based method (red). C.) Representative UPLC/MS chromatogram showing a total run time of 4.3 min, which represents a significant gain in speed compared to HPLC/MS.

The Absolute*IDQ* p180 Kit was utilized to determine differences in the serum metabolome between irradiated and non-irradiated mice. The identification of potential alterations in the levels of metabolites in the serum of mice exposed to gamma radiation is particularly significant because it could lead to the following: 1) a better understanding of the biochemical pathways involved in the response to gamma radiation; and 2) the discovery of biochemical indicators (biomarkers) of acute exposure to ionizing radiation. Rapid identification of biomarkers will be of particular importance in the case of accidental exposures and terrorist acts,[3,4] as classic cytogenetic methods available for biodosimetry are laborious and time-consuming. Using the Absolute*IDQ* p180 Kit, we were able to rapidly measure the serum levels of both polar and non-polar metabolites belonging to major biochemical pathways, as shown in Table 1.

Acylcarnitines (40)			
C0 Carnitine	C10:1 Decenoylcarnitine	C5:1-DC Glutaconylcarnitine	C16 Hexadecanoylcarnitine
C2 Acetylcarnitine	C10:2 Decadienylcarnitine	C5-DC (C6-OH) Glutarylcarnitine* (Hydroxyhexanoylcarnitine)	C16:1 Hexadecenoylcarnitine
C3 Propionylcarnitine	C12 Dodecanoylcarnitine	C5-M-DC Methylglutarylcarnitine	C16:1-OH Hydroxyhexadecenoylcarnitine
C3:1 Propenoylcarnitine	C12:1 Dodecenoylcarnitine	C5-OH (C3-DC-M) Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	C16:2 Hexadecadienylcarnitine
C3-OH Hydroxypropionylcarnitine	C12-DC Dodecanedioylcarnitine	C6 (C4:1-DC) Hexanoylcarnitine (Fumarylcarnitine)	C16:2-OH Hydroxyhexadecadienylcarnitine
C4 Butyrylcarnitine	C14 Tetradecanoylcarnitine	C6:1 Hexenoylcarnitine	C16-OH Hydroxyhexadecanoylcarnitine
C4:1 Butenylcarnitine	C14:1 Tetradecenoylcarnitine	C7-DC Pimelylcarnitine	C18 Octadecanoylcarnitine
C4-OH (C3-DC) Hydroxybutyrylcarnitine	C14:1-OH Hydroxytetradecenoylcarnitine	C8 Octanoylcarnitine	C18:1 Octadecenoylcarnitine
C5 Valerylcarnitine	C14:2 Tetradecadienylcarnitine	C9 Nonaylcarnitine	C18:1-OH Hydroxyoctadecenoylcarnitine
C5:1 Tiglylcarnitine	C14:2-OH Hydroxytetradecadienylcarnitine	C10 Decanoylcarnitine	C18:2 Octadecadienylcarnitine
Amino Acids and Biogenic Amines (40)			
Alanine	Leucine	Valine	Methioninesulfoxide
Arginine	Lysine	Acetylyornithine	Nitrotyrosine
Asparagine	Methionine	Asymmetric dimethylarginine	Hydroxyproline
Aspartate	Ornithine	Symmetric dimethylarginine	Phenylethylamine
Citrulline	Phenylalanine	Total dimethylarginine	Putrescine
Glutamine	Proline	alpha-Aminoadipic acid	Sarcosine
Glutamate	Serine	Carnosine	Serotonin
Glycine	Threonine	Creatinine	Spermidine
Histidine	Tryptophan	Histamine	Spermine
Isoleucine	Tyrosine	Kynurenine	Taurine
Sphingolipids (14)			
SM (OH) C14:1	SM C18:0	SM (OH) C22:2	SM C26:0
SM C16:0	SM C18:1	SM C24:0	SM C26:1
SM C16:1	SM C20:2	SM C24:1	
SM (OH) C16:1	SM (OH) C22:1	SM (OH) C24:1	

Table 1. List of metabolites analyzed using the kit.

Principal Component Analysis showed that the gamma irradiated group was well separated from the control group (data not shown). The signal intensities of the MRM pairs of the internal standards in the murine serum samples were compared to the values obtained for human plasma and to the values of the zero samples. Median and standard deviation values of the coefficient of variation (CV) were calculated for the different metabolite classes for all sample preparation conditions used in this study, as shown in Figure 5. Only levels of analytes with values above the limit of detection (LOD, defined as three times the median value of the zero samples) were considered. Exposure to gamma radiation induced significant changes in the levels of specific amino acids, such as arginine and serine, lyso-phosphatidylcholines (lyso-PC), phosphatidylcholines (PC), and acylcarnitines in mouse serum, as shown in Figure 6.

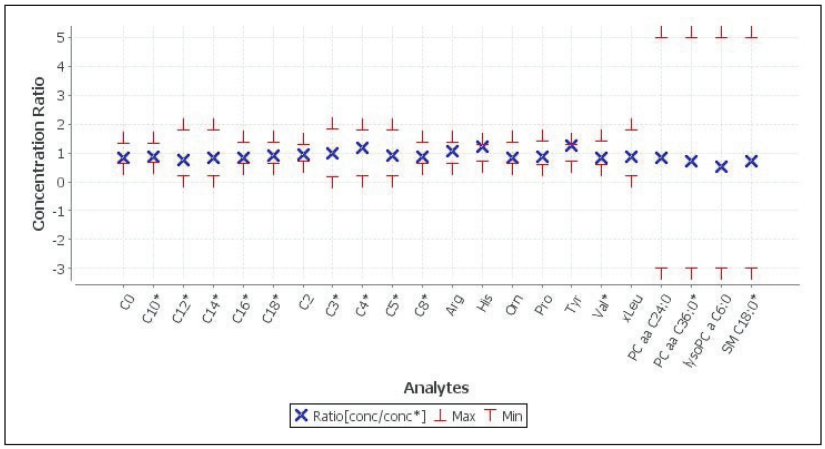

Figure 5. Quality control samples. Measured concentration/expected concentration ratios are displayed in the MetIDQ software, which is an integral part of the kit. Representative values for acylcarnitines (CO-C_{18}), amino acids, and lipids.

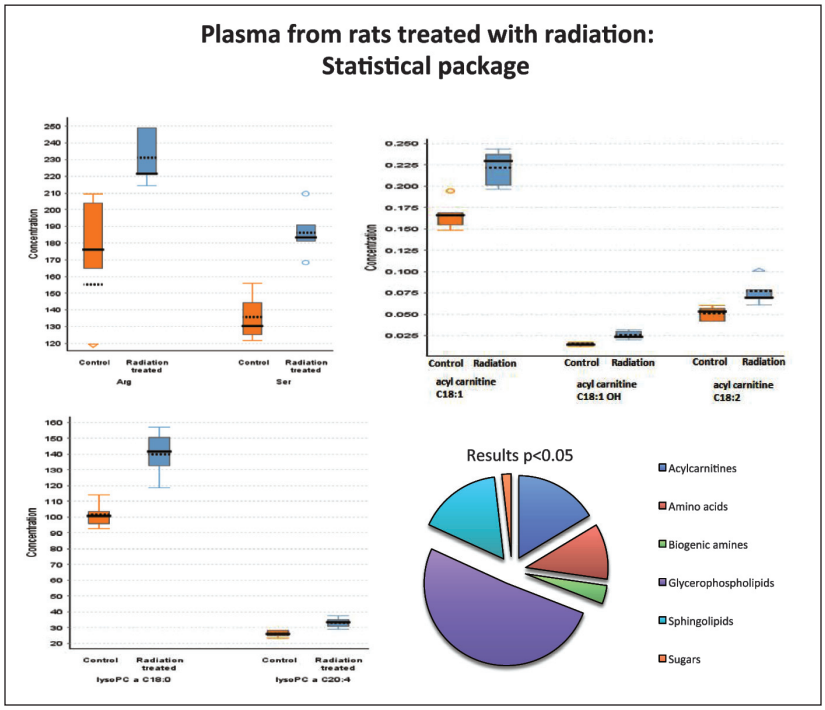

Figure 6. The box plots show examples of altered metabolites in the serum samples of gamma irradiated mice. The pie chart illustrates the kit metabolite panel separated into metabolite classes. Results of the statistically significant ions are presented as a percentage in each metabolic class.

CONCLUSIONS

By combining the ACQUITY UPLC System with the Xevo TQ or Xevo TQ-S Mass Spectrometers and the commercially available Absolute*IDQ* p180 Kit, rapid identification and quantification of more than 180 metabolites in murine serum were successfully attained. Similar applications could lead to novel mechanistic insight and biomarker discovery in drug development, diagnostics, and systems biology research.

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