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Cross-platform Q-TOF validation of global exo-metabolomic analysis: Application to human glioblastoma cells treated with the standard PI 3-Kinase inhibitor LY294002☆

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ABSTRACT

The reproducibility of a metabolomics method has been assessed to identify changes in tumour cell metabolites. Tissue culture media extracts were analyzed by reverse phase chromatography on a Waters Acquity T3 column with a 13 min 0.1% formic acid: acetonitrile gradient on Agilent and Waters LC-Q-TOF instruments. Features (*m/z*, RT) were extracted by MarkerLynx™ (Waters) and Molecular Feature Extractor (Agilent) in positive and negative ionization modes. The number of features were similar on both instruments and the reproducibility of ten replicates was <35% signal variability for ~50% and 40% of all ions detected in positive and negative ionization modes, respectively. External standards spiked to the matrix showed CVs <25% in peak areas within and between days. U87MG glioblastoma cells exposed to the PI 3-Kinase inhibitor LY294002 showed significant alterations of several confirmed features. These included glycerophosphocholine, already shown by NMR to be modulated by LY294002, highlighting the power of this technology for biomarker discovery.

1. Introduction

Metabolomics is defined as the quantitative profiling of endogenous metabolites in biofluids in order to characterize the metabolic phenotype or its response to stimulation [1,2].

Metabolomics strategies have been successful in a variety of applications, for example to characterize metabolic changes resulting from altered gene function in biological systems [3–5] or to assess the toxicological effect of chemical compounds [6–8]. Metabolic alterations in body fluids and human or animal tissues have the potential to improve clinical diagnostics and explore the return to cellular equilibrium following exposure to a particular drug, as well as highlighting off target effects and potential toxicity of novel chemical entities. The discovery of novel metabolic biomarkers, both disease-specific and pathway-specific, is a novel approach that offers exciting opportunities to guide the discovery and development of novel targeted agents in a number of therapeutic areas.

The large differences in physicochemical properties of metabolites require various analytical separation and detection methods. A series of analytical techniques are available including nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) typically coupled with gas or liquid chromatographic separation detection techniques [9–12]. In recent years, development of liquid chromatographic systems coping with higher pressure and smaller particle columns has provided increased chromatographic resolution, whilst reducing the run times [11,13,14]. In most cases, an electrospray interface (ESI) is used for the ionization step. Numerous types of mass analyzers are available and this study will describe two different quadrupole-time-of-flight (Q-TOF) instruments.

The technologies used in metabolomics produce large datasets, which need to be compared to identify those metabolites that vary between different groups. The critical information needs to be extracted from the mass of data [15,16]. Each chromatogram needs to be reduced to a set of variables each consisting of a molecular ion and a retention time. In addition, the intensity of the signal produced needs to be measured automatically and accurately for each variable. Different software packages are available to perform these operations. These initial data pre-processing steps are important as they determine the variables that will subsequently be compared by elaborate statistical methods. The process is designed to ensure that noise is eliminated from the analysis and that peak detection is optimal, taking into account possible shifts in retention time and mass accuracy.

Our aim is to use metabolomics to assist in the development of signal transduction inhibitors as novel therapeutic agents in

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oncology. We are using human cancer cells in culture to characterize the effects of treatment on the cellular exo-metabolome (extracellular metabolites). The first goal of this study was to demonstrate that the metabolomic analysis of tissue culture media was sufficiently robust to reliably and consistently identify specific changes in excreted or consumed metabolites. Using two different Q-TOF LC/MS instruments, the reproducibility of the analysis was assessed using automated and manual processing following replicate injections of tissue culture media spiked with external standards. The number and the reproducibility of each of these features were evaluated on both systems. The effect of the phosphatidylinositol 3-kinase (PI 3-Kinase) inhibitor LY294002 [17] on the exo-metabolome of U87MG human glioblastoma cells was then investigated using both systems. The features highlighted as being significantly altered in the samples were confirmed by integration with the associated quantitation software. The process allowing metabolite identification and deconvolution is illustrated, with the use of accurate mass and database searches, followed by LC/MS/MS to identify one of the metabolites significantly affected by treatment. The performance of both instruments throughout these different steps was monitored.

2. Experimental

2.1. Reagents and solutions

Water (HPLC grade), acetonitrile (HPLC grade) and formic acid (Aristar grade) were all purchased from Fisher Scientific (Loughborough, UK). Leucine enkephalin was purchased from Sigma (Poole, UK). The external standards [creatine (CAS no.: 57-00-1), carnitine (CAS no.: 541-14-0), colchicine (CAS no.: 64-86-8), hydrocortisone (CAS no.: 50-23-7), phenylalanine (CAS no.: 673-06-3) and hippuric acid (CAS no.: 495-69-2)] were purchased from Sigma (Poole, UK). Standard stock solutions of 1 mM were prepared in water or DMSO as appropriate and diluted with protein-precipitated cell culture media to 1 μM solutions. DMEM tissue culture medium and L-glutamine were purchased from Sigma (Poole, UK) and foetal calf serum (FCS) from PAA Laboratories (Somerset, UK).

2.2. Instrumentation

2.2.1. System 1: Agilent LC-Q-TOF

An Agilent 1200 Series Rapid Resolution LC system was connected to a hybrid quadrupole-time-of-flight Agilent 6510 mass spectrometer equipped with an electrospray ionization source, which was used in positive or negative mode (Agilent, Waldbronn, Germany).

2.2.2. System 2: Waters Micromass UPLC-Q-TOF Premier

A Waters Acquity UPLC system (Water, Herts, UK) was connected to a hybrid Q-TOF Premier equipped with an electrospray ionization source, which was used in positive or negative mode (Waters Micromass, Manchester, UK).

2.3. Analytical conditions

2.3.1. Chromatographic separation

On both systems chromatographic separation was performed on a Waters Acquity column HSS T3 C_{18} (100 × 2.1 mm, I.D. 1.8 μm). Mobile phase A was HPLC grade water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. The column and the autosampler were maintained at a temperature of 50 °C and 4 °C, respectively. A 13 min linear gradient elution was performed as follows: 100% mobile phase A for the first 0.5 min, changing to 100% B over 7.5 min, holding at 100% B up to 9.5 min and finally back to 100% A at 10 min and holding for 3 min. The flow rate was 0.4 ml/min on system 1 and 0.6 ml/min on system 2 and an injection volume of 10 μl was used.

2.3.2. Mass spectrometry conditions

2.3.2.1. System 1. MS source parameters were set with a capillary voltage of 4 kV in positive ionization mode and 3.5 kV for negative ionization mode. The fragmentor voltage was 100 V and skimmer was 65 V. The gas temperature was 300 °C, drying gas 8 l/min and nebulizer 40 psig. Nitrogen was used as a collision gas. MS spectra were acquired in full scan analysis over an *m*/*z* range of 100–1000 using extended dynamic range and a scan rate of 1.4 spectra/s. Data station operating software was MassHunter Workstation Software (version B.01.03). To maintain mass accuracy during the run time, a reference mass solution containing reference ions 121.0508 and 922.0097 was used in positive ionization mode and 112.39855 and 1034.9881 in negative ionization mode.

2.3.2.2. System 2. MS source parameters were set with a capillary voltage of 3.2 kV and 2.8 kV for the positive and negative ionization modes, respectively, with a cone voltage of 30 V. The cone and desolvation temperatures and gas flows (nitrogen) were 120 °C and 400 °C and 50 l/h and 900 l/h, respectively. TOF MS resolution was set to 4.7 for the low resolution mass filter and 15 for the high resolution mass filter. Argon was used as a collision gas with collision energy of 5 eV. MS spectra were acquired in full scan analysis over an *m*/*z* range of 100–1000. The detector potential was set to 2000 V. Scan times of 0.08 s/spectrum were chosen. Data station operating software was MassLynx 4.1. A LockSpray interface was used to maintain mass accuracy during the run time. For this a 1 ng/μl solution of leucine enkephalin (*m*/*z* 556.2771 or 554.2615 in positive and negative ionization modes, respectively) in acetonitrile:0.1% formic acid in water (1:1) was infused, post column, using an isocratic pump at a flow rate 40 μl/min.

2.4. Data processing

2.4.1. System 1

2.4.1.1. Molecular Feature extraction. The raw data (chromatograms) from the Agilent system were aligned and processed using the Molecular Feature Extractor (MFE) program within Agilent MassHunter Qualitative software (version B.02.00), which generates a list of features consisting of retention times (RT) and molecular masses. Sample processing used the following conditions: restrict retention time to 0.20–8.5 min, restrict *m*/*z* to 100–800, absolute height threshold: 10 000 and 500 for the positive and negative ionization mode, respectively, mass tolerance: 0.05, use peaks with height: >100 counts, isotope grouping: peak spacing tolerance: 0.0025 *m*/*z*, plus 7.0 ppm, isotope model: common organic model, mass filters: filter mass list: 20 ppm.

2.4.1.2. Statistical analysis. The data was analyzed using GeneSpring MS Analysis Platform (v1.2, Agilent Technologies, Inc., Santa Clara, CA). Extracted feature lists (neutral mass and retention time) were imported to GeneSpring MS where they were aligned and normalized. The data were then filtered by volcano plots, which combine the results of fold change filtering and *t*-tests in a single visualisation between treated and control groups, from which a mass list can be generated.

2.4.1.3. Confirmation of features affected. For those variables that showed a significant increase or decrease in GeneSpring MS following treatment with LY294002, raw data were re-integrated with MassHunter Quantitative analysis (version B.01.04) and *t*-test analysis performed to confirm significance.

2.4.2. System 2

2.4.2.1. Data alignment and peak detection. The raw data (chromatograms) from the Waters system were aligned and processed using MarkerLynx™ application manager software version 4.1 (Waters, Milford, USA) and tabulated as retention times and *m*/*z*. Samples were processed from 0.2 to 8.5 min with mass filters from 100 to 800, the retention time and molecular ion windows were ±0.15 min and *m*/*z* ±0.05 Da, respectively. Integration parameters were as follows: peak width at 5% height: automatic and 6 s, intensity threshold (counts) 50 (positive) and 25 (negative), noise elimination level: 6.00 and de-isotope filtering activated.

2.4.2.2. Statistical analysis. The obtained data matrix was then used for multivariate statistical analysis using the SIMCA-P v11.0 software (Umetrics AB, Umeå, Sweden). Orthogonal partial least-squares (OPLS) analysis was used as the classification method for modelling the discrimination between control and treated sample groups.

2.4.2.3. Confirmation of the variables affected. For those variables that showed a significant alteration in SIMCA following treatment with LY294002, raw data were re-integrated with QuanLynx Application Manager (v4.1) and statistical analysis performed with *t*-tests for unpaired variables (GraphPad Prism, version 4.3).

A summary of the overall analytical methods is shown in Fig. 1.

2.5. Metabolite deconvolution

In order to identify the metabolites, mass accuracy was determined on both systems and elemental formulae derived. A database search was then performed in Lipid Maps to elucidate the structure of the metabolite. In addition, MS/MS analysis was performed on both instruments using a collision energy of 15 V and acquiring data in the range of 80–500 *m*/*z*.

2.6. Sample preparation

Media samples were extracted using a 96 well protein precipitation plate (Whatman, Maidstone, UK) by adding 800 μl of acetonitrile followed by 200 μl of thawed media sample. The plate was vortexed for 1 min before a vacuum was applied. The filtered samples were collected in a 96 deep well plate.

2.7. Assessment of the analytical variability

Ten replicates of extracted tissue culture media were analyzed on both systems in the positive and negative ionization modes.

To continuously monitor the analytical variability of the system, external standard solutions (usually $n = 10$) were randomly added to any run. Colchicine, hydrocortisone, creatine and carnitine were spiked in extracted media at 1 μM for positive ionization mode, whereas hippuric acid and phenylalanine were used at 1 μM for negative ionization mode.

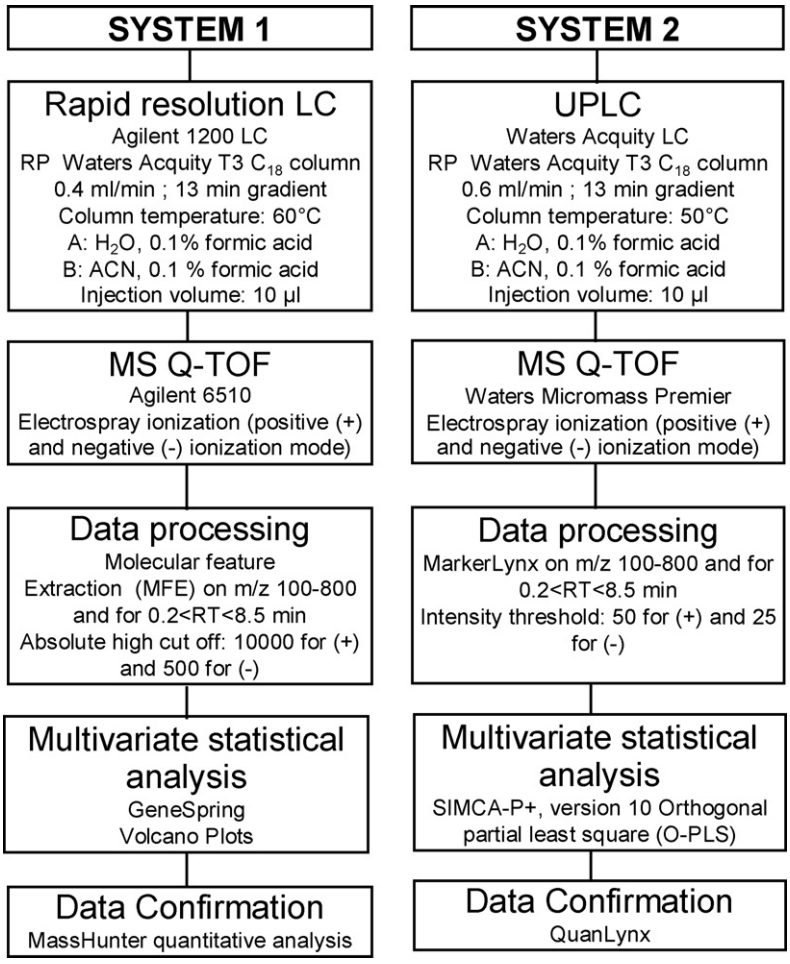

Fig. 1. Summary of sample analysis and data analysis workflow.

Table 1
Comparison of the number of features and reproducibility of data using systems 1 and 2 in positive and negative ionization modes.

ionization mode	Q-TOF	Total number of variables	% of variables whose CV is below	
			25%	35%
Positive	Agilent	1022	54	58
	Waters	1200	48	48
Negative	Agilent	335	31	47
	Waters	400	48	48

2.8. Cell culture and treatment with LY294002

U87MG cells (5×10^5) were grown for 48 h in T25 flasks (Corning, Surrey, UK) in DMEM tissue culture media containing 10% foetal calf serum. The media was then discarded, cells were washed with phosphate buffered saline (PBS) and media replaced with 4.5 ml of phenol red free DMEM medium containing either DMSO (control), 10 μM ($1 \times GI_{50}$) or 50 μM ($5 \times GI_{50}$) LY294002. Cells were incubated for a further 24 h with media aliquots taken at 30 min, 2 h, 6 h and 24 h. Samples were stored at −80 °C. In order to avoid analytical drift, samples were randomized throughout the run. Samples were analyzed using both systems and results processed and analyzed using the associated software packages.

3. Results and discussion

3.1. Validation

The ability to demonstrate that metabolomics data are of a high quality is crucial [18,19]. Important quality parameters are robustness, reliability and reproducibility. The analytical methodology employed in this study showed that it is possible to rapidly, consistently and reproducibly generate metabolomic profiles in positive and negative ionization modes. The advantage of a relatively short (10 min) run time is that large datasets can be analyzed in a timely fashion. By filtering metabolites with a molecular weight between 100 Da and 800 Da, both systems generated more features in positive than in negative ionization mode (Table 1). Although different flow rates were used, the column and gradients were similar, as were the number of features extracted on both systems. Peak areas were measured and the variability observed on both systems was similar, with approximately half the features showing less than 35% variation (Table 1). This suggests that in excess of 500 ions can be measured with acceptable reproducibility in positive ionization mode and around 200 in negative ionization mode. The knowledge of the degree of analytical variability is essential as it impacts on the biological increase or decrease required to detect statistically significant differences between groups. The higher the analytical variability, the greater the biological difference required to demonstrate statistical significance. For example, with 10% analytical variability (CV) on 10 replicates, an increase of 10% in the biological experiment produces statistical difference. With 25% analytical variation, this value increases to 25% and with 35% to 40%. The number of replicates impacts on both the analytical and biological variation. For example, with an analytical variation of 35%, the biological variation required to reach statistical significance increases to 60% for 6 replicates when compared to 40% for 10 replicates.

The analysis of the analytical reproducibility within a run was performed by monitoring a range of compounds with different physicochemical properties including known endogenous metabolites as well as xenobiotics. These compounds, referred to as external standards, eluted at various retention times throughout the analytical run. They were spiked in the matrix of 10 replicates, which were randomized in each analytical run. The study of the external standards ensured consistency of the LC system, evaluated by retention time, and of the mass spectrometer measured by mass accuracy. The maximum deviation in retention time for all the standard compounds was 0.03 min. The maximum mass accuracy deviation was 0.009 Da and 0.01 Da for positive and negative ionization mode, respectively.

Moreover, the results indicate that both systems showed high analytical intra-day reproducibility with less than 25% in both positive and negative ionization mode (Table 2). The use of the external standards provided a good estimate of inter-day variability with a coefficient of variation below 25% on five different days, allowing possible comparisons of samples analyzed on different days (Table 2). This is of critical significance if large databases of drug effects are to be compiled. In addition, knowledge of the different characteristics (retention time, peak area) of the external standards provides a rapid system suitability test that may be useful if analytical problems are encountered.

3.2. Metabolomics study of PI 3-Kinase inhibitor (LY294002) in U87MG cells

Phosphatidylinositol 3-kinase is a highly deregulated signalling pathway in many cancers. It phosphorylates the 3′ position of phosphatidylinositols. The negative regulator of PI 3-Kinase is the phosphatase PTEN which is absent in the human glioblastoma cell line U87MG. LY294002 is an inhibitor of PI 3-Kinase which has been a useful tool to understand the therapeutic potential of such inhibitors [20,21]. Circulating biomarkers of inhibition of the PI 3-Kinase pathway could help support the clinical development of PI 3-Kinase inhibitors.

Treatment of cells with 1× and 5× GI_{50} concentrations of LY294002 showed a significant number of variables that were increased or decreased on system 1 or system 2 as determined by integration generated with the associated software (GeneSpring MS or MarkerLynx). The validity of the features identified was then confirmed with manual integration with the associated quantitation software. The results of the number of features modified in positive ionization mode at 24 h by both treatments are shown in Table 3. GeneSpring MS identified 108 features significantly affected by both treatments and 58% of these features were reconfirmed by manual

Table 2
Intra- and inter-day variability of 6 metabolites used as external standards analyzed on systems 1 and 2 in both ionization modes. (n.a: non applicable).

ionization mode	Area (arbitrary units)				Intra-day CV (%) $n = 10$		Inter-day CV (%) $n = 5$	
	Positive		Negative					
	Agilent	Waters	Agilent	Waters	Agilent	Waters	Agilent	Waters
Colchicine	8.7×10^5	47.7	n.a	n.a	11.8	14.1	18.8	10.0
Hydrocortisone	1.5×10^5	9.9	n.a	n.a	13.9	13.2	8.3	11.2
Creatine	2.5×10^7	66.2	n.a	n.a	20.5	9.15	20.5	18.2
Carnitine	4.4×10^5	9.8	n.a	n.a	23	12.7	25	8.7
Hippuric acid	n.a	n.a	3.6×10^4	5.3	17.8	14.9	20.0	17.5
Phenylalanine	n.a	n.a	4.7×10^4	11.4	18.8	20	25	9.5

Table 3
Number of significant features affected by 1× and 5× GI_{50} concentrations of LY294002 at 24 h generated by GeneSpring and SIMCA for systems 1 and 2, respectively, in positive ionization mode and number of significant features confirmed with appropriate software.

	Agilent GeneSpring MS	Waters SIMCA
No. of significant features	108	150
No. of significant features confirmed after manual integration	MassHunter Quantitative analysis 63	QuanLynx analysis 69

integration with MassHunter Quantitative software. SIMCA analysis with OPLS identified 150 significant features and 46% of these features were reconfirmed by manual integration using QuanLynx. This corresponds to 63 features on system 1 and 69 features on system 2. Among these features, approximately 35% were found to be dose-dependent on both systems. The number of features specific to 1× and 5× treatment was 232 and 147, respectively, on system 1 and 120 and 58, respectively, of system 2. It has to be noted that system 1 takes into account adduct formation, therefore the lower number of significant variables identified does not reflect a lower performance of the overall analysis. Approximately 20% of these features were common to both systems. This may result from different ionization on each instrument detecting various metabolites. An alternative explanation is that the reproducibility of identical features may be different on the two systems.

3.3. Metabolite identification

Among the 20 common features significantly different in tissue culture media following treatment with LY294002, the molecular ion *m*/*z* 496.34 and *m*/*z* 540.33 in positive and negative ionization mode will be taken as an example for further characterization. This metabolite eluted at 6.75 min and 5.98 min on systems 1 and 2, respectively. Fig. 2 illustrates the peak area of this ion in the positive ionization mode in control and treated cells and shows a significant increase at 6 h and 24 h. A decrease of this ion was also observed over time in control cells.

Determination of the elemental formula of *m*/*z* 496.34 on both systems in the positive ionization mode gave $C_{24}H_{51}NO_7P$ as the most probable. The Agilent system gave a mass accuracy of −0.08 ppm with a score of 96 in positive ionization mode and −0.2 ppm with a score of 96 in negative ionization mode and the Waters system of 0.45 ppm with an i-fit of 21 in positive and −0.2 ppm with an i-fit of 15 in negative mode. The score includes both the mass calculated from the formulae as well as the isotope pattern match. It is rated on a scale of 100, the closer to a hundred the better. The i-fit provides a value for isotope fitting pattern and the lower the number the greater the mass accuracy. The databases Lipid Maps and Metlin both identified this metabolite as hexadecanoyl-*sn*-glycerophosphocholine (GPC1) which is naturally charged. However, two structures are possible for this hexadecanoyl-*sn*-glycerophosphocholine (Fig. 3). Unfortunately, we were unable to locate or purchase any analytical standard of this metabolite. We therefore performed MS/MS analysis on GPC1 in both positive and negative ionization modes. The interpretation of the fragmentation is shown in Fig. 4. In positive ionization mode, a choline fragment ($C_5H_{12}N^+$, *m*/*z* 86.10), choline ($C_5H_{14}NO^+$, *m*/*z* 104.10) and phosphatidylcholine ($C_5H_{15}O_4NP^+$, *m*/*z* 184.07) were observed. Additional information regarding the GPC1 back-

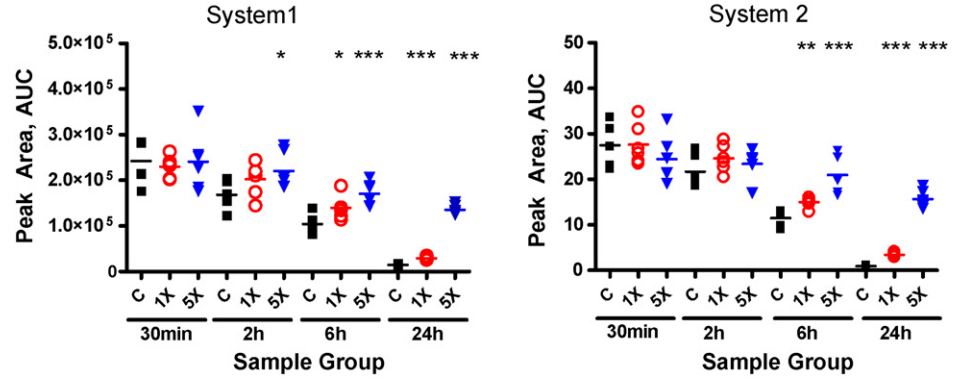

Fig. 2. Time course over 24 h of the ion *m*/*z* 496.34 in media of U87MG cells. Control (■), 1× (○) and 5× (▼) GI_{50} of LY294002 on systems 1 and 2. Significance for student *t*-test at 95%: * ($0.01 < P < 0.05$); ** ($0.001 < P < 0.01$); *** ($P < 0.0001$).

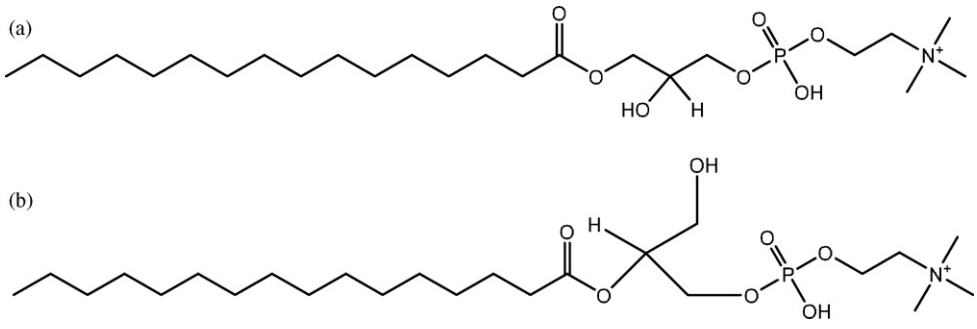

Fig. 3. Hypothetical structures of GPC 1: PC(16:0/0:0) (A) and PC(0:0/16:0). Note that this metabolite is naturally positively charged.

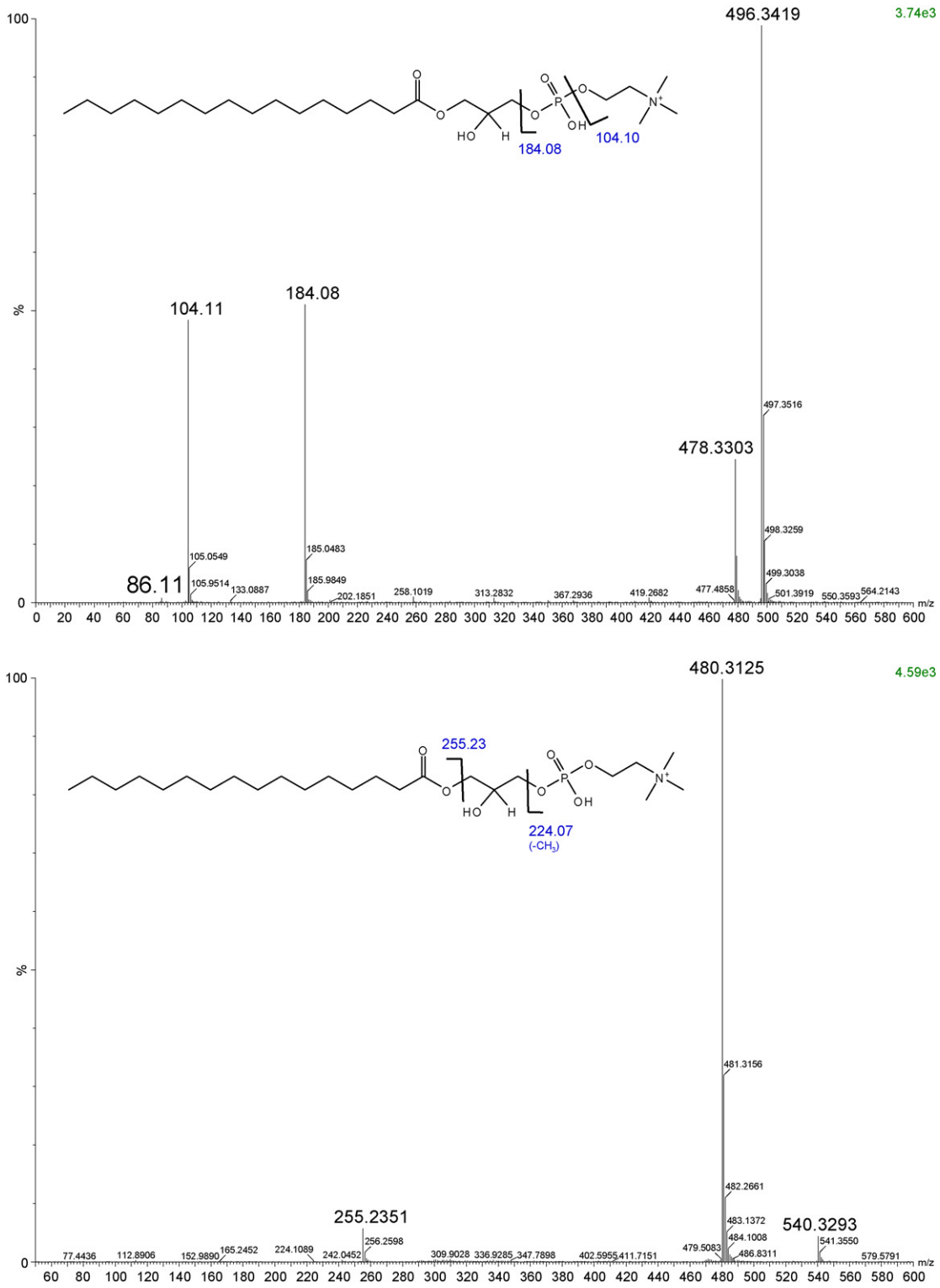

Fig. 4. MS/MS of GPC 1 in positive (A) and negative (B) ionization modes acquired on system 2 (collision energy = 15 V).

bone was obtained in negative ionization mode as the palmitic fragment (C16:0, *m*/*z* 255.2) was observed. Of note, the formation of a formic acid adduct allowed the detection of GPC1 in negative ionization mode. Although we were unable to discriminate between the two possible structures of GPC1 (Fig. 3) using MS/MS, the fragmentation confirmed this metabolite as a hexadecanoyl-*sn*-glycerophosphocholine. The mass accuracies of the fragments are shown in Table 4. The overall accuracy and variation were better on the Agilent system in positive ionization mode but the systems performed equally well in negative ionization mode. Altogether these data confirm the identity of this metabolite and indicate that both systems were able to identify it.

The increase of glycerophosphocholine observed upon treatment with LY294002 can be interpreted in several ways. Depletion

Table 4
Mass accuracy (in ppm) obtained in MS/MS for the fragment ion for GPC1 in positive and negative ionization mode (average on $n = 3$, best accuracy obtained).

	Mode +		Mode −	
Fragment ion	184.0734	104.1077	480.3090	255.2324
Agilent	1.4 (0.6)	0.7 (0.25)	3.5 (2)	3.4 (1.8)
Waters	1.6 (1.1)	6.4 (4.8)	2.2 (1.0)	3.13 (0.0)

of the metabolite from tissue culture media may suggest that it is a nutrient consumed by proliferating cells. The lower rate of decrease over time upon treatment would then reflect decreased proliferation. However, treatment of U87MG cells with 1× and 5× GI_{50} of the cytotoxic BCNU did not result in an increase in GPC1 levels (data not shown). Alternatively, this effect may be a specific effect of LY294002. LY294002 primarily inhibits PI 3-Kinase and mTOR but also other kinases such as casein kinase 2 (CK2), DNA-dependent protein kinase 2 (DNA PK) and Pim-1 [22–24]. The fact that similar experiments performed by NMR in breast cancer cells with this compound showed a decrease in intracellular phosphocholine and an increase in glycerophosphocholine signalling support the significance of our findings [25]. In addition, recent studies in the PC3 human prostate carcinoma cell line with the more specific PI 3-Kinase inhibitor PI103 [26] show similar results suggesting that a PI 3-Kinase and/or an mTOR inhibitor can deregulate choline kinase [27]. Whether this glycerophosphocholine will be a suitable circulating biomarker of PI 3-Kinase inhibitors *in vivo* remains to be established.

4. Conclusions

Our data show that metabolomic analyses of tissue culture media extracts performed on two Q-TOF instruments from different manufacturers are robust and that both perform reliably, generating very similar numbers of variables with high reproducibility within and between runs. In addition, automated data processing software and statistical packages provided with both systems generated very similar numbers of features affected in our experimental conditions. Finally, we were able to identify one of the metabolites previously shown to be altered with NMR based metabonomics, providing further evidence of the power of this technology. The mass accuracy provided by both instruments was key to the metabolite deconvolution and shows promise for the future of metabonomics in biomarker discovery.

Acknowledgments

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