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Metabolic profiling of serum using Ultra Performance Liquid Chromatography and the LTQ-Orbitrap mass spectrometry system

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ABSTRACT

Advances in analytical instrumentation can provide significant advantages to the volume and quality of biological knowledge acquired in metabolomic investigations. The interfacing of sub-2 μm liquid chromatography (UPLC ACQUITY®) and LTQ-Orbitrap mass spectrometry systems provides many theoretical advantages. The applicability of the interfaced systems was investigated using a simple 11-component metabolite mix and a complex mammalian biofluid, serum. Metabolites were detected in the metabolite mix with signals that were linear with their concentration over 2.5–3.5 orders of magnitude, with correlation coefficients greater than 0.993 and limits of detection less than 1 μmol L^{-1}. Reproducibility of retention time (RSD < 3%) and chromatographic peak area (RSD < 15%) and a high mass accuracy (<2 ppm) were observed for 14 QC serum samples interdispersed with other serum samples, analysed over a period of 40 h. The evaluation of a single deconvolution software package (XCMS) was performed and showed that two parameters (*snthresh* and *bw*) provided significant changes to the number of peaks detected and the peak area reproducibility for the dataset used. The data were used to indicate possible biomarkers of pre-eclampsia and showed both the instruments and XCMS to be applicable to the reproducible and valid detection of disease biomarkers present in serum.

1. Introduction

The metabolome is defined by the authors as the quantitative collection of endogenous and exogenous metabolites present in a cell or biological organism, whether synthesised and catabolised within the biological system or absorbed from its external environment (pharmaceuticals, food nutrients or the components of a growth medium or of symbiotic or passenger organisms). As well as the transcriptome and proteome, the metabolome is an appropriate functional level to explore [1,2] in the post-genomic era, given the desirability to study the interaction of all functional levels in systems biology investigations [3,4]. Further, as shown by Metabolic Control Analysis (MCA), changes in the concentration (but not necessarily the flux) of metabolites are amplified and thus detectable even when changes in the expression of proteins or transcripts are small or not detectable [5] and this has been observed experimentally [6,7]. Many metabolites are the final downstream products of the genome and reflect most closely the operation of the biological system, its phenotype. Finally, metabolomics can be described as a high-throughput strategy as the costs per analysis are low compared to those of proteomics and transcriptomics, and this allows greater numbers of samples to be analysed so to define technical and biological variance in a valid statistical manner [8].

Metabolomes are complex systems. The physical and chemical properties, their size and the ranges of their physiological concentrations are diverse, and these all influence the experimental strategy employed [9,10]. No single analytical methodology or platform is applicable to detect, quantify and identify all metabolites in a sample, the goal of metabolomics. Instead a strategy of metabolic profiling (or untargeted analyses) is commonly performed to allow the detection of a wide range of chemical classes and thereby obtain

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as broad a picture of metabolism as is achievable [2,9–11]. The combination of a range of analytical platforms is advantageous so to provide a greater coverage of the metabolome [6,12–16]. The strategy of metabolic profiling is advantageous as generally no *de novo* knowledge of the metabolites present is required. Instead a hypothesis-generating strategy is employed [17] where valid experiments are designed to collect a large volume of biological data that are interrogated so as to define metabolic differences related to the experimental objectives.

Within the toolbox, which researchers in metabolomics apply, are a range of analytical platforms that are employed [9,18–20]. These include gas chromatography–mass spectrometry (GC–MS) [6,21–23], comprehensive GC×GC–MS [24–26], liquid chromatography–mass spectrometry (LC–MS) [27–30] and variants including Ultra Performance Liquid Chromatography (UPLC) [31–33], capillary electrophoresis–mass spectrometry (CE–MS) [34–36], direct infusion mass spectrometry (DIMS) [37–40], Fourier transform infra red spectroscopy (FT-IR) and Raman spectroscopy [41–43] and NMR spectroscopy [13,44–46]. Of these, mass spectrometry and NMR have played a key role in the development of metabolomics (and the related discipline of metabonomics). Chromatography–mass spectrometry platforms offer a number of advantages to the study of complex metabolomes. High chromatographic resolution is obtainable, and this enables the partial or complete spatial separation of metabolites prior to detection, providing a 'pure' fragmentation mass spectrum or accurate mass for each metabolite to assist identification. The mass spectrometer provides the ability to detect metabolites at physiological concentrations (μmol L^{-1} to mmol L^{-1}) and the capability to identify metabolites through the application of retention times/indices and accurate mass or fragmentation mass spectra. The identification of metabolites detected is currently an important area where improvements are required, as many metabolites are currently classified as unidentified [47]. GC–MS was the first chromatographic platform employed in metabolic profiling studies in the 1960/1970s and more recently as metabolomics (a term first used in [48]) in 2000 for both the study of mammalian and plant systems [23,49–51]. Although LC–MS has lagged behind these early developments, its applications have expanded rapidly for the investigation of microbial, plant and mammalian metabolomes in the previous 5 years [19,27,52–54].

Recently, the introduction of two novel analytical platforms (UPLC [33] and the Orbitrap mass spectrometer [55]) has increased the volume of metabolic information obtained from any single sample compared to other LC–MS platforms currently used. UPLC operates with sub-2 μm chromatographic particles and a fluidics system capable of operating at pressures up to 15,000 psi, providing an increased chromatographic resolution compared to conventional HPLC using larger particles. The system allows the use of a wider range of linear velocities while maintaining good chromatographic resolution and therefore can provide more rapid analysis times. The high chromatographic resolution, which results in narrow peak widths and an increased S/N compared to conventional HPLC, is advantageous in metabolic profiling to allow the detection of a greater number of metabolites at physiological concentrations. A number of applications using UPLC have been described recently [13,32,56,57].

The Orbitrap mass spectrometer, otherwise defined as an electrostatic Fourier Transform mass spectrometer, is constructed of a spindle-shaped central electrode and two bell-shaped outer electrodes between which ions are constrained by a combination of electrostatic and centrifugal forces. Ions orbit the central electrode in both the axial and radial directions. The frequency of the harmonic oscillations in the radial direction is inversely proportional to the square root of the mass-to-charge (m/z) ratio. The image currents of these orbiting ions are detected by the outer electrodes and converted from time to frequency domain by Fourier transformation. The Orbitrap provides a higher mass resolution and mass accuracy over a wider dynamic range than is achievable with many other mass spectrometers [58], allowing the potential detection of a greater number of metabolites of similar accurate mass with a high level of confidence of metabolite identification, especially when coupled to retention times and the use of mass spectral libraries constructed with authentic standards. The Orbitrap is operated in combination with a linear ion trap in the hybrid LTQ-Orbitrap analytical platform and offers extra options of operation. The combination of two mass analysers allows two different scan types to be acquired simultaneously, similar to the process of MS^E described for the hybrid Q-TOF instrument by Plumb et al. [59] except that these scans were consecutive rather than in parallel. The two scan types are the collection of an 'accurate mass' spectrum in the Orbitrap in parallel to the collection of single or multiple MS/MS mass spectra using data-dependent analysis (DDA) in the linear ion trap. This provides multiple routes to identify metabolites on-line, though the software required to apply on-line identifications automatically is limited at present. With the coupling of UPLC and the LTQ-Orbitrap, fast scan or acquisition rates are required so as to provide sufficient data points across narrow chromatographic peaks. The mass resolution of the Orbitrap is correlated to acquisition time with longer acquisition times providing higher mass resolution. An acquisition time of 0.4 s provides a mass resolution of 30,000 (1.5–6 times greater than that observed for high resolution TOF instruments) and 25 data points across a peak of width at baseline of 10 s. Therefore, the acquisition of MS/MS spectra in the linear ion trap rather than the Orbitrap is advantageous, though collection of these data in the Orbitrap operating at a lower mass resolution of 7500 with a scan time of 0.1 s could be applied as an alternative with 20 data points collected across a peak. A limited number of studies report the use of the LTQ-Orbitrap instrument for metabolic profiling [60–63].

The validity and reproducibility of metabolic profiling data are influenced not only by the experimental and instrumental procedures but also by the data processing methodologies. A common approach for chromatography–mass spectrometry data is to apply deconvolution software to provide *in-silico* conversion of the three-dimensional raw data (m/z, retention time, ion current) to time and mass-aligned chromatographic peaks with associated peak areas [64,65]. This allows the alignment and fusion of data across multiple samples. An alternative is to employ feature selection strategies to define regions of metabolic difference between multiple sample classes [66–68]. There are a number of deconvolution software packages existing, available either freely (XCMS [69], MZmine [70], Metalign, MSFACTS [71], COMSPARI [72], MathDAMP [73] and METIDEA [74]) or commercially (ThermoFisher Scientific SIEVE, Waters MarkerLynx, Agilent GeneSpring and MDSSciex MarkerView). It has been observed for GC–MS and LC–MS data that processed data can be highly variable in respect to the number of peaks reported and reproducibility of peak areas, accurate mass and retention time [75]. Small changes in a single software parameter can greatly influence the validity and information content of results [76] and validation of software operation is required [77].

In this paper the combination of UPLC with an LTQ-Orbitrap mass spectrometry system for metabolic profiling of serum is evaluated with regards to sensitivity, reproducibility (retention time, peak area, mass accuracy) and discriminatory power for disease biomarker studies. The analytical advantages and limitations of both systems will be discussed with the use of an 11-component metabolite mix and with serum samples. The application of XCMS is also investigated for the processing of data obtained from the metabolic profiling of serum with a case–control study employing data obtained from a pre-eclampsia investigation.

2. Experimental

All reagents used were of HPLC Grade purity or Analytical Grade purity and purchased from Sigma–Aldrich (Gillingham, UK).

2.1. 11-Component metabolite mix study

Single-component solutions of each of eleven metabolites (leucine, glutamic acid, valine, phenylalanine, tryptophan, cytosine, adenosine, dopamine, galactose, glucose-6-phosphate and glucosamine-6-phosphate) were accurately prepared at concentrations in the range 15–22 mmol L^{-1} and aliquots of each combined to create a multi-component metabolite solution in which each metabolite was present at a concentration of 1–3 mmol L^{-1}. Dilution of this stock solution was performed to provide a series of calibration solutions in the concentration range 0.1–1000 μM.

Analysis of these calibration solutions was performed employing UPLC interfaced to the LTQ-Orbitrap mass spectrometer system, as described below. One technical replicate was analysed for each of the calibration solutions with the exception of the blank solution (water) where three replicates were analysed.

2.2. Preparation of serum and plasma samples

Serum samples (100 μL) were deproteinised by mixing with methanol (300 μL) at room temperature, vortex mixing for 15 s and centrifugation (13,487 × *g*, 15 min). Supernatants were transferred to Eppendorf tubes and lyophilised (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap (Jouan, Gydevang, Denmark)). Samples were reconstituted in 100 μL water prior to analysis.

2.3. Reproducibility study using biologically identical quality control (QC) samples

Two separate reproducibility studies of length 40 h were performed for the separate analysis of serum samples in positive and negative ion modes. The objectives of the studies were to both assess the reproducibility of the UPLC/LTQ-Orbitrap coupled system and report on the number of metabolites that can be detected using the platform. Fourteen biologically identical serum samples (designated as QC samples) prepared from a pooled serum sample (available commercially from Sigma-Aldrich, Gillingham, UK) were analysed at intervals during a 40 h period. Data for each QC sample was acquired as analysis number 1, 2, 3, 4, 5, 18, 29, 41, 52, 65, 76, 88, 99, 111 and were interdispersed with 97 other serum samples. Analysis of the samples was performed as described below.

2.4. Clinical case–control biomarker investigation

A small case–control clinical metabolomics study was performed to investigate the potential of the technologies described for application in the discipline of mammalian biomarker discovery. This study was designed as a validation study for previously published work on pre-eclampsia (a multi-system disorder of pregnancy [78]). Briefly, plasma samples were obtained from 20 primiparous Caucasian women with pre-eclampsia diagnosed according to ISSHP guidelines from a single maternity unit in England. Controls were obtained from the same antenatal population and were matched for maternal age, parity and BMI and for gestational age at sampling. Plasma samples were only retained from controls for this study if they subsequently experienced an uncomplicated pregnancy. The full sample collection and matching protocol has been reported [79]. Samples were prepared and analysed in a random order.

2.5. UPLC/LTQ-Orbitrap operation

Samples were analysed in positive and negative ion modes, separately, with a method developed using a closed-loop, multi-objective optimization experiment, similar to those described for GC–MS, GC×GC–MS and ESI–MS [80–82]. Samples were analysed in a randomized order using an Acquity UPLC (Waters, Elstree, UK) coupled to a LTQ-Orbitrap mass spectrometry system operating in electrospray ionization mode (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separations were performed employing an Acquity UPLC BEH 1.7 μm-C_{18} column at a flow rate of 0.4 ml min^{-1}. The column was eluted with 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The column was held at 100% A for 1 min and subsequently ramped to 100% B (curve 5) over 15 min, followed by a 4 min period at 100% B before a rapid return to 100% A and an equilibration period of 2 min. The column and samples were maintained at temperatures of 50 °C and 10 °C, respectively. A 10 μL sample volume was introduced onto the column. 50% of the column effluent was transferred to the mass spectrometer. Centroided mass spectra were acquired in the mass range of 50–1000 Th using the Orbitrap mass analyser operating with a target mass resolution of 30,000 (FWHM as defined at *m*/*z* 400) and a scan time of 0.4 s. All samples for each analytical run were prepared, stored at 10 °C in the UPLC autosampler and analysed within 48 h of reconstitution. Mass calibration was performed according to the manufacturer's guidelines using a manufacturer-defined mixture of sodium dodecyl sulphate, sodium taurocholate, the tetrapeptide MRFA and Ultramark 1621.

Data obtained in the instrument-specific data format (.RAW) were converted to NetCDF files for further data analysis using a conversion software program (file converter program available in ThermoFisher Scientific Xcalibur software).

2.6. Deconvolution using XCMS software

After analysis, each three-dimensional data matrix (intensity × *m*/*z* × time – one per sample) was converted (or deconvolved) into a vector of *peak responses*, where a *peak response* is defined as the sum of intensities over a window of specified mass and time range (e.g. *m*/*z* = 102.1 ± 0.01 and time = 130 ± 10 s). In this experiment the deconvolution was performed using the freely available XCMS software (http://masspec.scripps.edu/xcms/xcms.php).

XCMS is an LC–MS-based data analysis approach which incorporates novel nonlinear retention time alignment, matched filtration, peak detection, and peak matching [69,83]. The XCMS deconvolution process can be broken down into four basic steps: *peak picking*, *peak grouping*, and *retention time correction*, followed by a second *peak grouping*. After retention time correction, the initial peak grouping becomes invalid and the resulting data needs to be regrouped. A detailed description of the XCMS methodology is available (http://masspec.scripps.edu/xcms/xcms.php).

The operation of the XCMS software is complex but flexible allowing many parameters to be altered or 'tuned' by the user. The *peak picking* step provides eight parameters to optimise (*matched filter* method), the *peak grouping* provides five parameters, and the *retention time correction* also provides five parameters. Preliminary experiments (data not shown) showed that the raw data presented in this study were insensitive to the majority of these parameters (changing the parameter settings showed minimal changes in the number of peaks detected and the reproducibility of peak areas). Only two parameters significantly affected the number of peaks found and the reproducibility of peak areas when they were changed from their default settings. These two parameters were the signal to noise threshold in the *peak picking* algorithm, *snthresh*, and the "the inclusiveness of the grouping" parameter in the *peak group-*

ing algorithm, *bw* (used only in the second pass of this algorithm). In order to provide an indication of the effects of these two parameters on the data obtained from metabolic profiling studies described above, a set of *in-silico* experiments were performed. Each raw QC dataset (positive and negative mode, $n = 14$) was processed using XCMS such that *snthresh* was varied from 3–20 (default = 10) and *bw* was set to either 5, 10 or 30 (default 30). The optimal parameter settings were then compared to the default settings using the pre-eclampsia dataset.

2.7. Statistical analyses

In order to assess and compare the reproducibility of the metabolic profiles for different studies, or operating conditions, some sort of statistical analysis and/or graphical representation of response trends are required. Traditionally, in industrial process quality control (QC), only a small number of measurements need to be compared and thus the vast majority of QC analysis results are depicted using variants of the Shewhart's control chart plus associated statistics [84]; (see http://www.multiqc.com/shewhart.htm for demonstration). These charts independently monitor the variation in each QC measurement and then through the analysis of long and short term variations (LTV and STV, respectively) and the calculation of statistics such as *Capability Index* or *Performance Index*, the quality of the process is estimated.

The quality of a manufactured product centres on its uniformity about *a target*. There is no unique target in clinical chemistry, but there is a true concentration for each sample. So experimental quality is defined as uniformity of assayed concentrations about true concentrations. Tolerance limits tell us how much variation can be accepted. They are laid down by national or international regulations (for example, Food and Drug Administration, FDA) or derived from clinical needs or biological variation. In metabolomic analysis employing chromatography–mass spectrometry platforms, the absolute concentration (represented by the chromatographic peak area) of a particular metabolite in a QC serum sample cannot be predicted, thus analytical quality at a single metabolite level resolves down to setting tolerances on the variance of QC responses over a time period. The FDA suggests that variability of ±15% of the nominal value represents an acceptable degree of reproducibility, in addition the FDA allow 33% of QC samples to fall outside the acceptance criteria whilst accepting the analytical run as suitable for data analysis [85]. In research, rather than industry, the tolerances may need to be relaxed (acceptance of 20% and 40% are reported). So if the LTV or STV of the QC samples move outside the tolerance limits the process is said to be out-of-control and the cause/s of the intolerance need to be traced and eliminated, thus moving the process back into statistical control.

For the QC experiment described above the results will be reported in the following way. For each dataset generated by the XCMS process, any peak vector (response for that peak across all QC samples) with more than 40% missing values will be removed. This is performed for two reasons. Firstly, because such lack of consistency is a clear indication of poor reproducibility across the experiment. Secondly, any subsequent statistical measures for these peaks will be unrepresentative of the actual distribution of the data. For each peak vector that passes the missing values criteria the relative standard deviation (RSD – population standard deviation divided by the population mean) was calculated and 15% and 20% tolerance tests were performed. Also when comparing the different XCMS settings, the number of peak vectors with an RSD of less than 15% were compared with the number of peaks consistently detected. This RSD value was arbitrarily selected as a good threshold for a peak of suitable peak area reproducibility.

For the pre-eclampsia study the statistical analysis follows the protocols used in previous clinical metabolomics studies [22]. Univariate statistical analysis was performed in order to both pick and assess the characteristics of each independent peak generated using the above protocol. As the experimental design employed in this study was that of a matched case–control study, and taking into account that assumptions about population normality cannot be assumed, the non parametric rank-based Mann–Whitney *U*-test was used. For each metabolite, the null hypothesis (that the sample metabolite concentrations from both classes came from populations with the same mean) was tested. The critical *p*-value for rejecting the null hypothesis in a single test is usually 0.05 or 0.01 – i.e. there is a one in 20 chance or a one in 100 chance of randomly finding a significant biomarker (a false discovery). In this study many metabolites are tested in parallel. In order to compensate for the possibility of Type I errors (or false discoveries) the *p*-value for rejecting the null hypothesis for an individual metabolite must be reduced. Due to the high chromatographic and mass resolution of the technologies used in this study it is likely that many metabolites will have more than one peak (due to chemical adduction, isomerism, dimerization, etc); thus, in effect reducing the number of parallel tests. As a guide, the critical *p*-value was loosely set to 0.01. This value is still quite high but provides a useful threshold for the comparisons presented in this paper.

3. Results

3.1. Linear dynamic ranges, detection limits and mass accuracy for an eleven-component metabolite mix

The analytical capabilities of the combined UPLC/LTQ-Orbitrap system were investigated with a simple mixture of eleven metabolites present in a water matrix. This investigation was limited in the number of metabolites studied and is an initial descriptive study to define analytical capabilities. Data were collected as single technical analyses over the concentration range 0.1–1000 μM. Data for three technical replicates were also acquired for a blank sample (water only).

Typical chromatographic peak widths ranged from 7 to 15 s at baseline, demonstrating that the achievable chromatographic resolution is relatively high for the UPLC system. Peaks of narrower width have been reported [86], though the authors prefer these peak widths to ensure the satisfactory collection of data points across a chromatographic peak while operating the Orbitrap mass analyser with a mass resolution of 30,000 which requires a 0.4 s acquisition time. In general peak widths of 5–10 s allow the collection of an adequate number of data points across a chromatographic peak while maintaining good sensitivity and mass accuracy for a wide range of mass spectrometers. The authors operate the UPLC system at a less than optimal flow rate and temperature to achieve good data sampling across a chromatographic peak. However, wider chromatographic peaks can produce greater co-elution and a lower chromatographic resolution which can be surmounted with appropriate data processing of accurate mass data. The problems of matrix effects can be observed with co-eluting peaks. It is recommended to study narrower chromatographic peaks when collecting nominal mass data where data processing is less effective for co-eluting peaks. However, it should be noted that a number of peaks displayed a peak width considerable greater than this range (15–30 s) across the chromatogram. This is a compromise in metabolic profiling of a wide diversity of metabolite classes, of which not all classes are suited to the chromatographic phase and methodology employed in this study. The application of reversed phase and HILIC columns may be beneficial in metabolic profiling

Table 1
Data detailing the limits of detection (LOD), linear concentration ranges and correlation coefficients for 11 metabolites detected in positive and negative ion modes

Metabolite	Ion mode	Linear dynamic range (μmol L^{-1})	Correlation coefficient	LOD (μmol L^{-1})
Galactose	Negative	0.9–900	0.9992	0.84
Glutamic acid	Negative	0.8–800	0.9987	0.73
Glutamic acid	Positive	0.8–400	0.9935	0.79
Phenylalanine	Negative	0.8–800	0.9968	0.72
Phenylalanine	Positive	0.08–400	0.9988	0.07
Glucose-6-phosphate	Negative	0.3–600	0.9958	0.22
Adenosine	Negative	0.6–600	0.9959	0.58
Adenosine	Positive	0.6–360	0.9995	0.58
Dopamine	Negative	0.8–800	0.9991	0.78
Glucosamine-6-phosphate	Negative	0.4–400	0.9986	0.35
Leucine	Positive	0.7–700	0.9992	0.66
Cytosine	Positive	0.9–900	0.9999	0.86
Valine	Positive	0.7–700	0.9973	0.65
Tryptophan	Positive	0.2–200	0.9998	0.18

Table 2
Evaluation of the reproducibility of retention times (RT), accurate masses and peak areas for 14 QC samples analysed over a period of 40 h

Metabolite	Ion mode	RT range (min)	RT RSD (%)	Mean error in accurate mass (ppm)	Peak area RSD (%)
Valine/norvaline	Positive	0.97–1.04	2.6	−0.91	9.6
Leucine/isoleucine	Positivee	1.96–2.05	2.0	−0.38	7.7
Phenylalanine	Positive	2.90–2.94	2.9	0.04	14.7
Uric acid	Negative	1.04–1.08	1.1	−5.6 (−0.18)	13.6
3-Methyl-2-oxobutanoic acid	Negative	2.68–2.76	0.7	−4.9 (0.48)	12.5
Alpha-hydroxyisovaleric acid	Negative	3.45–3.48	0.4	−5.4 (0.02)	13.8
Glycerophosphocholine	Negative	14.89–14.94	0.1	−6.5 (−1.16)	10.5

The values for accurate mass mean error are acquired with external mass calibration and those values in parentheses are calculated using an internal mass calibrant to compensate for short-term variations in the mass calibration. Glycerophosphocholine is a possible identifier of a metabolite as defined by metabolite class. Other metabolites have been identified by comparison of retention time and accurate mass to authentic standards.

studies by providing a combined chromatographic resolution of a greater number of metabolites [29].

The data for linear dynamic ranges and detection limits are shown in Table 1, and were calculated using accurate mass single ion chromatograms (mass, approximately ±0.01 Da). Linear dynamic ranges extended over 2.5–3.5 orders of magnitude with correlation coefficients greater than 0.993. Deviation from linearity was observed at concentrations greater than the upper limit defined. The gradient decreased at these concentrations compared to the concentration range describing linearity. These are satisfactory though wider dynamic ranges have been reported for other instruments, including quadrupole mass spectrometers. Limits of detection were less than 1 μmol L^{-1}, which is important as the physiological concentrations of many primary metabolites are greater than this concentration (see http://www.hmdb.ca/). The chromatographic resolution and S/N achieved combined with the high sensitivity of the Orbitrap mass analyser is advantageous for metabolic profiling of mammalian biofluids. A number of metabolites were detected in both positive and negative ion modes.

An evaluation of the short-term mass accuracy for four metabolites across a wide concentration range was also calculated and is described in Fig. 1. The accurate mass was calculated using the single mass spectrum acquired at the chromatographic peak apex. These mass accuracies were observed with an external mass calibration only and show that sub-2 ppm mass accuracy is readily achievable over a wide concentration range (extending over 3 orders of concentration) to concentrations approaching the limit of detections for the four metabolites studied, though further investigations over a wider range of metabolites is required. This is important in metabolomics because of the wide range of physiological concentrations observed which allows narrow mass range windows to be employed in deconvolution software and also provide more definitive metabolite identifications. The application of an internal mass calibrant or stitching algorithms [87] may increase this mass accuracy further.

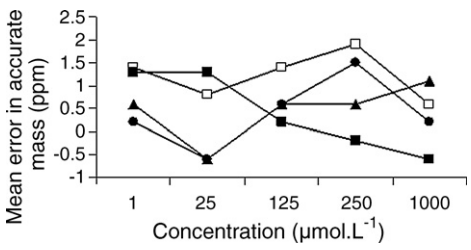

Fig. 1. Mass accuracies (ppm) observed for galactose (●), glutamic acid (□), phenylalanine (▲) and dopamine (■) acquired in positive and negative ion modes over a range of concentrations (1–1000 μmol L^{-1}).

3.2. Reproducibility of retention times, longer-term mass accuracy and peak areas for a 40 h analytical run

The assessment with a pure metabolite mixture described the analytical capabilities of the coupled UPLC and LTQ-Orbitrap platforms, though for a relatively simple sample. The next assessment investigated the reproducibility of retention times, accurate masses and peak areas for a single analytical batch acquired using serum, a considerably more complex sample. This was performed by assessing results obtained for the analysis of biologically identical QC samples, during an analytical run of 111 samples over a 40 h period. The data were assessed and integrated using the Xcalibur software package (ThermoFisher Scientific, Bremen, Germany) to determine retention times, mass accuracy and peak areas for a randomly chosen set of definitively identified metabolites (accurate mass and retention time match of authentic standard and metabolite in sample). Many of the metabolites are relatively polar with only one lipid-based metabolite chosen and metabolites were not chosen to represent all classes present in the serum metabolome. The accurate mass is calculated using the single mass spectrum acquired at the chromatographic peak apex. The data are shown in Table 2 and

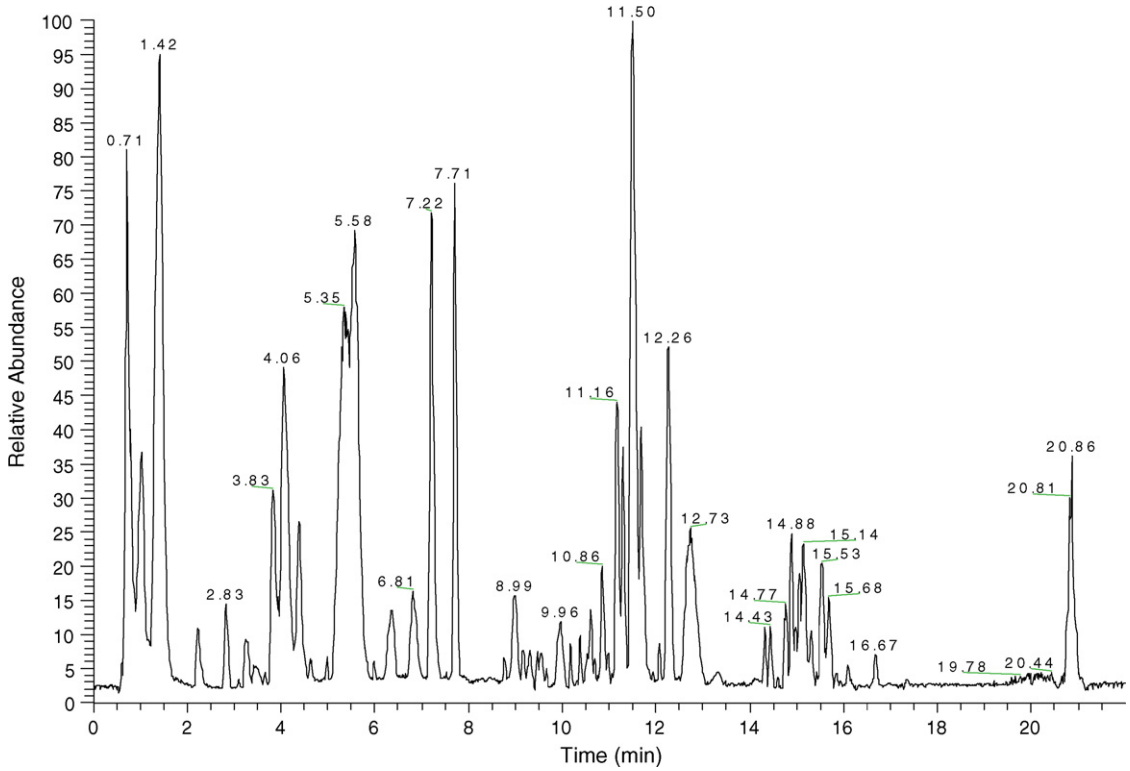

Fig. 2. A typical negative ion base peak chromatogram of serum showing the wide dynamic range of metabolites detected. More than 1000 peaks were reproducibly detected (RSD < 20%) in a set of QC serum samples across a 40 h batch analysis.

a typical base peak chromatogram is shown in Fig. 2. Retention time is adequately reproducible with relative standard deviations of less than 3%, which is acceptable for chromatographic peaks of typical width 7–15 s. The reproducibility of peak areas are also within an acceptable range of less than 15% RSD, a criterion described recently for metabolic profiling applications [85,88]. The mean errors in accurate mass were less than 3 ppm for positive ion mode employing external mass calibration only, though were typically between −4.9 and −6.5 for negative ion mode showing a systematic bias. This bias was compensated for with an internal mass calibrant (citric acid, $[M-H]^-$ mass 191.0192) and showed a decrease in the mean error to the range of −1.2 to 0.48 ppm. These levels of mass accuracy are acceptable and when combined with retention times are suitable for metabolite identifications by comparison of retention time and accurate mass to libraries constructed using authentic standards. The authors would like to stress that at least two orthogonal properties should be employed for metabolite identification when employing one analytical instrument, though multiple identification strategies should be employed [89]. In studies similar to those reported here, the authors apply a strategy of retention time, accurate mass and, where possible, MS/MS spectra for identification of metabolites.

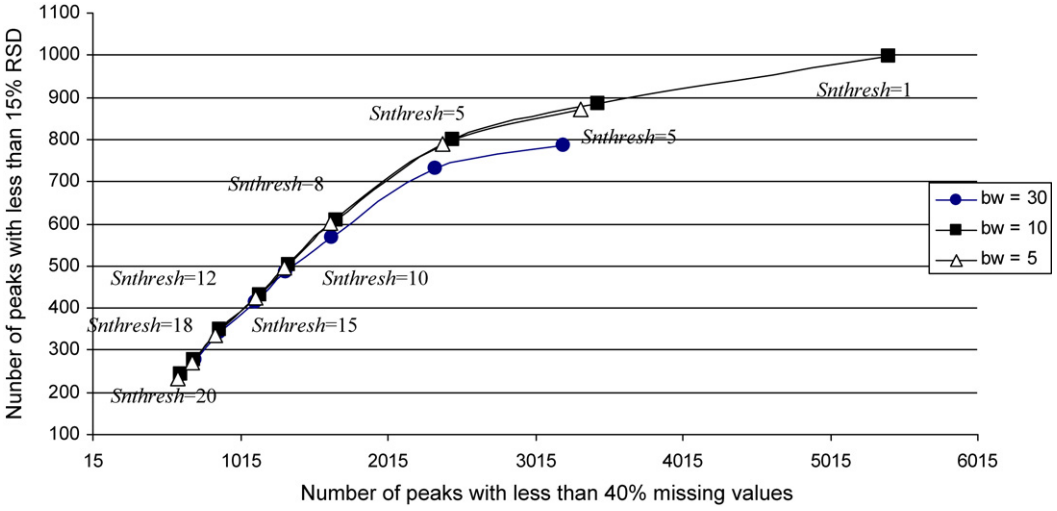

Fig. 3. Assessment of peak area reproducibility and number of peaks reproducibly detected in more than 60% of the samples for a range of values for the *bw* (30 (●), 10 (■) and 5 (△)) and *snthresh* (1–20) settings for negative ion data for all XCMS *in-silico* experiments. Labels show the different *snthresh* parameter settings for each processed dataset. Values for the parameter setting *snthresh* = 1 was calculated only for *bw* = 10.

3.3. Reproducibility study of the XCMS software using QC samples

The evaluations described above describe the reproducibility observed for the coupled analytical platforms for simple metabolite mixes and complex mammalian fluids, in this example serum. The process of converting analytical data to biological knowledge requires the conversion of the raw analytical data (*m*/*z vs* retention time *vs* ion response for chromatography–mass spectrometry-derived data) to a data matrix that is aligned in both the *m*/*z* and retention time dimensions. A range of deconvolution software packages are available to undertake this process [69–74]. The authors currently employ XCMS and an evaluation of the software was designed employing 14 QC samples analysed over a period of 40 h so as to mimic real experimental conditions and variations. A range of software settings are available and an initial study showed that two of these (*bw* and *snthresh*) were sensitive, changing the parameter settings showed large changes in the number of peaks detected and the reproducibility of peak areas. These settings were evaluated over a range of values to assess further the reproducibility of processed data and the volume of biological information acquired.

Fig. 3 shows the variability of the number of peaks detected with RSD less than 15% in comparison to the number of peaks detected in more than 60% of samples investigated, while varying both the *bw* and *snthresh* settings. The plot shows that as the *snthresh* increases fewer peaks are detected, though the ratio of peaks detected to peaks with RSD less than 15% remains relatively constant for all three *bw* values in the *snthresh* range of 5–20. In the range of *snthresh* below 5 this linear ratio is not observed with the increase in the number of peaks detected being greater than the increase in the number of reproducible peaks observed. One might expect that as the *snthresh* parameter is lowered (i.e. adding in relatively noisy peaks) the number of peaks with less than 15% RSD would level off to a constant value. However, although the gradient reduces as *snthresh* decreases to a value less than 5 both the number of peaks found and the number of 'reproducible' peaks continue to increase. Unexpectedly, employing a *snthresh* = 1 provides a greater number of reproducibly detected and reported peaks than using higher *snthresh* values. For example, an additional 100 reproducible peaks are detected for *bw* = 10 when comparing *snthresh* = 1 and *snthresh* = 3. This is a surprising result as *snthresh* = 1 is analytically defined as the measured response for noise and signal being of the same amplitude. The definition of *snthresh* for the software is ambiguous. Although improbable, these peaks could be reproducible noise peaks observed over a 40 h period although a number can be expected to be authentic metabolite peaks. In case–control studies where metabolic differences between sample classes are being investigated, noise peaks can be filtered during pre-processing steps using statistical methods, though this is not necessarily true for multivariate methods where appropriate cross-validation is required [8]. This unusual observation may be a result of minimal noise being detected in the Orbitrap with the noise

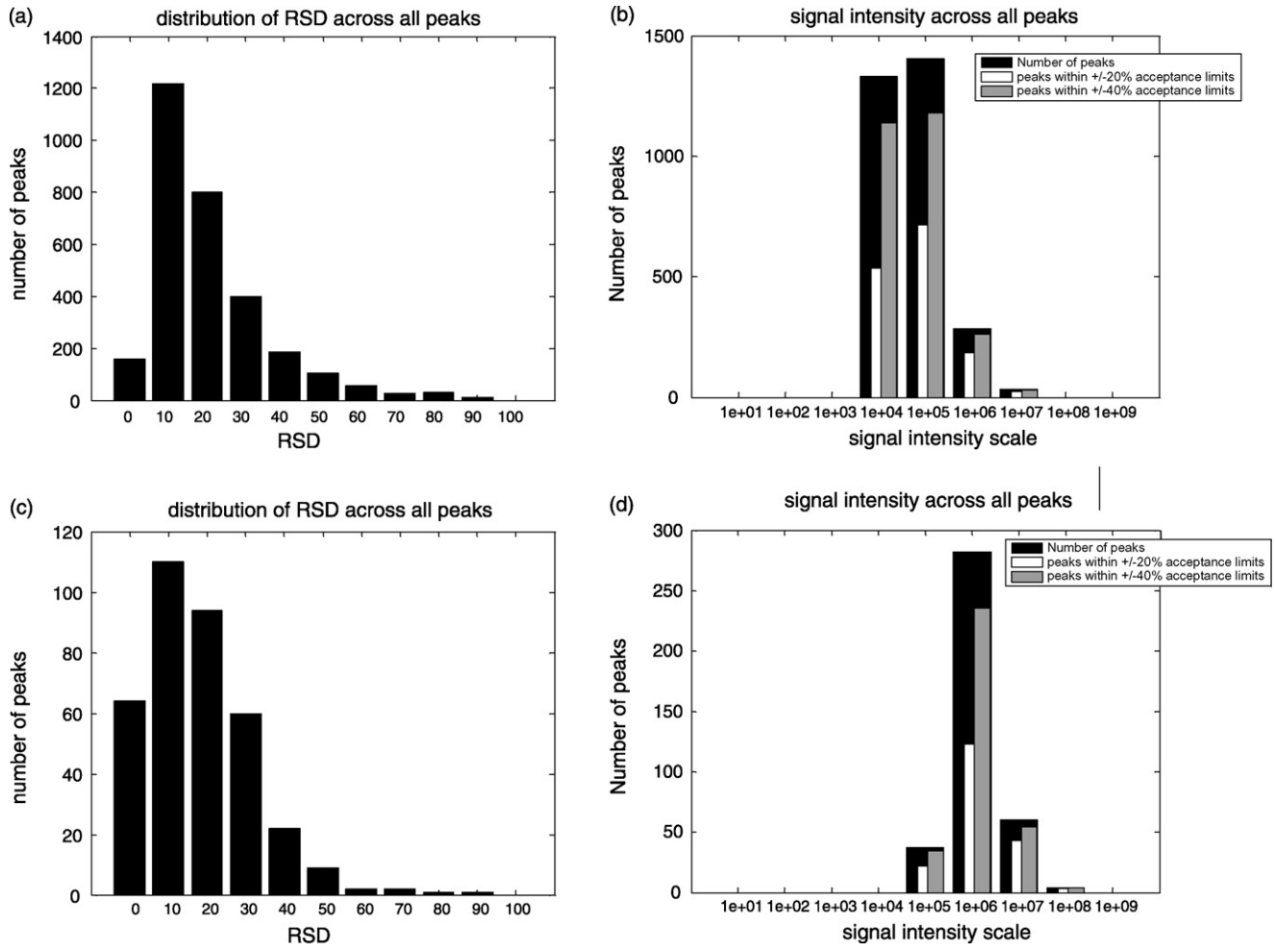

Fig. 4. The distribution of relative standard deviation (RSD) for all metabolites detected in negative ion mode in a minimum of 60% of all samples for *snthresh* = 1 (a) and *snthresh* = 10 (c). The distributions of peaks passing the tolerance test for RSDs of less than 20% and 40% for a range of signal intensities for *snthresh* = 1 (b) and *snthresh* = 10 (d) are also shown.

Table 3
The variability in the number of double peaks reported for a range of *snthresh* and *bw* settings

snthresh	*bw*	Number of peaks reported	Number of peaks reported more than once (%)
3	30	3557	727(20.4%)
3	10	3882	36(0.9%)
5	30	2566	377(14.6%)
5	10	2737	15(0.4%)
8	30	1794	183(10.2%)
8	10	1855	9(0.5%)
10	30	1479	120(8.1%)
10	10	1517	9(0.6%)
12	30	1257	79(6.3%)
12	10	1285	3(0.2%)
15	30	992	58(5.8%)
15	10	1014	1(0.01%)
18	30	796	41(5.2%)
18	10	807	2(0.25%)
20	30	695	28 (4%)
20	10	703	2(0.3%)

thresholds applied when acquiring accurate mass data. This is a relatively unique advantage of the Orbitrap mass analyser.

Fig. 3 also shows that across all *snthresh* values a *bw* parameter value of 10 slightly improves the number and reproducibility of the peaks found with respect to the default setting of 30. The *bw* setting was investigated in a stepwise manner for a range of *snthresh* values. The data is shown in Table 3 and indicates that for the data investigated the standard setting of *bw* = 30 is unsuitable as the number of double peaks is significantly greater than when compared to a *bw* = 10. The assumption can be made that the default setting of *bw* = 30 is suitable for HPLC studies where peaks are observed with greater peak widths but is unsuitable for UPLC conditions with narrower peak widths. This highlights the requirement to evaluate and validate software settings for the dataset of interest.

The distribution of peak area variability for the 14 QC samples was further assessed using the *bw* = 10 setting as shown in Fig. 4 for *snthresh* values of 1 and 10. The plots show the distribution of RSD for all peaks detected and also the number of all peaks observed with an RSD less than 15% and 20%, respectively, for different response ranges. It is evident that the majority of peaks detected were observed with an RSD less than 20%, though other peaks were detected with RSDs up to 100%. The reduction of the *snthresh* to 1 significantly increases the number of peaks reported with intensities less than 1e+05. However, the proportion of these low intensity peaks with RSDs less than 15% and 20% are similar as for peaks of greater peak areas. This result shows that reproducible data can be obtained for more than 1000 peaks over a 40-h analytical run for a wide concentration range, important in metabolic profiling where metabolite concentrations are varied. The low variability observed for both mass accuracy and retention times can be expected to underpin the low variability of peak areas observed across multiple samples.

Similar trends were observed for positive ion data (data not shown) as are shown for negative ion data.

From the results of the evaluation described above it was decided that the most appropriate XCMS operating parameters for these data (and given the type of univariate statistics being used in the case–control study) were *bw* = 10 and *snthresh* = 1, with all other settings used as described as default settings. It is recommended that a similar evaluation be performed for data acquired on different instruments or collected with different methodologies that influence peak width, retention time reproducibility and accurate mass reproducibility.

3.4. Case–control metabolic biomarker study for pre-eclampsia

The technologies described (UPLC, LTQ-Orbitrap and XCMS software) are to be employed in a range of metabolomic investigations

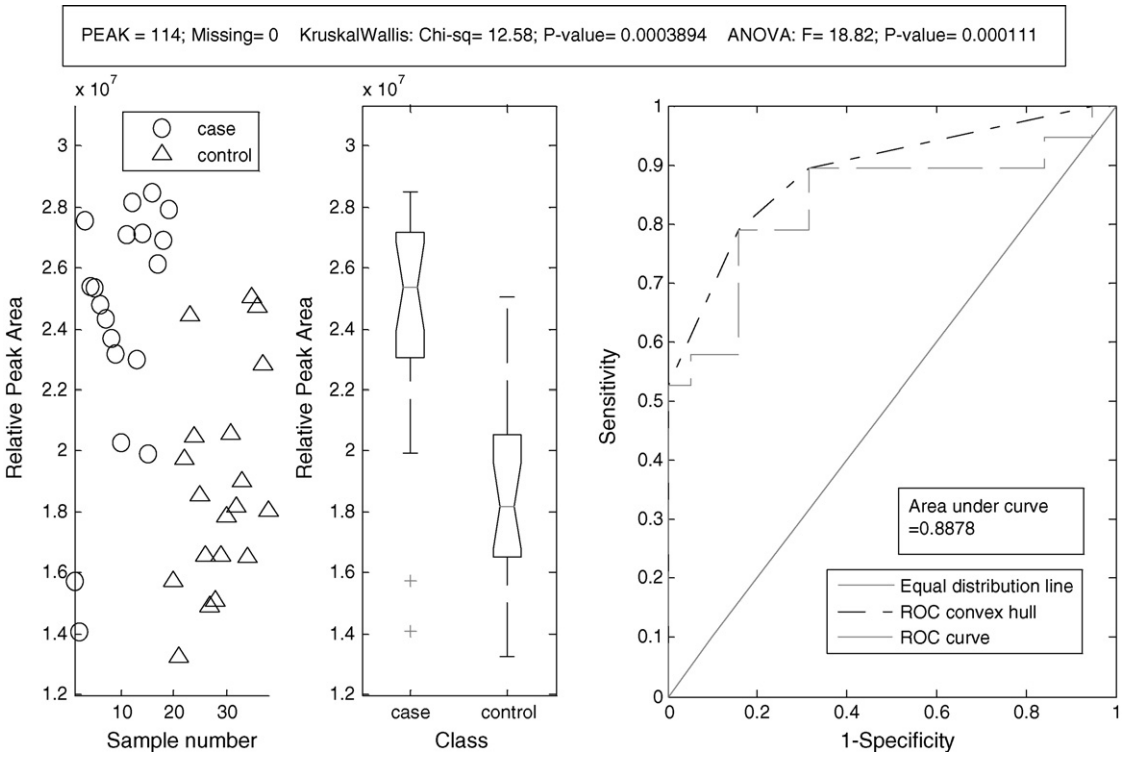

Fig. 5. An example of evaluating raw data for a single metabolite to ensure differences in the median peak areas for the case and control sample sets. The data shown is for uric acid using the data acquired with settings for *snthresh* of 1 and *bw* 10 in negative ion mode.

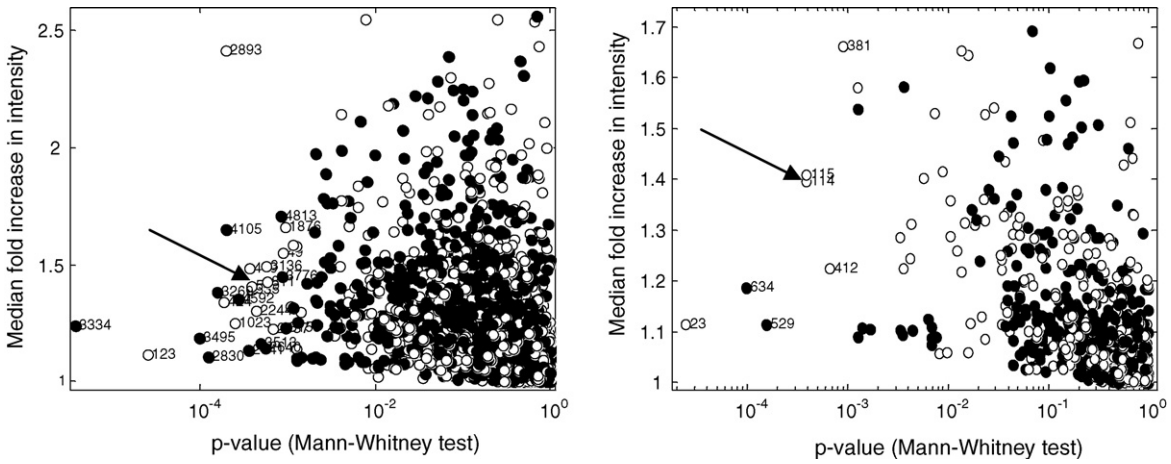

Fig. 6. The relationship between *p*-value and fold difference in intensity as a function of *snthresh* = 1 (a) and *snthresh* = 10 (b) for negative ion data. Filled circles indicate an increase in the intensity for the case group and empty circles indicate a decrease in the intensity for the case group. The peak identifiers are unique for each XCMS dataset and a peak is therefore identified with different identifiers in each diagram. Uric acid is indicated by the arrow (→).

in the future because of their combined advantages. One of these application fields will be that of the study of mammalian biofluids to determine biomarkers indicative of disease and drug toxicity or efficacy. A small case–control study was performed to assess the efficiency of the technologies in these types of studies. A set of 40 samples (20 case and 20 control) obtained from a pre-eclampsia study were analysed. The data and results are shown to evaluate the applicability of the technologies and not to obtain biological conclusions, since a larger sample set is recommended for this objective.

Due to the surprising results observed for the QC reproducibility study it was decided to test the XCMS algorithm for the case–control study using three *snthresh* settings (1, 5 and 10). Data were processed using XCMS default settings with the exception of *snthresh* (1, 5 and 10) and *bw* (10). Subsequent univariate data analysis was undertaken to define metabolites describing statistically significant differences between the two sample classes. The numbers of metabolite peaks defined as statistically different between the two classes using critical *p*-values of 0.01 and 0.001 are shown in Table 4. As expected, the number of discriminatory metabolites decreases when comparing results obtained using the two critical *p*-values and also decreases as the *snthresh* is increased. If the extra peaks reported are defined as metabolites it shows that setting a high *snthresh* can reduce the biological information obtained from the data. The authors recommend returning to the raw data to assess the possibility of false positives and this process can be performed in a number of ways. One of these is shown in Fig. 5 where both the raw data are plotted and also the area under the Receiver Operator Characteristic (ROC) curve is calculated. The data are shown for uric acid, a metabolite already identified as a possible metabolic biomarker [90]. A *p*-value of 0.0004 and a ROC area of 0.89 were observed for this metabolite. A similar trend is observed for positive ion data.

Table 4
The influence of *snthresh* and critical *p*-value on the number of metabolites defined as significantly different between the case and control samples for the negative and positive ion datasets

S/N	Mode	$p < 0.01$	$p < 0.001$
1	Negative	125	25
5	Negative	61	14
10	Negative	30	7
1	Positive	103	26
5	Positive	57	16
10	Positive	32	10

Fig. 6 shows the range of fold differences in mean peak area and critical *p*-value for all metabolites tested for *snthresh* = 1 and *snthresh* = 10. Critical *p*-values of less than 0.0001 were observed with typical fold differences in the peak area (related to metabolite concentration) being less than two. This indicates that the disease phenotype is reflected in the composition of individual components of the serum metabolome in a comparatively small way and that large changes in individual metabolite concentration are not observed. This may be taken to reflect the robustness of biological networks [91] and suggests that more successful diagnoses are likely to be based on multiple metabolites. As metabolic profiling studies require the technical variability to be lower than the biological variability these kinds of results show that low analytical variability is essential in these studies.

4. Conclusions

This study has highlighted the advantages of coupling a UPLC chromatographic system with the LTQ-Orbitrap mass spectrometry system. Peaks of width 7–15 s, high mass resolution and mass accuracy and linear dynamic ranges extending over 3 orders of magnitude to concentrations less than 1 μmol L^{-1} are all advantageous in metabolic profiling experiments. These advantages allow the technologies to be applied to the metabolic profiling of complex samples and this has been demonstrated for a case–control study of pre-eclampsia where a number of metabolites were shown to differ between the two classes in both positive and negative ion modes. We emphasise that this small study was used to demonstrate the applicability of the tools and is not intended to derive biological conclusions, where a larger sample set should be employed [8].

An important observation is that of the variability introduced to the results during the process of converting raw analytical data to a data matrix which is both retention time- and mass-aligned. The process of mass alignment, retention time alignment and peak picking and subsequent matching of peaks across multiple samples can all introduce error and variability into the final dataset. The reproducibility and validity of the final dataset is as dependent on the software used to perform this as on the analytical and clinical experimental design. In a number of studies, we have discovered that optimal software settings are problem-specific and are dependent on both the analytical platform used to acquire the data, the methods employed for any given analytical platform and the sample type. We have evaluated a single software package and chosen 'optimal' settings for the data described with the aim of using

univariate analysis methods. Different settings may be preferred if multivariate analysis is performed without any preprocessing to remove biologically irrevelant or non-reproducible peaks (e.g. remove those peaks which fail the tolerance test). This study was quite limited in the search space for optimal parameter settings. It may be prudent to expand out this optimization process to include all 18 XCMS settings, although this may be a very time consuming and expensive process.

We have demonstrated an appropriate experimental design to evaluate a single software but we would recommend evaluating several deconvolution software packages with one's own data. The authors do commend the XCMS software developers for making this process relatively straight forward. An appropriate experimental design employs the use of QC samples interspersed in the analytical run to assess reproducibility of the chromatograph and mass spectrometer, as discussed previously [85]. We highly recommend this approach and apply this in all our experiments so to assess the quality of our data acquired with UPLC–MS and GC–MS platforms.

The combination of these technologies has described a number of metabolites worthy of further investigation as time-of-disease biomarkers, though further validation studies are required employing larger numbers of samples. However, it is also worth assessing these biomarkers in a study investigating prognosis i.e. before clinical symptoms are observed [4]. The identification of these metabolic biomarkers is an ongoing complex process and will greatly benefit from the use of accurate mass and MS/MS spectra combined with confirmation of identity using authentic standards.

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