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Improvement in the Number of Analytic Features Detected by Non-Targeted Metabolomic Analysis: Influence of the Chromatographic System and the Ionization Technique

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1. Introduction

The development and optimisation of genomic, transcriptomic and proteomic technologies have significantly contributed to the assessment of biological systems and increased our understanding of gene function and regulation (Kitano 2002, Brown et al., 1999, Pandey and Mann 2000). In addition, metabolic fingerprinting or metabolomics complement these approaches by measuring low molecular weight chemicals in biological samples (Nicholson and Lindon, 2008). The elucidation of the links between genetic regulation, kinetic activities of enzymes and metabolic reactions is key to understanding homeostatic regulation of living organisms and the effects of food, diurnal variations disease and drugs (Nicholson et al., 2003, van der Greef et al., 2003, Plumb et al., 2003). Mapping of these various interactions is likely to result in applications in disciplines such as agriculture and medicine (Lee et al., 2007, Borodina and Nielson 2007, Wishart 200, Ducruix et al., 2006). Several analytic tools have been applied to profile the metabolome.

LC-MS studies are a more recent introduction to the field of metabolomics compared with the more established techniques of GC-MS and NMR. LC-MS can be used for the analysis of metabolites with a wide range of molecular weights than those detectable by GC-MS including polar and non-volatile compounds. With LC-MS, many different chromatographic phases and thus separation techniques are available when compared with GC-MS (Dunn, 2008).

Targeted metabolomic studies allow the identification and quantification of defined sets of metabolites and are performed using triple Quadrupole mass spectrometers which provide sensitivity and selectivity. Non-targeted global metabolomic studies are carried out on instruments with good mass accuracy such as time of flight and orbitrap mass analysers. In

metabolomic profiling, comparison of biological samples collected under different conditions is performed by multivariate statistical analysis in order to to identify significant differences between the groups. In metabolomics a "feature" is a molecular ion (*m/z*) coupled to a retention time (RT) that is generated following data processing, where feature finding is performed in conjunction with noise reduction and alignment of data. A drift in mass accuracy or retention time will affect the experimental results by creating additional numbers of novel features. An increase in variability of peak area or height may mask differences between experimental groups. The number of features, and their intensities in a number of replicate analysis of a given sample can define the robustness of an analytical run prior to complex statistical analysis of the data. . The acceptance criteria for reproducibility and repeatability differ between laboratories. In the studies presented in this chapter a coefficient of variation (CV) threshold of 25% was set which is in line with similar metabolomic studies published in the literature (Crews et al., 2009; Lai et al., 2010).

Plasma represents an important biofluid and global metabolite profiles have been derived from a variety of LCMS methods (Sabatine et al., 2005, Want et al., 2006, Bruce et al., 2008 and Zelena et al., 2009). We have previously shown that 2 different QTOF instruments produced the same number of reproducible features from tissue culture media extracts (Pandher et al., 2009). The goal of this study as to develop an efficient methodology using the Agilent LC Infinity system and Jet Stream Technology for metabolomic reverse phase LC-MS approaches. Here we describe the number of features obtained in human plasma extracts with a conventional rapid resolution chromatographic system and a QTOF mass spectrometer equipped with an electrospray ionization source in positive ionization mode. The improvement in the number of features and reproducibility following chromatographic separation with the Agilent 1290 Infinity LC system at various flow rates is also presented together with the peak capacity of a selected number of analytes. In addition, the impact of further optimization of the analytical conditions (temperature, flow rate) with the Jet Stream ionization technology on the number and reproducibility of the ions detected is presented.

2. Materials and method

Water (LC-MS grade), acetonitrile (LC-MS 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), tryptophan (CAS: 73-22-3) 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 in human plasma. Human plasma was collected in heparinised tubes from healthy donors and centrifuged at 1500 x g for 15 minutes at 4°C. Plasma was then stored at -80°C until analysis.

3. Sample extraction

Plasma samples (200 µl) were extracted with 4 volumes of acetonitrile, using a 96 well protein precipitation plate (Whatman, Maidstone, UK). The plate was vortexed for 1 min before a vacuum was applied. The filtered samples were collected in a 96 deep well plate and plasma extracts were pooled and aliquoted out for further analysis.

3.1 Sample analysis

For optimization of the analytical conditions, triplicate samples containing the spiked analytical standards were injected. For reproducibility studies, 3 replicates of unspiked and spiked plasma samples were analysed.

3.2 Liquid chromatographic separation

The HPLC systems used were the conventional Agilent 1200 and the Agilent 1290 Infinity LC system. Most of the analytic separation was achieved on a Waters Acquity column HSS T3 C_{18} (100 mm × 2.1 mm, I.D 1.8μm particles) and a 150 mm column was also tested.

Different chromatographic conditions were evaluated:

A mobile phase of 100% 0.1% formic acid was run isocratic for 0.5 minutes followed by a linear gradient ending in 100% acetonitrile over 7.5 minutes or 5.5 minutes or 3.5 minutes, followed by 100% acetonitrile over 2 minutes. After returning to the original conditions, the system was left to equilibrate for 3 minutes prior to the next injection. Different flow rates were evaluated (0.4 ml/min on the 1200 HPLC and 0.4, 0.6, 0.8 and 1 ml/min on the 1290 Infinity UHPLC). The same gradients were used on the 150 mm column. Columns were previously equilibrated with the injection of 5 plasma extracts.

3.3 Mass spectrometric analysis

Mass spectrometry was performed in positive ionization mode on a QTOF (6530, Agilent). Two different sources were evaluated: the classical electrospray ionization source and the Jet Stream technology. With the ESI source, parameters were set with a capillary voltage of 4 kV in positive ionisation mode. The fragmentor voltage was 140 V and skimmer was 65 V. The gas temperature was 250°C, drying gas 10 l/min and nebulizer 40 psig. Nitrogen was used as a drying gas. MS spectra were acquired in full scan analysis over an m/z range of 70-1000 using extended dynamic range and a scan rate of 1.4 spectra/second. To maintain mass accuracy during the run time, a reference mass solution containing reference ions 121.0508 and 922.0097 was used.

With the Jet Stream technology; parameters were set with a capillary voltage of 4 kV in positive ionisation mode. The fragmentor voltage was 140 V and skimmer was 65 V. The gas temperature was 250°C, drying gas 6 l/min and nebulizer 60 psig. Nitrogen was used as a drying gas. The sheath gas temperature was tested and optimized from 200°C to 400°C by increments of 50°C and the sheath gas flow rate was 11l/min. Total ion spectra were acquired in full scan analysis over an m/z range of 70-1000 using extended dynamic range 2GHz and an acquisition rate of 2Hz.

Sampling rates of 2, 4 and 6Hz were tested with the Jet Stream technology at 0.6 ml/min and 0.8 ml/min with the sheath gas at 200°C in order to evaluate the number of sampling points collected across each peak and the reproducibility of the analysis.

4. Data processing

Sample features were extracted with the molecular feature extractor (MassHunter Workstation Software (version B.01.03)). Data were processed using the following

conditions: restrict retention time to 0.20 - 8.5 min, restrict m/z to 100-800, absolute height threshold: 25000 or 2500, mass tolerance: 0.05, 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.

The list of features consisting of retention times (RT) and molecular masses was then analysed using GeneSpring MS Analysis Platform (v1.2, Agilent Technologies, Inc., Santa Clara, CA) where they were aligned and normalized.

Data were then imported into Excel spreadsheets and mean, SD and CV of all features was calculated.

5. Results and dicussion

There are a number of experimental variables, related to chromatography or mass spectrometry, that can impact the reproducibility of metabolomic profile data. This in turn can compromise the validity of the data's biological relevance and applicability. The importance of several variables, including flow rate, column length, mass spectrometric conditions were all evaluated in terms of the number of features found.

Following triplicate separation of human plasma on the conventional 1200 LC system at 0.4 ml/min with a 7.5 min gradient followed by 2 min isocratic on a 10 cm column with the ESI source, 1324 total features were detected out of which 795 (60%) showed less than 25% CV Table 1).

Method	System pressure	Total no. of features	No. of features <25% CV	% of features with <25% CV	% change in features with <25% relative to conventional system
LC1200 0.4ml/min	450	1324	795	60	n/a
LC1290 0.4ml/min	270	1714	925	54	+16%
LC1290 0.6ml/min	390	2559	1149	45	+44%
LC1290 0.8ml/min	502	2263	1074	47	+35%
LC1290 1.0ml/min	605	1805	305	17	-38%

aChromatography performed on a reverse phase Waters Acquity T3 column with a 7.5min 0.1% formic acid: acetonitrile gradient. Mass spectrometry analysis performed on 6530 QTOF in ESI mode. Data was extracted using MassHunter Qualitative software package and GeneSpringMS and then exported to Excel where statistics were performed.

Table 1. Effect of flow rate on the number and reproducibility of features present in technical replicates of human plasma extracts conventional 1200 LC and novel 1290 Infinity LC in ESI mode [a] (n=3).

Under similar conditions, the novel 1290 LC system generated 16% higher and reproducible features compared with the conventional system without any change in analytic conditions. Increasing the flow rate to 0.6 ml/min with the same gradient increased the number of reproducible features to 1149 allowing a 44% improvement when compared with the 1200 LC system. When the flow rate was increased from 0.4 to 0.6 ml/min many additional features were detected that were not previously observed following separation by the 1200 system. Careful examination of the features showed that they were mainly ions that had not previously eluted from the column at 0.4 ml/min. Further increase in flow rate showed that fewer reproducible features were detected. The pressure in the system with the column installed was significantly lower in the 1290 system with a back pressure of at 132 bar at 0.4 ml/min on the 1290 versus 450 bars on the 1200 respectively. At 1 ml/min, the pressure on the 1290 system was 605 bars only. It is possible that the different composition of the pistons and their independent operation together with the novel mixing technology used in the 1290 Infinity LC system can explain the decreased pressures compared with the conventional 1200 (data not shown)

The length of the gradient was then shortened to 5.5 minutes and 3.5 minutes respectively but this resulted in a significant decrease in reproducibility (Table 2). In fact, we noted that the isocratic segment of the gradient had to be extended in order to avoid carry-over from previous samples which defeated the purpose of a shorter analytic run (data not shown).

Method[b]	Total no. of features	No. of features <25% CV	% of features with <25% CV	% change in features with <25% relative to conventional system
LC1200 0.4ml/min *7.5min gradient*	1324	795	60	n/a
LC1290 0.6ml/min *7.5 min gradient*	2559	1149	45	+44%
LC1290 0.6ml/min *5.5 min gradient*	1989	1086	55	+37%
LC1290 0.6ml/min *3.5 min gradient*	1889	1017	54	+28%

[a]Chromatography performed on a reverse phase Waters Acquity T3 column with a 7.5min 0.1% formic acid: acetonitrile gradient. Mass spectrometry analysis performed on 6530 QTOF in ESI mode. Data was extracted using MassHunter Qualitative software package and GeneSpringMS and then exported to Excel where statistics were performed.
Each gradient was preceded by 0.5min of 100% A (0.1% formic acid in water) and followed by 2min of 100% B (0.1 formic acid in acetonitrile).

Table 2. The effect of gradient duration on the number and reproducibility of features present in technical replicates of human plasma extracts using conventional 1200 and novel 1290 Infinity LC systems in ESI mode [a] (n=3).

Following triplicate analysis of human plasma on the conventional 1200 LC system using the 150 mm column with our previously described gradient, there was no significant improvement in total or reproducible number of features when compared with the 100 mm column regardless of the flow rate (data not shown). Our conclusion for the data from the ESI source was that a flow rate of 0.6 ml/min was optimal with the 100 mm column with the original 7.5 minute gradient.

We then proceeded to evaluate the effect of the Jet Stream technology on the number of features detected and their repeatability. Incremental temperatures of 50°C of heated nitrogen sheath gas; from 200°C to 400°C were applied and evaluated. At 0.6 ml/min with a sheath gas of 200°C, both the total and reproducible features were more than doubled when compared to the equivalent result with the ESI source. Overall, 50% of features showed less 25% CV over triplicate analysis (Table 3). This represents a 173% increase in reproducible features when compared with the conventional 1200 LC system and the ESI source.

Method	Total no. of features	No. of features <25% CV	% of features with <25% CV	% change in features with <25% relative to conventional system
ESI LC1200 0.4ml/min	1324	795	60	n/a
JS LC1290 0.6ml/min *200°C*	4357	2176	50	+173%
JS LC1290 0.8ml/min *200°C*	4312	2512	58	+215%
JS LC1290 0.6ml/min *250°C*	4396	2294	52	+189%
JS LC1290 0.8ml/min *250°C*	4810	2708	56	+241%
JS LC1290 0.6ml/min *300°C*	4463	2565	57	+223%
JS LC1290 0.8ml/min *300°C*	5130	2869	56	+261%
JS LC1290 0.6ml/min *350°C*	4693	2762	59	+247%
JS LC1290 0.8ml/min *350°C*	5257	2994	57	+277%
JS LC1290 0.6ml/min *400°C*	4919	2707	55	+241%
JS LC1290 0.8ml/min *400°C*	5095	3310	65	+316%

[a]Chromatography performed on a reverse phase Waters Acquity T3 column with a 7.5min 0.1% formic acid: acetonitrile gradient. Mass spectrometry analysis performed on 6530 QTOF in ESI mode. Data was extracted using MassHunter Qualitative software package and GeneSpringMS and then exported to Excel where statistics were performed.

Table 3. The effect of Jet Stream (JS) sheath gas and flow rates on number and reproducibility of features present in technical replicates of human plasma extracts the novel 1290 Infinity LC system coupled to a 6530 QTOF using Jet Stream technology [a] (n=3)
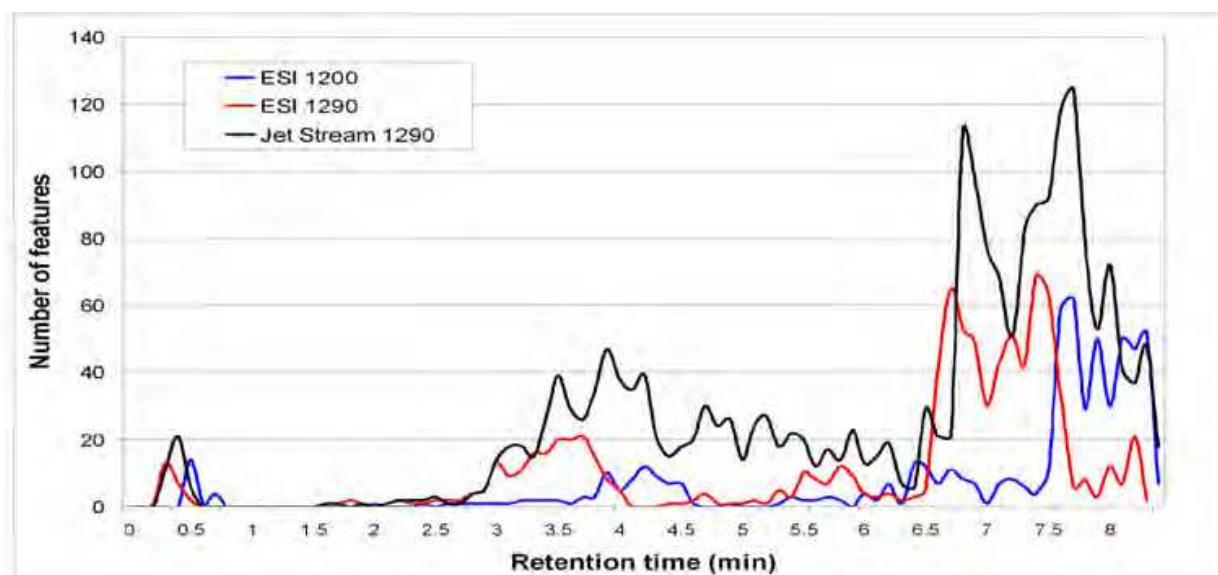
Fig. 1. Number of reproducible features against retention time. Number of reproducible features generated following data processing using the conventional 1200 LC system with ESI, the novel 1290 Infinity LC system with ESI and the novel LC system with Jet Stream technology.

The increased number of features ionized by the Jet Stream technology when compared with ESI is illustrated in Figure 1 which shows overlayed total ion chromatograms. In contrast to our results with the ESI source, increasing the flow rate to 0.8 ml/min further increased the number of reproducible features when compared with 0.6 ml/min (Table 3). Increasing the temperature of the sheath gas by increments of 50°C at both 0.6 and 0.8 ml/min gradually increased the number of features. At 400°C, a striking 5095 features were detected; 3310 of which showed less than 25% CV.

We were concerned that this significant increase in features with increase in temperature could be the result of thermal degradation of ions. . To address this, a Venn diagram derived from GeneSpring MS shows the ions present at at both temperatures, at 200°C or 400°C only. (Figure 2).

The heat plot in Figure 3 demonstrates that all the 1491 ions present at 400° C were weak whereas more than half of the 888 were stronger in intensity, suggesting that the increase in temperature fragmented the ions and that thermal degradation occurred. Further examination of the features at 200°C in Jet Stream and ESI at 0.6 ml/min, showed that >2000 features were specific to Jet Stream alone. By analysing the most intense features we found that they were not split features due to errors in the automatic processing software. We were concerned that some of these features may be detectable in ESI at a lower threshold. Therefore, we proceeded to lower the threshold to 2500 in both Jet Stream and ESI and found that 10599 and 15424 total features and 2792 and 4163 reproducible features were detected respectively in the two systems with 26% reproducibility obtained using both systems. Comparison of these features showed that a proportion was only present in Jet Stream and the remainder were detectable at low intensity and not reproducible.
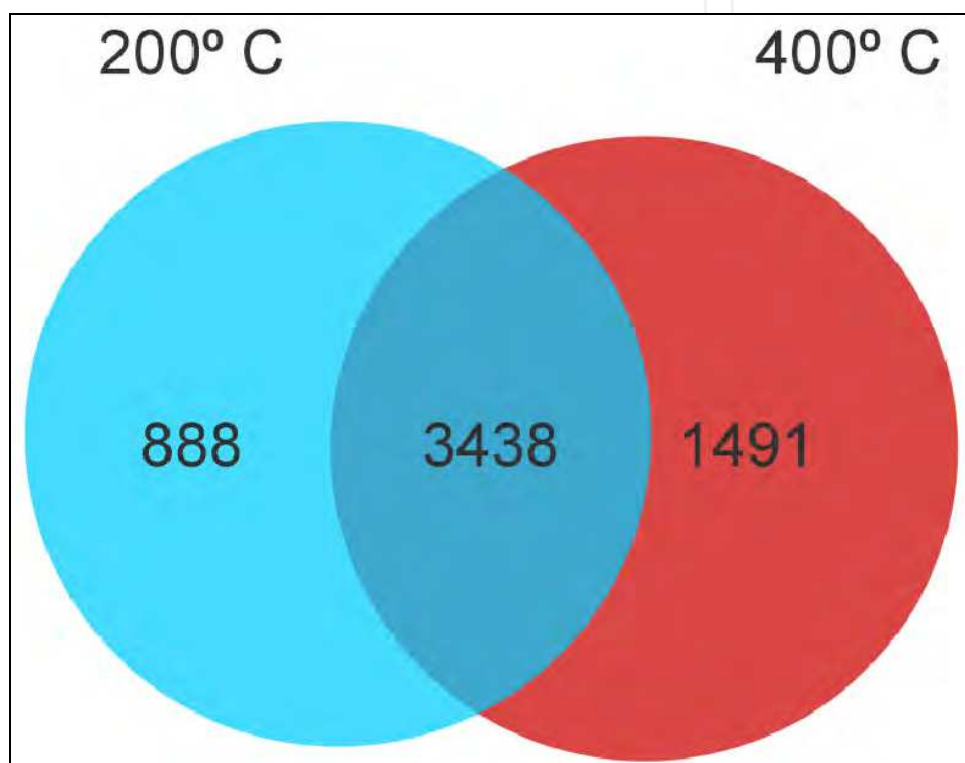
Fig. 2. Comparison of features observed with Jet Stream and ESI. Venn diagram showing features found in human plasma extracts using LC 1290 coupled to 6530 QTOF coupled with Jet Stream technology using sheath gas temperature of either 200°C or 400°C at 0.8 ml/min. The features present at 200°C were 888, 1491 were present only at 400°C and 3438 were present at both temperatures.
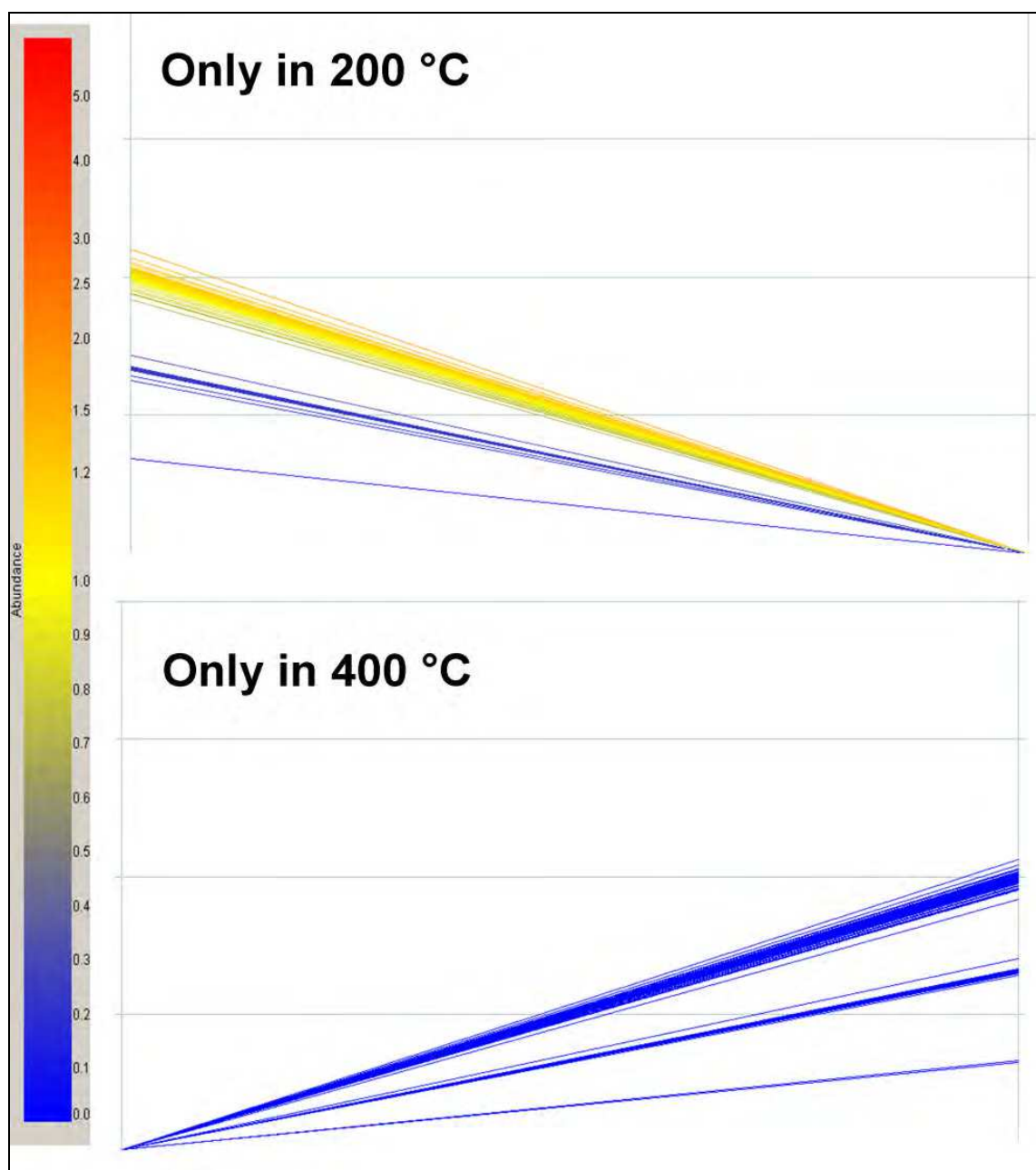
Fig. 3. Abundance of features observed exclusively at 200°C and 400°C. Heat plot depicting the abundance of features present in human plasma extracts using LC1290 coupled to 6530 QTOF coupled with Jet Stream technology at either 200°C or 400°C at 0.8 ml/min. Each line represents one feature found exclusively at either temperature, with red representing those features present in a low high intensity and in blue those present in low intensity.

In summary, the Jet Stream technology increased the overall number of features when compared with the ESI but thermal degradation occurred above 200°C, which is therefore the optimal temperature to use under the conditions studied.

Our data demonstrates the advantage of the new LC system which allowed operation at higher flow rates with low back pressure and very reproducible analysis. The increase in flow rate resulted in a predictable increase in peak capacity (Figure 4).
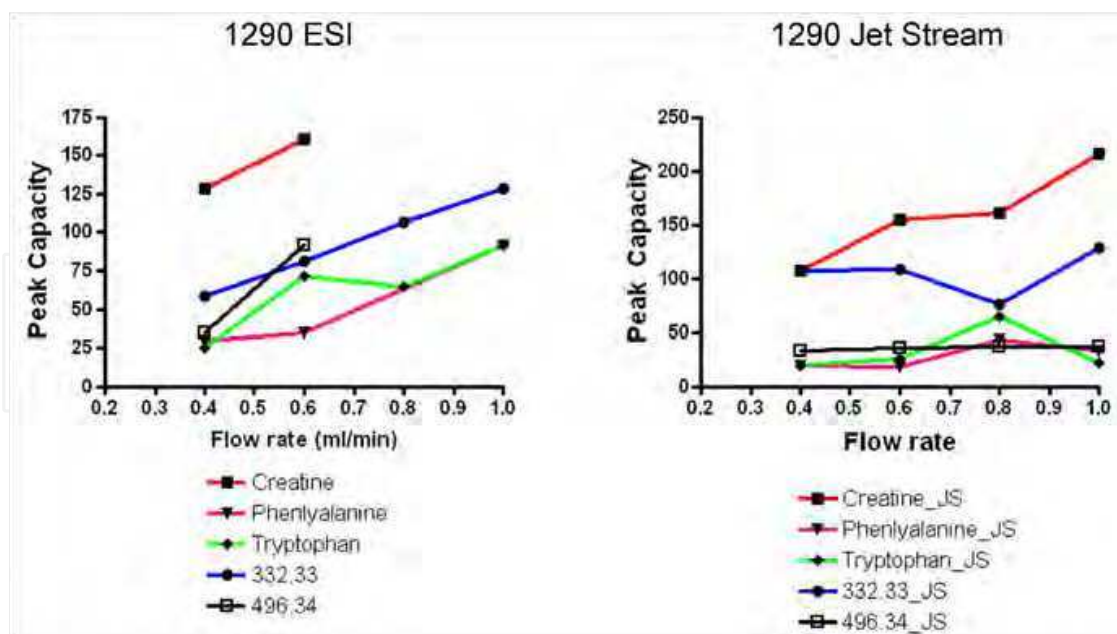
Fig. 4. Peak capacity versus flow rate determined for creatine (m/z 132.07), phenylalanine (166.08), tryptophan (m/z 204.10) and glycerophosphocholine (m/z 496.34) and ion m/z 332.33 using the conventional 1200 LC system and novel 1290 LC system coupled to ESI and Jet Stream technology. Chromatographic separation was carried out on a 100mmx2.1mm ID reverse phase Waters Acquity T3 column with a 7.5min 0.1% formic acid: acetonitrile gradient followed by 2 min isocratic.

However, saturation of desolvation may have occurred at flow rates of 0.8 ml/min and above, as mentioned previously, and features started to disappear. For example we could no longer detect creatine (*m/z* 132.07) and glycerophosphocholine (*m/z* 496.34) with ESI above a flow rate of 0.6 ml/min. The Jet Stream technology detected these features at higher flow rate when compared with the ESI and showed the expected linear increase in peak capacity with flow rate.

We were concerned however, that at higher flow rates, less data points were collected across chromatographic peaks and proceeded to test various scan rates (2, 4 and 6Hz) at 0.6 and 0.8 ml/min (Table 4a). For a limited number of compounds the number of points across peaks for early and late eluting metabolites (0.5-0.8 min, n=2, and 6-6.5 min, n=4) were measured). Decreasing the scan rate increased the number of points across the peaks but decreased the sensitivity of the analysis resulting in a significant decrease in the total and reproducible number of features (Table 4b).

For example, at 0.8 ml/min, at 2Hz; only 4566 features were detected and 2056 with less than 25%CV as opposed to 3716 and 1294 at 4Hz and 3233 and 893 at 6Hz. At 2Hz, there were an average number of 5 points across chromatographic peaks versus 15 at 4Hz, and in late eluting peaks an average of 10 points were monitored across peaks at 2Hz and 25 at 4Hz. Interestingly, the overall percentage of reproducible features was similar at all acquisition rates suggesting that the loss of sensitivity observed at 4 or 6GHz occurs equally across total features whether they are variable or not. The number of reproducible features before 2 min at 0.6 ml/min was: 28 at 2Hz versus 40 and 41 at 6Hz (data not shown). The lower number of reproducible features at 2Hz when compared to 4 and 6 suggests that early polar metabolites being defined with less than 10 points across peaks are less reproducibly detected.

m/z,RT	2GHz 200°C 0.6mlmin	4GHz 200°C 0.6mlmin	6GHz 200°C 0.6mlmin	m/z,RT	2GHz 200°C 0.8mlmin	4GHz 200°C 0.8mlmin	6GHz 200°C 0.8mlmin
116.07_0.47	8 (7.5)	22 (5.4)	38 (6.7)	116.07_0.36	6 (4.5)	17 (5.5)	22 (5.2)
182.08_0.50	8 (3.8)	9 (1.3)	11 (2.7)	182.08_0.38	7 (4.0)	7 (1.6)	9 (3.1)
312.36_6.20	11 (0.4)	27 (0.4)	33 (0.2)	312.36_5.95	10 (0.5)	15 (1.0)	30 (0.8)
496.34_6.00	10 (2.4)	23 (2.5)	27 (2.5)	496.34_5.70	8 (1.6)	19 (2.0)	24 (2.6)
520.34_5.80	9 (1.5)	19 (1.0)	25 (1.3)	520.34_5.50	7 (1.6)	13 (1.60)	23 (0.9)
522.34_6.30	10 (1.0)	29 (1.0)	37 (0.8)	522.34_7.20	10 (0.8)	28 (0.8)	32 (0.5)

[a]Chromatography performed on a reverse phase Waters Acquity T3 column with a 7.5min 0.1% formic acid: acetonitrile gradient. Mass spectrometry analysis performed on 6530 QTOF in ESI mode. Chromatograms were then analysed using MassHunter Qualitative software.

Table 4a. Evaluation of number of points across chromatographic peaks and mass accuracy of selected metabolites using 2Hz, 4Hz and 6Hz scan rates at 0.6ml/min and 0.8ml/min [a]

Method	Total no. of features	No. of features <25% CV	% of features with <25% CV
1290 Infinity LC 0.6ml/min 200°C 2HZ	4566	2056	45
1290 Infinity LC 0.6ml/min 200°C 4HZ	3058	1410	46
1290 Infinity LC 0.6ml/min 200°C 6HZ	2231	1073	48
1290 Infinity LC 0.8ml/min 200°C 2HZ	4460	2602	26
1290 Infinity LC 0.8ml/min 200°C 4HZ	3716	1294	35
1290 Infinity LC 0.8ml/min 200°C 6HZ	3233	893	28

[a]Chromatography performed on a reverse phase Waters Acquity T3 column with a 7.5min 0.1% formic acid: acetonitrile gradient. Mass spectrometry analysis performed on 6530 QTOF in ESI mode. Chromatograms were then analysed using MassHunter Qualitative software.

Table 4b. The effect of scan rate on number and reproducibility of features present in technical replicates of human plasma extracts the novel 1290 Infinity LC system coupled to a 6530 QTOF using Jet Stream technology [a] (n=3)

This study clearly demonstrates the considerable challenges associated with reproducibly and sensitively acquiring metabolomic data. Increasing the flow rates eluted more non-polar metabolites off the column but eventually at the detriment of polar metabolites that became undersampled. Whatever choice is made in analytic conditions cannot be optimal for all metabolites. It has to be noted that the number of features do not correspond to the number of metabolites. For example other studies have described up to 23 features for a given metabolite which further complicates matters (these may include multiply charged ions and in source fragmentation ions) (Evans 2009).

In conclusion, our study describes the much improved effect of the 1290 LC system together with the Jet Stream technology on the number of features detected compared with the 1200 LC system and ESI. It is clear that this increased number of features corresponds both to an increased number of metabolites eluting from the column at higher flow rates and an additional number of species being reproducibly ionized by the Jet Stream technology when compared with the ESI source. Our preferred analytic conditions use the 100 mm analytic column, a 7.5 min gradient (total run time 10 min plus 3 min equilibration) with a flow rate of 0.6 ml/min and an acquisition rate of 2Hz.

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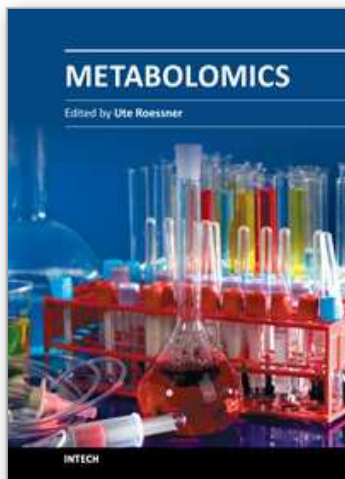
Metabolomics

Edited by Dr Ute Roessner

Metabolomics is a rapidly emerging field in life sciences, which aims to identify and quantify metabolites in a biological system. Analytical chemistry is combined with sophisticated informatics and statistics tools to determine and understand metabolic changes upon genetic or environmental perturbations. Together with other 'omics analyses, such as genomics and proteomics, metabolomics plays an important role in functional genomics and systems biology studies in any biological science. This book will provide the reader with summaries of the state-of-the-art of technologies and methodologies, especially in the data analysis and interpretation approaches, as well as give insights into exciting applications of metabolomics in human health studies, safety assessments, and plant and microbial research.

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