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High temperature-ultra performance liquid chromatography–mass spectrometry for the metabonomic analysis of Zucker rat urine☆

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ABSTRACT

The applicability and potential of using elevated temperatures and sub 2-μm porous particles in chromatography for metabonomics/metabolomics was investigated using, for the first time, solvent temperatures higher than the boiling point of water (up to 180 °C) and thermal gradients to reduce the use of organic solvents. Ultra performance liquid chromatography, combined with mass spectrometry, was investigated for the global metabolite profiling of the plasma and urine of normal and Zucker (fa/fa) obese rats (a well established disease animal model). "Isobaric" high temperature chromatography, where the temperature and flow rate follow a gradient program, was developed and evaluated against a conventional organic solvent gradient. LC–MS data were first examined by established chromatographic criteria in order to evaluate the chromatographic performance and next were treated by special peak picking algorithms to allow the application of multivariate statistics. These studies showed that, for urine (but not plasma), chromatography at elevated temperatures provided better results than conventional reversed-phase LC with higher peak capacity and better peak asymmetry. From a systems biology point of view, better group clustering and separation was obtained with a larger number of variables of high importance when using high temperature-ultra performance liquid chromatography (HT-UPLC) compared to conventional solvent gradients.

1. Introduction

The use of temperature as a variable in liquid chromatography, including the use of water as the sole solvent, in either isothermal or thermal gradient mode is an area of increasing interest [1–4]. Hot water (including water above 100 °C up to its supercritical temperature) as an eluent exhibits reduced viscosity, increased ability to dissolve non-polar compounds, and decreased polarity. These attributes result in the liquid offering properties comparable to an organic solvent. Thus, elevated temperatures can be used as a tool to reduce analysis time and operating pressure for the same solvent composition, or as a means of reducing the amount of organic solvent required, or indeed eliminating it entirely (e.g., see Refs. [5,6]). Separations based on superheated water include the LC analysis of barbiturates, steroids, herbicides, antioxidants and sulphonamides [7–12]. Recently, comprehensive reviews have covered the topic [13,14]. The ability of temperature to change eluotropic strength means that thermal gradients can be used to mimic the effects of solvent gradients [15,16]. As has been demonstrated [15–17] increasing the temperature increases the optimal flow rate, but also produces flatter van Deemter curves. Thus, maintaining a constant pressure throughout the run ensured that the chromatographic performance did not reduce substantially compared to using a constant flow rate. The application of thermal gradients could be of interest not only in targeted analysis but also in fingerprinting methodologies such as global metabolite profiling (metabonomics/metabolomcis) as it may provide an alternative way to perform sample profiling. Indeed, preliminary studies using elevated temperatures (90 °C) combined with conventional reversed-phase gradients have already shown considerable promise [17,18]. Also, since the application of LC–MS in metabolomics and metabonomics is often hampered by the poor retention of polar molecules in conventional reversed-phase LC it seems obvious that new selectivities and/or separation mechanisms should be investigated.

Here, we have studied the potential of using high temperature-ultra performance liquid chromatography (HT-UPLC) for the global metabolite profiling of urine and blood plasma samples obtained

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from two strains of the Zucker rat, a normal strain and the (fa/fa) obese strain. The (fa/fa) obese Zucker strain is leptin deficient, and characterised by obesity, insulin resistance, hyperinsulinaemia, dyslipidaemia and glucose intolerance. As such it is widely used as a model of Type II diabetes [19] and metabonomic studies can be expected to provide new insights into the global effects of the disease on metabolic pathways in addition to, e.g., simply those related to glucose and lipid metabolism. In the present study biofluids were analysed using both high temperature chromatography and conventional reversed-phase LC with MS detection in full scan mode using a hybrid triple quadrupole linear ion trap mass spectrometer. MS data was further treated by specially developed algorithms to extract features and allow the data to be further analysed by multivariate statistics (Principal Component Analysis, Partial Least Squares Discriminant Analysis: PCA and PLS-DA). The present study illustrates the potential of using temperature as a variable in method development for metabonomics and metabolomics. To the best of our knowledge this is the first report of the utilization of thermal gradients in metabonomic/metabolomic LC–MS investigations.

2. Experimental

2.1. Chemicals

Reagents were of analytical or higher grade and were used as supplied (Sigma, Poole, Dorset). Ultrapure water was obtained from a Purelab Ultra system from Elga (Bucks, UK). In all eluents 0.1% formic acid was added to assist ionisation in electrospray ionisation (ESI).

2.2. Sample treatment

Urine samples were collected from male Zucker lean and (fa/fa) obese rats (n = 9/group) reared at Alderley Park, at 20 weeks of age. Samples were stored at −20 °C until analysis. 50 μl aliquots of urine were diluted with 100 μl of pure water thoroughly mixed in a vortex mixer and were subsequently centrifuged at 7500 rpm for 15 min to remove particulate matter. Samples were transferred to autosampler vials and were kept in the autosampler at 4 °C. A "quality control" (QC) sample was prepared [20] by mixing equal volumes (50 μl) from each of the urine samples and then diluted (1:4, v/v) and centrifuged in the same way as the samples.

Blood plasma was obtained by centrifugation (3500 rpm, 10 min) from blood collected from the rats. All plasma samples were kept at −20 °C until they were defrosted immediately prior to analysis. To remove proteins, a protein precipitation step was applied as follows: an aliquot of 20 μl of plasma was thoroughly mixed in an Eppendorf tube with 40 μl acetonitrile. The mixture was vortexed (30 s) and subsequently centrifuged at 14,000 rpm for 5 min. An aliquot of 30 μl of the supernatant was withdrawn and diluted with 30 μl of water in an HPLC insert. The solution was vortex mixed and centrifuged again at 3500 rpm for 10 min and the autosampler vials were placed in the autosampler (4 °C) for analysis.

All sampling procedures were performed under the relevant UK regulations (Animals (Scientific Procedures) Act 1986),

2.3. Instrumentation

The chromatographic system comprised of an Acquity UPLC system (Waters, Milford, MA, USA) in conjunction with a GC-2014 GC oven (Shimadzu, Milton Keynes, UK) which was employed to heat the columns. Two 100 mm × 2.1 mm, 1.7 μm Acquity BEH C_{18} columns (Waters), connected in series, were used for the chromatographic separations. The eluent from the columns went first through a chiller and was then introduced to a QTRAP 4000 hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/Sciex, Toronto, Canada). LC–MS experiments were performed using electrospray ionisation in both positive and negative ionization modes (in separate experiments). Contact closures were used to trigger the LC–GC oven–MS systems simultaneously. The mass spectrometer source conditions were set as follows: source temperature 450 °C, with an ion spray voltage of 4200 V and gas settings of 40 for auxiliary gasses and 20 for curtain gas. Detection was performed in enhanced mass scanning mode (EMS) with a scan rate of 1000 amu/s from 100 to 800 amu.

2.4. Chromatographic conditions

2.4.1. Reversed-phase gradient LC

For conventional reversed-phase gradient elution with water–acetonitrile (each containing 0.1 vol% formic acid) the following conditions were employed. From 0 to 0.5 min 100% water, followed by a linear increase to 20% acetonitrile at 4 min, then linearly to 95% acetonitrile at 9 min where it was held isocratically for 1 min. This was then followed by a re-equilibration step of 3 min (100% aqueous). The flow rate was 0.25 ml/min and the column temperature was held constant at 58 °C.

2.4.2. Temperature gradient LC

A range of conditions for HT-UPLC were explored in this study with the final optimised conditions (shown in Table 1) as follows. For the period 0–3 min water (containing 0.1%, v:v, formic acid) was used as the mobile phase with the temperature maintained at 50 °C for the first 2 min. From 2.0 to 8.0 min the temperature was raised to 180 °C using a linear temperature gradient. At 10 min the temperature was then reduced to 50 °C and maintained at this temperature until 21 min post injection. From 4 min a linear gradient of acetonitrile was employed reaching 10% at 10 min, including a full organic step (100% acetonitrile) from 15.01 till 17 min after which the solvent composition returned to 100% aqueous formic acid. Along that program, the flow rate followed a program with flow increasing from 0.25 to 0.54 ml/min and then returning to 0.25 ml/min.

2.5. Data treatment and multivariate statistics

MS data acquired by Analyst® 1.4.1 software (Applied Biosystems) on the QTRAP 4000 were imported to MarkerView™ software version 1.2.0.1 (Applied Biosystems). In this way aligned peak tables were created according to specified peak finding and

Table 1
Temperature and flow rate gradient program

Time (min)	Water (%)	Acetonitrile (%)	Temperature (°C)	Flow rate (ml/min)
0	100	0	50	0.25
2	100	0	50	0.25
2.5	100	0	60.8	0.25
3	100	0	82.5	0.25
4	99	1	104.2	0.30
5	98	2	125.8	0.36
6	96	4	147.5	0.42
7	94	6	169.2	0.46
8	92	8	180	0.50
10	90	10	50	0.54
12	90	10	50	0.54
12.01	90	10	50	0.25
15	90	10	50	0.25
15.01	0	100	50	0.25
17	0	100	50	0.25
17.01	100	0	50	0.25

alignment parameters: Chromatographic peaks with base widths from 2 to 50 scans and spectral peaks with intensity of higher than 10^5 counts and minimum width of 0.25 amu were detected for all samples using a specific algorithm. Peaks were then aligned across the samples by recognising those of similar m/z values (0.25 amu tolerance) and retention time (with a 0.5 min tolerance).

3. Results and discussion

3.1. Chromatography

Previous studies [15–18] have demonstrated that using elevated temperatures for UPLC with conventional gradient chromatography provided a number of benefits in terms of, e.g., peak capacity and peak symmetry. In order to examine the potential of thermal, rather than solvent, gradients it was necessary to arrive at optimised chromatographic conditions for the samples (urine and blood plasma) of interest. In order to achieve this two different thermal gradients were investigated. In the initial studies the thermal gradient was used under constant flow conditions, representing a simple model, whilst the second developed with "isobaric" (constant pressure) elution conditions, which is somewhat more complex as the flow rates must be changed during the run. The derivation of these thermal gradients was based on previously published investigations [15,16] and is given briefly below.

3.1.1. Thermal gradients at constant eluent flow rate

Initial studies investigated the effect of using a simple thermal gradient, with no variation in the flow rate throughout the experiment. The thermal gradient began with isothermal conditions for 2 min at 50 °C, followed by a steep, linear, thermal gradient (over 6 min) to the maximum applied temperature of 180 °C, where it was held for 1 min before the re-equilibration step to the initial temperature (10 min). The re-equilibration step was longer than the whole thermal gradient program for two reasons: (1) firstly a GC oven heats at a much faster rate than it can cool and (2) a temperature lag exists between the oven temperature and the actual temperature within the column(s). Using these conditions we observed, as would be predicted, that the backpressure of the system declined as the temperature was increased [15,21]. Thus the maximum pressure observed, under the starting conditions of 60 °C and a flow rate of 0.25 ml/min with the two Acquity columns, was less than 10,000 psi falling to only 2900 psi at 180 °C.

However, under these conditions the analytical results (injections of blank, standards and real samples) were unsatisfactory: A high detector background signal observed with increasing temperature, probably due to changes in the pressure. It should be noted that the use of a post column chiller, to cool the eluent prior to entry into the ESI probe, was found to be essential. The reason for this is that, as we have noted previously [16], at the operating temperatures employed there is the potential for solvent phase transitions to occur, either within the column, or post column in the connecting tubes, with adverse effects on the analysis. Using the boiling point curve for water we determined the pressure at which a phase transition would take place and placed a post column restrictor, constructed by using a length of narrow bore stainless steel tubing (0.64 mm i.d., 26 cm long) to maintain a post column backpressure in excess of 600 psi at an oven temperature of 180 °C. This measure ensured that no phase transition could have occurred under the experimental conditions used.

3.1.2. Isobaric thermal gradient separations

Isobaric separations were investigated, by applying a gradual increase in the flow rate during the run to compensate for the drop in pressure across the column due to the increase in temperature thereby maintaining a relatively constant back pressure. The use of such isobaric separations potentially delivers two distinct advantages: (1) a reduction in analyte retention time and (2) improved peak capacity. The latter point is due to the fact that the optimal flow rate for chromatographic efficiency increases as the temperature of the chromatographic system is increased [21].

We have previously described a mathematical model, Eq. (1), which allowed the accurate prediction of column back pressure at a given temperature and flow rate.

$$\ln P = \ln\left(\frac{\varphi \eta_0 u L}{D_p^2}\right) + \frac{b}{T} \quad (1)$$

where P is the pressure across the column, L is the length of column, b is the constant dependant on the mobile phase only, u is the linear velocity of mobile phase, φ is the constant dependant on the packing efficiency of the HPLC column, D_p is the particle diameter of stationary phase material. H_0 is the viscosity of the mobile phase at a theoretical value of 0 K.

A detailed description of the derivation of this model can be found in Ref. [15]. This model was used to determine a temperature–flow rate profile that maintained a nominal constant pressure within the chromatographic system for isothermal conditions. We used the basic model to determine the flow conditions that would be required to maintain constant pressure (isobaric conditions) by the expedient of programming an increase in flow rate to compensate for the temperature-induced reduction in solvent viscosity, and hence pressure, with time.

The "required" pressure was fixed at 9000 psi, and the model derived flow rates were programmed into the LC system. Thus at the beginning of the run the flow rate was 0.25 ml/min, but at the end of the temperature gradient this had been increased to 0.54 ml/min (see Table 1).

3.2. Application to metabonomic analysis

As this thermal gradient program appeared to provide a good base for metabolite profiling we therefore proceeded to apply it to

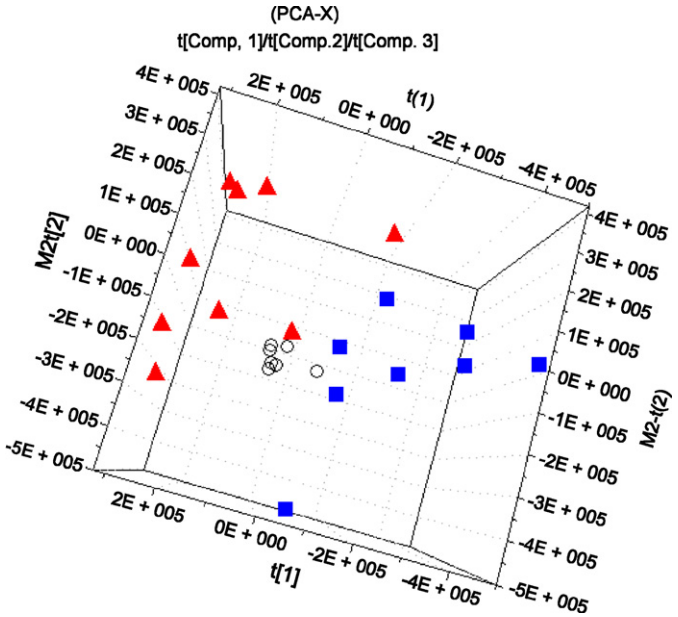

Fig. 1. PCA scores plot (PC1 vs. PC2 vs. PC3) from HT-UPLC-(+ESI) data. Empty circles represent the QC injections; blue boxes represent lean rats and red triangles represent fat rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

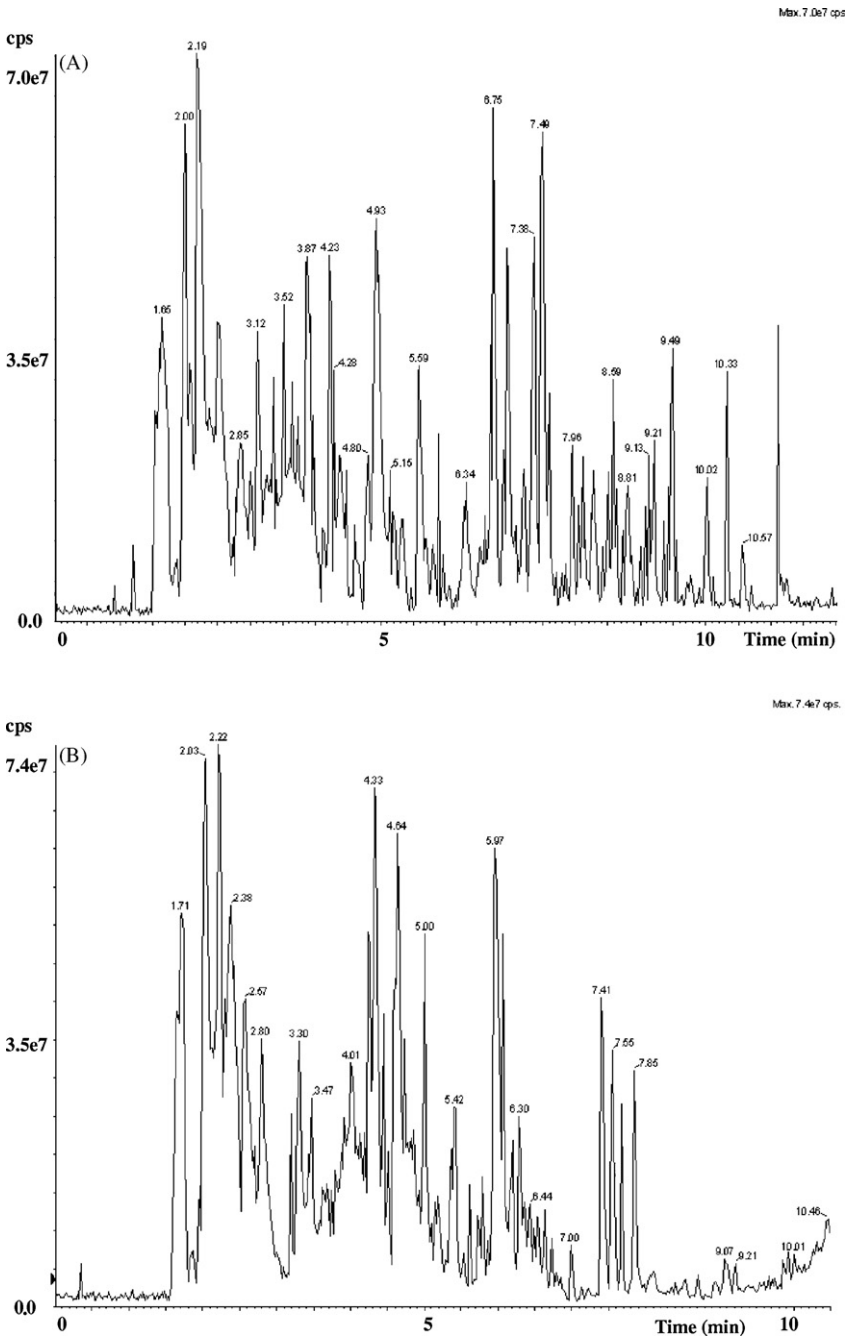

Fig. 2. Mass chromatogram (TIC) obtained from the LC–MS analysis of Zucker rat urine in +ESI. (A) Isobaric elevated temperature analysis with thermal and flow rate gradients; (B) conventional acetonitrile gradient under isothermal conditions (58 °C). Experimental details in text and Table 1.

the HT-UPLC–MS analysis of the biological samples. The pressure fluctuated over a narrow range (less than a hundred psi) and the chromatographic profile was satisfactory: blank runs provided a background signal without baseline drift as observed in the previous thermal gradient program. Next, urine samples were analysed to investigate the value of the method for the analysis of biological samples. Repeated analysis of the urine QC sample gave good results with regard to reproducibility and method sensitivity. In the case of blood plasma samples however, the resulting metabolic "fingerprint" was not satisfactory: the intensity and the number of peaks was rather poor compared to conventional LC–MS for such samples. This is probably due to the lipophilic nature of many of the plasma constituents and it is likely that the eluotropic strength of water, even at 180 °C was insufficient to elute them. The use of superheated water as an eluent for plasma, at least with C_{18} bonded phases, therefore seems impractical and was not studied further. Further work therefore concentrated on urine, which, as it consists largely of small polar molecules should not pose such problems. Indeed, the polar nature of these analytes is in itself often problematic for RPLC analysis making the development of a method based on pure or highly aqueous systems an attractive alternative (with potentially different chromatographic selectivity) and possibly a complementary profiling methodology. However, in order to avoid any build up of non-eluted sample constituents, and subsequent carry-over effects, a small portion of acetonitrile was added in the mobile phase in a shallow gradient program: 0–10% over 10 min,

with a 100% wash with acetonitrile at the end of the gradient. The addition of an acetonitrile wash step may also have advantages in resolvating the column and preventing stationary phase collapse in long analytical runs. The gradient program finally developed after several trials is given in Table 1. This program appeared to provide complete analyte elution and the regeneration of the stationary phase and was used for the rest of the study for the metabonomic analysis of Zucker rat urine.

There are many concerns about column stability when using elevated temperatures. However, whilst such concerns may be justified under some circumstances we noted no decline in column performance, using pure chromatographic criteria (operating pressure, retention times, peak widths, peak asymmetry). Good stability of this type of ethyl-bridged chromatographic material in such high temperatures has also been reported by other researchers [12]. The columns used in the study did not show loss of performance even after more than 250 injections of urine. This was also evident by the study of the QC samples, during the course of these investigations, which showed tight clustering (Fig. 1).

For comparison purposes a conventional gradient, of a type used extensively by us for metabonomic analysis of urine [20], was used in a separate experiment in the same instrumental configuration (employing the same Acquity columns) for the analysis of the Zucker urine sample set. Data was collected and was treated in the same way in order to provide a base for comparing and controlling the multivariate statistical results of the thermal gradient analysis. Comparing the performance of the two systems in a purely chromatographic manner, elevated temperature chromatography provided higher resolution power with higher peak capacity and better peak symmetry. These were calculated by extracting ion chromatograms for 15 ions selected randomly from the total ion current; the extracted ions were selected so that they would be representative of the collected mass range and also representative of the whole time frame of analysis; hence ions were selected from the front, the middle and the end of the mass chromatogram. In addition masses for certain known analytes were extracted: e.g., for hippuric acid (m/z 180), for tryptophan (m/z 205), etc. These results are given in Table 2 where peak widths (at the half peak height) and peak symmetry values are given for the thermal and the conventional acetonitrile gradient systems.

Peak capacity (PC) was calculated using the formula

$$\mathrm{PC} = 1 + \left(\frac{t_g}{w_{aver}}\right)$$

where t_g is the gradient time and w_{aver} is the average peak width [22]. The latter was calculated measuring the peak width at the base of 15 peaks and averaging their values. In HT-UPLC w_{aver} was found to be 9.8 s, whereas in conventional reversed-phase UPLC this was 15.2 s. Hence the corresponding peak capacity values were 62 and 41 for a gradient time of 10 min. Increased peak capacity is an important benefit arising from this development, as it represents a factor much sought after in metabonomic analysis. However, whilst the chromatographic performance for urine analysis appeared superior to conventional UPLC we did note that the signal for the mass spectrometer was somewhat noisier in high temperature chromatography. The selectivity of the HT-UPLC system seemed to be different from that of conventional RP-UPLC as indicated by the TIC obtained for typical profiles as shown in Fig. 2. However, since TIC are very complex profiles, this might not represent the best way to illustrate the elution of individual sample constituents. To illustrate this extracted ion chromatograms (XIC) for selected analytes, as described above, for peaks throughout the length of the chromatographic run are shown in Fig. 3. As shown also in Table 2 elevated temperature chromatography provided sharper peaks in general than those obtained at 58 °C. In addition

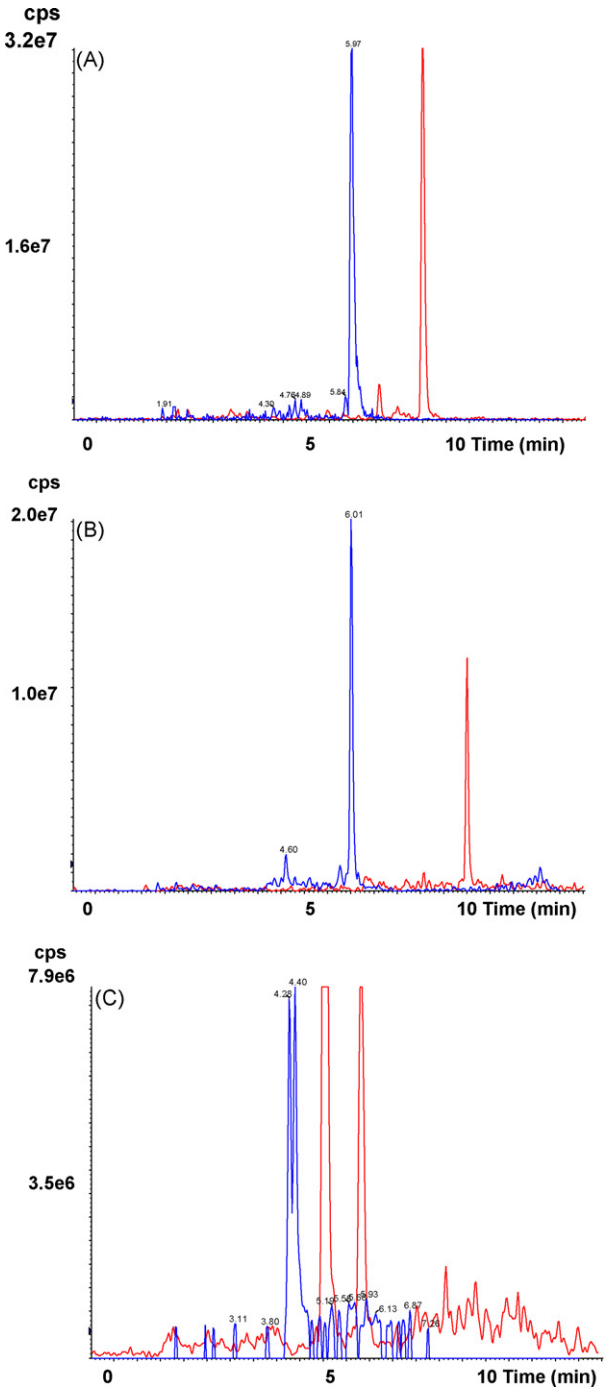

Fig. 3. Overlayed extracted ion chromatograms for three selected ions, obtained from the LC–MS analysis of the QC sample of Zucker rat urine in +ESI. In red the trace from the isobaric thermal gradient; in blue the trace of conventional acetonitrile gradient. Peak intensities are normalised to the blue trace (conventional reversed-phase LC run) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

superior peak separation was achieved as exemplified in Fig. 3c where the baseline resolution of the pair of peaks for the ion at m/z 239.0 is shown. In conventional reversed-phase LC coelution of the two peaks was observed.

As indicated above, Plumb et al. [17,18] have reported similar results working at 90 °C with UPLC-TOF MS for the analysis of urine samples and ginseng extracts, reaching peak capacities close to 500 for a 60 min analytical run.

Table 2
Peak characteristics for selected extracted ions from the conventional and the thermal gradient programs

Ion *m/z* (amu)	Analyte	HT-UPLC			Conventional acetonitrile gradient		
		Peak width 1/2 (min)	RT (min)	As	Peak width 1/2 (min)	RT (min)	As
114.1	Creatinine	0.08	2.00	2.15	0.09	2.04	2.57
136.1	Unknown	0.11	4.95	1.30	0.10	4.31	2.50
166.1	Phenylalanine	0.09	3.19	2.00	0.10	3.29	1.93
180.0	Hippuric acid	0.06	7.50	1.50	0.09	5.97	2.67
181.3	Unknown	0.06	7.21	1.27	0.07	5.42	1.64
188.1	Tryptophan	0.06	6.55	1.00	0.09	5.08	3.20
190.2	Kynurenic acid	0.08	7.36	1.43	0.13	5.41	2.00
206.1	Xanthurenic acid	0.07	6.92	2.00	0.09	5.03	1.50
216.0	Unknown	0.05	7.96	1.62	0.04	6.42	1.89
239.0	Unknown	0.10	5.81	1.20	0.04	4.40	2.20
271.2	Unknown	0.10	3.92	1.10	0.07	4.25	1.88
305.8	Unknown	0.07	8.61	1.10	0.06	5.55	1.67
319.1	Unknown	0.06	8.25	1.40	0.07	6.18	1.07
377.2	Unknown	0.07	8.50	1.20	0.07	6.01	1.80
590.3	Unknown	0.04	10.06	1.00	0.06	6.43	1.60

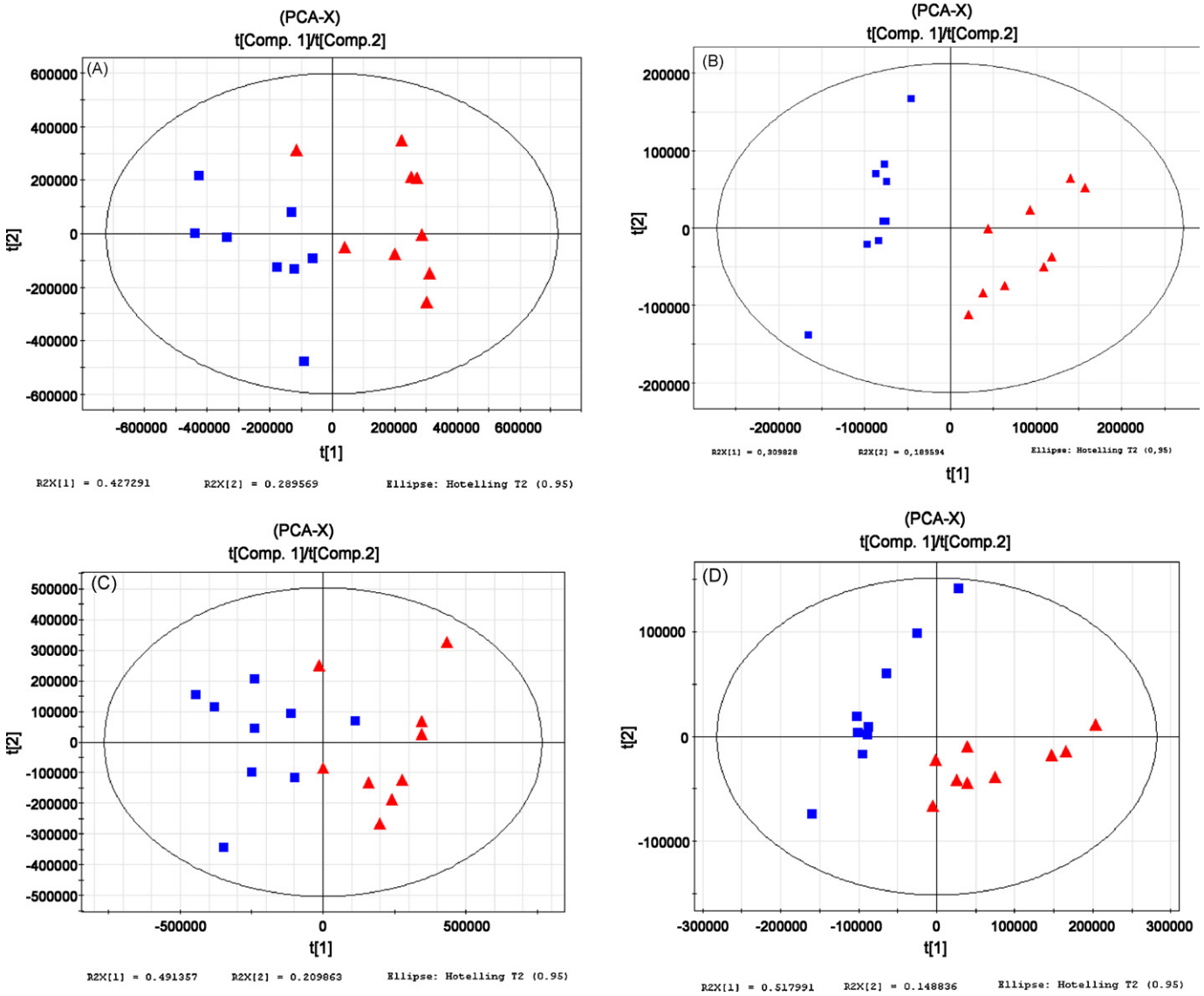

Fig. 4. PCA scores plots (PC1 vs. PC2), from the four datasets: (A) HT-UPLC (+ESI) model; (B) HT-UPLC-(−ESI) model; (C) conventional reversed-phase LC-(+ESI) model; (D) conventional reversed-phase LC-(−ESI) model. Blue boxes represent lean rats; red triangles represent (fa/fa) obese rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

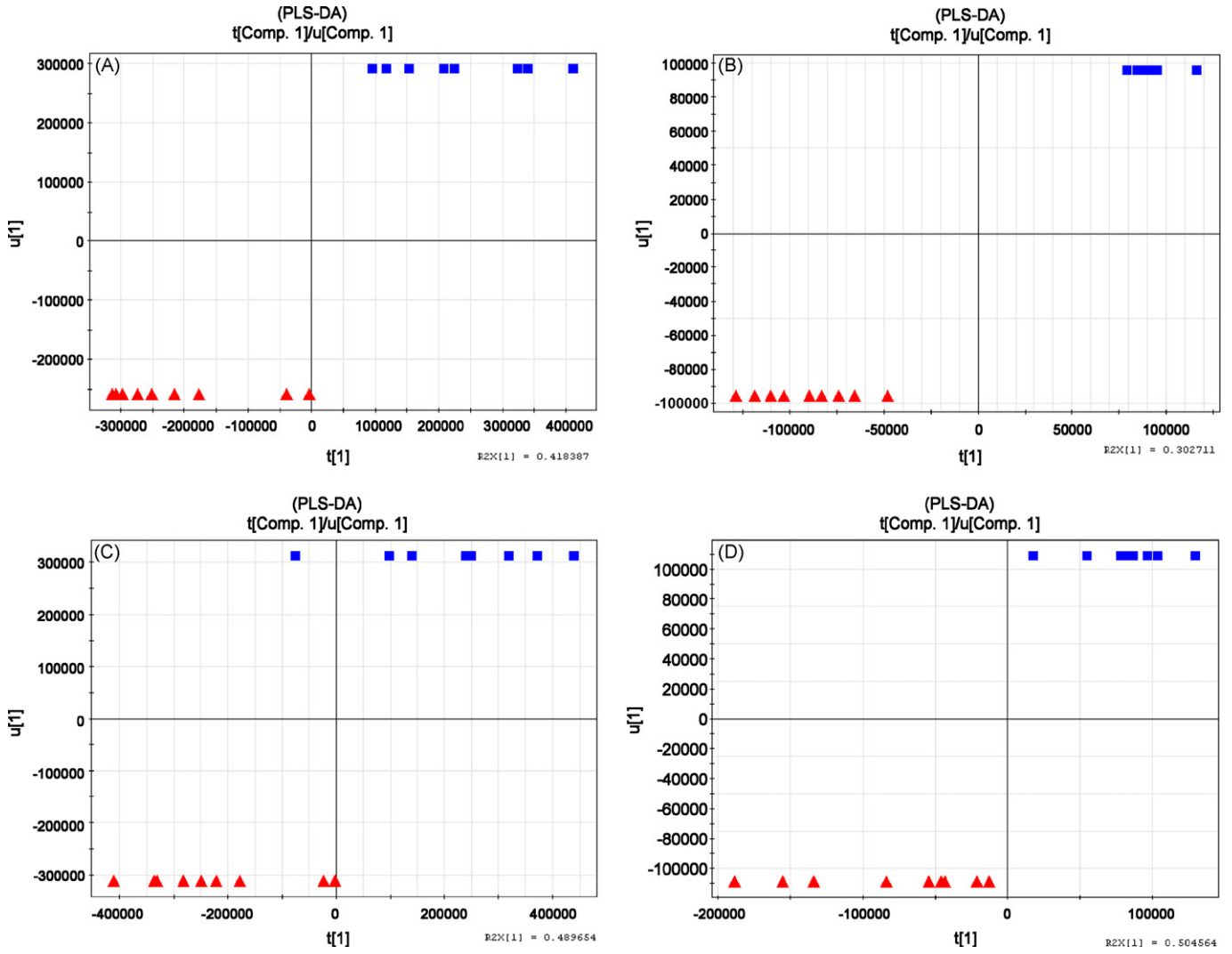

Fig. 5. Plots of t[1] vs. u[1] from the four datasets. (A) HT-UPLC-(+ESI) model; (B) HT-UPLC-(−ESI) model; (C) conventional reversed-phase LC-(+ESI) model; (D) conventional reversed-phase LC-(−ESI) model. Blue boxes represent lean rats; red triangles represent fat rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Multivariate statistics

All MS data collected were treated in MarkerView™ to generate two dimensional data matrices (peak tables) as described in Section 2. The peak tables were next imported to Simca P 11 (Umetrics, Umea, Sweden) for more advanced statistical analysis. Initially PCA (a non-supervised analytical tool) was applied. From the experiments performed here four datasets were created and studied: HT-UPLC-(+ESI)–MS, HT-UPLC-(−ESI)–MS, conventional reversed-phase LC-(+ESI)–MS, conventional reversed-phase LC-(−ESI)–MS.

To evaluate the goodness of fit and predictability of the models, R^2 and Q^2 values were calculated. R^2 values denote the percent of variation explained by the model. Q^2 values signify the percent of variation that can be predicted by the model. The values were: for HT-UPLC-(+ESI)–MS (5479 variables) $R^2 = 0.73$ and $Q^2 = 0.64$; for HT-UPLC-(−ESI)–MS (1891 variables) $R^2 = 0.67$ and $Q^2 = 0.47$. For the conventional reversed-phase LC-(+ESI) (5058 variables) $R^2 = 0.65$ and $Q^2 = 0.47$ and in conventional reversed-phase LC-(−ESI) (1426 variables) $R^2 = 0.78$ and $Q^2 = 0.57$.

Overall all four models presented satisfactory characteristics. The next step was to check how tight the QC injections clustered together. As we have explained elsewhere [20,23] a tight QC cloud in the scores plots is both a strong and a necessary evidence of a good analytical run. Fig. 1 gives an example from this study: as this shows, the QC samples (empty circles) cluster strongly together in the center of the PCA cube (PC1 vs. PC2 vs. PC3) and are very clearly separated from the two animal groups which are also clearly separated from each other. This indicated that the data was worth taking for further statistical analysis.

After establishing that the datasets were of good quality new PCA models were built with the exclusion of the QC samples. This was done in order to simplify the models and to be better able to focus on the differences between the two groups.

Fig. 4 depicts the scores plots of the first vs. the second principal component of the four investigated datasets. It is obvious that in all datasets the groups cluster together and the (fa/fa) obese rats can be easily distinguished from their lean counterparts. The best group clustering was obtained using HT-UPLC–MS in negative ionisation mode. The second best was obtained using conventional RP-gradient LC–MS in negative ionisation mode. In the remaining datasets the separation was not as clear cut and outliers appeared. The effect of scaling was also studied by applying other scaling modes in addition to Pareto. Centered scaling and Unit-variance scaling were also applied. Both scaling techniques provided scores

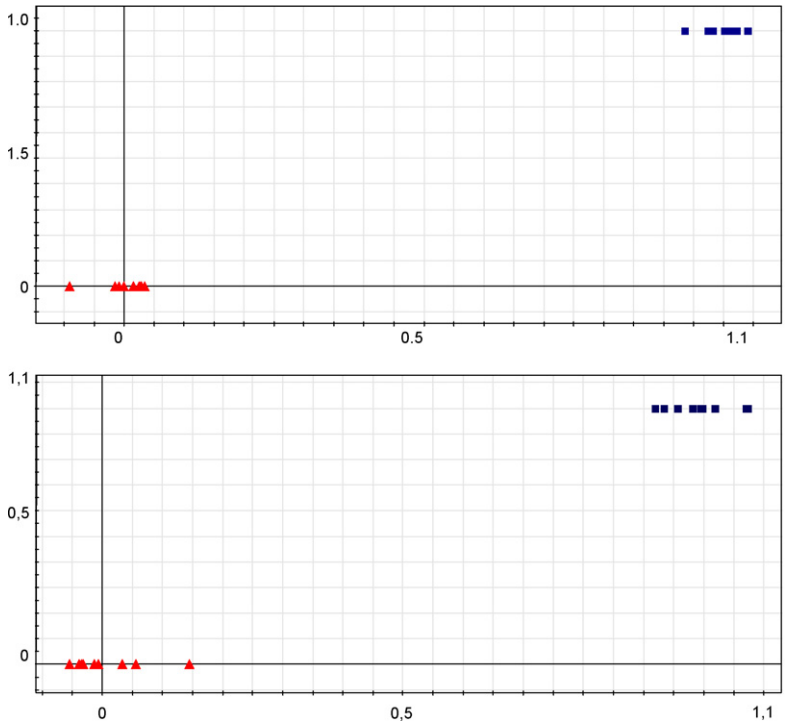

Fig. 6. Y predicted plots for the PLS-DA models generated from elevated temperature chromatography in +ESI top diagram and −ESI bottom diagram. The separation and predictability of the samples of each group is very satisfactory.

plots very similar to the Pareto scaled plots; the relative positions of the samples were not affected, and the separation of the two animal groups was still evident. The important finding was that, PCA alone was enough to separate the two groups, with clustering of the animals according to their weight group irrespective of the separation mode used. Following data analysis using PCA, the data was further examined using PLS-DA (a supervised analytical method), in order to extract additional information contained within the data. PLS-DA in general maximises the separation of two assigned populations and as would be expected provided a greater separation of the two age groups in this study. PLS-DA models were built using only the two classes, with the exclusion of the QC samples. In the same way as described above for PCA, quality characteristics were calculated for all four models: for the elevated temperature chromatography

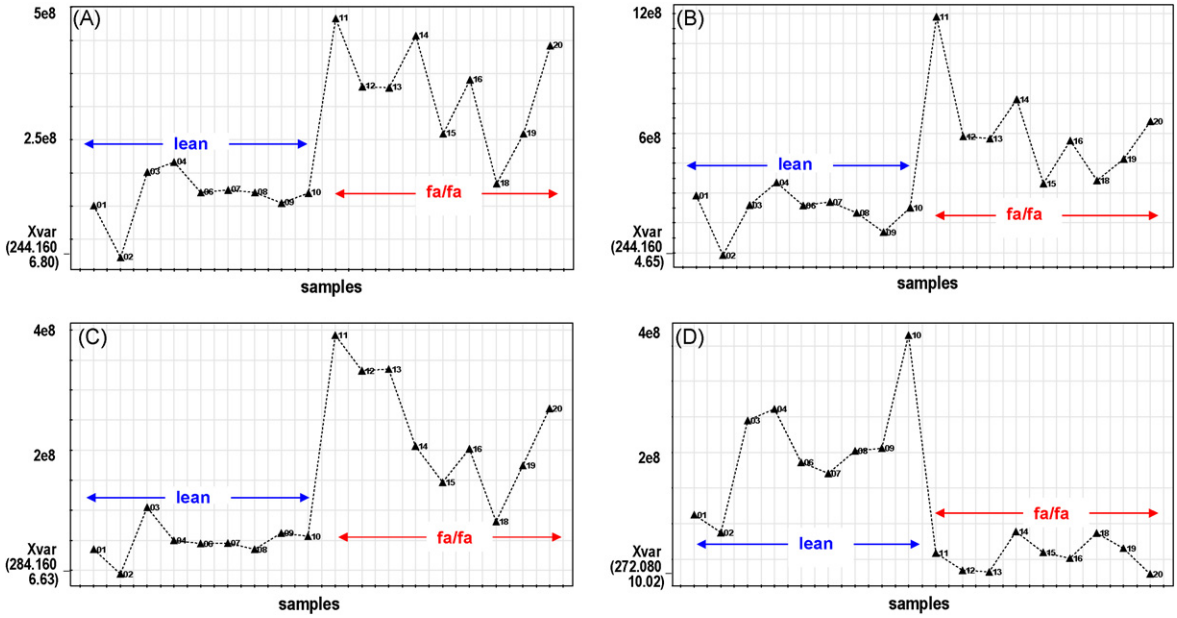

Fig. 7. Plots of the Xvar values for selected ions of high significance and strong contribution to group separation in PLS-DA. A, C and D are ions picked from the elevated temperature +ESI model. B is the same ion as in A but picked from the conventional reversed-phase LC-(+ESI) model. Ions A–C represent molecules with concentration higher in the fat rats. D exhibits higher concentration higher in the lean rats. Ion characteristics [m/z (amu)–retention time (min)]: (A) 244.160–6.80, (B) 244.160–4.65, (C) 288.160–6.63, and (D) 272.080–10.02.

positive ionization mode, the model gave $R^2 = 0.776$ and $Q^2 = 0.996$, whereas the negative ionization mode model gave $R^2 = 0.487$ and $Q^2 = 0.998$. With conventional RP-gradient LC, positive ionisation mode gave $R^2 = 0.742$ and $Q^2 = 0.968$, whereas negative ionization mode gave $R^2 = 0.776$ and $Q^2 = 0.99$.

As a whole the separation of the two animal groups was clear in all cases and as expected group separation was much better than PCA. This is depicted in Fig. 5 which illustrates t[1] vs. u[1] plots for the four models. These plots show the two groups and how well they are separated: the horizontal distance between the groups is a measure of their separation. As seen in the figure the HT-UPLC-(−ESI) model provides by far the best separation. In order to validate these PLS-DA models internal diagnostic strategies were applied. Calculation of the distance to model (DModX) was performed in all four PLS-DA models. DModX plots display the residual standard deviation (R.S.D.) of the observations in the X space; this is proportional to the distance of the observation to the model hyper plane. A DModX larger than the critical limit signifies that the observation is a possible outlier. All samples with the exception of sample 3 (lean rat) were under the critical limit, hence they were well described by the statistical PLS-DA model.

Further the prediction ability of the PLS-DA models was evaluated by studying classification lists and Y predicted values. The models managed to classify all samples in the correct groups: i.e., all lean rat samples were distributed within the lean group and all fat rat samples within the fat group, apart from two cases for the models of the conventional organic gradient. Thus, in (−ESI) one sample (lean rat sample 3) was not predicted clearly in the lean group and for the (+ESI) model a (fa/fa) obese rat (sample no 15) was not predicted clearly with the fat group. Y Predicted values show the distance of each model vs. the other. Fig. 6 gives a plot of the Y predicted for the HT-UPLC model obtained in positive ionisation and negative ionization modes. This plot signifies the quality of the predictive ability of the model and the clear cut separation of the groups.

In order to obtain insight on the contribution of the variables that were responsible for the group separation, the loadings plots of the PLS-DA models were studied. Ions were identified as responsible for the groups clustering and the variation of their intensity through the various analysed samples was plotted. Fig. 7 illustrates the Xvar of selected important ions: these were selected to represent ions (features) that were abundant in one of the two animal groups. The contribution of most of the studied ions was not found to be of the same significance in all the statistical models. This means that ions that are found to greatly contribute in, e.g., the elevated temperature (+ESI) were not found to be as significant in the conventional reversed-phase LC (+ESI) model. This was not unexpected based on our previous experience when comparing different ionization modes or different MS instruments (unpublished data) and can perhaps be explained by the observed differences in the chromatography and possible differences in ionisation and/or ion suppression. Apart from that, differences in the detection signal could be expected as the LC eluent differs to a great extent between the two chromatographic modes: predominantly aqueous in the elevated temperature chromatography vs. water–acetonitrile mixtures in the conventional RP LC gradient. This is also illustrated in Fig. 3, where varying responses are apparently observed for the same ions.

Finally the variables importance (VIP) was studied for the generated models. VIP values mainly reflect the correlation of the terms to all the responses and the importance of terms in the model both with respect to Y. Terms with large VIP, larger than 1, are the most relevant for explaining Y. HT-UPLC in either positive or negative ion mode provided a much higher number of high VIP variables. As an example the variables with VIP > 1, were 2003 for the HT-UPLC-(+ESI) dataset and only 1308 for the corresponding conventional acetonitrile gradient dataset (+ESI).

4. Conclusions

This work applied, for the first time in LC–MS-based metabonomics studies, thermal gradients and column temperatures higher than the boiling point of water. The potential of using thermal gradients for the LC–MS profiling of complex biological mixtures was clearly demonstrated with an increased number of ions detected and performance superior to conventional RPLC. Maintenance of both a minimum back pressure (to suppress phase transitions) and post column cooling was found to be critical to maximising the chromatographic performance of the system. The stability of the ethyl-bridged chromatographic material was satisfactory for these temperatures, and the columns that were used in the study did not show loss of performance even after more than 250 injections of urine. We believe that although the methodology needs further development it may provide a useful alternative for profiling in the metabonomics arena.

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