Talanta 83 (2011) 1769–1773

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

Urinary signature of anabolic steroids and glucocorticoids in humans by LC–MS

Agneta Kiss[a], Anne-Laure Jacquet[a], Olivier Paisse[a], Marie-Magdeleine Flament-Waton[a], Jacques de Ceaurriz[b], Claire Bordes[c], Jean-Yves Gauvrit[c], Pierre Lantéri[c], Cécile Cren-Olivé[a,*]

[a] Service Central d'Analyse (SCA), USR 59 CNRS, Chemin du Canal, Echangeur de Solaize, F-69390 Solaize, France
[b] Agence Française de Lutte contre le Dopage (AFLD), Département des analyses, 143, avenue Roger Salengro, F-92290 Châtenay-Malabry, France
[c] Laboratory of Chemometrics, UMR 5180, ESCPE Lyon, University Claude Bernard Lyon 1, Villeurbanne, France

ARTICLE INFO

Article history:
Received 6 June 2010
Received in revised form 17 October 2010
Accepted 26 October 2010
Available online 2 November 2010

Keywords:
Metabolic profiling
Metabonomics
Metabolomics
Doping products
LC–MS
Corticoids
Anabolics

ABSTRACT

A metabonomic strategy based on LC–MS was employed to investigate the metabolic profile of urine samples from 20 athletes who had been tested positive for corticoids and anabolic steroids and 29 controls. In this aim, different sample preparations and chromatographic conditions were compared. The acquired LC–MS data of doped athletes and controls were subjected to analysis of variance (ANOVA) and principal component analysis (PCA). Using this approach, molecular signature of human urine was obtained showing that metabonomics could be a complementary tool to discriminate different urinary profiles and to track down metabolic changes in humans.

1. Introduction

The doping is an old practice, probably as old as the practice of sports. Already in ancient Greece, athletes were following a diet adapted to the sport they practiced [1,2]. Doping is now generalised in all sports and at all levels due to political and economic pressures. Doping products have become more and more complex and the alimentation adapted to the practiced sport has become a product coming from the chemistry and the biotechnology.

The evolution of these illegal products or practices shows the importance and the obligation for the analytical techniques to evolve simultaneously. To date, the first analytical step of a doping control is a rapid screening that makes use of immunological or radio-immunological methods and chromatographic methods like gas chromatography (GC) and liquid phase (HPLC) techniques coupled or not to mass spectrometry (GC–MS and HPLC–MS) or atomic emission detection (GC-AED) [1,2]. The objective is to classify urine samples into different groups to select those that contain prohibited substances or, more generally, those with abnormal profiles. The aim of the analytical second step is to formally identify constituents (banned or not) detected during the first step, to determine concentrations and to try to define as precisely as possible the product initially used. Various chromatographic techniques coupled with liquid or gas mass spectrometry are used (GC–MS/MS, LC–MS/MS, GC/C/IRMS, etc.) [1,2].

The doping products analysis is a real challenge due to different problems [1–3] such as: (1) the analysis can be done only on "known compounds" and the molecules which have the same doping effect but have been structurally modified cannot be detected, (2) the concentration of the substance in the considered biological matrix is below the limit of detection of the analytical method and (3) the differentiation between exogenous and endogenous compounds remains extremely difficult. These examples underline the limits of the current analytical techniques and the need to develop new complementary methodologies.

Metabonomics is defined as "the quantitative measurement of multiparametric response of living systems to pathophysiological stimuli or genetic modification" [4,5]. This methodology is based on the determination of global metabolite profiles in biological fluids and tissues with subsequent data analysis via a range of multi-variate statistical approaches [4]. Metabonomics is typically performed on biofluids such as serum, urine, saliva and cerebrospinal fluid [4,6,7]. This methodology has been applied in different domains such as plant genotype description [8], stress induced plant metabolome modifications [9], characterization of genetically modified animal models [10,11], toxicology [12–14] (in particular, the study of metabolic profile after administration of xenobiotics [12,13], heavy metals [14]), pharmaceutical

* Corresponding author. Tel.: +33 4 78 02 22 04; fax: +33 4 78 02 71 87.
E-mail address: c.cren@sca.cnrs.fr (C. Cren-Olivé).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.10.030

research and development [7,15] and human disease diagnosis [15,16]. Recently, metabonomic approaches tempting to investigate anabolic steroid doping in animals have been reported [13,17]. Metabonomics, therefore, seems an attractive approach to track down doping in sportsmen.

There is no single way of performing a metabonomic profiling. A number of analytical tools are currently employed including nuclear magnetic resonance spectroscopy, mass spectrometry, high-performance liquid chromatography, optical spectroscopic analyses or a combination of techniques, each with their own advantages and drawbacks [4,18–20]. To date, the vast majority of work in this field used nuclear magnetic resonance (NMR) [4,21,22] but the technique is relatively insensitive and subtle variations of trace constituents might not be evidenced with this method [23]. On the contrary, liquid chromatography coupled with mass spectrometry has a greater sensitivity and enables good resolution of the different metabolites based on both their molecular weights and polarities. Therefore, it is considered to have a bright future in the research of trace-level metabonomics [24,25].

In this context, the present work describes, to our best knowledge, the first metabonomic profiling attempting to emphasize urinary metabolic signatures of athletes having taken anabolic steroids and glucocorticoids. The objective of this study was to investigate differences between athletes who have been tested positive for corticoids and anabolic steroids and controls by analysing their urine metabolite profiles by LC–MS. For this purpose, we evaluated different sample preparations and optimized the LC–MS analysis using different types of columns and chromatographic conditions. Data sets generated by the optimized LC–MS analysis were evaluated using analysis of variance (ANOVA) and principal component analysis (PCA). Using this approach, urinary signature of anabolic steroids and glucocorticoids was obtained showing that metabonomics could be a complementary tool to discriminate different urinary profiles and to track down metabolic changes in humans.

2. Experimental

2.1. Chemical and reagents

Water was obtained from a Millipore Milli-Q water purification system. Methanol and acetonitrile of HPLC–MS grade were purchased from Carlo Erba (Milan, Italy), the formic acid was purchased from Fluka Sigma–Aldrich (St Quentin Fallavier, France) and the ammonium acetate added as an ionic additive to the eluents was purchased from Merck (Fontenay-sous-Bois, France).

2.2. Urine collection

Twenty-nine urine samples were collected from volunteers and the 20 urine samples collected from doped athletes were provided by the French anti-doping accredited laboratory (AFLD, Agence Française de Lutte contre le Dopage, Châtenay-Malabry). The nature and the concentration of the prohibited substances used are shown in Table 1. All urine samples were stored at −24 °C until LC–MS analysis, as recommended for stability reasons [26].

2.3. Sample preparation

A "quality control" (QC) sample was prepared, as recommended in different publications [26–28], by mixing equal volumes (500 μL) from each of the samples as they were being aliquoted. This "pooled" urine was used to provide a representative "mean" sample containing all the analytes that will be encountered during the analysis. This "pooled" urine was used (i) to optimize sample preparation before analysis and (ii) as quality control sample during analysis.

Table 1
Doping features of the urine of the 20 tested positive athletes.

Sample	Detected substance No. 1	Concentration (ng/mL)	Detected substance No. 2	Concentration (ng/mL)
1	Betamethasone	342		
2	Prednisolone	573	Prednisone	212
3	Prednisolone	703	Prednisone	157
4	Stanozolol			
5	Prednisolone	11 923	Prednisone	2174
6	Prednisolone	48		
7	Budesonide	253		
8	Prednisolone	62		
9	Boldenone			
10	Budesonide	45		
11	Budesonide	215		
12	Prednisolone	25	Prednisone	66
13	Dexamethasone	106		
14	Budesonide	109		
15	Betamethasone	92		
16	Budesonide	109		
17	Triamcinolone	567		
18	Stanozolol			
19	Triamcinolone	1085		
20	Stanozolol			

Pooled urine was prepared using four different approaches. Firstly, urine was put directly in the vials and injected neat. Then, urine was centrifuged to 10 000 rpm at 20 °C for 5 min and the supernatant was injected. The third tested preparation was a SPE extraction on HLB 30 Oasis cartridge. The cartridge was conditioned with 3 mL methanol and then with 3 mL formic acid aqueous 0.1%. 2.5 mL of sample was loaded and the cartridge was washed by 6 mL formic acid aqueous 0.1%. The elution is performed with 3 mL methanol. The wash and the eluted phases are then separately injected. Finally urine was protein precipitated using acetonitrile (1:3, v/v) and centrifuged (10 000 rpm, 20 °C, 5 min) as several authors reported an increased in the urinary protein concentration induced by exercise [32,33]. The supernatant was removed for dryness. The evaporated samples were reconstituted in 1000 μL of water, centrifuged (10 000 rpm, 20 °C, 5 min) and then injected.

At the beginning of each batch, six QC samples were run to ensure that the analytical system had come to equilibrium. But these first data were not used in the statistical analysis since we and others [26–28] established that up to this point the urinary profiles produced by LC–MS are not reproducible.

2.4. Chromatographic and mass spectrometry conditions

The LC–MS system consisted of an Agilent HP 1100 MSD autosampler equipped with a UV diode-array detector and a mass detector with atmospheric pressure chemical ionization and electrospray ionization (MSD Hewlett Packard 1100 Series Quadrupole). The chromatographic separation was evaluated on two different columns: an Uptisphere BP 2 C_{18} (150 mm × 3 mm, 5 μm) and an Uptisphere C4 (150 mm × 2 mm, 3 μm, 300 Å) column. The column oven temperature was set to 50 °C; injection volume at 5 μL and flow-rate at 0.3 mL/min. Different mobile phases were evaluated: (A1) ammonium acetate 1 mM adjusted to pH 9.3, (A2) ammonium acetate 1 mM adjusted to pH 2.8 with formic acid and (B) acetonitrile. Gradient on Uptisphere BP 2 C_{18} consisted: 100% (A1) or (A2) for 5 min followed by a linear increase from 0% to 100% (B) over 15 min, isocratic cleaning step at 100% B for 10 min and column equilibration step at 0% (B) for 20 min. Gradient on Uptisphere C4 (150 mm × 2 mm, 3 μm, 300 Å) consisted of 100% (A2) for 5 min, linear increase from 0% to 100% (B) over 20 min, 100% B for 10 min and 100% (A2) for 20 min.

Table 2
List of detected masses and their intensities depending on the chromatographic and ionization conditions.

	pH 2.8 Positive mode	pH 2.8 Negative mode	pH 9.03 Positive mode	pH 9.03 Negative mode
Number of detected masses	3543	3270	4195	4571
Minimal intensity	1412	1434	1431	1577
Maximal intensity ($\times 10^7$)	5.63	8.72	7.32	95.0
Average intensity ($\times 10^4$)	10	3	0.9	1

Detection was performed in both positive and negative electrospray mode under the following conditions: nebulizer pressure 60 psi; drying gas (N_2) temperature 350 °C; drying gas flow 13 L/min; capillary voltage 4000 V; fragmentor voltage 70 V. Mass spectrometric data was collected over a range from 100 to 800 m/z. A post-column composed of 99% methanol and 1% formic acid at a flow rate of 0.3 mL/min was added to improve the ionization of molecules.

2.5. Data collection, processing, and multivariate data analysis

Collected data were processed by MZmine [29,30] and GeneSpring MS (Agilent). Peak detection in MZmine is performed in a three-step manner: first, mass values are detected within each spectrum. In the second step, a chromatogram is constructed for each of the mass values which span over certain time range. Finally, deconvolution algorithms are applied to each chromatogram to recognize the actual chromatographic peaks. These parameters were set as follows for peak detection: m/z bin size at 0.250, noise level (absolute value) at 250, minimum peak eight (absolute value) at 15, minimum peak duration at 3 s, tolerance for m/z variation at 0.2 Da and tolerance for intensity variation at 20%. Then, several modules are used for further processing of peak detection results, including deisotoping, filtering and alignment. In this way, aligned peak tables were created according to specified peak finding and alignment parameters: balance between m/z and RT at 10.0; m/z tolerance size at 0.2; RT tolerance size at 1%.

Data were also processed by GeneSpring MS using peak detection, alignment with a RT tolerance set at 0.2 and a match factor at 0.6. The ion intensities for each detected peak were then normalized in a three-step manner: (i) data transformation which sets intensity values inferior to 0.01 to 0.01, (ii) normalization to 50th percentile per run, and (iii) normalization to median per mass. Firstly, an univariate method called ANOVA followed by multiple testing corrections (Benjamini and Hochberg False Discovery Rate) was used. This parametric statistical test has already been reported in metabonomic studies [34] for its ability to select those variables significant for discrimination. Finally, the resulting data set was analysed by principal component analysis (PCA) available in Gene Spring MS (Agilent).

3. Results and discussion

The objective of this study was to investigate differences between 20 athletes who have been tested positive and 29 controls by analysing their urine metabolite profiles by LC–MS. The nature and the concentration of the prohibited substances detected in the urine by the French anti-doping accredited laboratory (AFLD) are shown in Table 1. Four athletes were tested positive for anabolic steroids (stanozolol or boldenone) and 16 for glucocorticoids (betamethasone, prednisolone, prednisone, budesonide, dexamethasone and triamcinolone). Among these 16 last ones, 4 athletes presented prednisolone and its pro-drug prednisone.

3.1. LC–MS analysis optimization

The literature [6,14,23,25–27,31] about the analysis by LC–MS of human urine shows a very wide range of experimental conditions including the ionization mode, the mobile phases and the pH of the latter. In order to obtain as much information as possible and the best mass spectra quality, chromatographic and ionization conditions have been evaluated to reduce ion suppression by reducing the number of competitive analytes entering simultaneously into the ion source as suggested by J.Boccard et al. [34].

Two aqueous mobile phases (basic A1 and acidic A2, B being acetonitrile) were compared in positive and negative ionization mode by injecting the pooled urine sample on the C18 column. Due to the complexity of the chromatogram obtained, the choice of the best condition was made on the basis of the molecular fingerprints obtained with MZmine (Table 2).

When the mobile phase pH is basic, whatever the ionization mode, the signal is very noisy (many masses detected with very weak intensities) as shown in Table 2. Working with acidic mobile phase A2, the noise is reduced: lower number of detected masses but higher average intensity (Table 2). Moreover, in positive ionization mode, the average intensity is 10 times higher than in negative ionization mode. Thus to obtain better sensitivity and the most informative molecular fingerprints, we have decided to work in positive ionization mode with acidic mobile phase A2.

3.2. Sample preparation evaluation

For the sample preparation optimization, four protocols were compared using the pooled urine sample and the optimized analytical conditions described above: (i) solid phase extraction, (ii) protein precipitation, (iii) centrifugation and (iv) direct injection.

Depending on the preparation performed, the fingerprints obtained with MZmine were different (Table 3). A comparative study of these fingerprints showed that protein precipitation and SPE extraction led to a significant loss of metabolites, as expected since SPE is more used for partial metabolic profiling. On the other hand, injection after centrifugation and direct injection do not differ significantly: 1469 masses were detected after direct injection and 1416 after centrifugation with comparable maximal and average intensities. Under these conditions, the direct injection seems to be the best method: it reduces the time dedicated to sample

Table 3
List of detected masses and their intensities depending on the sample preparation tested.

	SPE	Proteins precipitation	Centrifugation	Direct injection
Number of detected masses	993	1208	1416	1469
Minimal intensity	5200	5045	5202	5166
Maximal intensity ($\times 10^7$)	2.41	1.08	1.13	1.05
Average intensity ($\times 10^5$)	1.01	1.01	1.19	1.18

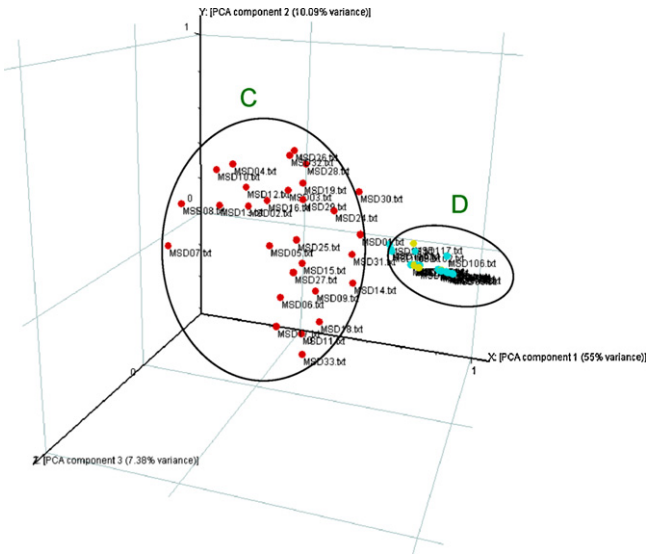

Fig. 1. PCA score plot (PC1 × PC2 × PC3) of the LC–MS data set obtained on the C_{18} column. C, control subjects, D, doped subjects.

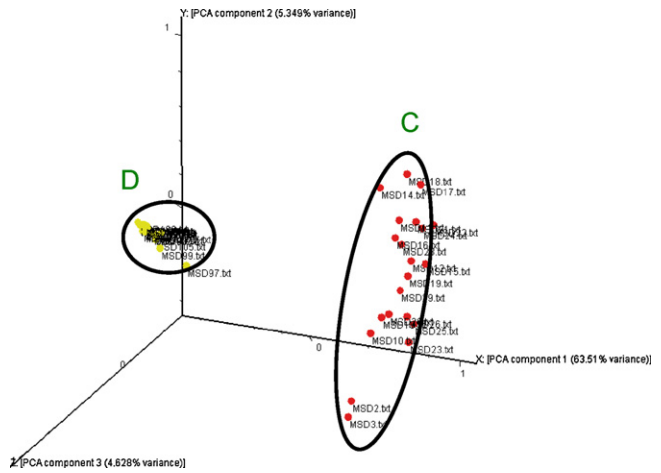

Fig. 2. PCA score plot (PC1 × PC2 × PC3) of the LC–MS data set obtained on the C_4 column. C, control subjects, D, doped subjects.

preparation as well as the risk of contamination or human errors and so leads to a simplification of procedures in the context of a subsequent legal framework. However, in order to remove the particulates and, thus, preserve the wealth of the system, a cleaning step was necessary. Samples were first vortexed, left to decant and then the supernatant was injected. Each sample was injected three times in order to assure reliable results.

3.3. Urinary metabolite profile discrimination

The 20 doped urine samples and the 29 controls were first analysed on the C_{18} column (Uptisphere BP 2 C_{18}) dedicated to bio analysis of crude samples without the risk of contaminating the column head or clogging. As expected in metabonomic studies, the LC-MS analysis leads to a huge amount of data (900 to 6000 variables in the 49 urine samples). Each variable is characterized by its m/z value and its retention time and in front of this challenge, the classical statistical and graphical methods are powerless. We therefore, turned our attention to chemometrics methods that could simplify the data and unhide important information.

First, PCA was applied on the totality of detected peaks (without prior ANOVA filtration), the separation exist but is less clear (data not shown). This is probably due to the fact that raw data are very noisy (analytical noise, case-irrelevant information such as: nutrition, environment, gender, lifestyle, etc.). Then, the two groups were compared by means of a significance statistical test. It is a univariate method that statistically asses the probability that a difference is due to chance alone. The lower this probability, the higher the chance that the assessed variable plays an important role in the discrimination between the two groups (negative tested athletes and athletes tested positive for anabolic steroids and glucocorticoids). A critical p-value of 0.05 was chosen. Another technique was the Principal Component Analysis, a multivariate technique that allowed the graphical visualization of the most important variation directions of the data set.

The PCA score plot of the whole ANOVA-filtered data set is presented in Fig. 1. Control samples (C, negative tested for glucorticoids or anabolis steroids) appear to have different profiles from doped samples (D, positive tested for glucorticoids or anabolic steroids). PC1 and PC2 describe about 65% of the total data variability remaining after ANOVA filtering [34]. The ANOVA selected variables have different trends in the positive tested samples than in the negative tested ones. They are currently under study as they may be related to metabolites that are up or down-regulated as a consequence of the anabolic steroids and/or glucocorticoids use. The major ions contributing to profile differences (m/z from 197.7 and 305.4) were eluted between 12 and 22 min, e.g., with ~40–100% of acetonitrile, indicating hydrophobic small molecules.

In the light of these first results and in order to better investigate the differences between the metabolic profiles of the two groups we decided to drastically change the chromatographic conditions using a C_4 column. The LC–MS dataset generated on the 49 samples were subjected to the same chemometric analysis and Fig. 2 presents the PCA score plot of the whole ANOVA-filtered data set. The difference between the two groups of athletes, doped (D, positive tested for glucorticoids or anabolic steroids) and controls (C, negative tested for glucorticoids or anabolis steroids) is most obvious along PC1, which describes alone about 64% of the total data variability remaining after ANOVA filtering [34]. The number of potential biomarkers detected is high and these compounds are currently under investigation. Further analytical and biological studies are needed to select the most relevant molecules and to prove their association with anabolic steroids or glucocorticoids administration.

4. Conclusion

This preliminary investigation supports the concept of a global approach for doping-to-non-doping-investigation for the development of a rapid screening method in doping control procedures. Profile differences were, indeed, signaled by PCA and ANOVA filtered datasets. Score plots allow easy interpretation of the results. Further studies are still needed (i) to analyse, by UPLC–HRMS, samples from larger sets of athletes, but also samples which present particular difficulties in classical control strategies and (ii) to thoroughly investigate the potential biomarkers compounds. With time, the improvements should enable this innovative technique to discriminate doped athletes. This work suggests that metabonomic-based strategies may play an invaluable role in the future of rapid screening methods used in doping control strategies.

References

[1] P. Hemmersbach, Journal of Mass Spectrometry 43 (2008) 839–853.
[2] G.J. Trout, R. Kazlauskas, Chemical Society Reviews 33 (2004) 1–13.
[3] F. Botre, Journal of Mass Spectrometry 43 (2008) 903–907.
[4] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Nature Reviews Drug Discovery 1 (2002) 153–161.
[5] J.K. Nicholson, J.C. Lindon, E. Holmes, Xenobiotica 29 (1999) 1181–1189.

[6] X. Lu, X.J. Zhao, C.M. Bai, C.X. Zhao, G. Lu, G.M. Xu, Journal of Chromatography B—Analytical Technologies in the Biomedical and Life Sciences 866 (2008) 64–76.
[7] J.C. Lindon, E. Holmes, M.E. Bollard, E.G. Stanley, J.K. Nicholson, Biomarkers 9 (2004) 1–31.
[8] O. Fiehn, Plant Molecular Biology 48 (2002) 155–171.
[9] E. Grata, J. Boccard, G. Glauser, P.A. Carrupt, E.E. Farmer, J.L. Wolfender, S. Rudaz, Journal of Separation Science 30 (2007) 2268–2278.
[10] J.C. Lindon, E. Holmes, J.K. Nicholson, Current Opinion in Molecular Therapeutics 6 (2004) 265–272.
[11] M.J. Chen, L.P. Zhao, W. Jia, Journal of Proteome Research 4 (2005) 2391–2396.
[12] M.A. Constantinou, M. Vertzoni, C. Reppas, A. Tsantili-Kakoulidou, E. Mikros, Molecular Pharmaceutics 4 (2007) 258–268.
[13] M.E. Dumas, C. Canlet, J. Vercauteren, F. Andre, A. Paris, Journal of Proteome Research 4 (2005) 1493–1502.
[14] A. Lafaye, C. Junot, B. Ramounet-Le Gall, P. Fritsch, E. Ezan, J.C. Tabet, Journal of Mass Spectrometry 39 (2004) 655–664.
[15] J.C. Lindon, E. Holmes, J.K. Nicholson, Expert Review of Molecular Diagnostics 4 (2004) 189–199.
[16] G.A.N. Gowda, S.C. Zhang, H.W. Gu, V. Asiago, N. Shanaiah, D. Raftery, Expert Review of Molecular Diagnostics 8 (2008) 617–633.
[17] J.C.W. Rijk, A. Lommen, M.L. Essers, M.J. Groot, J.M. Van Hende, T.G. Doeswijk, M.W.F. Nielen, Analytical Chemistry 81 (2009) 6879–6888.
[18] W.B. Dunn, N.J.C. Bailey, H.E. Johnson, Analyst 130 (2005) 606–625.
[19] J.L. Griffin, Current Opinion in Chemical Biology 7 (2003) 648–654.
[20] S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, J. Nielsen, Mass Spectrometry Reviews 24 (2005) 613–646.
[21] H.C. Keun, T.M.D. Ebbels, H. Antti, M.E. Bollard, O. Beckonert, E. Holmes, J.C. Lindon, J.K. Nicholson, Analytica Chimica Acta 490 (2003) 265–276.
[22] N.V. Reo, Drug and Chemical Toxicology 25 (2002) 375–382.
[23] S. Wagner, K. Scholz, M. Donegan, L. Burton, J. Wingate, W. Volkel, Analytical Chemistry 78 (2006) 1296–1305.
[24] D.J. Crockford, J.C. Lindon, O. Cloarec, R.S. Plumb, S.J. Bruce, S. Zirah, P. Rainville, C.L. Stumpf, K. Johnson, E. Holmes, J.K. Nicholson, Analytical Chemistry 78 (2006) 4398–4408.
[25] I.D. Wilson, J.K. Nicholson, J. Castro-Perez, J.H. Granger, K.A. Johnson, B.W. Smith, R.S. Plumb, Journal of Proteome Research 4 (2005) 591–598.
[26] H.G. Gika, G.A. Theodoridis, I.D. Wilson, Journal of Chromatography A 1189 (2008) 314–322.
[27] H.G. Gika, G.A. Theodoridis, J.E. Wingate, I.D. Wilson, Journal of Proteome Research 6 (2007) 3291–3303.
[28] T. Sangster, H. Major, R. Plumb, A.J. Wilson, I.D. Wilson, Analyst 131 (2006) 1075–1078.
[29] M. Katajamaa, J. Miettinen, M. Oresic, Bioinformatics 22 (2006) 634–636.
[30] M. Katajamaa, M. Oresic, BMC Bioinformatics 6 (2005).
[31] R. Williams, E.M. Lenz, A.J. Wilson, J. Granger, I.D. Wilson, H. Major, C. Stumpf, R. Plumb, Molecular Biosystems 2 (2006) 174–183.
[32] A. Clerico, C. Giammattei, L. Cecchin, A. Lucchetti, L. Cruschelli, G. Penno, G. Gregori, O. Giampietro, Clinical Chemistry 36 (1990) 562–564.
[33] M. Kohler, K. Walpurgis, A. Thomas, M. de Maree, J. Mester, W. Schanzer, M. Trevis, Proteomics Clinical Applications 4 (2010) 568–576.
[34] J. Boccard, E. Grata, A. Thiocone, J.Y. Gauvrit, P. Lantéri, P.A. Carrupt, J.L. Wolfender, S. Rudaz, Chemometrics and intelligent Laboratory Systems 86 (2007) 189–197.