Metabolomics of biofluids : from analytical tools to data interpretation

Nevedomskaya, E.

Chapter 3

CE-MS for metabolic profiling of volume-limited urine samples: application to accelerated aging TTD mice

Nevedomskaya E., Ramautar R., Derks R., Westbroek I., Zondag G., van der Pluijm I., Deelder A.M., Mayboroda O.A.

Journal of Proteome Research **2010**, *9*, 4869–4874

ABSTRACT

Metabolic profiling of biological samples is increasingly used to obtain more insight into the pathophysiology of diseases. For translational studies, biological samples from animal models are explored; however, the volume of these samples can be a limiting factor for metabolic profiling studies. For instance, only a few microliters of urine is often available from small animals like mice. Hence, there is a need for a tailor-made analytical method for metabolic profiling of volume-limited samples. In the present study, the feasibility of capillary electrophoresis time-of-flight mass spectrometry (CE-ToF-MS) for metabolic profiling of urine from mice is evaluated. Special attention is paid to the analytical workflow; that is, such aspects as sample preparation, analysis, and data treatment are discussed from the metabolomics viewpoint. We show that metabolites belonging to several chemical families can be analyzed in mouse urine with the CE-ToF-MS method using minimal sample pretreatment and an in-capillary pre-concentration procedure. This exemplifies the advantages of CE-ToF-MS for metabolic profiling of volume-limited samples as loss of material is minimized. The feasibility of the CE-ToF-MS-based workflow for metabolic profiling is illustrated by the analysis of urine samples from wild-type as well as from TTD mutant mice, which are a model for the accelerated aging, with osteoporosis being one of the main hallmarks.

INTRODUCTION

Metabolomics is a rapidly developing field in the "postgenomic" research that focuses on the global profiling of small endogenous molecules present in body fluids.(1) As end-points of biochemical processes, endogenous metabolites are directly associated with the phenotype of the organism.(2) Consequently, an overview of the metabolic composition of body fluids and urine in particular can be helpful for the phenotypic characterization of genetically modified animals and eventually for the understanding of the link between phenotypic trait and genetic background.

At present, high-resolution ^{1}H NMR spectroscopy is one of the key technologies for body fluid investigations as it is capable of producing fast and highly reproducible metabolic profiles in body fluids without the need for the preselection of analytical parameters or sample derivatization procedures. For volume-limited samples, a miniaturized NMR probe coil can be implemented for the analysis of volumes as low as a few microliters.(3) However, a major limitation of NMR still is the relatively poor concentration sensitivity compared to MS-based techniques. For GC-MS, 10-50 μL of sample is often a minimum volume requirement for sample pretreatment steps (such as for example, derivatization), and in LC-MS, injection volumes of 5-10 μL are commonly used for metabolic profiling studies.

With regard to miniaturization, capillary electrophoresis-mass spectrometry (CE-MS) (4-6) is a well-suited method, as recently illustrated by single-cell and even subcellular level analyses.(7) Moreover, it is possible to simultaneously concentrate and separate analytes in a biological sample without any sample pretreatment, which is very advantageous for volume-limited samples.(8)

In the present study, we describe an analytical workflow based on CE-MS for metabolic profiling of urine samples from mice. As a model we use TTD mice: fast-aging mice which carry a mutation in the XPD gene that is involved in the Nucleotide Excision Repair (NER) pathway.(9) At a first glance, the phenotype of these animals would appear to require no additional analysis. They exhibit a series of clear phenotypical changes such as osteoporosis and kyphosis, osteosclerosis, early greying, cachexia, infertility, and reduced life-span. However, such a complex phenotype may conceal some basic physiological changes in animal metabolism essential for understanding the effect of the mutation. Using a previously developed CE-MS method for amino acid profiling in human urine as a starting point, (10) we here extend this method to metabolic profiling of urine samples from mice, including all steps required for the processing and statistical analysis of complex samples.(11;12)

MATERIALS AND METHODS

Chemicals. Methanol (MeOH) (HPLC-grade, Biosolve B.V., The Netherlands), ultrapure water (18.2 MΩ/cm), and formic acid (FA) (Fluka, Germany) were used for solvent preparation. A standard solution of 17 amino acids at 1 mM each in 1 M HCl was purchased from Sigma (Sigma-Aldrich, Germany). Dopamine hydrochloride, folic acid, and Phe-Gly hydrate were purchased from Fluka (Germany). Sarcosine, theophylline, caffeine, nortriptyline hydrochloride, creatinine, 4-O-methyldopamine hydrochloride, homovanillyl alcohol, glutathione, thyroxin, and 5-hydroxyindole-3-acetid acid (5-HIAA) were acquired from Sigma (Germany). Stock solutions of the 30 reference compounds were prepared in water at a concentration of 200 μM.

Mouse Urine Samples. Animals were housed at the Animal Resource Center (Erasmus University Medical Center), which operates in compliance with the "Animal Welfare Act" of the Dutch government, using the "Guide for the Care and Use of Laboratory Animals" as its standard. As required by Dutch law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities. All animal studies were approved by an independent Animal Ethical Committee.

Experiments were performed in accordance with the "Principles of laboratory animal care" (NIH publication no. 86-23) and the guidelines approved by the Erasmus University animal care committee. The generation of XPD^{TTD} alleles has been previously described.(13) XPD^{TTD} homozygous mutant animals were obtained by crossing $XPD^{TTD/+}$ with $XPD^{TTD/+}$ mice in a pure C57Bl6J background. Wild-type littermates were used as controls. Mice were housed in individual ventilated cages with *ad libitum* access to AIN93 M synthetic food.

In total, 75 mice were used; 38 TTD mutants and 37 wild-type. Groups were gender matched. The urine was collected at 4 time points, 26, 45, 52, and 65 weeks. Each mouse urine was collected on a piece of Parafilm between 11.00 and 13.00 h and stored at -70 °C. Four microliters of urine was mixed with 4 μL of MeOH, 11 μL of water, and 1 μL of background electrolyte (BGE); centrifuged to eliminate any possible sediment remaining; and put into vials for injection into the CE instrument.

Instrumentation. CE was performed on a P/ACE ProteomeLab PA 800 (Beckman Coulter, Fullerton, CA). Fused silica capillaries (BGB-Analytik, Germany) with total length of 100 cm with 50 μm inner diameter were used for separation. Twenty percent MeOH with 2 M FA was used as background electrolyte. Sample injection was performed hydrodynamically with pH-mediated stacking: a small plug (50 mbar, 9 s) of 12.5% ammonium hydroxide (NH_4OH) was injected before the sample plug (50 mbar, 90 s). The sample plug injected corresponds to a volume of ca. 69 nL, which corresponds to 3.9% of total capillary volume. A washing step of 5 min with 0.1 M NaOH was included between the

runs. During rinsing with 0.1 M NaOH, the end plate voltage, capillary voltage, and the nebulizer were set to 0, which prevented the solution to enter the vacuum part of the MS. The separation voltage was +30 kV (yielding a current of ca. 23 μA).

MS was performed using a micrOTOF (Bruker Daltonics, Bremen, Germany). Transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany). Spectra were collected with a time resolution of 1 s. CE-MS coupling was realized by a coaxial sheath liquid interface (Agilent Technologies, Waldbronn, Germany) with methanol-water-formic acid (50:50:0.1, *v/v/v*) as sheath liquid. The following spray conditions were used: sheath liquid flow, 4 μL/min; dry gas temperature, 180 °C; nitrogen flow, 4 L/min; nebulizer pressure, 0.5 bar. Electrospray in positive ionization mode was achieved at -4.5 kV. The analytical performance of the CE-MS method was evaluated by the analysis of a standard mixture of metabolites throughout the experiments.

Tandem MS analysis was performed using a micrOTOF-Q (Bruker Daltonics, Bremen, Germany) instrument.

Data Analysis. All data files were recalibrated on the masses of sodium formiate clusters. For estimation of the number of detected compounds, Find Molecular Features algorithm within DataAnalysis (DA) software package (Bruker Daltonics) was used (signal-to-noise cutoff set to 10, correlation coefficient 0.9).

The alignment of electropherograms was performed using XCMS software (The Scripps Research Institute, La Jolla, CA).(14) The resulting table included the detected ion features and their peak areas, which were then normalized using nonparametric normalization.(15) Afterwards, data was imported into Simca-P+ software package, version 12.0 (Umetrics, Umeå, Sweden) for further multivariate analysis. Following principal component analysis (PCA), partial least-squares discriminant analysis (PLSDA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA), compounds responsible for group separation were found. To identify metabolites of interest, rational chemical formulas were generated based on internally calibrated monoisotopic masses within 10 mDa mass error, using the SmartFormula tool within the DA. The chemically reasonable formulas were submitted to metabolome databases: Kyoto Encyclopedia of Genes and Genomes (KEGG) ligand database,(16) the Human Metabolome Database (HMDB),(17) and the METLIN database.(18) The isotopic distribution patterns of the matched metabolite candidates were simulated with the Simulate Pattern tool of DA and compared with observed mass spectra to reduce further the number of potential elemental compositions.(19)

The results of MS/MS analysis confirmed previously acquired identity of the compounds with the help of public databases: HMDB, METLIN, and MassBank.(20)

RESULTS AND DISCUSSION

Recently, we have developed a CE-ToF-MS method for the highly efficient and sensitive analysis of amino acids in human urine.(10) The application of this method for phenotype characterization using metabolic profiling demands broader coverage of the metabolites. Therefore, we first evaluated whether this method can be used for the profiling of diverse metabolites. For this, we have analyzed a standard mixture composed of compounds from different chemical families: amino acids, alcohols, xanthines, amines, dipeptides, and so forth (Supplementary Materials, Table S1). Under the conditions used for separation, pH 1.8, all basic and amphoteric compounds are positively charged, allowing their migration toward the MS detector. Except for glycine, which is outside the selected mass window, all the compounds in the standard mix have been analyzed within 30 min (Supplementary Materials, Figure S1). Compounds that are doubly charged under the used conditions (*e.g.*, lysine, creatinine, histidine) migrated first, followed by amino acids carrying a single charge (*e.g.*, threonine, serine, valine, *etc.*) as well as small peptides (e.g., phenylalanyl-glycine, glutathione). The next group is formed by compounds containing phenyl group as well as acidic groups (*e.g.*, thyroxin and folic acid). The clear separation of compounds present in our standard mix indicates that this CE-MS method can be used for the analysis of various classes of metabolites as required in metabolic profiling studies.

For the evaluation study and testing of the CE-MS method for volume-limited samples, a cohort of urine samples from accelerated aging TTD mice was chosen. One of the model's features is significantly reduced body weight: as a consequence the sampling of body fluids is difficult and only volume-limited samples can be collected. Thus, CE-MS is the method of choice for this particular set of samples. Our workflow included CE-ToF-MS measurement, data preprocessing (alignment and normalization), data analysis, and identification of metabolites of interest (Figure 1).

By using pH-mediated stacking as an in-capillary pre-concentration step, around 600 compounds were detected on average in mouse urine (see Supplementary Materials, Figure S2), which is an improvement of 50% compared to the analysis without pH-mediated stacking. A substantial fraction of those compounds could be identified on the basis of their accurate mass and isotopic pattern.

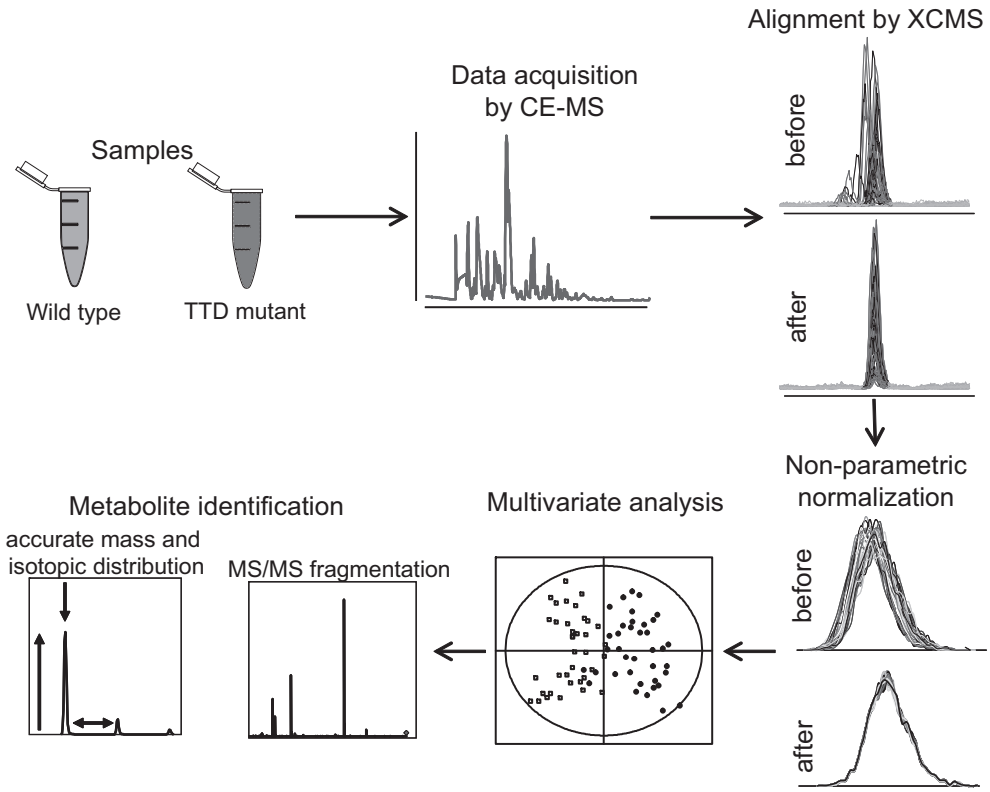

Figure 1. Analytical workflow.

One of the frequently discussed drawbacks of CE-MS using bare fused silica capillaries is the lack of migration time reproducibility. Therefore, XCMS, which is a package combining alignment, peak picking, and statistical analysis, was used for data analysis.(14) The output contains detected ion features and their areas across all samples. To find features responsible for the separation of the groups of animals, a multivariate data analysis approach was used, including unsupervised as well as supervised methods. Prior to statistical analysis, nonparametric normalization was done, as previously described, (15) which unifies distributions of variables. Performing principal component analysis (PCA) on the set of standards run between blocks of mouse urine samples, it is clear that the variation present in the group of biological samples is much larger than the variation between standards (Figure 2A). This means that the analytical variation does not interfere with natural variation between the samples under study. PCA models were also computed for the whole cohort (Supplementary Materials, Figure S3) as well as for samples of male and female mice separately (Supplementary Materials, Figure S4). None of the PCA scores plots showed clustering of the samples according to genotype. This is not unusual in metabolic

profiling studies as the differences of interest might be small and obscured by the relatively large, intrinsic variation between biological samples. To reveal this information, supervised methods were used. Partial least squares discriminant analysis (PLS-DA) model for the whole cohort was quite poor (R2Y = 0.71, Q2 = 0.28). However PLS-DA models, computed for males and females separately, showed that for females the model was quite satisfactory with R2Y and Q2 parameters equal to 0.977 and 0.5, respectively, unlike for males with very low predictive ability (R2Y) 0.78, Q2 < 0, Supplementary Materials, Figure S5). Female mice were chosen for modeling differences between wild-type and mutant phenotypes and retrieval of compounds responsible for separation between them. Next, orthogonal PLS-DA (OPLS-DA) was used to separate systemic noise from variation correlated with the studied classes' discrimination. The scores plot for OPLS-DA model (Figure 2B) shows that the wild-type and mutants are clearly separated from each other.

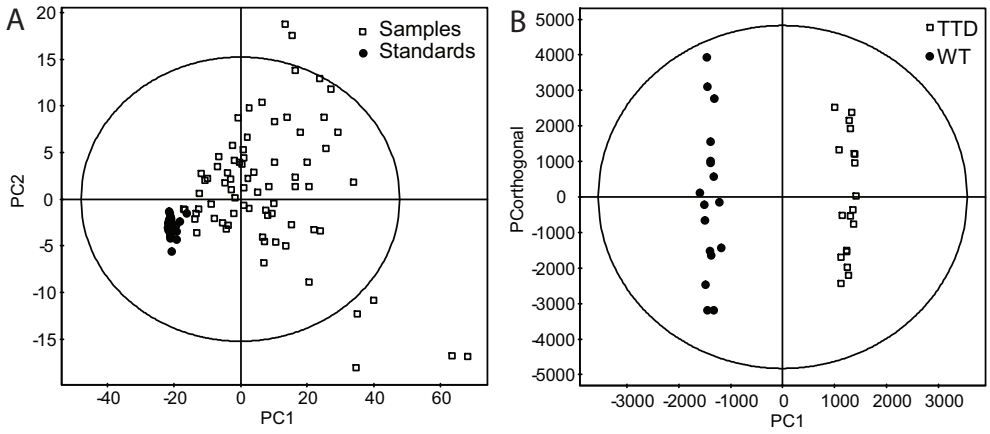

Figure 2. Multivariate data analysis of obtained CE-MS data. (A) PCA scores plot on samples (white boxes) and standards (black circles). First two principal components cover 56.8% of the variation. (B) OPLS-DA scores plot discriminating wild-type female mice (black circles) from mutant TTD female mice (white boxes).

The compounds responsible for discrimination between classes have been chosen based on an S-plot, which shows the compounds' importance and reliability (modeled covariance higher than 0.13 or less than -0.13, modeled correlation more than 0.2 or less than -0.2). Seven compounds fulfilled these criteria and were used for further analysis as possible markers of TTD phenotype (Table 1).

Table 1. Identified classifiers between female TTD mutants and female wild type animals.

measured mass	migration time, min (RSD, %)[a]	identified compound	formula	theoretical mass	error, mDa	mSigma	fold change[b]	p-value[c]	modelled covariation	modelled correlation
399.1459	22.5 (2)	S-Adenosyl-L-Methionine	$C_{15}H_{23}N_6O_5S$	399.1445	-1.4	25	9.5	0.0009	0.22	0.51
156.0769	22.3 (2.5)	Histidine	$C_6H_{10}N_3O_2$	156.0768	-0.2	5.2	3.2	0.014	0.14	0.36
189.1589	22.05 (1.7)	Trimethyl-L-Lysine	$C_9H_{20}N_2O_2$	189.1589	0.8	8.4	6.4	0.012	-0.16	-0.4
204.1349	28.3 (1.8)	Gly-Lys, Gly-Lys	$C_8H_{17}N_3O_3$	204.1343	-0.6	10.8	2.5	0.034	0.12	0.3
147.113	21.3 (1.3)	Lysine	$C_6H_{14}N_2O_2$	147.1128	-0.2	7.1	-1.9	0.064	-0.13	-0.27
188.1747	20.8 (1.7)	N-acetylspermidine	$C_9H_{21}N_3O$	188.1757	1	10.4	1.5	0.086	0.11	0.24
151.1443	20.5 (3.5)	Unidentified					-1.6	0.107	-0.09	-0.2

[a] RSD – relative standard deviation in migration time

[b] calculated as difference in mean areas in TTD mutant relative to wild type animals (negative values indicate that the compound is decreased in TTD compared to WT, positive that it is increased in TTD compared to WT)

[c] unpaired t-test using a Benjamini–Hochberg correction for the p-values

Identification of compounds of interest was performed using the SmartFormula tool within the Bruker Daltonics' DataAnalysis software package and various databases. As some of the tentatively identified compounds were also present in the standard mixture, their migration times were compared and confirmed. The chemical structures of these compounds have been confirmed by tandem mass spectrometry. A sample MS/MS spectra for the compound with the mass 399.1459 (S-Adenosyl-L-Methionine) is shown in Figure 3. Only one compound with mass 151.1443 has not been identified.

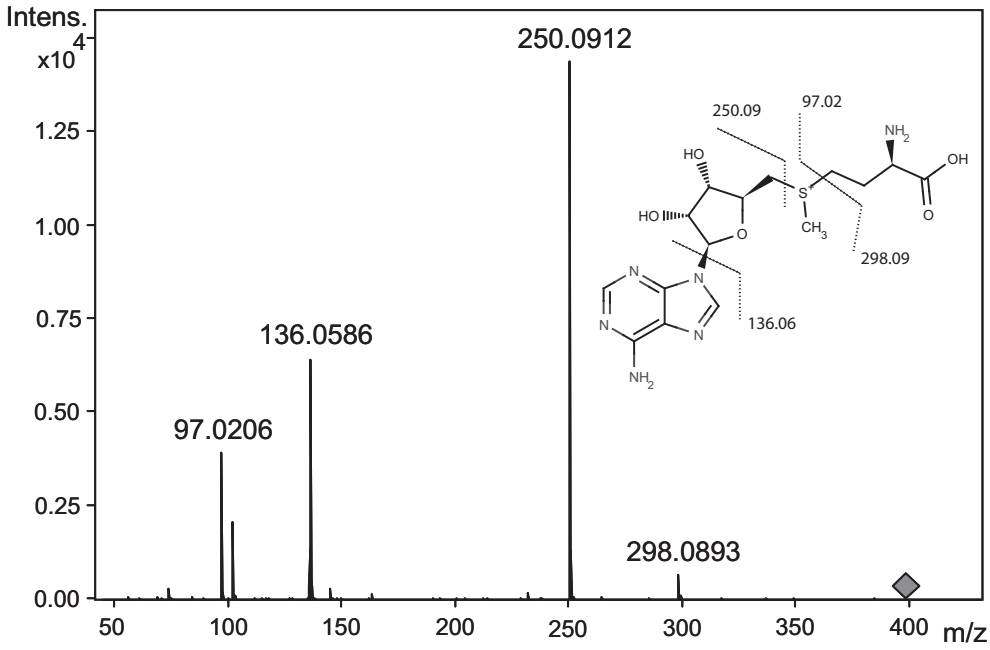

Figure 3. MS/MS spectrum for the compound of interest with mass 399.1459.

Regardless of the analytical method used (NMR, LC-MS, GC-MS), the interpretation of the biological significance of metabolic profiles is based only on a fraction of the metabolome accessible to a particular technique, and CE-MS is not an exception. Despite the mentioned above bias toward polar, positively charged metabolites, our method was capable of revealing gender specific effects of the mutation. The identified classifiers contribute most significantly to the stability of OPLS model, but an attempt to build a biological interpretation solely on those classifiers could be misleading. The measured perturbation in the metabolic composition of mouse urine is a result of complex interplay of many physiological processes. Thus, it is more practical to treat those metabolites as a part of the metabolic "signature of the phenotype" rather than the fingerposts to the

particular pathways. Nevertheless, some of the identified metabolites deserve at least a brief comment.

For example, one of these compounds, histidine, is a classical "usual suspect".(21) Such compounds are often reported as differential metabolites in many pathological states (*e.g.*, (22;23)), but because they are involved in a large number of biochemical processes, it is difficult to assign them to a specific pathway. L-lysine has been associated with effects on bone health, as it stimulates intestinal calcium absorption and cross-linking of bone collagen. Because TTD mice exhibit spontaneous development of osteoporosis, this particular molecule and its relation to bone density, for example, would be interesting to investigate further. N-acetylspermidine and trimethyl-L-lysine are linked in their biosynthesis to another identified classifier, S-adenosyl-L-methionine. Their simultaneous change can indicate the existence of an underlying mechanism regulating them. N-Acetylspermidine is involved in processes of cell growth and differentiation, in adaptation of the cell to a range of stress conditions, and in protection of DNA, lipids, and proteins from oxidative damage. S-Adenosyl-L-methionine is a methyl donor; methylation is one of the mechanisms of regulation of cell growth and differentiation as well as the main epigenetics gear. It is also important for generation of an antioxidant glutathione. As these TTD mice suffer from oxidative damage of DNA that is not repaired by DNA-repair mechanism, up-regulation of the compounds related to antioxidant defense may indicate activation of other protective mechanisms.

To summarize, we have shown that CE-ToF-MS is a very attractive method for metabolic profiling in urine especially in studies with mice where the sample amount is rather limited. Using this method, we have demonstrated differences in metabolic composition between wild-type and mutant animals. These differences were found to be more prominent within female mice, which is in accordance with other phenotypical observations. The identified discriminating compounds would appear to be an interesting group of molecules and need further investigation.

CONCLUSIONS

In this work, we have outlined an analytical workflow based on CE-MS for metabolic profiling of volume-limited samples, that is, mouse urine. We have shown that with a limited amount of sample a wide array of metabolite classes can be covered in mouse urine with the CE-MS method using minimal sample pretreatment and in-capillary preconcentration. The CE-MS method outlined here is especially suited for the profiling of cationic metabolites. In the near future, we will extend this methodology to the profiling of

acidic compounds at high pH separation conditions to further increase the detection coverage of metabolites in urine.

The feasibility of the CE-MS-based workflow for small-sized samples was shown for urine samples from TTD mutant mice. Multivariate analysis of the preprocessed data showed that changes in cationic metabolites are more prominent in female mice. A number of classifiers were identified based on high-resolution MS data as well as fragmentation pattern in MS/MS experiments. These compounds are interesting and the alterations in their concentrations can be related to oxidative defense in the mutant animals.

ACKNOWLEDGEMENTS

This work was supported by Senter Novem (ScreenOP IS061028; All parties), EU FP 7 (MarkAge 200880; Dept. of Genetics and DNage) and EU FP6 (Lifespan, (EC-LSHG-CT-2007-036894); Dept. of Genetics). Gerben Zondag and Ingrid van der Pluijm work for DNage B.V. The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Table S1. Compounds included in the standard mixture.

Amino acids	Alanine
	Arginine
	Aspartic acid
	Cysteine
	Glutamic acid
	Glycine
	Histidine
	Isoleucine
	Leucine
	Lysine
	Methionine
	Phenylalanine
	Proline
	Serine
	Threonine
	Tyrosine
	Valine
	Sarcosine
	Thyroxine
Alcohols	Homovanillyl alcohol
Pterins	Folic acid
Xanthines and related coumpounds	Caffeine
	Theophylline
Compound with Indoles group	5-hydroxyindole-3-acetic acid
Amines	Nortriptyline
Compounds with Hydroxyl and Amine groups	Dopamine
	4-O-Methyldopamine
Compounds with Imidazol groups	Creatinine
Dipeptides	Phe-Gly
Tripeptides	Glutathione

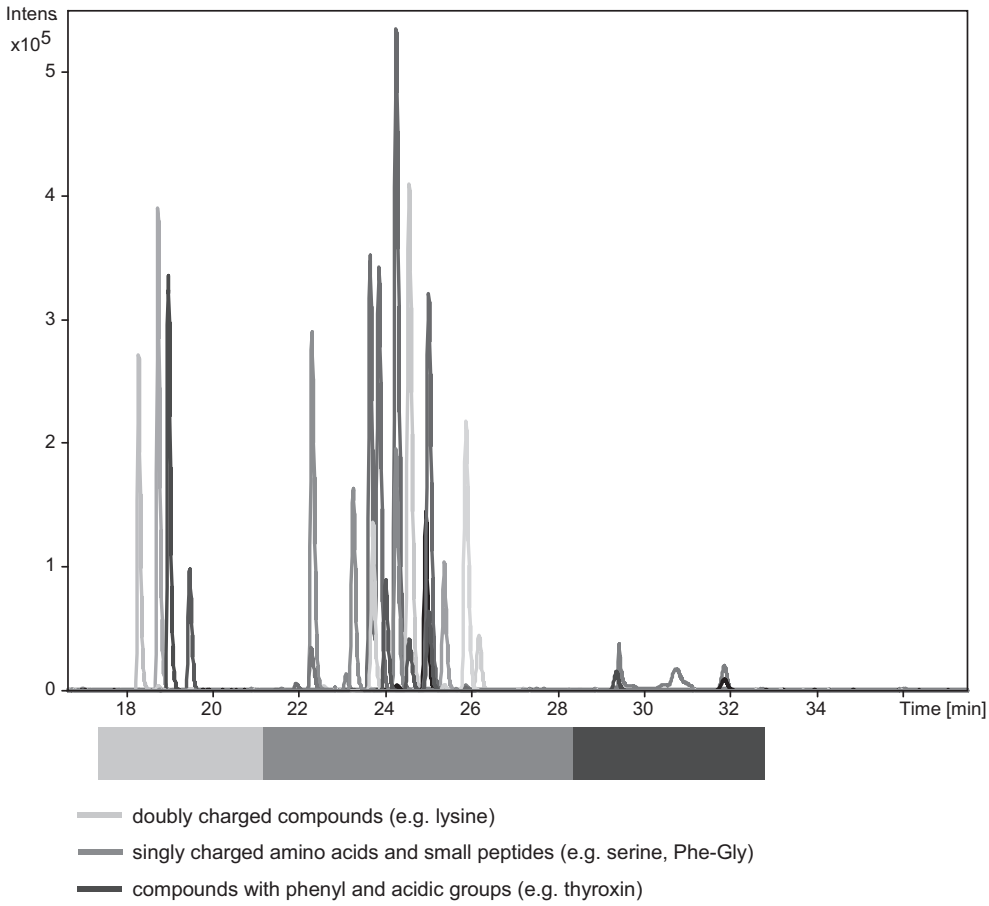

Figure S1. Combined extracted ion electropherograms of compounds present in standard mixture.

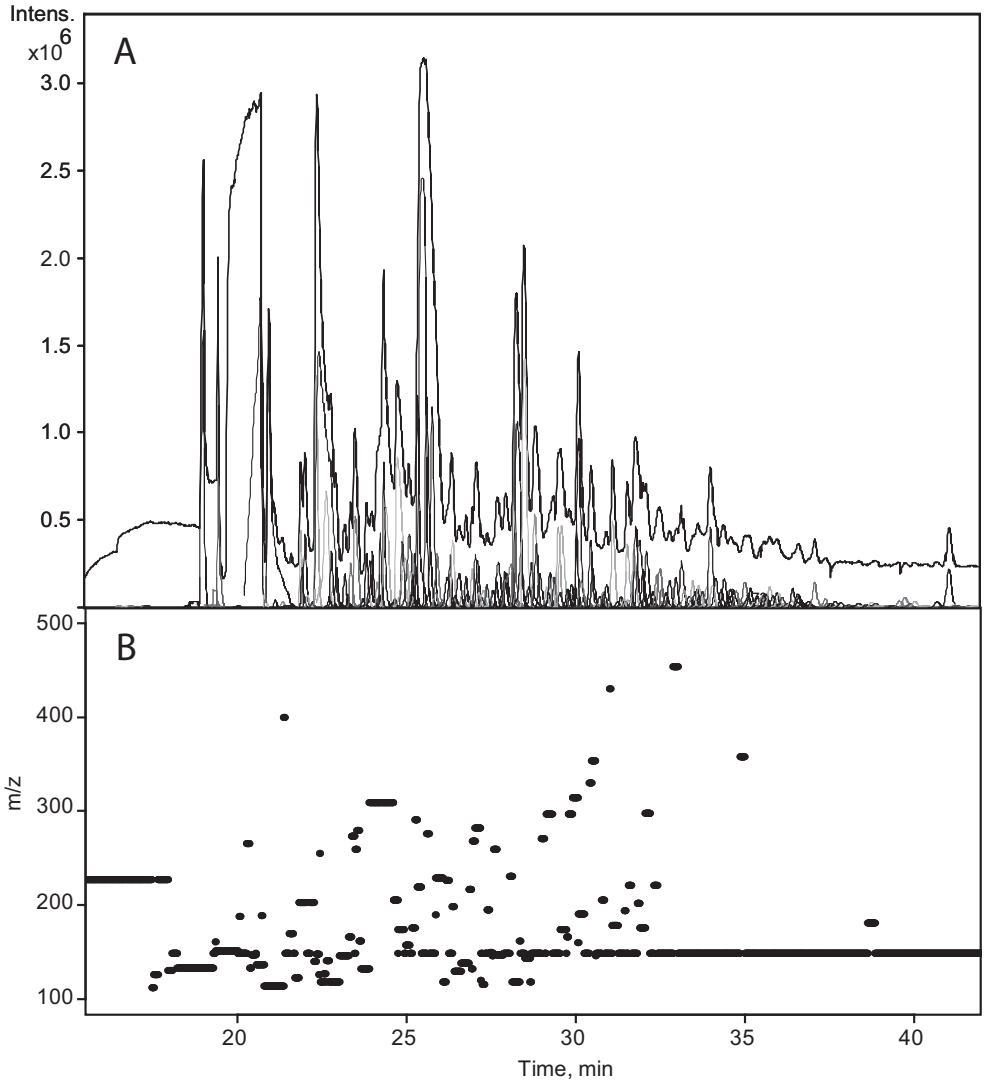

Figure S2. Typical metabolic profile of mouse urine by CE-MS. (A) Total ion electropherogram and extracted ion electropherograms. (B) Masses of the most intense peaks in the spectrum.

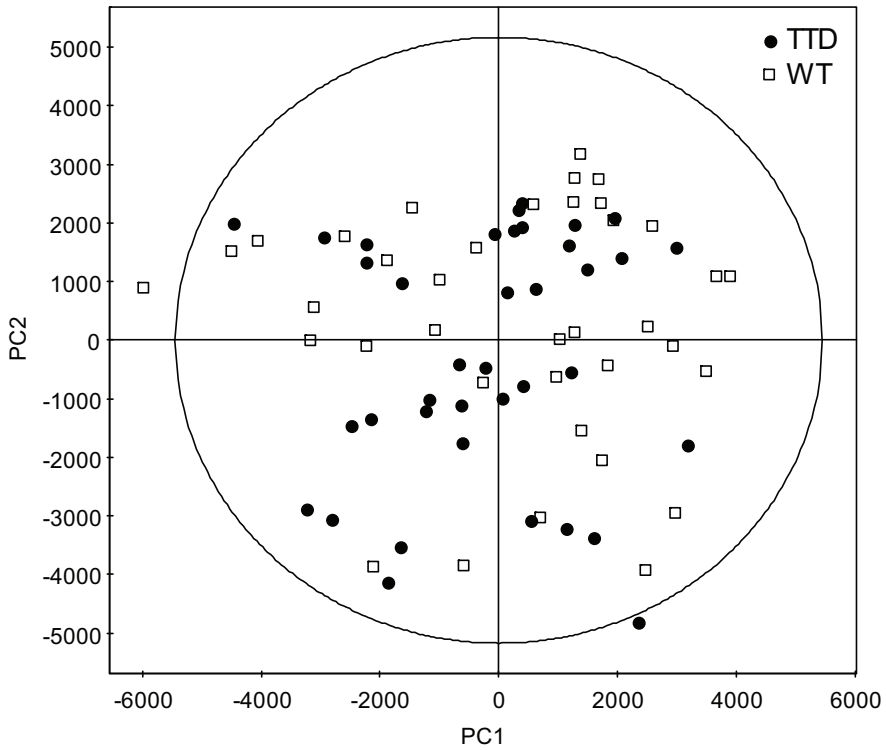

Figure S3. PCA scores plot for 75 mouse urine samples.

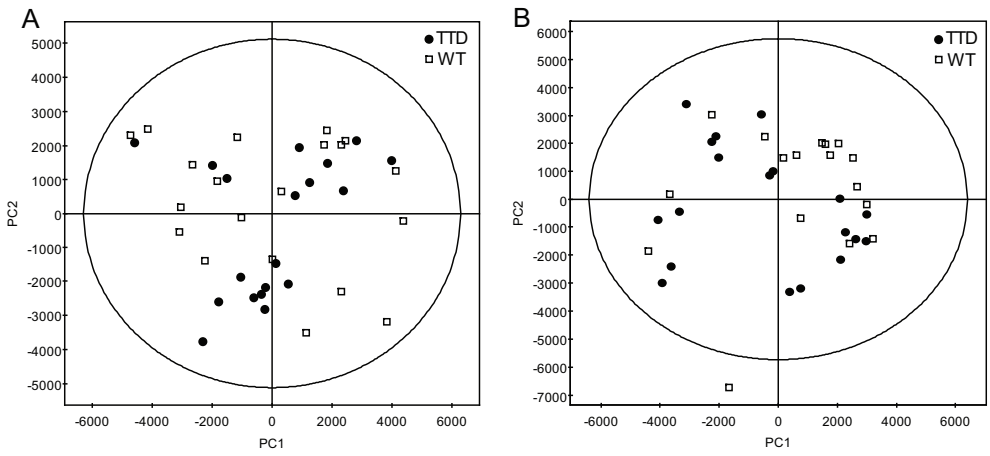

Figure S4. PCA scores plot for male (A) and female (B) mice.

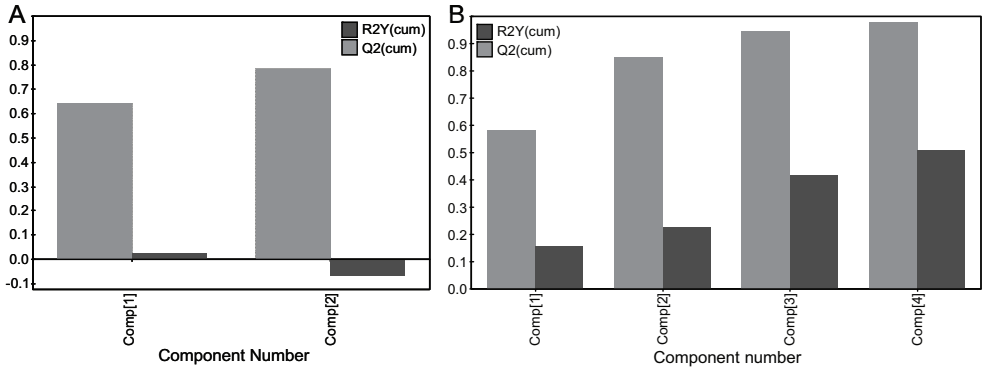

Figure S5. Overview of PLS-DA models for male (A) and female (B) mice.