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Serial-omics characterization of equine urine

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Abstract

Horse urine is easily collected and contains molecules readily measurable using mass spectrometry that can be used as biomarkers representative of health, disease or drug tampering. This study aimed at analyzing microliter levels of horse urine to purify, identify and quantify proteins, polar metabolites and non-polar lipids. Urine from a healthy 12 year old quarter horse mare on a diet of grass hay and vitamin/mineral supplements with limited pasture access was collected for serial-omics characterization. The urine was treated with methyl tert-butyl ether (MTBE) and methanol to partition into three distinct layers for protein, non-polar lipid and polar metabolite content from a single liquid-liquid extraction and was repeated two times. Each layer was analyzed by high performance liquid chromatography—high resolution tandem mass spectrometry (LC-MS/MS) to obtain protein sequence and relative protein levels as well as identify and quantify small polar metabolites and lipids. The results show 46 urine proteins, many related to normal kidney function, structural and circulatory proteins as well as 474 small polar metabolites but only 10 lipid molecules. Metabolites were mostly related to urea cycle and ammonia recycling as well as amino acid related pathways, plant diet specific molecules, etc. The few lipids represented triglycerides and phospholipids. These data show a complete mass spectrometry based—omics characterization of equine urine from a single 333 μL mid-stream urine aliquot. These omics data help serve as a baseline for healthy mare urine composition and the analyses can be used to monitor disease progression, health status, monitor drug use, etc.

Introduction

Over the last two decades, mass spectrometry has been used extensively to characterize the protein and small molecule content in biological samples [1]. Mass spectrometry including gas chromatography and liquid chromatography has been used extensively to profile molecules in urine, both from animals and humans [2, 3]. Methods have been developed and evaluated for preparing horse urine samples for small molecule analysis including precipitation and liquid-liquid extraction methods [4]. High resolution mass spectrometry and speed of fragmentation scanning has allowed proteins to be profiled from many diseases tissues and mammalian proteomes including relative protein levels and post-translational modifications of proteins [1, 5–7]. In addition, lipidomics profiling has recently become popular in present day—omics
technologies using mass spectrometry (MS) [8]. The tri-ome (proteomics, metabolomics and lipidomics) has been studied by our group from human cell lines in diseases such as cancer as well as mouse tumor tissues [9]. The tri-ome can reveal active pathways that are driving the biology of the organism or tissue in question at the point in time of sampling. Information can be obtained from metabolic pathways, protein synthesis and expression, signal transduction and fatty acid biosynthesis. When studying biofluids such as urine or blood serum, one can also garner information about secreted molecules that are indicative of external influences or internal influences on an organism’s biological state. Equine urine has been tested for several decades in the performance horse industry for the presence of drugs and illegal substances in the racing, showing, eventing and Olympic industries using both GC-MS and LC-MS [10–29]. However, one can also begin to think about profiling the widely accessible fluid for performance markers or measures of health. A recent article utilized a multi-omics proteomic and metabolomic strategy to probe urinary diseases [30]. In this study, we incorporate a “serial-omics” or triomics approach to help create a baseline of normal horse urine in terms of its protein, metabolite and lipid content for the potential diagnosis, treatment and prevention of equine diseases. It uses four different LC-MS/MS platforms including targeted metabolomics, untargeted metabolomics, untargeted lipidomics and untargeted proteomics including phosphoproteomics. The requirement of all platforms is that there is fragmentation to support chemical structure and amino acid sequence with the help of commercial software and publicly available databases for their identification. JF Sierra Flame is an American Quarter Horse Association (AQHA) registered mare foaled in May, 2004. She is fed a daily diet of primarily forage (~20–22 lb) with 2 cups of a fortified grain pellet supplement containing protein, vitamins, minerals, probiotics, etc. in addition to occasional feeding of natural treats such as apples and/or carrots. She is ridden approximately three times per week and has access to a pasture containing grass and some weeds. On rare occasion, if soreness is suspected, the horse is fed Bute-Less pellets (Absorbine) containing the natural anti-inflammatory herbs Devil’s claw and Yucca extract. This study represents molecules that we could accurately detect in horse urine sample using various mass spectrometry approaches that are common to our laboratory and some may be potentially used as biomarkers with rigorous testing.

Material and methods

Liquid-liquid extraction

~45 mL of horse mare urine was collected in mid-stream using a 50 mL polypropylene tube. A 333 μL aliquot of horse urine was taken and 2.475 mL HPLC grade methanol (Pharmco-Aaper, #33900HPLC) was added and vortexed vigorously for 1 min. After the addition of 8.25 mL of 99.8% MTBE (Sigma Aldrich, #306975-1L), the samples were shaken for 1 hr at RT. We added 2.06 mL of water, vortexed for 1 min and spun for 10 min. The resulting upper (lipid) and lower (metabolite) liquid phases were collected separately in 1.5 mL glass vials and dried out in a SpeedVac.

Proteomics

The protein pellet on the bottom was re-suspended in 200 μL 0.5x sample buffer (6X SDS Sample Buffer (0.375M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6M DTT, 0.06% bromophenol blue) transferred to a microcentrifuge tube and dried down to 50 μL in a SpeedVac. The protein samples were loaded on a 4–12% gradient gel (Lonza, #58520) and ran until the loading dye reached the bottom of the gel. The gel was stained with GELCODE Blue (Fisher Scientific, #PI24590) for 30 minutes and each lane with sample was cut into 10 equal pieces. Gel sections were reduced with 55 mM dithiothreitol (DTT) (Sigma-Aldrich), alkylated with 10 mM DTT.
iodoacetamide (Sigma-Aldrich), and digested overnight with TPCK modified trypsin (Pro-mega) at pH = 8.3. Peptides were extracted, dried out in a SpeedVac, re-suspended in 10 μl of 50% ACN, 6% TFA and rocked on a shaker for 15 min. The TiO$_2$ TopTip (PolyLC, # TT10TIO) were washed with 50% ACN, 6% TFA, spin at 1500 rpm 0.5 min for four times. The samples were loaded on the TiO$_2$ tips and incubated for 30 min followed by wash with 10 μl 50% ACN, 1% TFA (spin at 1500 rpm 0.5 min), repeated two times, eluted with three times 10 μl 40% ACN, 15% NH$_4$OH, added 60 μl buffer A (0.1% formic acid/99.9% water) and dried out to 5 μL. The protein sample was analyzed by positive ion mode LC-MS/MS using LTQ Elite hybrid ion trap-Orbitrap mass spectrometer (Thermo Fisher Scientific) in a data-dependent analysis (DDA Top8). Peptides were delivered and separated using an EASY-nLC nano-flow HPLC (Thermo Fisher Scientific) at 300 nL/min using self-packed 15 cm length × 75 μm i.d. C$_{18}$ fritted microcapillary columns. Solvent gradient conditions were 120 minutes from 3% B buffer to 38% B (B buffer: 100% acetonitrile; A buffer: 0.1% formic acid/99.9% water). MS/MS spectra were analyzed using Mascot search engine v2.5.1 (Matrix Science) by searching the reversed and concatenated Equus caballus (Horse) protein database (UniProt, version 20161102, 20,312 entries) with a parent ion tolerance of 18 ppm and fragment ion tolerance of 0.80 Da. Carboxymethylation of cysteine (+57.0293 Da) was specified as a fixed modification and oxidation of Methionine (+15.9949), phosphorylation of Serine/Threonine/Tyrosine (+79.97) as variable modifications. Results were imported and analyzed using ScaffoldQ+S 4.6 software (Proteome Software, Inc.) resulting in a peptide false discovery rate (FDR) of ~1%. Further pathway analysis was performed by Panther (http://www.pantherdb.org/).

**Lipidomics**

The dried lipid layer was re-suspended in 30 μL of 1:1 LC/MS grade isopropanol:methanol prior to LC-MS/MS analysis, 5 μL were injected. A Cadenza 150 mm x 2 mm 3 μm C$_{18}$ column (Imtakt) heated to 40 ºC at 260 μL/min was used with a quaternary pump HPLC with room temperature autosampler (Agilent 1100 series). Lipids were eluted over a 20 min. gradient from 32% B buffer (90% IPA/10% ACN/10 mM ammonium formate/0.1% formic acid) to 97% B. A buffer consisted of 59.9% ACN/40% water/10 mM ammonium formate/0.1% formic acid. Lipids were analyzed using a hybrid QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific) in DDA mode using positive/negative ion polarity switching with 1 MS1 scan followed by 8 MS2 HCD scans per cycle (Top 8). DDA data were acquired from m/z 200–1450 in MS1 mode and the resolution was set to 70,000 for MS1 and 35,000 for MS2. MS1 and MS2 target values were set to 5e5 and 1e6, respectively. Lipidomics data were analyzed using LipidSearch 4.1.9 software (Thermo Fisher Scientific) for identification and validation.

**Metabolomics**

Half of the metabolite layer was re-suspended in 20 μL LC/MS grade water, 5 μL were injected over a 15 min gradient using a 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) via SRM of a total of 287 SRM transitions using positive and negative polarity switching corresponding to 259 unique endogenous water soluble metabolites. Samples were separated using a Amide XBridge HPLC hydrophilic interaction liquid chromatographic (HILIC) column (3.5 μm; 4.6 mm inner diameter (i.d.) × 100 mm length; Waters) at 300 μL/min. Gradients were run starting from 85% buffer B (LC/MS grade acetonitrile) to 40% B from 0–5 min; 40% B to 0% B from 5–16 min; 0% B was held from 16–24 min; 0% B to 85% B from 24–25 min; 85% B was held for 7 min to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate (pH = 9.0) in 95:5 water/acetonitrile. Peak areas from the total ion current.
for each metabolite SRM transition were integrated using MultiQuant version 2.1.1 software (AB/SCIEX). The other half of the metabolite layer was re-suspended in 20 μL LC/MS grade water, 5 μL were analyzed by positive/negative polarity switching mode using a hybrid QExactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific) via a data-dependent analysis (DDA Top8). Metabolites were delivered and separated using an EASY-nLC nanoflow HPLC (Thermo Fisher Scientific) at 225 nL/min using self-packed 15 cm length × 75 μm i.d. C18 fritted microcapillary columns. Solvent gradient conditions were 25 minutes from 3% B buffer to 38% B (B buffer: 100% acetonitrile; A buffer: 0.1% formic acid/99.9% water). The data were analyzed using Elements (Proteome Software) with the NIST MS/MS spectral database (http://chemdata.nist.gov/mass-spc/msms-search/) and HMDB metabolite database followed by statistical analysis and pathway analysis with Excel 2013 and MetaboAnalyst 3.0 software (http://www.metaboanalyst.ca/).

Results and discussion

We utilized a new approach whereby a single aliquot of urine is used for each individual—omics analysis via a liquid-liquid extraction with methyl tert-butyl ether (MTBE). When MTBE, methanol and water are added to urine, the protein precipitates to the bottom while the aqueous polar metabolites form a middle layer and the non-polar lipids form the top layer. Each layer including the protein precipitate was removed and analyzed separately. Fig 1 shows the workflow from the horse anatomy to urine collection, sample preparation, mass spectrometry analysis and the data analysis. The protein mixture was separated into 10 fractions using SDS-PAGE gel [31] and in gel digested using trypsin to produce sequenceable peptides. Each peptide mixture was analyzed using a 1hr LC-MS/MS data dependent acquisition (DDA) run with nanoflow HPLC and a hybrid Orbitrap Elite high resolution mass spectrometer in biological triplicates.

The results after database searching and interpretation are shown in Table 1. We identified 46 unique proteins and they are ranked according to the number of total peptide spectra identified (spectral count) which can be indicative of relative protein quantity.

Proteomics is very common for detecting biomarkers, quantifying protein levels across sample conditions and for identifying proteins and post-translational modifications (PTMs) [6, 32, 33] including urinary proteomics [34]. Over 500 proteins were reported from canine urine [35], though other articles report far less proteins in mammalian urine [36]. For example, one report showed less than 100 proteins in human urine [37]. The urinary proteome varies greatly from animal to animal or patient to patient, etc. due to many factors including health, diet, medications, lifestyle, etc. and there is no benchmark of expected numbers. We identified a total of 46 unique proteins from 12 yr old quarter horse mare urine (Fig 2A and 2B). We required at least 2 unique peptides per protein and a false discovery rate (FDR) of less than 1% for a positive identification from the Uniprot Equus caballus (horse) protein database. The top five proteins other than keratin, a common cytoskeletal protein related to skin and hair, are common to the urinary tract and kidneys and include uromodulin, calcium activated chloride channel protein, protein AMBP, kininogen-1, the cell adhesion cadherin molecules 1, 6, 15 and epithelial cell adhesion molecule, etc. In addition, structural and cytoskeletal proteins were identified as well as other glycoproteins commonly expressed in connective tissue such as tenascin-X. Pantetheinase is secreted from the mucous membranes of the intestine to hydrolyze pantothene to pantothenate (Vitamin B) ([38, 39]. Butyrophilin-like protein 10 is an intestinal epithelial protein containing an Ig domain and may regulate T lymphocytes [40, 41]. Trypsinogen is a precursor of the enzyme trypsin and is produced in the pancreas and its levels are sometimes used as marker for pancreatitis [42]. The corticosteroid-binding globulin
protein binds circulating plasma cortisol and may play hormonal roles, possibly in mares, especially during pregnancy when its plasma concentration decreases [43, 44]. The bottom portion of Fig 2A shows a scatterplot that demonstrates the molecular weight distribution vs relative intensity between lipids, polar metabolites and proteins identified across all LC-MS/MS based—omics experiments from the urine aliquot. These data show that the majority of urinary components are small molecules followed by very large protein molecules and far fewer moderately sized lipids. A detailed description of the Fig 2B shows a simple number distribution of the various omics results and the bottom portion of Fig 2B lists a number of topics that we can address using serial-omics. For example, a single aliquot of microliter levels of urine, blood, etc. can be used collect a catalog of molecules from small polar metabolites to non-polar lipids to large proteins and the collection of these molecules may be used to develop biomarkers for tracers of drugs, feed, plants, supplements, etc. in addition to monitoring health or disease status and markers of performance and recovery. However, defining and validating biomarkers of this sort requires the collection and analysis of urine from a large number of horses.
### Table 1. Equine mare urine proteins identified by LC-MS/MS from a serial-omics liquid-liquid extraction.

| Protein name                                                                 | Accession          | MW       | # of spectra |
|------------------------------------------------------------------------------|--------------------|----------|--------------|
| Uromodulin OS = Equus caballus                                              | F7BM54_HORSE       | 70 kDa   | 376          |
| Calcium-activated chloride channel regulator 1 OS = Equus caballus           | CLCA1_HORSE        | 100 kDa  | 101          |
| Keratin, type I cytoskeletal 10 OS = Equus caballus                         | F6WDW3_HORSE       | 43 kDa   | 66           |
| Protein AMBP OS = Equus caballus                                            | F6UZH0_HORSE       | 39 kDa   | 56           |
| Immunoglobulin lambda light chain OS = Equus caballus                       | F6QA5_HORSE        | 11 kDa   | 47           |
| Keratin, type II cytoskeletal 1 OS = Equus caballus                         | F7B7X0_HORSE       | 66 kDa   | 44           |
| Kininogen-1 OS = Equus caballus                                             | F7C0Z0_HORSE       | 72 kDa   | 42           |
| Keratin, type II cytoskeletal 5 OS = Equus caballus                         | F6W7V0_HORSE       | 62 kDa   | 33           |
| Cadherin 1 OS = Equus caballus                                              | F6YO09_HORSE       | 89 kDa   | 26           |
| Poly-Ig receptor OS = Equus caballus                                         | F6W2K5_HORSE       | 83 kDa   | 26           |
| EGF-containing fibulin-like extracellular matrix protein 1 OS = Equus caballus | F6PVG3_HORSE      | 55 kDa   | 25           |
| Cytokeratin-2e OS = Equus caballus                                          | F6SHJ8_HORSE       | 61 kDa   | 24           |
| Pro-epidermal growth factor OS = Equus caballus                             | F7B762_HORSE       | 133 kDa  | 23           |
| Keratin, type II cytoskeletal 6A OS = Equus caballus                        | F7AGY4_HORSE       | 60 kDa   | 21           |
| Keratin, type I cytoskeletal 16 OS = Equus caballus                         | F6ZEG3_HORSE       | 51 kDa   | 20           |
| Ubiquitin A-52 residue ribosomal protein fusion product 1 OS = Equus caballus | A0A0B4J1C5_HORSE  | 15 kDa   | 19           |
| Pantetheinase OS = Equus caballus                                           | F6Z1Z9_HORSE       | 58 kDa   | 17           |
| Antithrombin protein OS = Equus caballus                                    | F7CYR1_HORSE       | 52 kDa   | 17           |
| Trypsinogen OS = Equus caballus                                             | F6VNT7_HORSE       | 26 kDa   | 16           |
| Epithelial cell adhesion molecule OS = Equus caballus                       | F6R6Z6_HORSE       | 33 kDa   | 16           |
| Keratin, type II cytoskeletal 73 OS = Equus caballus                        | F7CY11_HORSE       | 59 kDa   | 16           |
| Cadherin-6 OS = Equus caballus                                              | F6X2O0_HORSE       | 88 kDa   | 14           |
| Corticosteroid-binding globulin OS = Equus caballus                         | F7DRS2_HORSE       | 45 kDa   | 14           |
| Complement C4-A OS = Equus caballus                                         | F6XSF7_HORSE       | 193 kDa  | 13           |
| Heparan sulfate proteoglycan 2 OS = Equus caballus                          | F7C0I7_HORSE       | 466 kDa  | 12           |
| Butyrophilin-like protein 10 OS = Equus caballus                            | F6VGK4_HORSE       | 51 kDa   | 12           |
| Collagen alpha-3(VI) chain OS = Equus caballus                             | F6R735_HORSE       | 342 kDa  | 11           |
| IgG heavy chain OS = Equus caballus                                         | H9GZT5_HORSE       | 36 kDa   | 11           |
| Cadherin-15 OS = Equus caballus                                             | F6WEI6_HORSE       | 82 kDa   | 11           |
| Tenascin-X OS = Equus caballus                                              | F7CCQ6_HORSE       | 445 kDa  | 9            |
| Desmocollin-2 OS = Equus caballus                                           | F6UVP2_HORSE       | 100 kDa  | 8            |
| Keratin, type I cytoskeletal 17 OS = Equus caballus                         | F6YIA9_HORSE       | 48 kDa   | 8            |
| Mucin-5AC OS = Equus caballus                                               | F6QCB3_HORSE       | 477 kDa  | 7            |
| Aminopeptidase N OS = Equus caballus                                        | F7BB47_HORSE       | 110 kDa  | 6            |
| Major allergen Equ c 1 OS = Equus caballus                                  | ALL1_HORSE         | 22 kDa   | 6            |
| Cartilage intermediate layer protein OS = Equus caballus                    | F7C2J3_HORSE       | 133 kDa  | 5            |
| Tetratricopeptide repeat protein 28 OS = Equus caballus                     | F6WBY4_HORSE       | 257 kDa  | 5            |
| Protein FAM151A OS = Equus caballus                                         | F6WIV4_HORSE       | 64 kDa   | 4            |
| Serum albumin OS = Equus caballus                                           | ALBU_HORSE         | 69 kDa   | 4            |
| Microtubule-actin cross-linking factor 1 OS = Equus caballus                | F6YMD9_HORSE       | 827 kDa  | 3            |
| Collagen alpha-1 type I chain OS = Equus caballus                           | F7A3F7_HORSE       | 141 kDa  | 3            |
| Nuclear receptor co-repressor 2 OS = Equus caballus                         | F6UWM2_HORSE       | 275 kDa  | 3            |
| Synapsin-1 isoform Ib OS = Equus caballus                                   | F6XVE9_HORSE       | 58 kDa   | 3            |
| AXL receptor tyrosine kinase OS = Equus caballus                            | F6VEV4_HORSE       | 98 kDa   | 3            |
| Nuclear receptor-interacting protein 1 OS = Equus caballus                  | F6QWB9_HORSE       | 127 kDa  | 3            |
| Far upstream element-binding protein 2 OS = Equus caballus                  | F7A984_HORSE       | 70 kDa   | 3            |

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(>50) of healthy horses of varying age and gender in order to generate a baseline of “normal” serial-omics profiles [45]. However, using targeted mass spectrometry for the quantification of potential biomarkers is a routine and robust approach [46].

Fig 3A shows the enriched representative protein pathways using the Panther gene ontology tool [47]. Many other proteins were detected in the normal mare urine and are listed in the S1 Dataset containing the peptide sequences identified as well as the database search criteria and scoring information.

After characterizing the urine proteome of the 12 year old quarter horse mare, we analyzed the aqueous polar metabolite composition of the MTBE extraction middle layer. We used two different approaches to identify small polar metabolites, a targeted method that utilized positive/negative polarity switching via selected reaction monitoring (SRM) [48] and an untargeted method using a high resolution QExactive HF mass spectrometer in positive/negative polarity switching mode via DDA using Elements identification software and the NIST small...
molecule spectral database. From 474 total unique metabolites identified from both experiments in triplicate, the major identifiable pathways are attributable to protein biosynthesis due to an abundance of amino acids likely broken down from hay and grain products. In addition, amino acid related pathways such as methionine, alanine, aspartate, glycine, serine, threonine, arginine and proline metabolism amongst several others are well represented in the metabolome of normal urine. These amino acids are breakdown products from digested proteins in hay and grain as well as amino acids that may be present in feed supplements and not utilized by the horse. In addition, prominent urine and kidney related pathways are well-represented including the urea cycle and ammonia recycling and many other major metabolite pathways such as glutathione metabolism, betaine metabolism, fatty acid oxidation, NAD metabolism, etc.

We then took the top 10 metabolites identified from each experiment (targeted and untargeted) for a total of 20 metabolites and performed a pathway enrichment analysis using MetaAnalyst software [49]. The analysis revealed the major pathways are citric acid cycle, urea cycle, betaine metabolism, taurine and hypotaurine metabolism followed by amino acid metabolism. To better resolve the metabolite analysis, we identified the top 3 most intense
metabolites from each metabolomics dataset and characterized them. From the untargeted experiment, hippuric acid, a common carboxylic acid found in the urine of horses and other plant eating species was the most abundant molecule identified followed by creatinine, a breakdown product of creatine that is commonly secreted through urine and is used to monitor normal kidney function and can be used as a urine control metabolite for normalization [50]. Phenylacetlyglycine was also very abundant and is a gut microbial co-metabolite with hippuric acid and well-studied using LC-MS/MS methods from urine as phospholipidosis markers [51, 52]. p-Cresol glucuronide is another common urine metabolite derived from intestinal bacteria. The targeted SRM based mass spectrometry experiment which is limited to less than 300 metabolites, shows the top molecules as creatinine, betaine, urea, taurine, citrate, etc.; metabolites which are known to be present in high concentrations in urine [4, 53]. The same strategy of taking the top 20 metabolites in MS1 peak intensity from the non-targeted experiment did not yield any known enriched metabolic pathways. The targeted metabolite method can be used to target specific drugs for quantification if one is testing for a particular compound or several compounds [54–57]. Our lab routinely performs that test from mouse tumor tissue and there are many reports in urinalysis [58].

In addition to the most abundant metabolites in horse urine, some interesting metabolites were identified that are indicative of the feeding habits of both humans and horses (Fig 3D). One metabolite, phlorizin, is a common flavonoid metabolite in high concentration in apples, a common treat fed to horses as well as being present in other vegetative plants, leaves, bark, etc. [63]. Mammeigin is another common fruit and vegetable specific neoflavinoid compound and may have antiproliferative activity in some cancer cells [64]. Buntansin A is a coumerin compound commonly found in plants [65]. A surprising finding is that the second most abundant metabolites from the non-targeted metabolomics search was acetohydroxamic acid (AHA), a urea-like molecule that is synthetically made to treat urinary tract infections, prevent kidney stones and studied in human as well as canine urine [66]. Since we did not administer this drug to the horse, this may be evidence that AHA is produced naturally in the horse and present in the kidneys and subsequently secreted in the urine. A high level of Monomethyl phenylphosphonate was found to be present and this compound is a breakdown product of leptophos, a no longer used pesticide. It is important to note that Bronco Gold fly spray (Farnum) is routinely used on the horse during summer months during fly season. The main active ingredients in the fly spray are pyrethrins and pyrethroids, piperonyl butoxide and butoxy poly propylene glycol. Many other abundant natural metabolites that do not fit into distinct pathways may be derived from either the feed byproducts or plant derivatives and include N’-Hydroxysaxitoxin, 4-hydroxyphenyl-2-propionic acid, cis-mulberroside A, neoisoquirtin, etc.

The last—omics component that we analyzed was lipidomics or any identifiable fatty acid containing molecules in the horse mare urine. Urine typically does not contain an appreciable amount of lipid unless nephrotic syndromes are present in either humans or animals [67]. A study in human prostate cancer yielded only approximately 100 lipid molecules using LC-MS/
Table 2. Top 60 equine mare urine metabolites by intensity from 474 total identified metabolites by LC-MS/MS from a serial-omics liquid-liquid extraction.

| Polar metabolite name (Untargeted, Top 30) | Accession | Molecular Formula | MS1 Log10 Peak Area |
|-------------------------------------------|-----------|-------------------|---------------------|
| Hippuric acid                             | HMDB00714 | C9H9NO3           | 11.8                |
| Phenylacetylglucose                       | HMDB00821 | C10H11NO3         | 11.6                |
| 2-Hydroxy-4-trifluoromethyl benzoic acid  | HMDB60715 | C8H5F3O3          | 11.6                |
| Creatinine                                | HMDB00562 | C4H7N3O           | 11.4                |
| p-Cresol glucuronide                      | HMDB11686 | C13H16O7          | 11.3                |
| Buntansin A                               | HMDB35086 | C11H8O5           | 10.9                |
| Acetoxyhydroxamic Acid                    | HMDB14691 | C2H5NO2           | 10.8                |
| Mesoridazine                              | HMDB15068 | C21H26N2OS2       | 10.7                |
| Mammeegin                                 | HMDB30785 | C25H24O5          | 10.6                |
| Monomethyl phenylphosphonate              | HMDB31868 | C7H9O3P           | 10.6                |
| Neoisoliquiritin                          | HMDB37317 | C21H22O9          | 10.5                |
| Geranylgeranylcysteine                    | HMDB11678 | C23H37NO3S        | 10.4                |
| 4-Hydroxy-8-methoxy-2H-furo[2,3-h]-1-benzopyran-2-one | HMDB32659 | C12H8O5          | 10.4                |
| N'-Hydroxysaxitoxin                       | HMDB41683 | C9H10O3           | 10.4                |
| acis-Mulberroside A                       | HMDB31726 | C26H32O14         | 10.3                |
| N-Methylphthalimide                       | CASNO:550-44-7 | C9H7NO2      | 10.3                |
| Phlorizin                                 | HMDB36634 | C21H24O10         | 10.3                |
| L-leucyl-L-proline                        | HMDB11175 | C11H20N2O3        | 10.2                |
| Benzocaine                                | HMDB04992 | C9H11NO2          | 10.2                |
| Vanilloloside                             | HMDB32013 | C14H20O8          | 10.2                |
| apo-[3-methylcrotonoyl-CoA:carbon-dioxide ligase (ADP-forming)] | HMDB59607 | C7H15N3O2        | 10.1                |
| Trimethylamine N-oxide                    | HMDB00925 | C3H9NO            | 10.1                |
| Acetaminophen                             | HMDB01859 | C8H9NO2           | 10.1                |
| D-(-)-Isosorbic acid                      | CASNO:89-65-6 | C6H8O6         | 10.1                |
| Butyrylcarnitine                          | HMDB02013 | C11H21NO4         | 10.1                |
| DOPA sulfate                              | HMDB02028 | C9H11N7O5         | 10.0                |
| Pilocarpine                               | HMDB15217 | C11H16N2O2        | 10.0                |
| Pyrogallol-2-O-glucuronide                | HMDB60017 | C12H14O9          | 10.0                |

| Polar metabolite name (Targeted, Top 30) | Accession | Molecular Formula | Q3 Log10 Peak Area |
|------------------------------------------|-----------|-------------------|--------------------|
| 2-Hydroxy-2-methylbutanedioic acid       | C02612    | C5H8O5            | 7.7                |
| citrate                                  | C00158    | C6H8O7            | 7.3                |
| 2-Isopropylmalic acid                    | C02504    | C7H12O5           | 7.3                |
| 1-Methyl-Histidine                       | C01152    | C7H11N3O2         | 7.3                |
| betaine                                  | C00719    | C5H11NO2          | 7.2                |
| aconitate                                | C00417    | C6H6O6            | 7.1                |
| oxaloacetate                             | C00036    | C4H4O5            | 7.1                |
| Acetylcarnitine DL                       | C02571    | C9H18NO4          | 7.1                |
| allantoin                                | C01551    | C4H6N4O3          | 7.1                |
| Urea                                     | C00086    | C4H4N2O           | 6.9                |
| Acetyllysine                             | C02727    | C8H16N2O3         | 6.8                |
| N6-Acetyl-L-lysine                       | C02727    | C8H16N2O3         | 6.7                |
| 2-hydroxyglutarate                       | C02630    | C5H8O5            | 6.7                |
| D-sedoheptulose-1-7-phosphate            | C05382    | C7H15O10P         | 6.6                |
| Phenylpropionic acid                     | HMDB00563 | C9H6O2            | 6.6                |
| DL-Pipecolic acid                        | C00408    | C6H11NO2          | 6.5                |

(Continued)
MS [68]. In general, non-polar lipids should be barely detectable in healthy mammals. We used a QExactive Plus Orbitrap mass spectrometer in polarity switching mode with reversed-phase chromatography with LipidSearch identification software [69, 70]. The results in Table 3 and Fig 3C show that very few lipids were identified. We identified five triglyceride (TG) lipids, two phosphatidic acid (PA) lipids, two phosphatidylethanolamines (PE) and two phosphatidylethanol (PEt) lipids. To put that in perspective, we routinely identify ~1000 lipids or more from cells, plasma, tumors, etc, with our lipidomics platform [8]. The fatty acid chains associated with the identified lipid molecules primarily consisted of the basic fatty acid building blocks of palmitate (C16:0), oleate (C18:1) and stearate (C18:0). The very few identified lipids in horse urine are consistent with expectations for a healthy mammal. The S3 Dataset contains the detailed lipid search results including scoring information and peak area quantification.

Conclusions

It is important to note that the majority of global urine -omics studies have taken place from human and mouse urine samples while most horse urine studies have focused on specific targeted compounds. However, we expect that many mammals should have a somewhat similar urine profile as far as it concerns the major metabolites and proteins. However, diet also plays

Table 3. Equine mare urine non-polar lipids identified by LC-MS/MS from a serial-omics liquid-liquid extraction.

| Lipid name               | Accession | Ion Formula       | MS1 Log10 Peak Area |
|--------------------------|-----------|-------------------|---------------------|
| PA(16:0/18:1)-H          | LMGP10010032 | C37H70O8N0P1      | 7.473               |
| PA(18:0/18:1)-H          | LMGP10010037 | C39H74O8N0P1      | 6.740               |
| PE(16:0/16:0)+H          | LMGP02010037 | C37H75O8N1P1      | 6.842               |
| PE(18:0/16:0)+H          | LMGP02011205 | C39H79O8N1P1      | 6.407               |
| PE(16:0/14:0)-H          | N/A        | C35H68O8N0P1      | 6.613               |
| TG(16:0/14:0/16:0)+NH4   | LMGL03012786 | C49H98O6N1        | 6.224               |
| TG(16:0/16:0/16:0)+NH4   | LMGL03010001 | C51H102O6N1       | 6.480               |
| TG(18:0/16:0/16:0)+NH4   | LMGL03010004 | C53H106O6N1       | 6.213               |
| TG(16:0/18:1/18:1)+NH4   | LMGL03010100 | C55H106O6N1       | 6.415               |
| TG(18:1/18:1/18:1)+NH4   | LMGL03012612 | C57H108O6N1       | 6.402               |

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a crucial role as well as differences in the digestion system between horses and humans. As a result, we found a significant number of plant related metabolites due to a horse’s diet of hay and vegetation. As expected, the number of identified proteins (46) from horse mare urine was significant but far less than analyses in other biological tissues such as cells, plasma, solid tissue where thousands of proteins are the norm. The number of small molecule polar metabolites was high with 474 molecules identified. Only 10 non-polar lipid molecules were identified. These show that a single aliquot of horse urine can be used for a liquid-liquid extraction for a multi-omics analysis. We chose to perform lipidomics, proteomics and metabolomics on normal 12 year old horse mare urine. These analyses demonstrate a baseline for omics analyses from horse urine and can be used as a reference for expected future results. This technique can be applied in discovering the presence of a diseased or drug administered-horse from the norm by comparing alterations in the metabolites, lipids and proteins identified. If repeated over a time-course, these analyses can potentially be used to monitor disease progression, health status, inflammation or used to develop biomarkers for performance indicators. Not only do we expect levels of common metabolites and proteins to vary across sample conditions, but unique molecules to be identified in specific cohorts. The proteomic analysis can reveal disease-specific proteins or reveal a relative quantitative change in protein levels. The untargeted metabolomic analyses can identify illicit drug molecules or reveal metabolic changes that indicate disease. In addition, the lipidomics analysis can reveal diseases in the nephrotic system based on the number and types of lipids present. While horses urinate in several liter volumes per episode, we chose to use a very small aliquot of 333 μL in order to demonstrate that we can acquire comprehensive—omics data on small volumes of biological fluid. This can be applied to as little as a few drops of blood, saliva, tears, etc [71]. The current state of high resolution tandem mass spectrometry is extraordinarily sensitive down to sub nanogram levels [72]. These data represent the first comprehensive multi-omics report from normal and healthy horse urine and is intended to be used as a reference tri-ome for further comprehensive equine urine research. We anticipate follow-up studies will include various equine age groups, sexes, in addition to various time points related to pre and post riding exercises from events such as show jumping, dressage, racing, rodeo, polo, etc.

Supporting information

S1 Dataset. Proteomics dataset containing all of the identified horse urine proteins from LC-MS/MS analysis and Mascot database searching. (XLSX)

S2 Dataset. Complete polar horse urine metabolomics dataset from both targeted and untargeted LC-MS/MS with polarity switching from Elements database searching. (XLSX)

S3 Dataset. Non-polar horse urine lipidomics dataset from untargeted LC-MS/MS with polarity switching and LipidSearch database search results. (XLS)

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References

1. Cox J, Mann M. Quantitative, high-resolution proteomics for data-driven systems biology. Annu Rev Biochem. 2011; 80:273–99. https://doi.org/10.1146/annurev-biochem-061308-093216 PMID: 21548781.

2. Ehrich JH, Schiffer E, Drube J. [Urinary proteomics: the diagnostic key for urinary tract abnormalities and kidney diseases in children?]. Urologe A. 2011; 50(2):170–9. PMID: 21161159.

3. Mikami T, Aoki M, Kimura T. The application of mass spectrometry to proteomics and metabolomics in biomarker discovery and drug development. Curr Mol Pharmacol. 2012; 5(2):301–16. PMID: 22122469.

4. Stojiljkovic N, Paris A, Garcia P, Popot MA, Tabet JC, et al. Evaluation of horse urine sample preparation methods for metabolomics using LC coupled to HRMS. Bioanalysis. 2014; 6(6):785–803. https://doi.org/10.4155/bio.13.324 PMID: 24702112.

5. Eliuk S, Makarov A. Evolution of Orbitrap Mass Spectrometry Instrumentation. Annu Rev Anal Chem (Palo Alto Calif). 2015; 8:61–80. https://doi.org/10.1146/annurev-anchem-071114-040325 PMID: 26161972.

6. Breitkopf SB, Asara JM. Determining in vivo phosphorylation sites using mass spectrometry. Curr Protoc Mol Biol. 2012; Chapter 18:1–27. https://doi.org/10.1002/0471142727.mb1819s98 PMID: 22470061

7. Walthier TC, Mann M. Mass spectrometry-based proteomics in cell biology. J Cell Biol. 2010; 190(4):491–500. https://doi.org/10.1083/jcb.201004052 PMID: 20733050.

8. Breitkopf SB, Ricoult SJH, Yuan M, Xu Y, Peake DA, Manning BD, et al. A relative quantitative positive/negative ion switching method for untargeted lipidomics via high resolution LC-MS/MS from any biological source. Metabolomics. 2017; 13(3):30. https://doi.org/10.1007/s11306-016-1157-8 PMID: 28496395.

9. Breitkopf SB, Yuan M, Helenius KP, Lyssiotis CA, Asara JM. Triomics Analysis of Imatinib-Treated Myeloma Cells Connects Kinase Inhibition to RNA Processing and Decreased Lipid Biosynthesis. Anal Chem. 2015. https://doi.org/10.1021/acs.analchem.5b03040 PMID: 26434776.

10. Chan GH, Ho EN, Leung DK, Wong KS, Wan TS. Targeted Metabolomics Approach To Detect the Misuse of Steroidal Aromatase Inhibitors in Equine Sports by Biomarker Profiling. Anal Chem. 2016; 88(1):764–72. https://doi.org/10.1021/acs.analchem.5b03165 PMID: 26632865.

11. DiMaio Knych HK, Arthur RM, Taylor A, Moeller BC, Stanley SD. Pharmacokinetics and metabolism of dantrolene in horses. J Vet Pharmacol Ther. 2011; 34(3):238–46. https://doi.org/10.1111/j.1365-2885.2010.01214.x PMID: 21492188.
12. Dumasia MC, Houghton E, Hyde W, Greulich D, Nelson T, Peterson J. Detection of fenspiride and identification of in vivo metabolites in horse body fluids by capillary gas chromatography-mass spectrometry: administration, biotransformation and urinary excretion after a single oral dose. J Chromatogr B Analotechnol Biomed Life Sci. 2002; 767(1):131–44. PMID: 11863284.

13. Escalona EE, Leng J, Dona AC, Merrifield CA, Holmes E, Proudman CJ, et al. Dominant components of the Thoroughbred metabolome characterised by 1H-nuclear magnetic resonance spectroscopy: A metabolite atlas of common biofluids. Equine Vet J. 2015; 47(6):721–30. https://doi.org/10.1111/evj.12333 PMID: 25130591.

14. Grace PB, Drake EC, Teale P, Houghton E. Quantification of 19-nortestosterone sulphate and boldenone sulphate in urine from male horses using liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2008; 22(19):2999–3007. https://doi.org/10.1002/rcm.3698 PMID: 18777513.

15. Guan F, Uboh C, Soma L, Hess A, Luo Y, Tsang DS. Sensitive liquid chromatographic/tandem mass spectrometric method for the determination of beclometasone dipropionate and its metabolites in equine plasma and urine. J Mass Spectrom. 2003; 38(8):823–38. https://doi.org/10.1002/jms.495 PMID: 12938103.

16. Ho EN, Leung DK, Leung GN, Wan TS, Wong HN, Xu X, et al. Metabolic studies of mesterolone in horses. Anal Chim Acta. 2007; 596(1):149–55. https://doi.org/10.1016/j.aca.2007.05.052 PMID: 17616252.

17. Kieken F, Pinel G, Antignac JP, Monteau F, Christelle Paris A, Popot MA, et al. Development of a metabolomic approach based on LC-ESI-HRMS measurements for profiling of metabolic changes induced by recombinant equine growth hormone in horse urine. Anal Bioanal Chem. 2009; 394(8):2119–28. https://doi.org/10.1007/s00216-009-2912-8 PMID: 19585110.

18. Kolliais-Baker C, Maxwell L, Stanley S, Boone T. Detection and quantification of cocaine metabolites in urine samples from horses administered cocaine. J Vet Pharmacol Ther. 2003; 26(6):429–34. PMID: 14962054.

19. Kuuranne T, Thomas A, Leinonen A, Delahaut P, Bosseloir A, Schanzer W, et al. Insulins in equine urine: qualitative analysis by immunoaffinity purification and liquid chromatography/tandem mass spectrometry for doping control purposes in horse-racing. Rapid Commun Mass Spectrom. 2008; 22(3):355–62. https://doi.org/10.1002/rcm.3360 PMID: 18181226.

20. Lechner AF, Hughes CG, Karpiesiuk W, Harkins JD, Dirikolu L, Bosken J, et al. Development of a method for the detection and confirmation of the alpha-2 agonist amitraz and its major metabolite in horse urine. J Anal Toxicol. 2004; 28(7):553–62. PMID: 15516314.

21. Leung GN, Kwok WH, Wan TS, Lam KK, Schiff PJ. Metabolic studies of formestane in horses. Drug Test Anal. 2013; 5(6):412–9. https://doi.org/10.1002/dta.1444 PMID: 23339113.

22. McKinney AR, Suann CJ, Stenhous AM. The detection of modafinil and its major metabolite in equine urine by liquid chromatography/mass spectrometry. Rapid Commun Mass Spectrom. 2005; 19(10):1217–20. https://doi.org/10.1002/rcm.1910 PMID: 15834965.

23. Scarth JP, Spencer HA, Timbers SE, Hudson SC, Hillyer LL. The use of in vitro technologies coupled with high resolution accurate mass LC-MS for studying drug metabolism in equine drug surveillance. Drug Test Anal. 2010; 2(1):1–10. https://doi.org/10.1002/dta.88 PMID: 20878880.

24. Spyridaki MH, Lyris E, Georgoulakis I, Kouretas D, Konstantinidou M, Georgakopoulos CG. Determination of xylazine and its metabolites by GC-MS in equine urine for doping analysis. J Pharm Biomed Anal. 2004; 35(1):107–16. https://doi.org/10.1016/j.jpba.2003.12.007 PMID: 15030885.

25. Taylor P, Scarth JP, Hillyer LL. Use of in vitro technologies to study phase II conjugation in equine sports drug surveillance. Bioanalysis. 2010; 2(12):1971–88. https://doi.org/10.4155/bio.10.135 PMID: 21110741.

26. Wieder ME, Brown PR, Grainger L, Teale P. Identification of etamiphylline and metabolites in equine plasma and urine by accurate mass and liquid chromatography/tandem mass spectrometry. Drug Test Anal. 2010; 2(6):271–7. https://doi.org/10.1002/dta.133 PMID: 20564606.

27. Yamada M, Aramaki S, Hosoe T, Kurosawa M, Kijima-Suda I, Saito K, et al. Characterization and quantification of fluoxymesterone metabolite in horse urine by gas chromatography/mass spectrometry. Anal Sci. 2008; 24(7):911–4. PMID: 18614835.

28. Yamada M, Kinoshita K, Kurosawa M, Saito K, Nakazawa H. Analysis of exogenous nandrolone metabolite in horse urine by gas chromatography/combustion/carbon isotope ratio mass spectrometry. J Pharm Biomed Anal. 2007; 45(4):654–8. https://doi.org/10.1016/j.jpba.2007.07.005 PMID: 17714906.

29. Yu NH, Ho EN, Leung DK, Wan TS. Screening of anabolic steroids in horse urine by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2005; 37(5):1031–8. https://doi.org/10.1016/j.jpba.2004.08.041 PMID: 15862683.
30. Chen Z, Kim J. Urinary proteomics and metabolomics studies to monitor bladder health and urologic diseases. BMC Urol. 2016; 16:11. https://doi.org/10.1186/s12894-016-0129-7 PMID: 27000794

31. Yang X, Friedman A, Nagpal S, Perrimon N, Asara JM. Use of a label-free quantitative platform based on MS/MS average TIC to calculate dynamics of protein complexes in insulin signaling. J Biomol Tech. 2009; 20(5):272–7. PMID: 19949701

32. Doll S, Burlingame AL. Mass spectrometry-based detection and assignment of protein posttranslational modifications. ACS Chem Biol. 2015; 10(1):63–71. https://doi.org/10.1021/cb500904b PMID: 25541750

33. Olsen JV, Mann M. Status of large-scale analysis of post-translational modifications by mass spectrometry. Mol Cell Proteomics. 2013; 12(12):3444–52. https://doi.org/10.1074/mcp.O113.034181 PMID: 24187339

34. Thomas S, Hao L, Ricke WA, Li L. Biomarker discovery in mass spectrometry-based urinary proteomics. Proteomics Clin Appl. 2016; 10(4):358–70. https://doi.org/10.1002/prca.201500102 PMID: 26703953

35. Brandt LE, Ehrhart EJ, Scherman H, Olver CS, Bohn AA, Prenni JE. Characterization of the canine urinary proteome. Vet Clin Pathol. 2014; 43(2):193–205. https://doi.org/10.1111/vcp.12147 PMID: 24773128.

36. Sun W, Li F, Wu S, Wang X, Zheng D, Wang J, et al. Human urine proteome analysis by three separation approaches. Proteomics. 2005; 5(18):4994–5001. https://doi.org/10.1002/pmic.200401334 PMID: 16281181.

37. Thongboonkerd V, McLeish KR, Arthur JM, Klein JB. Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int. 2002; 62(4):1461–9. https://doi.org/10.1111/j.1523-1755.2002.kid565.x PMID: 12234320.

38. Naquet P, Pitari G, Dupre S, Galland F. Role of the Vnn1 pantetheinase in tissue tolerance to stress. Biochem Soc Trans. 2014; 42(4):1094–100. https://doi.org/10.1042/BST20140092 PMID: 25110008.

39. Nitto T, Ondera K. Linkage between coenzyme a metabolism and inflammation: roles of pantetheinase. J Pharmacol Sci. 2013; 123(1):1–8. PMID: 23978960.

40. Yamazaki T, Goya I, Graf D, Craig S, Martin-Orozco N, Dong C. A butyrophilin family member critically inhibits T cell activation. J Immunol. 2010; 185(10):5907–14. https://doi.org/10.4049/jimmunol.1000835 PMID: 20944003.

41. Pathan S, Gowdy RE, Cooney R, Beckly JB, Hancock L, Guo C, et al. Confirmation of the novel association at the BTNL2 locus with ulcerative colitis. Tissue Antigens. 2009; 74(4):322–9. https://doi.org/10.1111/j.1399-0039.2009.01314.x PMID: 19659809.

42. Mayumi T, Inui K, Maetani I, Yokoe M, Sakamoto T, Yoshida M, et al. Validity of the urinary trypsinogen-2 test in the diagnosis of acute pancreatitis. Pancreas. 2012; 41(6):869–75. https://doi.org/10.1097/MPA.0b013e318248ab7 PMID: 22481290.

43. Martin B, Silberzahn P. Concentration decrease of corticosteroid binding globulin (CBG) in plasma of the mare throughout pregnancy. J Steroid Biochem. 1990; 35(1):121–5. PMID: 23083323.

44. Rosner W, Hryb DJ, Khan MS, Singer CJ, Nakhla AM. Are corticosteroid-binding globulin and sex hormone-binding globulin hormones? Ann N Y Acad Sci. 1988; 538:137–45. PMID: 3056181.

45. Mordente A, Meucci E, Martorana GE, Silvestrini A. Cancer Biomarkers Discover y and Validation: State of the Art, Problems and Future Perspectives. Adv Exp Med Biol. 2015; 867:9–26. https://doi.org/10.1007/978-94-017-7215-0_2 PMID: 26530357.

46. Lau TYK, Collins BC, Stone P, Tang N, Gallagher WM, Pennington SR. Absolute Quantific ation of Toxi-cological Biomarkers via Mass Spectrom etry. Methods Mol Biol. 2017; 1641:33 7–48. https://doi.org/10.1007/978-1-4939-7172-5_19 PMID: 28748474.

47. Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, et al. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res. 2017; 45(D1):D138–D9. https://doi.org/10.1093/nar/gkw1138 PMID: 27899595

48. Yuan M, Breitkopf SB, Yang X, Asara JM. A positive/negative ion-switching, targeted mass spectrometry platform for bodily fluids, cells, and fresh and fixed tissue. Nat Protoc. 2012; 7 (5):872–81. https://doi.org/10.1038/nprot.2012.024 PMID: 22498707

49. Xia J, Wishart DS. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. Curr Protoc Bioinformatics. 2016; 55:14 0 1–0 91. https://doi.org/10.1002/9780471703755.cbi111 PMID: 27603023.

50. Warrack BM, Hnatiyshyn S, Ott KH, Reilly MD, Sanders M, Zhang H, et al. Normalization strategies for metabolomic analysis of urine samples. J Chromatogr B Analyt Technol Biomed Life Sci. 2009; 877(5-6):547–52. https://doi.org/10.1016/j.jchromb.2009.01.007 PMID: 19185549.

51. Zhang Y, Gu L, Jiang Y, Bi K, Chen X. Quantitative analysis of biomarkers of liver and kidney injury in serum and urine using ultra-fast liquid chromatography with tandem mass spectrometry coupled with a
hydrophilic interaction chromatography column: Application to monitor injury induced by Euphorbia pekinensis Radix. J Sep Sci. 2016; 39(20):3936–45. https://doi.org/10.1002/jssc.201600470 PMID: 27697729.

52. Kamiguchi H, Yamaguchi M, Murabayashi M, Mori I, Hirouchi A. Method development and validation for simultaneous quantitation of endogenous hippuric acid and phenylacetylglycine in rat urine using liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J Chromatogr B Analayl Technol Biomed Life Sci. 2016; 1035:76–83. https://doi.org/10.1016/j.jchromb.2016.09.036 PMID: 27697729.

53. Gao Y, Lu Y, Huang S, Gao L, Liang X, Wu Y, et al. Identifying early urinary metabolic changes with long-term environmental exposure to cadmium by mass-spectrometry-based metabolomics. Environ Sci Technol. 2014; 48(11):6409–18. https://doi.org/10.1021/es500750w PMID: 24834460.

54. Dams R, Murphy CM, Lambert WE, Huestis MA. Urine drug testing for opioids, cocaine, and metabolites by direct injection liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2003; 17(14):1665–70. https://doi.org/10.1002/rcm.1098 PMID: 12845594.

55. Eichhorn J, Etter M, Lepage J, Lehotay DC. Urinary screening for methylphenidate (Ritalin) abuse: a comparison of liquid chromatography–tandem mass spectrometry, gas chromatography–mass spectrometry, and immunoassay methods. Clin Biochem. 2004; 37(3):175–83. https://doi.org/10.1016/j.clinbiochem.2003.11.006 PMID: 14972638.

56. Fitzgerald RL, Griffin TL, Yun YM, Godfrey RA, West R, Pesce AJ, et al. Dilute and shoot: analysis of drugs of abuse using selected reaction monitoring for quantification and full scan product ion spectra for identification. J Anal Toxicol. 2012; 36(2):106–11. https://doi.org/10.1093/jat/bkr024 PMID: 22337779.

57. Wu AH, Gerona R, Armenian P, French D, Petrie M, Lynch KL. Role of liquid chromatography-high-resolution mass spectrometry (LC-HR/MS) in clinical toxicology. Clin Toxicol (Phila). 2012; 50(8):733–42. https://doi.org/10.3109/15563650.2012.713108 PMID: 22889997.

58. Gonzalez-Billalabeitia E, Seitzer N, Song SJ, Song MS, Patnaik A, Liu XS, et al. Vulnerabilities of PTEN-TP53-deficient prostate cancers to compound PARP-Pi3K inhibition. Cancer Discov. 2014; 4(8):896–904. https://doi.org/10.1158/2159-8290.CD-13-0230 PMID: 24866151.

59. Kienzl E, Eichinger K, Sofic E, Jellinger K, Riederer P, Kuhn W, et al. Urinary dopamine sulfate: regulations and significance in neurological disorders. J Neural Transm Suppl. 1990; 32:971–9. PMID: 2089119.

60. Koeberl DD, Young SP, Gregersen NS, Vockley J, Smith WE, Benjamin DK Jr., et al. Rare disorders of metabolism with elevated butyryl- and isobutyryl-carnitine detected by tandem mass spectrometry newborn screening. Pediatr Res. 2003; 54(2):219–23. https://doi.org/10.1203/01.PDR.0000074972.36356.89 PMID: 12736383.

61. van de Merbel NC, Tinke AP, Oosterhuis B, Jongma JH, Bohle JF. Determination of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid in human plasma and urine by high-performance liquid chromatography with tandem mass spectrometric detection. J Chromatogr B Biomed Sci Appl. 1998; 708(1–2):103–12. PMID: 9653952.

62. Hansen P, Clerc B. Anisocoria in the dog provoked by a toxic contact with an ornamental plant: Datura stramonium. Vet Ophthalmol. 2002; 5(4):277–9. PMID: 12445299.

63. Judge H, Nguy D, Moller I, Cooney JM, Atkinson RG. Isolation and characterization of a novel glycosyltransferase that converts phloretin to phlorizin, a potent antioxidant in apple. FEBS J. 2008; 275(15):3804–14. https://doi.org/10.1111/j.1742-4658.2008.06526.x PMID: 18573104.

64. da Cunha MG, Rosalen PL, Franchin M, de Alencar SM, Ikegaki M, Ransom T, et al. Antiproliferative Constituents of Geopropolis from the Bee Melipona scutellaris. Planta Med. 2016; 82(3):190–4. https://doi.org/10.1055/s-0035-1558142 PMID: 26544117.

65. Manayi A, Saedinia S, Ostad SN, Hadijakhooi A, Ardekani MR, Vazirian M, et al. Chemical constituents and cytotoxic effect of the main compounds of Lythrum salicaria L. Z Naturforsch C. 2013; 68(9–10):367–75. PMID: 24459770.

66. Lake KD, Brown DC. New drug therapy for kidney stones: a review of cellulose sodium phosphate, acetohydroxamic acid, and potassium citrate. Drug Intel Clin Pharm. 1985; 19(7–8):530–9. PMID: 3896714.

67. Tipthara P, Thongboonkerd V. Differential human urinary lipid profiles using various lipid-extraction protocols: MALDI-TOF and LIFT-TOF/TOF analyses. Sci Rep. 2016; 6:33756. https://doi.org/10.1038/srep33756 PMID: 27646409.

68. Min HK, Lim S, Chung BC, Moon MH. Shotgun lipidomics for candidate biomarkers of urinary phospholipids in prostate cancer. Anal Bioanal Chem. 2011; 399(2):823–30. https://doi.org/10.1007/s00216-010-4290-7 PMID: 20953865.
69. Narvaez-Rivas M, Zhang Q. Comprehensive untargeted lipidomic analysis using core-shell C30 particle column and high field orbitrap mass spectrometer. J Chromatogr A. 2016; 1440:123–34. https://doi.org/10.1016/j.chroma.2016.02.054 PMID: 26928874

70. Breitkopf SB, Yuan M, Xu Y, Asara JM. A Quantitative Positive/Negative Ion Switching Method for Shotgun Lipidomics via High Resolution LC-MS/MS from any Biological Source. American Society for Mass Spectrometry; June 9, 2016; San Antonio. JASMS2016.

71. Karamichos D, Zieske JD, Sejersen H, Sarker-Nag A, Asara JM, Hjortdal J. Tear metabolite changes in keratoconus. Exp Eye Res. 2015; 132:1–8. https://doi.org/10.1016/j.exer.2015.01.007 PMID: 25579606

72. Thomas A, Geyer H, Schanzer W, Crone C, Kellmann M, Moehring T, et al. Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. Anal Bioanal Chem. 2012; 403(5):1279–89. https://doi.org/10.1007/s00216-011-5655-2 PMID: 22231507.