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Metabolic Phenotype of the Healthy Rodent Model Using In-Vial Extraction of Dried Serum, Urine, and Cerebrospinal Fluid Spots

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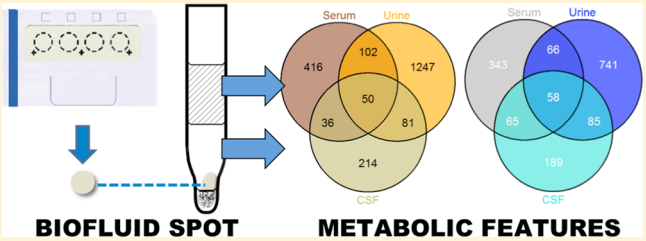
ABSTRACT: High-throughput multiplatform metabolomics experiments are becoming an integral part of clinical and systems biology research. Such methods call for the adoption of robust sample storage and transport formats for small volumes of biofluids. One such format is the dried biofluid spot, which combines small volume requirements with easy portability. Here, we describe ultra high-performance liquid chromatography–mass spectrometry (UHPLC–MS) metabolomics of dried rodent serum, urine, and cerebrospinal fluid spots. An in-vial extraction and UHPLC–MS analysis method was first developed and validated by fingerprinting two test fluids, rat serum and RPMI cell nutrient medium. Data for these extracts were compared in terms of (i) peak area measurements of selected features to assess reproducibility and (ii) total fingerprint variation after data pretreatment. Results showed that percentage peak area variation was found to range between 1.4 and 9.4% relative standard deviation (RSD) for a representative set of molecular features. Upon application of the method to spots bearing serum, urine or cerebrospinal fluid (CSF) from healthy rats and mice, a total of 1 182 and 2 309 reproducible molecular features were obtained in positive and negative ionization modes, respectively, of which 610 (positive) and 991 (negative) were found in both rats and mice. Feature matching was used to detect similarities and differences between biofluids, with the biggest overlap found between fingerprints obtained in urine and CSF. Our results thus demonstrate the potential of such direct fingerprinting of dried biofluid spots as a viable alternative to the use of small (10–15 μL) volumes of neat biofluids in animal studies.



Metabolomics is quickly establishing itself as an integral part of research efforts in the life sciences.[1,2] Metabolomics-based studies have already contributed to major advances in biomedical knowledge, e.g., the correlation of diet and gut microbial activities with high blood-pressure using NMR metabolomics,[3] and the association of gut flora metabolism with cardiovascular disease through liquid chromatography–mass spectrometry (LC–MS) based metabolomics.[4] However, as an "omics" approach it lags behind more established methods such as proteomics and genomics, particularly since it deals with the small-molecule fingerprinting of complex biological samples and aims to identify metabolites in samples containing a multitude of similar molecules.

Typically, an untargeted metabolomics experiment includes a comprehensive metabolite fingerprinting step, either by NMR or LC–MS methods. The primary challenges of such untargeted experiments include detecting intact small molecules (<1500 Da) over a wide concentration range as well as ensuring sufficient metabolite coverage to shed light on the metabolic problem being investigated. Hence, untargeted metabolomics studies often use multiple analytical technologies to study the same samples, and metabolomics protocols are now available for a variety of biofluid and tissue types. For large-scale metabolomics experiments, LC–MS[5,6] and NMR[7] methods are predominantly used, followed by gas chromatography/mass spectrometry (GC/MS)[8] and capillary electrophoresis–mass spectrometry (CE–MS)[9] approaches. One advantage of LC/GC/CE–MS methods is that metabolites can be separated based on their physicochemical characteristics prior to detection and analysis, thus reducing the number of molecules that enter the MS at a given time. Multiple components entering the MS at the same time can impact ionization and ion suppression, especially when using electrospray ionization; hence, better separation tends to improve measurement sensitivity and accuracy.

The diversity of analytical approaches used in large-scale metabolomics studies emphasizes the need for standardized and robust sample storage methods. At the discovery stage of metabolomics, the use of small amounts of biofluids also confers several advantages and is the ethically preferred route (particularly when dealing with animal models).[10] One small-volume sampling approach involves profiling dried blood spots (DBS), blood samples which have been collected onto different types of absorbent paper. Such spots have a number of practical

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benefits, including the added ease of storage and transportation at ambient conditions. Spots have been used in preclinical drug development, diagnostics, and treatment since the 1960s,[11−17] and DBS studies are becoming increasingly popular in drug analysis as well.[14,18−21] Pioneering metabolomics studies have shown the utility of DBS fingerprinting with ^{1}H NMR spectroscopy, ultra high-performance liquid chromatography−mass spectrometry (UHPLC−MS), and GC/MS based methods.[22−24] The UHPLC−MS investigation cited was not limited to blood but also showed that urine and serum spotting are viable alternatives when compared directly with classic metabolite profiling and data treatment. Another study by Michopoulos et al. proved that best results were achieved when spots are stored frozen on collection cards which are additive-free, as the latter could interfere with the quality of the raw data.[24,25] Spot profiling can thus result in a more economical experimental design, particularly when the costs of animals, sampling, storage, and shipment are factored in.

While we have previously succeeded in devising a small volume (20 μL) plasma fingerprinting method that yields comprehensive metabolite coverage,[26] optimizing coverage per volume or mass unit of sample is also critical in metabolomics. In the current work, we describe a method for biofluid spot sample preparation, in-vial and in-vial dual extraction (IVE and IVDE), and UHPLC−MS analysis for use in metabolic fingerprinting. We have investigated the robustness and capabilities of this method for metabolite phenotyping by testing it on rat serum and RPMI cell nutrient broth analysis and have applied the method to the study of serum, urine, and CSF from two separate rodent (rat and mouse) models. As previously demonstrated, the IVDE method yields reproducible data with low analytical variation and requires very small amounts of biofluid for successful implementation. The study described herein thus combines the benefits of spot analysis with the analytical reproducibility of the in-vial extraction (IVE) method for metabolomics.

METHODS

Reagents and Materials. LC−MS grade water and methanol, analytical-grade formic acid and methyl *tert*-butyl ether (MTBE), as well as rat serum used for validation experiments were purchased from Sigma-Aldrich (U.K.). Samples were spotted onto Whatman FTA DMPK-C Cards from GE Healthcare Bio-Sciences (NJ). All sample extractions were performed in amber glass HPLC vials with fixed 0.3 mL inserts (from Chromacol, Welwyn Garden City, U.K.). RPMI-1640 cell nutrient broth was purchased from Invitrogen.

Rodent Biofluid Harvesting and Spot Preparation. Three adult male and three adult female Sprague−Dawley (SD) rats (250−280 g), and three normal c57 mice (25 g) were obtained from the Laboratory Animal Unit of the LKS Faculty of Medicine in the University of Hong Kong and were maintained in a temperature-controlled room with a 12 h light/dark cycle. The animals were handled according to protocols approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. The rats were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (ketamine 80 mg/kg and xylazine 8 mg/kg; from Alfasan, Woerden, Holland). The skin at the back of the neck and on the chest and the lower abdomen was shaved, and alcohol pads were used to clean the skin. A horizontal incision opened the lower abdominal wall, and a 1 mL syringe was used to withdraw urine directly from the bladder. A longitudinal incision in the back of the neck skin was then made, through which the foramen magnum was found. A 1 mL syringe was used to puncture through the dura and withdraw CSF. Finally, through an incision in the chest, blood was withdrawn from the right atrium using a heparinized 5 mL syringe. The blood samples were centrifuged at 4000 r.c.f. for 20 min at 4 °C and plasma was collected.

Spots were prepared by dropping 15 μL of each biofluid onto sample spots on Whatman FTA DMPK-C cards, with the exception of murine CSF, which was diluted 10× in Milli-Q water before spotting. Between 2 and 4 replicates were prepared for each biofluid type per animal. Spots were air-dried for 2 h at room temperature and then placed in PE plastic packaging for shipment. Samples were shipped in the absence of desiccant, at ambient temperature. A total of 30 spots bearing murine biofluids (urine = 12, serum = 12, CSF = 6) and 47 spots bearing rat biofluids (urine = 12, serum = 22, CSF = 13) were extracted and analyzed. Samples for validation were prepared by creating spots with 10 μL of commercially obtained rat serum (×10) and 10 μL of RPMI1640 (cell nutrient fluid) (×10). Validation samples were prepared to investigate analytical reproducibility of the spot-IVDE methodology at 10 μL volumes. Mice and rat biofluids were 15 μL in keeping with conventional blood collection volumes (max 0.6 mL/kg/day).

Sample Spot Extraction. The extraction methods were adapted from a procedure previously used for the analysis of plasma by Whiley et al.[26] Spots with a diameter of 6 mm were punched out of the Whatman cards and then introduced into a Chromacol HPLC vial (with a 300 μL insert). All subsequent extraction steps were performed in the same vial, allowing analytical samples to be withdrawn from the extract with minimal sample loss. Extraction protocols for different biofluids are described below.

Serum IVDE. Figure 1 illustrates the use of the IVDE method for the extraction of serum spots. A volume of 10 μL of water was added to each vial containing a sera spot, followed by 40 μL of methanol for protein precipitation. Samples were vortex-

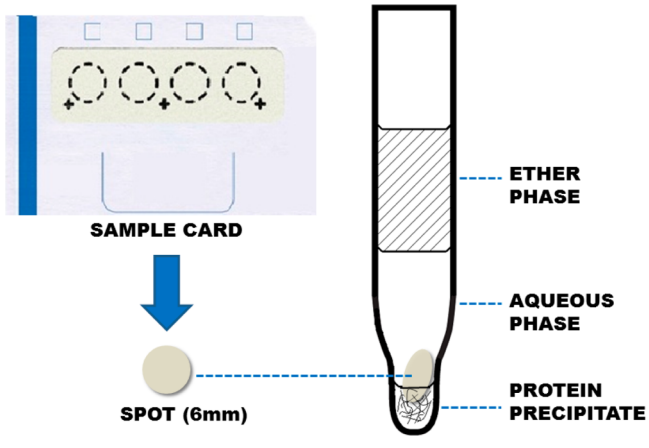

Figure 1. In-vial dual extraction (IVDE) of serum spot samples. The top left is a Whatman FTA DMPK-C sample card, with each circled area bearing 10−15 μL of biofluid. A 6 mm (diameter) spot (bottom left) is punched out from the card and inserted into a Chromacol vial with a 300 μL glass insert (right). Extraction solvents are added in the manner described in the Methods section, and the resultant solvent bilayer serves for nontargeted general extraction of polar and nonpolar analytes.

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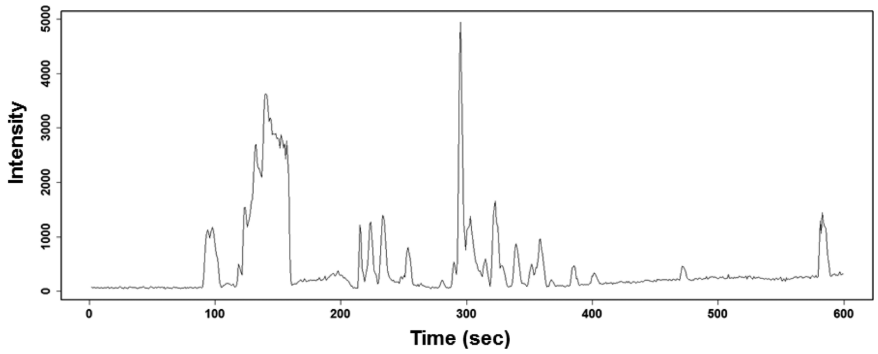

Figure 2. Example base peak intensity ion chromatogram of mouse urine extract (positive ionization mode).

mixed for 2 min, after which 200 μL of MTBE was added to each sample. Samples were vortexed at room temperature for 60 min. Following addition of 50 μL of water per vial, the samples were centrifuged at 3000 r.c.f. for 10 min. This resulted in a clear separation of MTBE (upper) and aqueous (lower) phases, with protein precipitate aggregated at the bottom of the vial. Upper and lower phases could thus be injected directly into the LC−MS system in two separate runs, by adjustment of the injection needle height (15 mm for upper phase, 5 mm for lower).

In-Vial Extraction (IVE) of Urine, CSF, and Cell Nutrient Fluid (RPMI). Water (50 μL) was added to each vial containing urine, CSF, or cell nutrient fluid spots. Proteins were precipitated with 150 μL of methanol and vortex-mixed for 2 min, followed by centrifugation at 3000 r.c.f. for 10 min. The sample was then injected into the LC−MS system directly from the vial.

Analytical Setup. LC−MS analysis was completed using a Waters ACQUITY UPLC coupled to a Waters Xevo G1 QTOF (Waters, Milford, MA). Sample extract (5 μL) was injected onto a reversed-phase C18 column (Supelco Discovery HS C18, 150 mm × 2.1 mm i.d., 3 μm particle size). Separation was performed at 25 °C with a flow-rate of 0.2 mL/min, solvent A composed of 0.1% formic acid in water, and solvent B composed of 0.1% formic acid in methanol. Metabolites were eluted using the following gradient elution method: from 0% B at 0 min to 100% B in 10 min, returning to initial conditions in 1 min, maintained at initial conditions for 4 min. A feature is any m/z signal maximizing at an individual retention time. Figure 2 shows an example of a chromatogram from rat urine that clearly illustrates the density of molecular features obtained using this gradient elution method.

MS analysis was completed in separate positive and negative mode experiments. In both modes, all analyses were acquired in LockSpray mode to ensure accuracy and reproducibility; leucine-enkephalin was used as a lock mass (m/z 556.2771) at a concentration of 500 ng/mL and a flow rate of 10 μL/min. Data were collected in the centroid mode over the mass range m/z 100−1000 with an acquisition time of 0.1 s a scan. In positive ion mode, the XEVO QTOF was operated with a capillary voltage of 3.2 kV and a cone voltage of 35 V. The extraction cone was set to 2.0 V. The desolvation gas flow was 400 L/h and maintained at 400 °C. The source temperature was 120 °C, and the cone gas was set to 50 L/h. In negative ionization mode, the XEVO QTOF was operated with a capillary voltage of −2.6 kV and a cone voltage of 45 V. The extraction cone was set to 2.0 V. The desolvation gas flow was 800 L/h and maintained at 350 °C. The source temperature was 120 °C.

MS^e Data Collection. All data were collected using Waters MS^e technology, at two constantly interchanging energies. This enables data collection at two "levels". Level one is traditional MS raw data with parent molecular ionization. Level two initiates high collision energies within the MS source, which has the effect of causing molecular fragmentation, meaning accurate mass fragmentation data can be collected simultaneously with accurate mass precursor ion data. In this instance, data was acquired at level one with a low collision energy of 5 V, whereas level two acquired the data at collision energy of 50 V.

Data Processing and Analysis. All data was processed within the Masslynx application manager 4.1 (Waters, Milford, MA) and exported into SIMCA version 13 (MKS Umetrics AB, Sweden) for multivariate analysis. Data files collected in MassLynx were imported into MarkerLynx (a module within MassLynx) for data pretreatment, including peak alignment and normalization to total peak area (per chromatogram). Processed data was subsequently filtered using an in-house algorithm, which allowed the selection of normalized features present in >50% of samples within each biofluid group (e.g., within the rat CSF group, within the mouse urine group, etc.). Filtered data was then pareto-scaled and subjected to principal components analysis (PCA) in SIMCA. Note that data is mean-centered as a matter of course during PCA modeling in SIMCA software. Graphical outputs were prepared in SIMCA as well as in the XCMS[27−29] and VennDiagram[30] packages in R.[31]

RESULTS AND DISCUSSION

The use of dried biofluid spots for the acquisition of semiquantitative metabolomics data is advantageous for a number of reasons. First, there are the practical benefits associated with easier storage and safer transport alternatives available to spot samples. Second, there are important ethical and experimental reasons for pursuing the use of viable small-volume sample spot methods. Burnett[10] summarizes the ethical benefits of spot analysis, which include a reduction in the number of animals or participants needed (such as the use of single animals for TK studies) as well as method refinement via the use of less invasive collection procedures. The latter is possible as 10−20 μL samples can be acquired using anticoagulant-coated glass capillaries, which is infeasible for conventionally collected 0.3−0.5 mL blood volumes. It is worth noting that the long-term stability of components in spots has not been thoroughly investigated, even though spot freezing upon acquisition has been recommended for metabolomics applications.[25] However, the aforementioned practical and

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ethical advantages associated with spot analysis, when combined with the concomitant decrease in man-hours needed for sample collection and processing, could lead to broad adoption of the spot methodology in the near future.

Here, we applied the in-vial extraction protocol to the analysis of dried biofluid spots. The combination of small volume spots format with the low loss, high coverage IVDE method yields reproducible results in a validation set of samples, with low intersample variability.

Spot-IVDE Reproducibility Study. To test the reproducibility of the extraction and analysis protocols when applied to the spot samples, 10 spots bearing 10 μL of rat serum and 10 spots bearing 10 μL of cell nutrient medium were studied. Spots that were 6 mm in diameter were punched out of the Whatman cards and extracted using either a modification of the previously published IVDE method[26] (rat serum) or a methanol/water extraction solvent (RPMI cell nutrient medium). The combination of aqueous and organic phases in the IVDE method permits the extraction of both polar and nonpolar metabolites without transfer-associated sample loss.

The 10 replicates from each method were analyzed using identical LC–MS protocols, and fingerprints with >4000 distinct features per chromatogram were obtained. After data processing for noise reduction and normalization to total intensity, a set of six features from each type of extract (i.e., organic and aqueous extracts for serum, aqueous extract for RMPI), representing a range of retention times and masses, was chosen for the analysis of reproducibility. In order to select peaks for reproducibility analysis, the raw data was inspected for m/z-retention time features that were present in every sample of the groups in Table 1. The raw data was then processed using the Waters QuanLynx application's ApexTrack algorithm, which performed mean peak smoothing and baseline correction prior to peak detection and integration for selected masses. Output integrated peak areas were then used for the analysis of variation within the validation groups. Relative standard deviation (RSD) for the peak areas of these metabolites ranged from 1.4 to 9.4% RSD for these metabolites, an acceptable level of variation (see Table 1). Processed data from both positive and negative modes was further parsed using an unsupervised multivariate analysis (MVA) PCA model. A PCA model best explains the variability in the data, and to quantify this variability, the distance between the two principal vectors was measured for the different types of extract. This vector distance was obtained from the difference between the maximum and minimum vector scores for each group of replicates. In Figure 3, the PCA scores can be observed for the data collected in positive ionization mode. The variability within the swarm of replicates for each group (calculated as the maximum and minimum vector distance in both PC1 and PC2 directions) corresponds to the serum ether extracts (averaged RSD of 15.1%), followed by aqueous serum extracts (8.1%) and RPMI extracts (5.7%), respectively (see Table 2). The PCA analysis shows that the cumulative fingerprint of the ether group was less reproducible (i.e., showed more spread) than the other sample extracts. However the univariate variation (as measured by the % RSD) for the six analytes chosen for the serum ether extracts was similar to that of the metabolites from the other extracts. This higher variability in the lipophilic extracts and fingerprint could be attributed to increased peak overlapping at the higher organic gradient conditions. In our previous work, injection of the ether layer was highly reproducible for single features, showing 821 features with variation RSD < 5% when triplicate extractions were performed.[26]

Since small changes in metabolite concentrations can indicate important abnormalities in metabolic pathways, analytically robust methods for metabolomics fingerprinting are essential in order to extract meaningful biomarker data. However analytical variation of some magnitude will always be a factor when assessing potential markers in metabolic fingerprint data. LC–MS as a tool for quantitative analysis can generally show inaccuracies of 10% to 15% RSD, depending

Table 1. Peak Area Variation for Six Selected Analytes from Each Extract Type in the Test Set

(a) Serum-Upper Layer				
m/z	RT	avg peak area	RSD (%)	RSD (%) for ratio[a]
178.12	5.11	458.79	5.48	3.70
647.25	**5.87**	48.69	5.98	
491.18	5.92	52.45	3.83	3.36
205.04	6.18	887.27	9.41	9.97
674.51	11.93	449.04	6.61	7.26
765.49	12.23	527.25	6.96	7.78
(b) Serum-Lower Layer				
m/z	RT	avg peak area	RSD (%)	RSD (%) for ratio[a]
378.90	1.79	224.35	1.39	3.38
182.08	**5.82**	1271.42	3.50	
250.04	7.01	117.34	3.59	3.81
188.07	7.79	6860.07	4.46	1.83
801.03	9.73	130.01	2.50	3.13
550.63	12.66	66.56	6.44	6.83
(c) RPMI1640				
m/z	RT	avg peak area	RSD (%)	RSD (%) for ratio[a]
242.93	1.75	304.85	4.74	7.20
497.06	2.05	62.80	3.47	5.31
188.07	**8.04**	294.01	3.59	
349.18	8.34	30.56	5.92	5.86
171.10	9.16	1179.99	8.90	8.52
413.27	14.58	3264.73	5.18	6.89

[a]Calculated ratio = peak area of feature/peak area of bolded features in parts a, b, and c (chosen with medium % RSD). This allows for comparison in each group in a normalized fashion.

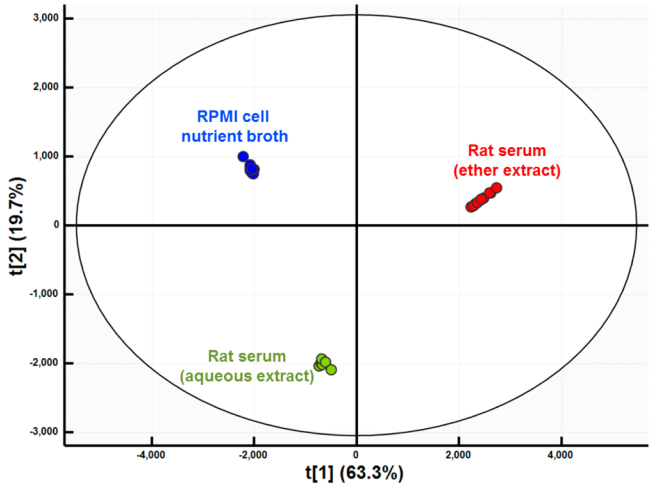

Figure 3. PCA plot depicting the variation of spots for rat serum (both organic and aqueous extracts) and for the aqueous (methanolic) extract of the RPMI cell nutrient fluid.

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Table 2. Variation for Each Extract Type in the Test (Validation) Set As Calculated from the PCA Scores Plot, Where Variation Is Calculated Per Group of 10 Replicates As Dispersion Observed for the Two First PC Vector Scores (t[1] and t[2])

	(a) serum-upper layer		(b) serum-lower layer		(c) RPMI1640	
	M1.t[1]	M1.t[2]	M1.t[1]	M1.t[2]	M1.t[1]	M1.t[2]
mean scores value	2450.87	379.16	−642.67	−2015.97	−2065.26	830.42
Δ[a]	504.69	284.91	241.63	160.24	211.39	255.70
RSD (%)	6.55	23.75	13.60	2.70	2.85	8.55

[a] Δ = max vector score − min vector score (for each group of replicates, per component).

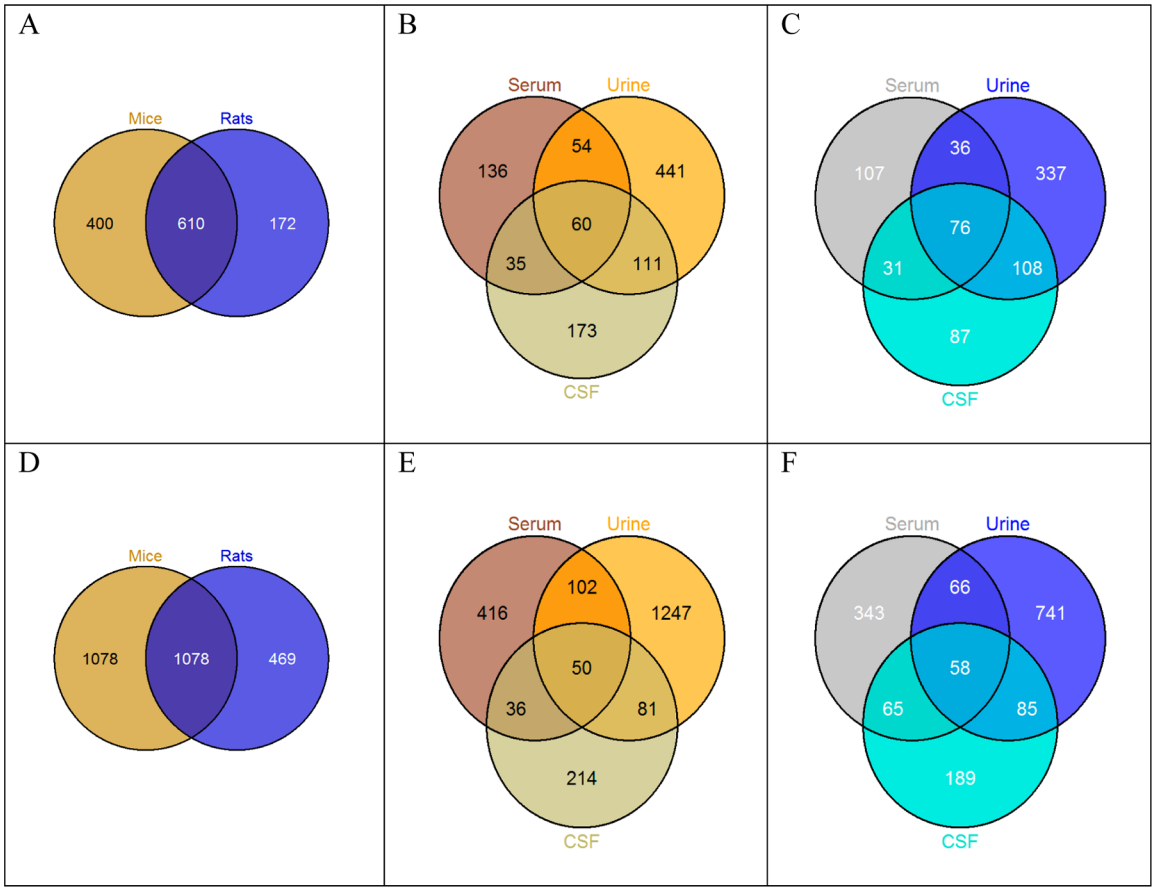

Figure 4. Venn diagrams depicting the distribution of molecular features extracted from rodent biofluids. A total of 1 182 and 2 625 distinct features were observed. (A) Overlap between features found in mice and rats (positive ionization mode). (B) Overlap between features found in all three murine biofluids (positive mode). (C) Overlap between features found in all three rat biofluids (positive mode). (D) Overlap between features found in mice and rats (negative mode). (E) Overlap between features found in the three murine biofluids (negative mode). (F) Overlap between features found in the three rat biofluids (negative mode). Figures were prepared using the VennDiagram package[30] in R.[27−29].

on factors such as the type of molecule being detected, absolute concentrations, and matrix effects,[32] and this variability depends upon the relationship between these factors and molecular ionization behaviors (e.g., ion suppression or enhancement phenomena).[33] In recent studies with UHPLC−MS/MS,[34] 13% RSD variation was accounted for in a conventional drug pharmacokinetic assay in plasma. While a small sample analysis where sensitivity is compromised, such as in hair analysis, variation of 11.5% was recorded in another validated method.[35] In addition, the recoveries of tacrolimus from blood spots can have a wider (135%) allowed bracket in validation studies.[36]

Interanimal and Intra-Animal Fingerprinting Comparison. As a proof of concept the method described above was applied to spots bearing three different biofluids (CSF, urine, and serum) from healthy rats and mice. After a systematic process of data cutoff and mining, which consisted of recording features that were present in at least 50% of the group (per biofluid and per animal), a total of 1182 (positive ionization) and 2625 (negative) features were recorded.

Figure 4 illustrates the overlap in molecular features acquired per animal and per biofluid in the form of Venn diagrams. Feature selection was performed on data processed in the Waters MarkerLynx application, using a mass tolerance of 0.05 Da and a retention time window of 0.2 min as threshold values for peak detection and grouping. Features were considered matched if they had the same m/z and retention time values after processing, and the Venn Diagrams were produced based on these matched features. From Figure 4B,C,E,F, it is evident that the greatest overlap was seen for metabolites from urine and CSF.

Numbers of features in serum were decreased when compared to our previous work where +1500 features were

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reproducibly acquired from 20 μL of plasma.[26] It must be noted that the 15 min gradient used for spot analysis is much shorter than the 60 min gradient used for the previous analysis of plasma by IVDE. This reduction in gradient length is a factor worth considering since the number of distinguishable features diminishes greatly with the length of the run. Such a diminishment can mean an inability to detect small changes in metabolite profiles, such as those associated with lower dose treatments or with changes in low-concentration metabolites. For spots to be a viable alternative for metabolomics studies in animal models, extensive additional work with validation steps will need to cover all aspects of sample handling, shipment, storage, processing (e.g., biofluid temperature and viscosity, punch size, etc.) which have not been considered in the experimental procedure presented here. However, the presented combination of faster screening methods with low sample volumes is likely to be preferred when hundreds or thousands of samples are being investigated in a single experimental design.

CONCLUSIONS

An analytical method for dried biofluid spot metabolomics was designed and tested using samples from rat serum and RPMI cell nutrient medium. Volumes of 10 μL of serum and 10 μL of cell medium spot samples were extracted using in-vial extraction protocols, and selected metabolites of varying lipophilicity and mass were chosen for evaluation of method reproducibility. Peak area variations for these selected metabolites were found to be 1.4−9.4% RSD, an acceptable range for conventional LC−MS procedures. When cumulative fingerprints were subjected to standard multivariate statistical data treatment, fingerprints from the serum ether extracts had the highest variability (15.1% variation), while the serum aqueous extracts and RPMI cell nutrient liquid aqueous extracts accounted for 8.1% and 5.7% variation, respectively. To test the utility of the method in an animal model, the spot-IVDE method was used in the analysis of 86 spots of three different biofluids, which were obtained from healthy rats and mice. A total of 1182 features were recorded in positive ionization mode and 2625 in negative, and from those, 610 and 1078, respectively, were found to be shared in mice and rats. Furthermore, a higher overlap of molecular features was observed between urine and CSF spots for both animals. With this work we have clearly shown that the application of the IVDE method to dried biofluid spots results in reproducible metabolic fingerprints, with sufficient feature coverage for high-throughput metabolomics experiments. The small sample sizes (10−15 μL) required for the spots, and the low transfer losses associated with the IVE/IVDE method, suggest that the spot-IVDE procedure should be widely adopted, in particular for large-scale animal model studies.

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Author Contributions

§A.S. and Y.W. contributed equally to the work performed.

Notes

The authors declare no competing financial interest.

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