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Impact of four different extraction methods and three different reconstitution solvents on the untargeted metabolomics analysis of human and rat urine samples

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ABSTRACT

Unsuitable sample preparation may result in loss of important analytes and consequently affect the outcome of untargeted metabolomics. Due to species differences, different sample preparations may be required within the same biological matrix.

The study aimed to compare the in-house sample preparation method for urine with methods from literature and to investigate the transferability of sample preparation from human urine to rat urine. A total of 12 different conditions for protein precipitation were tested, combining four different extraction solvents and three different reconstitution solvents using an untargeted liquid-chromatography high resolution mass spectrometry (LC-HRMS) metabolomics analysis. Evaluation was done based on the impact on feature count, their detectability, as well as the reproducibility of selected compounds.

Results showed that a combination of methanol as extraction and acetonitrile/water (75/25) as reconstitution solvent provided improved results at least regarding the total feature count. Additionally, it was found that a higher amount of methanol was most suitable for extraction of rat urine among the tested conditions. In comparison, human urine requires significantly less volume of extraction solvent.

Overall, it is recommended to systematically optimize both, the extraction method, and the reconstitution solvent for the used biofluid and the individual analytical settings.

1. Introduction

Metabolomics focuses on a wide variety of low molecular weight metabolites (<1500 Da) in a biological system [1]. Since the metabolome is not limited to endogenous metabolites but also to metabolites from exogenous sources such as drugs, diet, and gut microbiota, a high chemical diversity and complexity of metabolites exists. Especially in untargeted metabolomics, it is desirable to use methods that are not biased for or against specific analyte classes but cover a broad range of metabolites [2]. Amongst others, sample preparation is a critical step with consequences for metabolite extraction and their subsequent detection to obtain high quality and comprehensive metabolomics data [2,3]. Several studies showed that inadequate sample preparation can lead to significantly different metabolites and contradictory biological interpretation or erroneous conclusions [2,4–6]. To obtain as many unknown metabolites with various physicochemical properties as possible, the integrity of the samples should be altered as little as feasible. Hence, an ideal sample preparation should be non-selective, reproducible, simple, and fast [2,7]. Therefore, the general purpose of sample preparation is to reproducibly convert the sample into a format that is compatible with the analytical system while maintaining the original metabolite composition of the sample as much as possible [5]. Since there is no one method that is optimal for all requirements, a compromise is often necessary. Typically accomplished non-selective methods used in untargeted approaches are dilution, solvent precipitation, or solvent extraction [5,7–9]. More selective methods such as solid phase extractions are generally avoided in untargeted approaches [5,8,10]. Additionally, to minimize metabolite loss, it is important to reduce the number of process steps and use short sample preparation time to enable high sample throughput [5].

Due to several advantages such as non-invasive sample collection, large volumes, possibility of repeated sampling, low sample complexity

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compared to plasma, and reflection of the endogenous as well as exogenous metabolic profile, urine has been established as a key biological matrix in metabolomic studies [11,12]. Despite these benefits, urine has a wide range of metabolite concentrations and is thus subjected to variable and unpredictable dilution [12]. Due to this fact and the extreme chemical diversity of the metabolites, appropriate sample preparation is required. For urine, recommendations for very simple sample preparations such as filtration, centrifugation, dilution, or combinations thereof are found in literature, since most analytes are present in sufficiently high concentrations and the protein levels are quite low [1,7,13,14]. However, there are certain classes of compounds such as biogenic amines, lipids, or steroids that are present in lower concentrations and require additional pre-analytical concentration steps [13]. Numerous studies have already shown that sample preparation is highly dependent on the analytical platform [15–22]. There is no consensus and no standard operation procedure (SOP) for the preparation of biological samples, which makes it difficult to compare inter-laboratory studies. Martias et al. have therefore addressed this issue and attempted to describe such an SOP for four different biological matrices including human urine using multiple platforms. As extraction methods for urine, monophasic extraction with two different solvents (MeOH or ACN) and dilution preparation were compared. Results showed that the preparation using MeOH had the largest coverage of metabolite sets [15]. Furthermore, due to species differences, different sample preparations may be required within the same biological matrix from different origins to cover the respective metabolome.

The aim of this study was, on the one hand, to compare an in-house sample preparation method for urine with methods for several analytical platforms derived from literature and, on the other hand, to investigate the transferability of sample preparation from human urine to rat urine. The focus was primarily on protein precipitation with subsequent evaporation to be able to detect metabolites in lower concentration and to achieve a better clean-up of the samples. Therefore, the impact of four different extraction solvents for protein precipitation in combination with three different reconstitution solvents were evaluated using an untargeted liquid-chromatography high resolution mass spectrometry (LC-HRMS) metabolomics analysis of rat and human urine samples to find the most suitable method for each biofluid.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium formate, ammonium acetate, DL-aspartic acid-d_3 (DL-aspartic acid-2,3,3-d_3), cortisol-d_4 (cortisol-9,11,12,12-d_4), creatinine-d_3, formic acid, d-glucose-d_7 (D-glucose-1,2,3,4,5,6,6-d_7), glycine-^{15}N, palmitic acid-d_{31}, and succinic acid-d_4 were obtained from Merck (Darmstadt, Germany). Acetonitrile, ethanol, and methanol (all LC-MS grade) were from VWR (Darmstadt, Germany). Water was purified with a Millipore filtration unit (18.2 Ω x cm water resistance). l-Tryptophan-d_5 was obtained from Alsachim (Illkirch-Graffenstaden, France). l-Carnitine-d_9, cytosine-d_2, d-fructose-$^{13}C_6$, hypoxanthine-d_4, kynurenic acid-d_5, prostaglandin-E_2-d_9, stearic acid-^{13}C, and thymidine-d_4 were purchased from Cayman Chemical (Ann Arbor, MI, USA). DL-Glutamic acid-d_3 (DL-glutamic-2,4,4-d_3 acid), l-arginine-d_7 (L-arginine-2,3,3,4,4,5,5-d_7), and l-lysine-d_3 (L-lysine-2,6,6-d_3) were obtained from Toronto Research Chemicals (Toronto, Canada).

2.2. Sample collection and preparation

Rat urine ($n = 5$) included in this study was used from the control group of a previously published study [23]. Human urine was collected from 10 healthy individuals. Samples were aliquoted and stored at −80 °C. Aliquots were thawed at 4 °C over night and pooled for each species. Pooled urine was centrifugated at 15,000 x *g* at 4 °C for 10 min. For each preparation, 100 µL of supernatants of pooled rat or pooled human urine were transferred into a reaction tube. A total of 12 sample preparations (Table 1) were tested, based on four different extraction solvents. After precipitation, samples were shaken for 2 min at 1500 rpm, precipitated at −20 °C for 30 min, and then centrifuged at 15,000 x *g* and 4 °C for 10 min. The supernatant was transferred in new reaction tubes and evaporated to dryness using a vacuum centrifuge at 1400 rpm and 24 °C. The obtained residues were reconstituted in 50 µL using three different reconstitution solvents (Table 1). Each sample was prepared in quintuplets ($n = 5$). Pooled quality control (QC) samples were prepared by transferring 10 µL of each sample into one MS vial. Extraction solvents and reconstitution solvents were spiked with a total of 19 different isotope labeled endogenous compounds of various compound classes.

2.3. LC-HRMS/MS apparatus

Analysis was performed according to previous published studies [23,24]. Analyses were performed using a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 RS pump consisting of a degasser, a quaternary pump, and an UltiMate Atosampler, coupled with a TF Q Exactive Plus equipped with a heated electrospray ionization (HESI)-II source [24–26]. Performance of the columns and the mass spectrometer was tested using a test mixture described by Maurer et al. [27]. Gradient reversed-phase (RP) elution was performed on a Waters (Eschborn, Germany) ACQUITY UPLC BEH C_{18} column (100 mm x 2.1 mm, 1.7 µm) and gradient hydrophilic interaction chromatography (HILIC) elution using a Merck (Darmstadt, Germany) SeQuant ZIC HILIC (150 mm x 2.1 mm, 3.5 µm). The mobile phase for the RP chromatography consisted of 10 mM aqueous ammonium acetate containing acetonitrile (1 %, *v/v*) and formic acid (0.1 %, *v/v*, pH 3, eluent A) and acetonitrile containing formic acid (0.1 %, *v/v*, eluent B). The flow rate was set from 0 to 10 min to 500 µL/min and from 10 to 13.5 to 800 µL/min using the following gradient: 0–1 min hold 99 % A, 1–10 min to 1 % A, 10–11.5 min hold 1 % A, and 11.5–13.5 min hold 99 % A. The gradient elution for HILIC was performed using aqueous ammonium acetate (200 mM, eluent C) and acetonitrile containing formic acid (0.1 %, *v/v*, eluent D). The flow rate was set to 500 µL/min using the following gradient: 0–1 min hold 2 % C, 1–5 min to 20 % C, 5–8.5 min to 60 % C, 8.5–10 min hold 60 % C, and 10–12 min hold 2 % C. Injection volume was set to 1 µL for all samples. For preparation and cleaning of the injection system, isopropanol:water (90:10, *v/v*) was used. The following settings were used: wash volume, 100 µL; wash speed, 4000 nL/s; loop wash factor, 2. Column temperature for every analysis was set to 40 °C, maintained by a Dionex UltiMate 3000 RS analytical column heater. HESI-II source conditions were as follows: ionization mode, positive or negative; sheath gas, 60 AU; auxiliary gas, 10 AU; sweep gas,

Table 1
Overview of the used sample preparation conditions. Ratio and percent refer to urine or solvent volume. MeOH = methanol, ACN = acetonitrile, and H_2O = purified water. $^+$ according to previous published studies [23,26]; * according to Martias et al. [15].

Preparation	Extraction solvent	Reconstitution solvent
1_1$^+$	Urine:MeOH (1:4)	ACN/MeOH (70/30)
1_2	Urine:MeOH (1:4)	ACN/H_2O (+0.1 % formic acid) (75/25)
1_3	Urine:MeOH (1:4)	ACN/H_2O (+0.1 % formic acid) (25/75)
2_1	Urine:MeOH (1:8)	ACN/MeOH (70/30)
2_2*	Urine:MeOH (1:8)	ACN/H_2O (+0.1 % formic acid) (75/25)
2_3	Urine:MeOH (1:8)	ACN/H_2O (+0.1 % formic acid) (25/75)
3_1	Urine:ACN (1:8)	ACN/MeOH (70/30)
3_2*	Urine:ACN (1:8)	ACN/H_2O (+0.1 % formic acid) (75/25)
3_3	Urine:ACN (1:8)	ACN/H_2O (+0.1 % formic acid) (25/75)
4_1	Urine:ACN:MeOH (2:1:1)	ACN/MeOH (70/30)
4_2	Urine:ACN:MeOH (2:1:1)	ACN/H_2O (+0.1 % formic acid) (75/25)
4_3	Urine:ACN:MeOH (2:1:1)	ACN/H_2O (+0.1 % formic acid) (25/75)

3 AU; spray voltage, 3.5 kV in positive and −4.0 kV in negative mode; heater temperature 320 °C; ion transfer capillary temperature, 320 °C; and S-lens RF level, 50.0. Mass spectrometry for untargeted metabolomics was performed according to a previously optimized workflow [24,28]. The settings for full scan (FS) data acquisition were as follows: resolution 140,000 at *m/z* 200; microscan, 1; automatic gain control (AGC) target, 5e5; maximum injection time, 200 ms; scan range, *m/z* 50–750; spectrum data type; centroid. All samples were analyzed in randomized order, to avoid potential analyte instability or instrument performance to confound data interpretation. Additionally, one QC injection was performed every ten samples to monitor batch effects, as described by Wehrens et al. [29]. TF Xcalibur software version 3.0.63 was used for data handling.

2.4. Data evaluation

According to previously published workflows, data processing was performed in an R environment [26,28]. Thermo Fisher Scientific LC-HRMS raw files were converted into mzXML files using ProteoWizard [30]. XCMS parameters were optimized according to Manier et al. [28] and peak-picking and alignment parameters are summarized in Table S2. Peak picking was performed using XCMS 3 (version 3.20.0) [31] in an R environment and the R package CAMERA [32] was used for annotation of adducts, artifacts, and isotopes. Feature abundances containing the value zero were replaced by the lowest measured abundance as a surrogate limit of detection and the whole dataset was then log 10 transformed. Peak areas were normalized to the different ratios of extraction solvents. To evaluate the number of features that can be detected by the analysis used, total feature count was assessed. Therefore, the number of features which peak area was not declared as not available ("NA") was summed up for each analysis. The autosampler stability of the analytes was tested by determining the coefficient of variation (CV) of the total feature count in the technical replicates ($n = 5$) for each preparation. For the reproducibility, the coefficient of variation (CV) was determined from the peak areas of each sample preparation. In addition to the total feature count, peak areas of spiked internal standards were evaluated to compare each preparation in terms of different compound classes. Statistical evaluation was done using one-way ANOVA as well as Welch's two sample *t*-test for significance comparing total feature count of each group in rat or human urine samples.

R script can be found on GitHub (https://github.com/sehem/urine_preparation.git) and mzXML files are available via Metabolights (study identifier MTBLS8237).

3. Results and discussion

Results of analysis using hydrophilic interaction chromatography (HILIC) with positive and negative ionization are displayed in Figs. 1–4. Those of the analysis using reversed-phase chromatography (RP) are shown in Figure S1–4 in *Supporting information*.

3.1. Study design

Different extraction and reconstitution solvents derived from literature were compared. As already mentioned in the introduction, the focus of sample preparation was on evaporation after protein precipitation in order to pre-concentrate metabolites that are present in lower concentrations, such as fatty acids or biogenic amines, and to ensure a better clean-up of the samples for the subsequent measurement [13,16]. The combination urine:MeOH (1:4) and reconstitution solvent MeOH/ACN is an in-house method and should be used as reference [23,26]. The monophasic extractions urine:MeOH (1:8) and urine:ACN (1:8) were selected based on the study by Martias et al. [15]. The study aimed amongst others to test whether these combinations postulated for multianalytical platforms might be transferred to other laboratories in the sense of an SOP. The study further aimed to test whether this method might also be transferable to the same matrices derived from another species. The fourth extraction consisting of urine:ACN:MeOH (2:1:1) was used based on the publication by Zou et al. who had also investigated different analytical platforms [17]. This kind of extraction was used to utilize the advantages of both solvents. Since not all reconstitution solvents showed good solubility of metabolites and insufficient compatibility with the downstream analytical setup, these should also

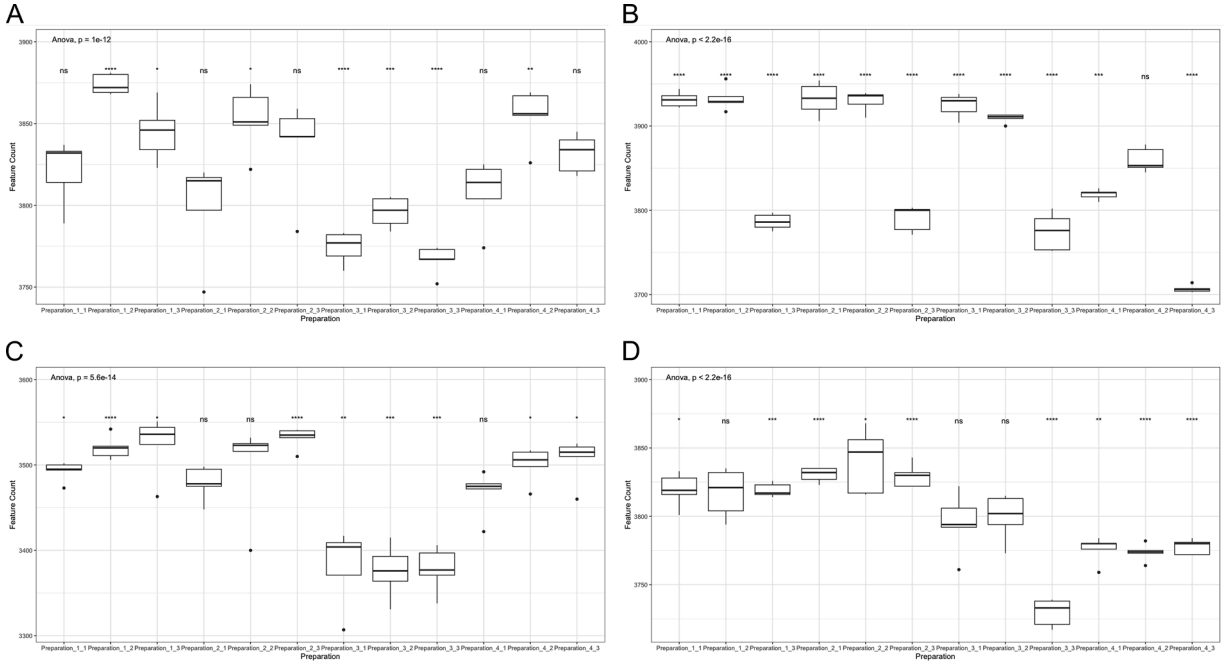

Fig. 1. Results of statistical evaluation using one-way ANOVA and Welch's two sample *t*-test comparing total feature count of each group in rat and human urine samples. Analysis was done using hydrophilic interaction chromatography (HILIC) in positive (pos) and negative (neg) ionization mode. *A* = rat urine pos, *B* = rat urine neg, C = human urine pos, *D* = human urine neg. ns not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

be compared with the different extraction solvents. The reconstitution solvent ACN/MeOH (70/30) is the in-house reconstitution solvent and was considered the best solution by Manier and Meyer based on plasma extracts [18]. Since elevated concentrations of water (H_2O) might impair the performance of HILIC, cause instability, or poor solubilization of certain analytes, different ratios for ACN and H_2O were evaluated in addition to the previously described optimized composition of ACN and MeOH [16,17]. According to Fernández-Peralbo and Luque de Castro, the composition of reconstitution solvent for urine varies from 5 % ACN to 50 % and 75 % up to 100 % [16]. Martias et al. also used different reconstitution agents for the various analytical columns [15]. The ratio ACN/H_2O (75/25) was selected for HILIC among others. As preliminary tests had shown that not all of the residues could be reconstituted with this proportion of water, the ratio ACN/ H_2O (25/75) was also tested. In preliminary studies, we tested various ACN/H_2O ratios with and without formic acid. No significant differences were found neither for the total feature count nor for the selected analytes when formic acid was present or absent. Due to the lack of difference, we decided to use formic acid as additive, since several studies have shown that the addition of formic acid can improve chromatographic performance, increase MS signal response, and can lead to better reproducibility. Additionally, formic acid can overcome matrix effects and thereby increase the sensitivity of MS detection [33–37]. For the reconstitution solvent ACN/MeOH no formic acid was added since it corresponds to our in-house method and should be used for comparison.

To monitor both extraction and reconstitution, isotope labeled endogenous compounds that occur in the matrix under investigation were added to the respective solutions. Therefore, two different standard solutions representing the analytes' physiological concentrations according to human metabolome database (HMDB) [38] were spiked (Table S1). At least one compound from each substance class was added to the corresponding extraction or reconstitution solvent. The concentrations were selected so that they corresponded to the physiological concentrations in matrix.

3.2. Total feature count

Untargeted metabolomic studies aim to detect as many metabolites as possible to describe the metabolome best. Therefore, the size of total feature count was an important parameter to compare the influence of (pre-)analytical methods. Regarding the total feature count, each preparation condition was able to provide a huge number of features and it appears that there is no condition that is ideal for all four analytical methods or both species. However, it appears that the total feature count mainly depends on the used reconstitution solvent and that extraction solvent urine:ACN (1:8) showed the lowest count (Fig. 1 and S1). For rat urine (Fig. 1A+B and S1A+B), the highest feature count was observed for reconstitution solvent ACN/H_2O (+0.1 % formic acid) (75/25) using all four analytical methods. Compared to rat urine, differences were observed for human urine with respect to the used chromatographic method (Fig. 1C+D and S1C+D). For HILIC, reconstitution solvent ACN/ H_2O (+0.1 % formic acid) (25/75) shows the highest effect using positive ionization and for RP reconstitution solvent ACN/H_2O (+0.1 % formic acid) (75/25) using both polarities. Nevertheless, the total feature count was also described as inappropriate parameter since it can be widely differ due to artifactual interference and therefore a method that detects the maximum number of features is not always the method that provides the broadest metabolome coverage. Such artifactual interference can be caused by contamination during metabolite extraction, carryover from previous experiments, background noise detected by MS, or misannotation of data during bioinformatic processing, amongst others [39]. Since this study followed a highly standardized procedure and almost the same conditions for each sample, the variability in the total feature count caused by different mechanisms is rather small compared to the variability caused by different concentrations of the metabolites. Thus, the increase in the total feature count in this study is most likely the result of additional metabolites being detected by using different sample preparations.

To investigate the autosampler stability of the extracts, the total feature count was used. Therefore, the CV of the total feature count in each preparation was calculated to validate stability (Table S3). For each species, column, and polarity, the CV ranged from 0.1 to 3.8 %. This suggests that the features are stable at least on the time scale of an analytical sequence (24 h).

In addition to total feature count, the reproducibility of the features was also evaluated using CV $<$ 20 % (Fig. 2 and S2). Overall, no clear trend was recognizable for one single sample preparation for all four analytical methods. Nevertheless, it can be assumed that for rat urine the reproducibility is higher when using a ratio of 1:8 instead of 1:4 urine: MeOH, and for human urine less extraction solvents is required. This is most probably because rat urine contains a higher protein concentration than human urine and therefore requires a larger amount of solvent for protein precipitation [40]. Again, reconstitution solvents exerted a major impact. In rat urine, the highest reproducibility in positive ionization was for reconstitution solvent ACN/H_2O (+0.1 % formic acid) (25/75) across all extractions, whereas using negative ionization no clear trend can be observed. For human urine, the reproducibility of the peak areas after using different reconstitution solvents highly depended on the used analytical method.

3.3. Metabolite recovery

Since the meaningfulness of the feature count and its reproducibility are to be discussed, the peak areas of selected internal standards were also investigated in every analysis. For this purpose, various isotope labeled endogenous compounds, typically present in the investigated matrix, were spiked into the extraction or reconstitution solvent at physiological concentrations according to the Human Metabolome Database (HMDB) (Table S1) [38]. Results of the mean peak areas of each isotope labeled compound for each preparation in rat and human urine are shown in Fig. 3 for HILIC and in Figure S3 for RP as heat maps. Cortisol-d_4, DL-aspartic acid-d_3, glycine-^{15}N, hypoxanthine-d_4, and stearic acid-^{13}C were not detected at their physiological concentrations in any species or analytical setup. Even in the neat solvents, they could not be detected, which may be due to either low analyte concentration, matrix effects, or poor ionizability. Since most analytes in urine are hydrophilic, the investigated compounds are also primarily detected by HILIC (Fig. 3). Additionally, most hydrophilic compounds eluate within the first 60 s on RP columns which led to an increased risk of ion suppression. As already described for total feature count, the reconstitution solvents showed a greater impact compared to extraction solvents. For rat urine, reconstitution solvent ACN/H_2O (+0.1 % formic acid) (75/25) shows the best results, since no fatty acids were detected using a higher amount of water and very hydrophilic compounds shows quite smaller peak areas using ACN/MeOH (70/30). With respect to extraction solvent, MeOH resulted in the highest peak areas. In human urine, a similar trend was observed as for rat urine, except for the two analytes cytosine-d_2 and thymidine-d_4. Both analytes were not detected, possibly due to specific matrix effects as the analytes were detected in the respective neat solvents. To sum up, reconstitution solvents show the highest impact in case of the investigated analytes.

3.4. Intraday precision

Since the validation of analytical methods for biological samples in targeted analysis is clearly established, standardized guideline for validation of untargeted metabolomics methods is not yet available [41]. However, it is crucial to consider the precision as a key parameter of a method in untargeted metabolomics [15,41,42]. To determine reproducibility, samples were extracted five times and subsequently analyzed. The CV of the peak areas for each isotope labeled endogenous compound in each preparation was calculated to validate reproducibility

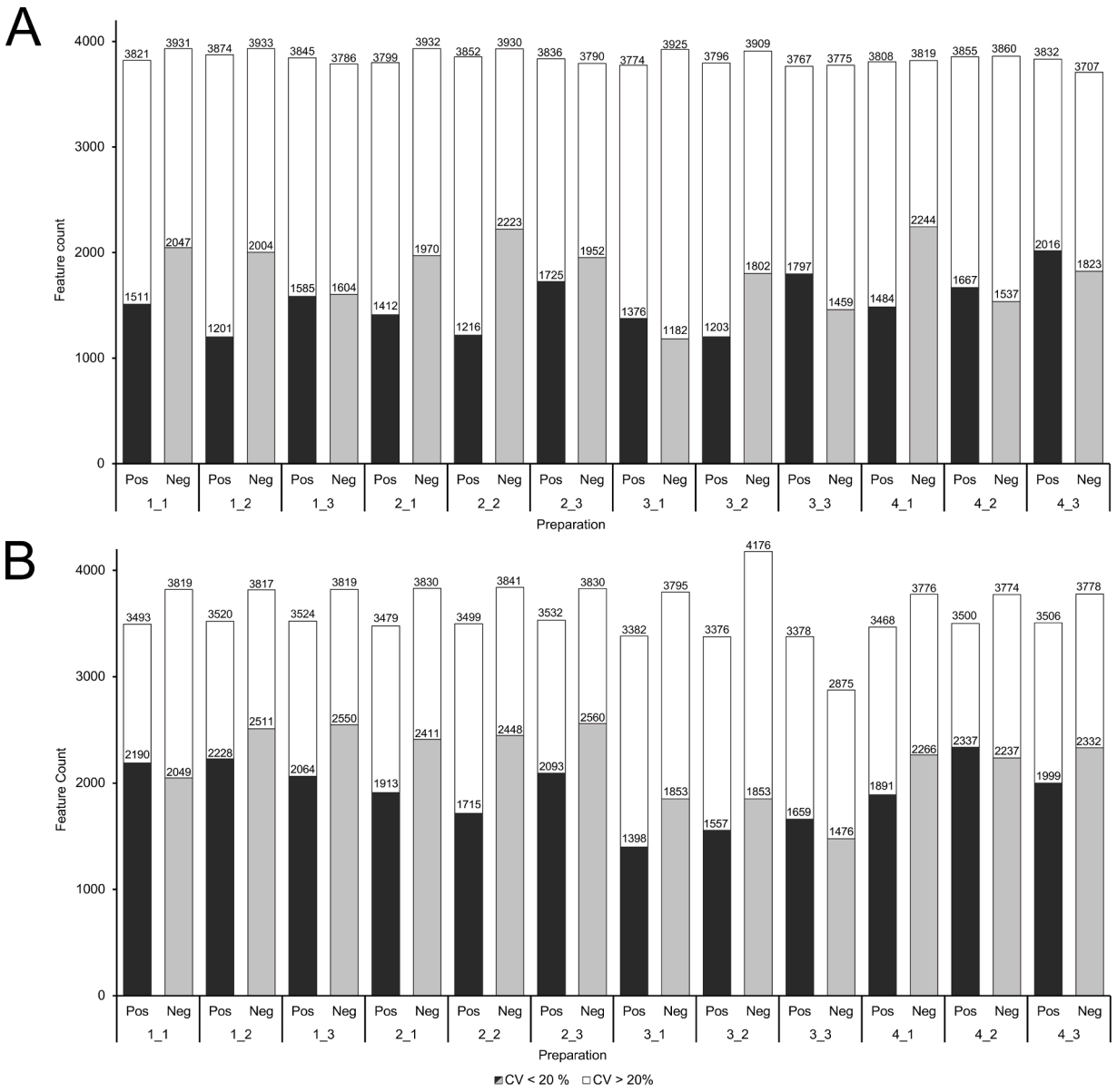

Fig. 2. Histogram of the total feature count extracted for each preparation and their respective reproducibility evaluated by coefficient of variation (CV) in rat urine (A) and human urine (B) using hydrophilic interaction chromatography (HILIC) in positive (pos) and negative (neg) ionization mode. Black or gray filled area indicates the number of features with a CV < 20 %.

(Table S4–7). To optimize sample preparation methods, it was assumed that a CV of less than 20 % would be acceptable for the analytical variability. To ensure better comparability, the mean value of all CVs of the peak areas of all detected isotope labeled endogenous compound were calculated for each column. Results of the mean CV of each isotope labeled compound for each preparation in rat and human urine are shown in Fig. 4 for HILIC and in Figure S4 for RP. However, it is important to note that in sample preparations using reconstitution solvent with a higher content of water, the detection of palmitic acid-d_{31} was not possible. Thus, the mean CV values for the respective methods were calculated without it compared to the other sample preparations. As mentioned above, the spiked compounds are hydrophilic and do not retain efficiently on the RP column. Therefore, the following discussion focuses on the analysis using HILIC column. The results for RP column can be found in the supplementary.

For rat urine, none of the compounds showed a CV higher than 20 % for preparation 2_3. In terms of the mean CV for all compounds, only preparation 3_1 and 3_2 show a CV higher than 20 % (Fig. 4A). Regarding human urine, no analyte exceeded a CV higher than 20 % for preparation 1_3. Upon examining the mean CV for each individual preparation, preparations with ACN/H_2O (+0.1 % formic acid) (75/25) as reconstitution solvent showed a higher CV compared to the other ones (Fig. 4B). Additionally, palmitic acid-d_{31} could not be detected in human urine for preparation 4_2. This might be explained due to possible matric effects in human urine using this preparation, as palmitic acid-d_{31} could be detected in rat urine.

3.5. Summary

Based on all the results described above, it can be summarized that various reconstitution solvents have a greater impact on compound recovery compared to extraction solvents during sample preparation of urine. Even the used chromatography plays a crucial role in the selection of the extraction or reconstitution solvent. However, the use of multiple extraction and/or reconstitution solvents is expected to be unfeasible in most circumstances, as it is time-consuming and costly. It therefore

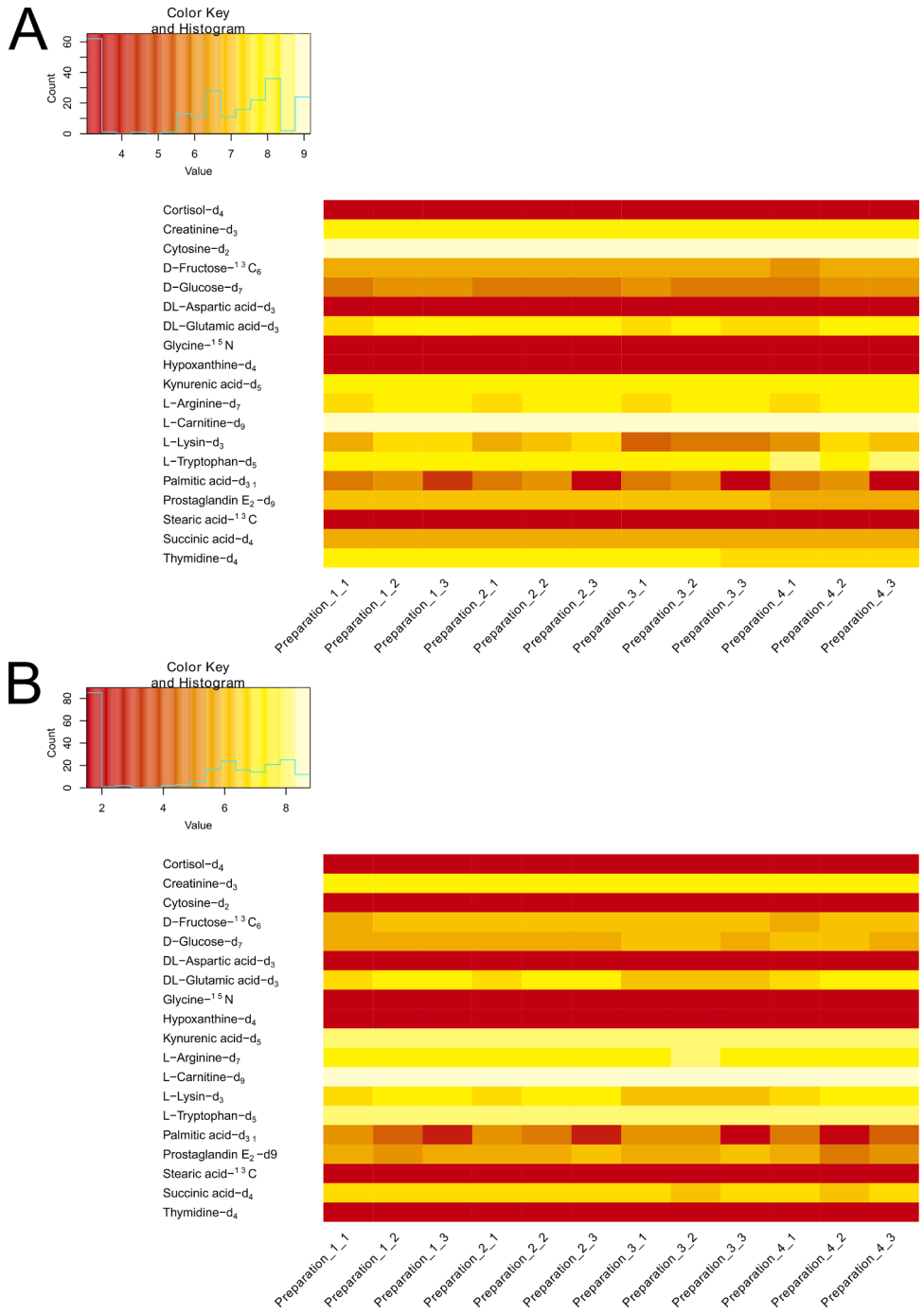

Fig. 3. Heat map of the mean peak areas of isotope labeled endogenous compounds (log 10 transformed) for each preparation in rat urine (A) and human urine (B) using hydrophilic interaction chromatography (HILIC) in positive or negative ionization mode depending on isotope labeled endogenous compounds.

appears to be more reasonable to select solvents that are optimal for each chromatographical method, even if this implies compromising on some performance. Compared to the results of Martias et al. [15], the present study has shown that no SOP is yet available for sample preparation of biological matrices for untargeted metabolomics. Nevertheless, this study also showed that it is not recommended to use the same sample preparation for the same matrix of different species. Even if rat and human urine differ slightly, it should not be automatically assumed that the same preparation will lead to the same results.

However, the results of this study are clearly limited to the investigated compounds and matrix. Therefore, it was not the aim of the study to describe one preparation which is universally applicable. Conversely, this type of study must be conducted for each workflow to evaluate the most suitable solvents.

4. Conclusion

The aim of this study was to compare the in-house urine sample preparation with preparations for different analytical platforms from the literature and to investigate the transferability of sample preparation from human urine to rat urine. Therefore, the impact of four different extraction solvents in combination with three different reconstitution solvents was tested to assess the metabolome by HILIC- and RP-LC in positive and negative ionization HRMS in terms of metabolic coverage

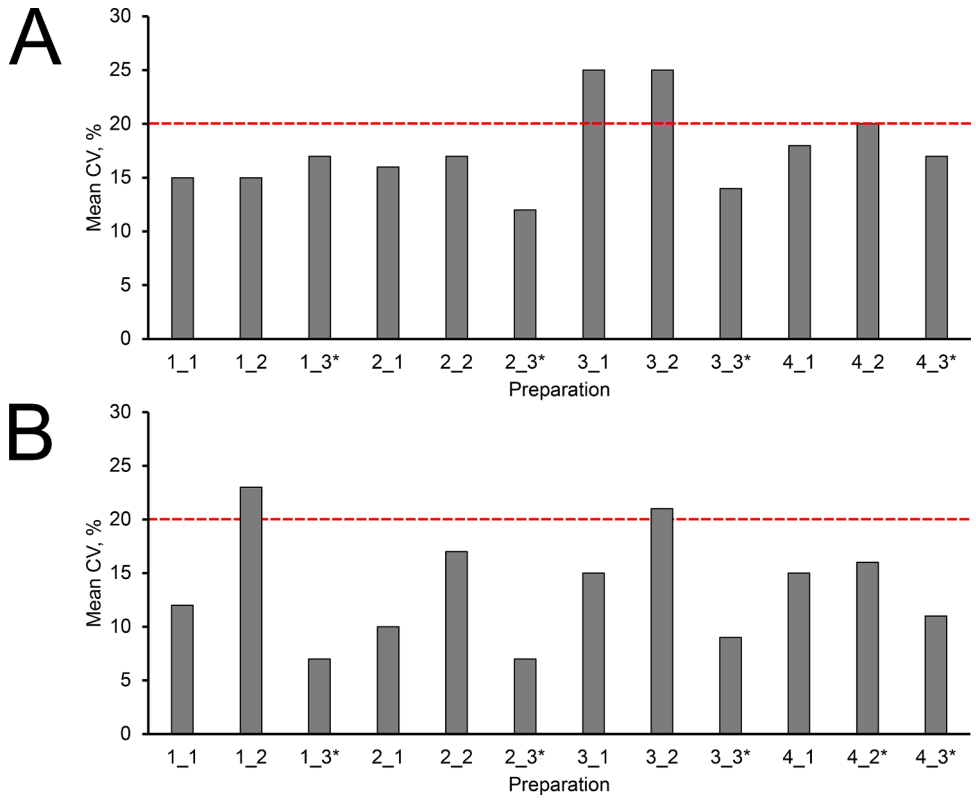

Fig. 4. Bar chart of the mean coefficient of variation (CV) values of the peak areas from all detected isotope labeled endogenous compounds for each preparation in rat urine (A) and human urine (B) using hydrophilic interaction chromatography (HILIC) in positive or negative ionization mode depending on isotope labeled endogenous compounds. The corresponding CVs of each compound can be found in Table S4 and S6. Red dotted line marks the set limit of CV < 20 %. * = CV values were calculated without palmitic acid-d_{31}.

regarding the number of detectable metabolites and highest reproducibility in rat and human urine. Results of this study shows that the feature count and selected compounds mainly depends on used reconstitution solvents. The selection of the solvents is based on analyte properties and therefore, it should be considered in advance which analytes shall be detected. In addition, even the same matrix was investigated, rat urine required higher ratio of extraction solvents. Based on the results of this study, the overall recommendation is a combination of methanol for extraction and acetonitrile/water (75/25) for reconstitution solvent. However, to describe the best metabolome coverage, it is essential to adapt the preparation not only to the investigated species but also to the used chromatographic conditions.

CRediT authorship contribution statement

Selina Hemmer: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Sascha K. Manier:** Writing – review & editing, Conceptualization. **Lea Wagmann:** Writing – review & editing, Conceptualization. **Markus R. Meyer:** Writing – review & editing, Resources, Project administration, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The R script can be found on GitHub (https://github.com/sehem/urine_preparation.git) and the mzXML files used in this study are available via Metabolights (www.ebi.ac.uk/metabolights/MTBLS8237).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.464930.

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