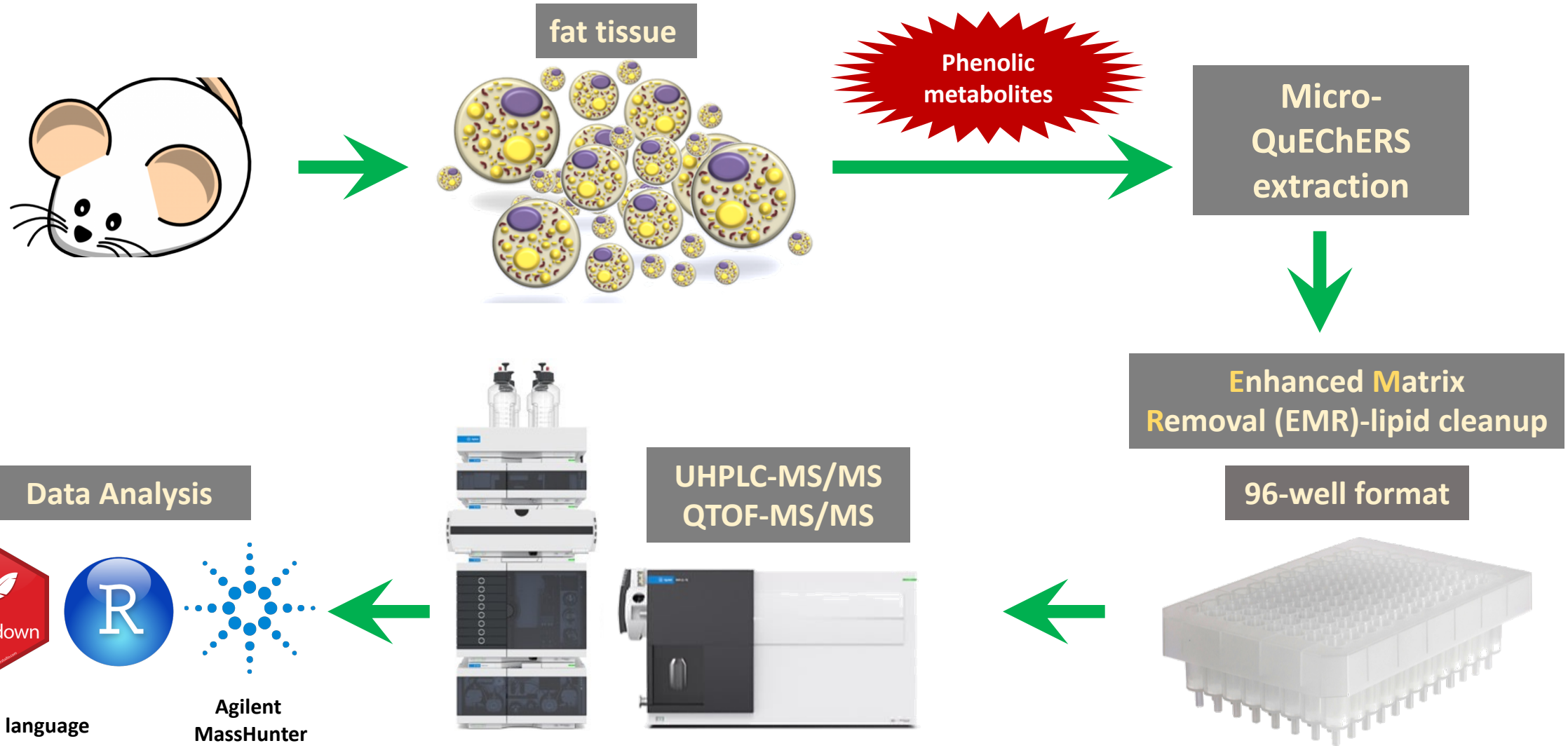
Novelty Statement

This work reported a reliable and high-throughput sample preparation method for analysis of phenolic acids in adipose tissue, using micro-QuEChERS and enhanced matrix removal in 96-well plate format. We also reported the novel use of reversed phase silica sorbent in SpeedVac drying to improve analytes recovery.

Highlight

1. Method developed to quantify raspberry ketone (RK) metabolites in adipose tissue.
2. Enhanced-matrix-removal in 96-well plate applied for efficient sample cleanup.
3. Reversed-phase C18 sorbent exploited for improved recovery from SpeedVac drying
4. Stepwise recovery monitoring technique developed for validation.



Development and validation of a micro-QuEChERS method with high-throughput enhanced matrix removal followed with ultra-high performance liquid chromatography-tandem mass spectrometry for analysis of raspberry ketone-related phenolic compounds in adipose tissues

Bo Yuan [a, b], Danyue Zhao [a, e *], Weiting Lyu [a, c], Zhiya Yin [a, b], Dushyant Kshatriya [d], James E. Simon [a, c], Nicholas T. Bello [d], Qingli Wu [a, b, c, *]

[a] New Use Agriculture and Natural Plant Products Program, Department of Plant Biology and Center for Food Systems Sustainability, Rutgers, The State University of New Jersey, 59 Dudley Road, New Brunswick, NJ 08901, USA

[b] Department of Food Science, Rutgers, The State University of New Jersey, 65 Dudley Road, New Brunswick, NJ 08901, USA

[c] Department of Medicinal Chemistry, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA

[d] Department of Animal Sciences and Nutritional Sciences, School of Environmental and Biological Sciences, Rutgers University, 84 Lipman Drive, New Brunswick, NJ 08901, USA

[e] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, China.

[*] Corresponding authors at:

Department of Plant Biology, Rutgers University, 59 Dudley Road, New Brunswick, NJ 08901, USA.

E-mail addresses: daisydy.zhao@polyu.edu.hk (D. Zhao); qlwu@sebs.rutgers.edu (Q. Wu).

Abstract

Raspberry ketone (RK), a major flavor compound in red raspberries, has been marketed as a popular weight-loss dietary supplement with high potential in targeting adipose tissues. However, challenges in extracting and characterizing phenolic compounds in fatty tissues persist due to the excessively high lipid content and the vast concentration ranges of phenolic constituents accumulating in adipose tissues. In this work, we reported a high-throughput sample preparation method for RK and 25 related phenolic compounds in white adipose tissues using a modified QuEChERS (quick, efficient, cheap, easy, rugged and safe) approach with enhanced matrix removal (EMR)-lipid cleanup in 96-well plates, followed by UHPLC-MS/MS analysis. The absolute recovery at the extraction step was 73~105%, and achieved 71~96% at the EMR cleanup step. The EMR cleanup removed around 66% of total lipids in the acetonitrile extract as profiled by UHPLC-QTOF-MS/MS. The innovative introduction of a reversed-phase C18 sorbent into the extract significantly improved the analytes' recovery during SpeedVac drying. The final accuracy achieved 80~120% for most analytes. Overall, this newly developed and validated method could serve as a powerful tool for analyzing RK and related phenolic compounds in fatty tissues.

Key words: bioavailability, phenolic compound, fatty tissues, lipid removal, solid phase extraction, UPLC-QqQ-MS/MS

1 Introduction

Accurate and efficient analyte determination in high-fat matrices has always been a challenging task. Analyte extraction is typically achieved using solid-liquid, semisolid-liquid or liquid-liquid extraction. Yet, the presence of excessive amount of lipids in the matrix could lead to low extraction recovery and repeatability due to various binding effects. Moreover, the undesired lipids co-extracted from the matrix may contribute significantly to the deterioration of chromatographic column and the suppression of ionization in the mass spectrometer. High efficiency and consistency in both analyte extraction and sample cleanup procedures are crucial for successful downstream analysis particularly in fatty tissues. In the recent decade, the QuEChERS (quick, easy, cheap, effective, rugged and safe) methodology has become widely applied for analyzing a diverse range of analytes in different matrices (**Table 1**). The QuEChERS method was first published by Anastassiades et al. in 2001 for analysis of multi-residue pesticides in fruits and vegetables [1, 2], and later standardized as the official pesticide analysis method by the Association of Official Analytical Collaboration International (AOAC) [3] and European Committee for Standardization (CEN) [4] in 2007 and 2008, respectively. This approach typically involves extraction of analytes using acetonitrile (ACN) (occasionally with other organic solvents) from an aqueous matrix, and the ACN layer is then salted out from the aqueous base by extraction salt of magnesium sulfate and sodium chloride (also referred to as the polishing salt). The high ionic strength in the aqueous phase forces more analytes to enter the ACN phase resulting in high recovery. The ACN extract is then cleaned up with one or a mixture of different types of dispersive solid phase extraction (d-SPE) sorbents. The most used d-SPE sorbents include primary secondary amine (*N*-isopropylethylenediamine or PSA), graphitized carbon black (GCB), octadecyl (C18) silica, Z-Sep (zirconium oxide ZrO_2 bonded to silica) and Z-Sep Plus (ZrO_2 and C18 dual bonded to silica) [5-12]. Other reported sorbents include chitin derived from shrimp shell waste [13], multi-walled carbon nanotubes (MWCNT) [14], composite of MWCNT with amine-

functionalized magnetic Fe_3O_4 nanoparticles [15], and amine modified graphene [16]. With particular regard to cleanup of extract from fatty and other complex matrices, many of these sorbents have been applied with success, such as for cleanup of high-fat fish and meat, oil-enriched plants and beeswax, as summarized in **Table 1**. The use of these dispersive sorbents has also been reported to provide better results than traditional cleanup methods. For example, mixed PSA and C18 were found more effective for coextractives removal from acetonitrile extract of flaxseed oil than gel permeation chromatography, and reduced the co-extractive weight *ca.* four times more than the traditional low-temperature fat precipitation method [5].

Among the various d-SPE sorbents, EMR-lipid stands out as a next-generation sorbent introduced in recent years and is especially designed for removing lipids from sample matrices for improved recovery [17]. Based on size-exclusion and hydrophobic interaction chromatography, it selectively traps the lipids while leaving the analytes in the eluent [18]. EMR-lipid has been applied for cleanup of the extract from various matrices such as fish, meat, oil crops, beeswax and cosmetics (see **Table 1**). Compared with mixed C18 and PSA, Z-Sep and Z-Sep plus, EMR has been shown to give the highest analyte recovery and the lowest matrix effect when dealing with lipid-rich materials, such as olive, olive oil and avocado for pesticide analysis [19]. In a separate study on the analysis of pesticides in spices, EMR sorbent better clarifies sample extracts than PSA, Z-Sep, Oasis®Prime HLB, and Supelclean™Ultra cartridges respectively comprising a top bed of PSA, C18 and GrashsphereTM2031 and a bottom bed of Z-Sep [20].

Raspberry ketone (RK; 4-(4-hydroxyphenyl)-butan-2-one) is a key phenolic aroma compound naturally present in red raspberries (*Rubus idaeus*), and it is generally recognized as safe (GRAS) as a flavoring agent added to cosmetics and food products [21]. In recent years, RK has been marketed in the US as a dietary supplement for weight control given its efficacy in limiting fat accumulation in white adipose tissue in rodents [22-24]. Our previous bioavailability studies on RK and its structurally associated

phenolic metabolites in mice provide important insight into its metabolism and pharmacokinetic behaviors [25]. Notably, we found that adipose tissues tend to take up higher amount of RK and its structurally related metabolites with longer half-life in obese mice than the normal-weight ones. Although we have reported a validated UHPLC-MS/MS method for RK and 25 associated phenolic compounds in mouse plasma and brain samples [26], this sample preparation method involving ethyl acetate extraction was inappropriate for processing adipose and other fatty tissues due to the excessive amount of lipids extractable into ethyl acetate. Thus, improved sample preparation and cleanup method was in much need. In this current work, we reported a novel sample preparation method for analysis of RK and 25 other phenolic compounds in white adipose tissues. To address the problem with the complex bio-matrix highly rich in lipids, we developed and validated a new high-throughput analyte extraction and sample cleanup method by applying the QuEChERS methodology and the next-generation EMR sorbent in a 96-well plate form. In addition, we also reported the use of silica gel and reversed-phase C18 sorbent for their protective effect against analytes loss during SpeedVac concentration and reconstitution.

2 Experiment

2.1 Chemicals and reagents

Twenty-six phenolic compounds were investigated [25, 26], including **phenolic aldehyde and derivatives**: 4-(4-hydroxyphenyl)-butan-2-one (RK) (**1**), 4-(4-methoxyphenyl)-2-butanone (RK-Me) or anisylacetone (**2**), benzylideneacetone (PhLiAce) (**3**), 3, 4-dihydroxybenzylideneacetone (3, 4-DHPhLiAce) (**4**), vanillylacetone (VLAce) (**5**), vanillylidenacetone (VLiAce) (**6**); **phenolic alcohol derivatives**: 4-(4-hydroxyphenyl)-2-butanol (ROH) or rhododendrol (**7**), 2-(4-hydroxyphenyl) ethanol or tyrosol (4-HPE) (**8**), 2-(3, 4-dihydroxyphenyl) ethanol (3, 4-DHPE) or 3-hydroxytyrosol (**9**), 4-hydroxybenzyl alcohol (4-HBOH) (**10**); **phenylpropionic derivatives**: 3-(3-hydroxyphenyl) propionic

acid (3-HPPA) (**11**), 3-(4-hydroxyphenyl) propionic acid (4-HPPA) (**12**), 3-(3, 4-dihydroxyphenyl) propionic acid (3, 4-DHPPA) (**13**), 3-(3-methoxy, 4-hydroxyphenyl) propionic acid or dihydroferulic acid (DFA) (**14**); **cinnamic acid derivatives**: 4-hydroxycinnamic acid (4-HCA) or *p*-coumaric acid (**15**), ferulic acid (FA) (**16**), caffeic acid (CA) (**17**); **phenyl acetic derivatives**: 3-hydroxyphenylacetic acid (3-HPAA) (**18**), 4-hydroxyphenylacetic acid (4-HPAA) (**19**), 3, 4-dihydroxyphenylacetic acid (3, 4-DHPAA) (**20**); **benzoic acid derivatives**: 3-hydroxybenzoic acid (3-HBA) (**21**), 4-hydroxybenzoic acid (4-HBA) (**22**), 3, 4-dihydroxybenzoic acid (3, 4-DHBA) or protocatechuic (**23**), vanillic acid (VA) (**24**), homovanillic acid (HVA) (**25**), and hippuric acid (HA) (**26**). 4-Hydroxybenzoic-d_4 acid (**27**) and *trans*-cinnamic acid-d_7 (**28**) were used as internal standards (IS). The association of analytes with IS refers to our prior report [26]. The reference standards for all analytes and β-glucuronidase (from limpets (*Patella vulgate*), ≥85,000 units/mL, in contamination with sulfatase) were purchased from Sigma-Aldrich (St. Louis, MO, USA), except that standards of **4** and **14** were acquired from Alfa Aesar (Tewksbury, MA, USA), **7** from USP (Rockville, MD, USA), and **16** and **23** from ChromaDex (Irvine, CA, USA). Other reagents including LC/MS grade formic acid, water, acetonitrile, isopropanol and ammonium formate, and ACS grade anhydrous magnesium sulfate and sodium chloride were obtained from Fisher Scientific (Waltham, MA, USA). The sorbents investigated were silica gel, 28~200 mesh (640~74 μm), from Sigma-Aldrich; thin layer chromatography (TLC) silica gel 60 H, 15 μm, from Millipore (Burlington, MA, USA); and Partisil octadecyl C18 silica gel (ODS), 40 μm, from Whatman (Maidstone, United Kingdom).

2.2 Analytical instruments and equipment

Analysis of phenolic compounds was performed using Agilent 1290 Infinity II UHPLC interfaced with 6470 triple quadrupole mass spectrometry (QqQ-MS/MS) with an electrospray ionization (ESI) source with jet stream (Santa Clara, CA). Nitrogen supplied from Parker Balston NitroFlow 60 NA

nitrogen generator (Lancaster, NY) was used as the ESI gas and collision gas. The column used was Waters Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm) with a VanGuard Acquity C18 guard column (5 × 2.1 mm, 1.7 μm) (Milford, MA). The software was MassHunter Workstation Data Acquisition B.08.00, Qualitative Analysis B.07.00 and Quantitative Analysis B.07.01. Sample cleanup was achieved using Agilent Captiva EMR-lipid in 96-well plate (40 mg) and the associated collection plate (Santa Clara, CA), and a Waters 96-well extraction plate vacuum manifold (Milford, MA). The vacuum pump used was a Brinkmann water-recirculating vacuum aspirator, with aspiration hose connected with valves for fine-control of the vacuum. Solvent evaporation and removal were conducted using Thermo Scientific Savant SpeedVac concentrator AES2010 (Waltham, MA).

Analysis of lipids in the extract prior to and after EMR cleanup was conducted using Agilent 1290 Infinity II UHPLC interfaced with 6546 quadrupole time-of-flight mass spectrometry (QTOF). The column used was Agilent Zorbax SB-C18 (100 × 2.1 mm, 3.5 μm) with an SB-C18 guard column (5 × 2.1mm, 1.8 μm). Qualitative Analysis B.10.00, Profinder B.10.00 and PCDL manager B.08.00 was used for data processing.

2.3 Adipose tissue collection

Seven-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were fed a polyphenol-free diet (D12450H; Research Diets, Inc., New Brunswick, NJ; 10% fat, 20% protein, 70% carbohydrate; 3.85 kcal/g) for 14 days before euthanasia for sample collection. The mice were deeply anesthetized with isoflurane and euthanized for collection of the epididymal white adipose depot. The adipose tissue was mixed with 0.2% formic acid at 1:2 w/v, homogenized and snap frozen in liquid nitrogen. Before analysis, the samples were thawed and, if necessary, re-homogenized into a fine creamy and fluid smoothie. All

protocols involving animals were approved by the Institutional animal Care and Use Committee of Rutgers University (OLAW #A3262-01).

2.4 Sample extraction (micro QuEChERS approach) and clean-up

An aliquot of 200 μL of thawed adipose slurry was mixed with 260 μL NaH_2PO_4 buffer solution (pH 5.4) and 80 μL beta-glucuronidase (2000 U, prepared in NaH_2PO_4 buffer, in contamination with sulfatase), and incubated under 37 ℃ for 45 min . After enzymatic digestion, an aliquot of 600 μL acetonitrile (ACN) with 4% formic acid (FMA) was added and vortexed for 20 seconds to extract the analytes. Approximately, 240 mg (220~250 mg weighed in practice) of pre-mixed extraction salt of anhydrous magnesium sulfate and sodium chloride of mass ratio of 4:1 was added into the sample, vigorously vortexed and manually shaken for 1 min. The sample was centrifuged at 10,000 rpm for 10 min. After centrifuge, the fat matrix appeared to be tightly sandwiched between the upper ACN layer (slightly over 600 μL) and the bottom salt-containing aqueous layer. To avoid disturbing the fat matrix, an aliquot of 600 μL of the ACN layer was taken and transferred to an EMR-lipid 96-well plate, and carefully mixed in-well with the pre-added 100 μL water containing 4% FMA. The EMR sorbents were activated with 400 μL 80% ACN containing 4% FMA before the addition of water and ACN. To accommodate the different elution rate in wells of different positions in the plate, elution was conducted under progressively increased vacuum from 1 to 5 inch mercury (in.Hg) at an increment rate of 0.5 in.Hg, and each step was held for 1 min. The plate was covered with a rubber seal to maintain a vacuum environment. A second wash was applied using 200 μL 80% ACN with 4% FMA and eluted under gradually increased vacuum condition in like manner, and finally purged under higher vacuum at 10 in.Hg for 5 seconds to ensure complete elution. The eluent was transferred to 1.5 mL Eppendorf tubes pre-loaded with 2~5 mg reversed-phase C18 sorbent powder, and subjected to dryness using the SpeedVac concentrator under high drying rate. The dried residue was then

reconstituted in 200 µL 60% methanol with 0.1% FMA, vigorously vortexed and then centrifuged at 10,000 rpm for 10 min. An aliquot of 50 µL of the supernatant was transferred to an HPLC vial with insert and was ready for UHPLC-QqQ-MS/MS analysis (*section 2.5*). The sample preparation procedure is summarized in **Fig. 1**.

2.5 UHPLC-QqQ-MS/MS method for phenolic compound analysis

The method was based on our earlier report with modifications [26]. For chromatographic separation, mobile phase A was water with 0.05% FMA and mobile phase B was ACN with 0.05% FMA. The flow rate was 0.45 mL/min. The gradient elution (noted as B%) was 5% at 0 min, 10% at 0.5 min, 28% at 3.8 min, 40% at 3.9 min, 55% at 5.5 min, 80% at 5.6 min and then kept isocratically until 7 min. The column was equilibrated for 2 min before the next injection. The column was thermostatted at 30 °C and the autosampler was maintained at 4°C. The injection volume was 3 µL. For the MS part, the drying gas temperature was at 250℃ with a flow rate of 12 L/min, sheath gas at 250 ℃ and 8 L/min, nebulizer pressure at 30 psi, capillary voltage at +3000 V/ -2500 V, and nozzle voltage of +1500 / -1000 V. The MS was operated in dynamic multiple reaction monitoring (dMRM) mode with switching polarities, at a cycle time of 600 ms. The precursor-to-product ion transitions were the same as previously reported [26].

2.6 Micro QuEChERS-EMR-lipid method validation

The sample preparation method was validated for recovery, matrix effect and accuracy. A mixture of 200 µL of adipose homogenate was digested with the deconjugating enzyme solution and further processed using the QuEChERS and EMR method as described in *Section 2.4* and **Fig. 1**. The blank samples (unprocessed tissue homogenate) were spiked with 10 µL analyte standards and 10 µL of IS. To investigate the stepwise recoveries along the sample processing procedure, spiking was conducted at

different steps: 1) before ACN extraction (noted as the step X), 2) post-extraction and prior to EMR cleanup (PX), 3) post-cleanup and prior to SpeedVac concentration (PC), and 4) after SpeedVac drying and reconstitution and prior to QqQ-MS/MS analysis (PR). Samples spiked at steps of X, PX and PC were eventually recovered in 200 μL reconstitution solvent of 60% methanol with 0.1% FMA, and samples spiked at PR were recovered in 180 μL solvent (plus the spiked 10 μL analyte standards and 10 μL IS to make a total of 200 μL). The analyte recovery at different processing steps, i.e., extraction, EMR-cleanup, and SpeedVac drying and reconstitution, was computed and respectively denoted as X/PX, PX/PC, and PC/PR, with each term here referring to the peak area of analytes spiked at the corresponding step. In addition, 10 μL of analyte standard and 10 μL of IS were spiked into 180 μL of the reconstitution solvent (note as PRneat) in the absence of biomatrices, and the matrix effect was calculated as PR/PRneat. The IS-corrected recovery and matrix effect was calculated using the same formula, but with each term divided by the peak area of the respective IS (selection of the IS refers to our earlier work [26]). Accuracy was determined as the calculated concentration divided by the theoretical value. Accuracy was validated at four concentration levels noted as A, B, C and D, corresponding to respectively *ca.* 1000, 500, 100 and 20 ng/mL in the final reconstituted sample before UHPLC-MS/MS analysis. Recovery and matrix effect were validated at level B.

2.7 Assessment of lipid removal efficiency

To evaluate the removal efficiency of lipids and other matrix following EMR filtration, 600 μL of the ACN supernatant acquired after extraction, and the total eluent collected after EMR cleanup (*ca.* 800 μL) were each brought to a final volume of 1 mL with ACN. The samples were centrifuged at 10,000 rpm for 10 min, and then the supernatant was ready for analysis of lipids in the ACN extract using UHPLC-QTOF/MS (*section 2.8*). The workflow is depicted as the magenta line in **Fig. 1**. The lipid removal

efficiency was calculated as the total lipid peak area after EMR cleanup divided by the peak area before cleanup.

2.8 UHPLC-QTOF/MS for lipid analysis

For the chromatographic part, the mobile phase A was 60% ACN in water, and mobile phase B was 9.5% ACN and 0.5% water in isopropanol (IPA), both mobile phases modified with 10 mM ammonium formate with 0.1% FMA. The flow rate was 1.2 mL/min. The linearly programmed gradient was 15% B at 0 min, 45% B at 2 min, 60% B at 3 min and held to 10 min, 99% B at 15 min and held to 20 min, then back to 15% B at 20.5 min. The column was equilibrated with 15% B for 1 min between injections. The column was thermostatted at 60℃. The autosampler was set to 4℃. The injection volume was 1 μL. Needle wash was conducted using IPA for 10 seconds. For the MS part, the nebulizer was set at 35 psi and the nozzle voltage at 1 kV. The drying gas was 200 ℃ with a flow rate of 13 L/min. The sheath gas was 350 ℃ with a flow rate of 11 L/min. The scan was performed in positive mode from 399 to 1700 *m/z* with an acquisition speed at 6 spectra per second.

2.9 Statistics analysis

Data analyses were conducted using R and the script refers to https://yuanbofaith.github.io/RK_adipose_QuEChERS_EMR/.

3 Results and discussion

3.1 Micro QuEChERS EMR-method development

This work reported for the first time the application of a QuEChERS method in conjunction with a next-generation EMR sorbent in a 96-well plate system for extraction and cleanup of RK and 25 related

phenolic compounds in mouse white adipose tissues. While most published QuEChERS methods involve grams of material to extract from and the extraction is usually performed in a 50 mL tube, in this work, given the limited amount of adipose tissues available from each mouse, the newly-developed micro QuEChERS extraction method was scaled down to perform in 2.0 mL eppendorf tubes. FMA was added to ACN at 4% to bring down the pH of NaH_2PO_4 buffer from 5 to *ca.* 2.5 for enhanced stability of most phenolic compounds. The ratio used among the quantities of biomatrix (including buffer and enzyme), extraction solvent ACN and extraction salt followed the AOAC standard protocol [3] and those practices commonly cited in the literature (**Table 1**).

The QuEChERS method typically involves the use of dispersive SPE (d-SPE) for sample cleanup, and the dispersive format of SPE allows for "on-line" cleanup that is more streamlined than traditional cartridge-based SPE method. When handling a large number of samples, however, d-SPE processing could still be time-consuming. In this work, we report the use of SPE in the 96-well plate format for further improved throughput. In addition, to achieve selective lipid removal, the novel sorbent of EMR was used. While EMR sorbent activation and conditioning (column wetting with solvent before sample loading) was not applied in earlier reports [18, 27], we found that activation and conditioning with 200 ~ 400 μL of 80% ACN resulted in higher recovery and reproducibility. Analyte elution was conducted with 80~85% of ACN (with 4% FMA to be consistent with the extraction solvent), as previously reported [18], which sufficed to recover the majority of the analytes while in the meanwhile trapping the undesired lipids in the sorbent. A second wash with 200 μL of 80% ACN with 4% FMA further improved the recovery for most of the analytes by 5~25%. A volume of second wash beyond 200 μL did not significantly increase the recovery. Another critical parameter to be controlled during EMR cleanup is the elution rate that should be slow enough to allow the lipids to have sufficient time to interact with the sorbent for effective trapping.

Wells in different plate positions presented noted difference in elution rate. To accommodate such variance, vacuum pressure was applied incrementally to ensure consistent result across wells.

3.2 Reducing analyte loss during SpeedVac drying via sorbent addition

While SpeedVac drying before the subsequent reconstitution with a small volume of solvent is a widely applied sample concentration procedure for phenolic compounds and their derivatives, this step alone was found to be a major source of analyte loss in this work. Among all analytes investigated, the recovery of some early to midtime-eluted compounds, 4-HBOH, CA, 3, 4-DHPPA and 3, 4-DHPAA, in particular, was only *ca.* 10~20%, and recovery of the later eluted RK-Me and PhLiAce was less than 1% (in the absence of biomatrical coextractives), as shown in **Fig. 2A**. The cause of analyte loss remains elusive.

Interestingly, the addition of silica gel or reversed-phase C18 sorbent to the analyte extract (*ca.* 2~10 mg per 600 μL ACN extract) before SpeedVac drying, i.e., drying and reconstitution with the sorbent, remarkably protected against analyte loss and therefore significantly improved the recovery. For example, in the presence of the normal phase silica gel (74~640 μm), the recovery of 4-HBOH was improved from 22% to 73%, RK-Me from 0.9% to 66%, and PhLiAce from 0.4% to 16%. Using silica gel of smaller particle diameter (15 μm) led to further improvement in the majority of the 26 analytes with recovery mostly above 80%, including CA and 4-HBOH. This was probably a result of the increased surface area provided by the smaller sized sorbent and thus better protective adsorption effect during the drying step. In addition, sorbent with smaller particle size was also found to significantly reduce the required drying time by *ca.* one hour than using those with larger particle size. Blending with reversed-phase C18 sorbent (40 μm) appeared to further improve the analyte recovery by providing an opposite adsorptive affinity. Such improvement was particularly efficient for certain analytes while marginal to moderate for most

others. For example, 3,4-DHPAA and 3,4-DHPPA showed the highest recovery of *ca.* 55~70% at a 25:75 blending ratio of the normal and C18 sorbent, while PhLiAce showed the most improved recovery up to 62.9% when using the C18 sorbent alone (**Fig. 2A**, without the presence of biomatrical coextractives). Based on our optimization results, the reversed-phase C18 sorbent was applied in the final method.

While the presence of the coextracted biomatrical substance in the extract is generally considered unfavorable to analyte recovery, the adipose coextractives investigated in this work appeared to be protective against analyte loss during SpeedVac drying and reconstitution, resulting in comparable or higher recovery for most compounds than without the coextractives (**Fig. 2B**, red vs. grey bars). In particular, PhLiAce and RK-Me, which were less than 1% recovered in pure solvent after SpeedVac drying as aforementioned, showed much improved recovery (up to 17% and 48%, respectively) in the presence of coextractives. This observation indeed lead us to use sorbents to achieve enhanced adsorptive protection, which provided an efficient solution for analyte loss during sample concentration. In the presence of coextractives and the addition of C18 sorbents (2~5 mg per *ca.* 700 μL ACN extract), the recovery of all compounds was further improved by 3~71%, with the final recovery of PhLiAce and RK-Me elevated to 78% and 90% (**Fig. 2B**, blue vs. red bars; also see **Fig. S1**).

3.3 QuEChERS-EMR and UHPLC-MS/MS method validation

The developed QuEChERS-EMR and UHPLC-MS/MS method was validated for its stepwise recovery, matrix effect and accuracy (refer to **Fig. 1** for individual steps). The absolute recovery (AR) reflects the analytes loss during sample preparation, and mirrors the decreases in signal response and detection sensitivity. The AR, however, does not account for the loss adjusted by IS, and is not directly associated with the final method's accuracy. The IS-corrected recovery (CR) is calculated similarly as AR, except that the analytes peak area is divided by the peak area of the respective IS. Therefore, CR reflects

the final recovery after loss-adjustment. It is not informative of analyte loss that occurred during sample preparation, but is directly relevant to the method's final accuracy. The AR at the extraction step for all 26 analytes ranged from 73 to 105%, showing ideal extraction efficiency. The IS adjusted for such loss and allowed CR to reach 79~125%. The AR at the sample cleanup step using EMR was 71~96%, and the CR for all analytes at this step was 79~99%. The AR and CR at the step of SpeedVac drying and reconstitution was 80~120% for most analytes, but for some analytes the loss was significant, such as 4-HBOH and 3, 4-DHPE with AR and CR of only 28~61% (even with the use of C18 sorbent). For the significantly lost analytes, since the loss of IS at this step was much less, IS did not provide sufficient correction effect on recovery. The overall AR for the entire sample preparation process, from extraction to final reconstitution, was mostly 50~90%, and the CR was 60~110% (**Fig. 3A**).

The matrix effect (ME) in the ESI chamber reflects ionization suppression of the analytes caused by coeluted biomatrical substances in the injected sample, and IS can be used for correction of such effects in like manner as for recovery. While EMR was an efficient method for removal of lipids and other matrices (*section 3.4*), a noticeable ME still existed in the range of 60~91% for most analytes. The use of IS corrected for such effect and gave the corrected ME (CME) at 75~119%. While the biomatrical coextractives usually suppress ionization in the ESI chamber, it is interesting to note in this work that the coextractives generated remarkable signal boosting effects for compounds CA, 4-HBA and 3,4-DHBA, with their ME calculated to be 119~128%, and the CME reaches 147~158%.

The processing efficiency (PE) manifests the collective effect of overall AR and ME, and reflects the analyte and signal loss or gain end-to-end from sample preparation to instrumental detection. The PE for most analytes were 33~66%, and the IS-corrected PE (CPE) were 51~105%. Analytes of 4-HBOH and 3, 4-DHPE, however, had low PE of 12~15% and CPE of 18~23% due to significant loss during the drying

step. For CA, 4-HBA and 3,4-DHBA, though having a good PE of 94~101%, had much elevated CPE (144~163%) after IS-correction due to the excessive ionization enhancement exerted by the ME.

A stepwise dissection of the recovery and matrix effect with or without the IS correction as aforementioned is of great value for validation analysis. In this work, we visualized such analytes / signal loss and gain as a streamlined flow chart throughout the processing procedure (see **Fig. 3B** for RK and ROH; for all the other phenolic compounds, refer to https://yuanbofaith.github.io/RK_adipose_QuEChERS_EMR/Stepwise_Recovery_linePlot.html). As clearly shown from the streamlined plot analysis, although the analyte loss (except those special cases with positive ME effects) at each processing step may not constitute a concern, the accumulated loss across all steps can be very significant. The inclusion of IS was of vital importance to compensate for such loss, with overall correction efficacy (i.e., regarding the CPE) for most analytes by 20~40%. In the meanwhile, it is important to recognize that the IS did not always follow the same percentage of loss/gain as the analytes, with under-correction and over-correction to various extent at different steps, and the overall correction was the accumulated corrective effect across all steps.

The validated accuracy was calculated taking the entire dynamic linear range into consideration. It would be identical to CPE at the same spike level if perfect linearity, i.e., passing the origin, $R^2 = 1$, and error-free experimental conditions could be achieved. The accuracy assessed at four spike levels (*ca.* 2000, 500, 100 and 20 ng/mL in injected samples) for the analytes was mostly 80~120% (**Fig. 3B**), though for analytes such as CA and 3, 4-DHBA exhibiting signal-enhancing ME, the recovery was much higher (up to 160%), and for analytes with considerable loss during the drying step, such as 4-HBOH and 3, 4-DHPE, the accuracy was undesirably below 40%, as in similar case of CPE. It is worth mentioning that the accuracy variance was closely and negatively associated with the spike amount, and the lower spike amount lead to higher variance (**Fig. 3C**). A major contributor to such effects arose from the error

associated with measurement of the background, i.e., endogenous compounds in the blank samples, which needs to be subtracted in calculation.

3.4 Assessment of lipid removal efficiency following EMR cleanup

While the QuEChERS method allowed for easy removal of the majority of lipids from adipose tissues, a moderate amount of undesired lipids can still partition into the ACN layer. As analyzed by UPLC-Q-TOF/MS, the major lipids coextracted into the ACN layer included phospholipids conjugated with choline, ethanolamines and serines; sphingolipids; and di- and triacylglycerols. The lipid molecules were found to have acyl groups with 14 to 24 carbons and up to five double bonds (**Fig. 4A** and **B)**. After EMR clean-up, the early eluted lipids (peaks **1**-**6**) were significantly reduced, and the later eluted lipids (peaks **7**-**10)** were no longer detectable. The lipids removal efficiency was *ca.* 66% calculated by comparing the total integrated peak area (**1**-**10**) before and after EMR clean-up. Meanwhile, the baseline intensity was also much decreased, showing the removal of a wide range of lipids as well as other matrical substances after EMR cleanup.

4 Conclusion

In the present work, a high-throughput sample preparation method employing micro QuEChERS and enhanced matrix removal (EMR)-lipid cleanup in 96-well plate was developed and validated for 26 phenolic compounds in mouse adipose tissues. In particular, sample cleanup using EMR sorbent successfully removed 66% of total lipids, and the application of reversed-phase C18 silica gel sorbent significantly reduced analyte loss during the SpeedVac drying and reconstitution procedures. The accuracy validated at four spike levels showed 80~120% accuracy for most analytes. Our results demonstrated that the newly developed method can be applied for efficient extraction and accurate

quantitation of RK and related phenolic compounds in fatty tissues. The application of our method could be further expanded to other phenolic compounds with similar structures as the 26 analytes studied in this work for future bioavailability studies.

One limitation of this work is the analyte loss during SpeedVac drying despite the protective effect from the use of reversed-phase C18 silica gel sorbent. Future research could involve a thorough investigation of the issue with analyte loss by comparing different drying apparatus and methods. In addition, for phase II metabolites deconjugation, the excessive enzymes used in this work, while allowing for efficient metabolites hydrolysis, introduced substantial background interference and resulted in significant measurement variability for trace analysis. As such, future work is needed to investigate ways to reduce such background interference.

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Table 1. Representative QuEChERS and related methods in the literature for various analyte analyses in high-fat matrices.

No.	Analytes	Matrix	Extraction solvent #	Extraction salt	Dispersive sorbent *	Instrument	Reference
1	34 pesticides	Flaxseeds, peanuts and dough (11.4 ~ 42.2% lipid in sample)	ACN	$MgSO_4$ & NaCl (4:1)	PSA & C18 (3:1)	GC-TOF MS	Thailand & USA [5]
2	33 polycyclic aromatic hydrocarbons	High-fat smoked salmon (3~11% fat in sample)	Acetone, ethyl acetate & isooctane (2:1:1)	$MgSO_4$ & NaCl (4:1); or $MgSO_4$, NaCl, TC & DH (4:1:1:0.5)	PSA, C18 & $MgSO_4$ (1:1:3)	GC-MS (SIM)	USA [6]
3	3 pesticides	Meat and bovine fat	ACN	$MgSO_4$ & NaCl (4:1)	PSA & C18 (1:1) for meat; PSA alone for bovine fat (following hexane defatting)	GC-MS(SIM)	Brazil [7]
4	136 pesticides	Avocado	ACN with 1% AA	$MgSO_4$ & NaOAc (4:1)	PSA, C18 & $MgSO_4$ (1:1:3) §	LC-MS (79 analytes) & GC-MS (57 analytes)	USA [8]
5	28 pesticide	Rapeseed, soybean, peanut & sesame seeds	ACN	NaCl	Amine-modified graphene	UHPLC-MS/MS	China [16]
6	15 polycyclic aromatic hydrocarbons	Salmon	ACN	$MgSO_4$ & NaCl (4:1)	EMR-lipid ¶	GC-MS	USA [17]
7	7 neonicotinoid insecticides	Cocoa beans and shells	ACN	$MgSO_4$ & NaOAc (4:1)	PSA, C18 & GCB (1:1:1)	HPLC-MS/MS	Ghana & Denmark [28]
8	67 pesticides	Olive, olive oil and avocado	ACN	$MgSO_4$ & NaCl (4:1)	EMR-lipid	UHPLC-MS/MS	Spain [19]
9	28 carbamates	High-fat cheese	ACN with 1% AA	$MgSO_4$ & NaCl (4:1)	Z-Sep plus (ZrO_2 and C18 dual bonded to silica)	UHPLC-MS/MS	Spain [10]
10	205 pesticides	Spices	ACN	$MgSO_4$, NaCl, TC & DH (4:1:1:0.5)	EMR-lipid	GC-MS/MS	Spain [20]
11	7 neonicotinoid insecticide	Beeswax	Methanol and ethyl acetate (7:3)	No salt used (no liquid-liquid partition)	EMR-lipid	UHPLC-QTOF	Spain [29]
12	39 veterinary drugs	Pork and bovine muscle, porcine and chicken liver and kidney, etc.	EDTA solution, ACN with 2% FMA and 2% DMSO	No salt used (no liquid-liquid partition)	EMR-lipid (cartridge format)	UHPLC-MS/MS	USA [18]
13	methyldibromo glutaronitrile (a now-prohibited preservative)	Creamy cosmetic products	ACN	No salt used (no liquid-liquid partition)	EMR-lipid	GC-MS	Italy [30]
14	Cholesterol and its 7 oxidative products	Meat from pork, chicken & fish; fat of tallow, lard and butter	Acetone	$MgSO_4$ & NaOAc (4:1)	PSA, C18 & $MgSO_4$ (1:1:3)	GC-MS	China [9]
15	5 heterocyclic amines	Cooked beef, chicken and bacon (3~43% fat in samples)	ACN with 1% FMA (for beef) or 2% FMA (for chicken and bacon)	$MgSO_4$ & NaCl (4:1)	EMR-lipid ¶	UHPLC-MS/MS	USA [31]
16	flubendiamide (a pesticide)	bee pollen	ACN	NaCl	EMR-lipid (4 min dry ice chill before clean up)	HPLC-MS	Spain [32]
17	21 polycyclic aromatic hydrocarbons	Smoked mackerel fish	ACN	$MgSO_4$ & NaCl (4:1)	EMR-lipid	GC-MS	Czech & Poland [33]
18	26 phenolic compounds	Mouse white adipose tissue	ACN	$MgSO_4$ & NaCl (4:1)	EMR-lipid (96-well plate)	UHPLC-MS/MS	**Our method**

Abbreviations: PSA, primary secondary amine (*N*-isopropylethylenediamine); GCB, graphitized carbon black; EMR, enhanced matrix removal; NaOAc, sodium acetate; TC, trisodium citrate dihydrate; DH, disodium hydrogencitrate sesquihydrate; AA, acetic acid. SIM,

selected ion monitoring. #, mixed extraction solvent is noted by the volume ratio in the parenthesis. Apart from the organic solvent, water is often added to moisten the dried material as needed and to increase the aqueous base (not shown here). *, the sorbents are in dispersive format unless otherwise specified. Mixed sorbents of different types are noted by the mass ratio in the parenthesis. The $MgSO_4$ in the sorbents are for removal of water in the extract prior to GC analysis, not for the purpose of adsorption and removal of coextractives in the extract. In contrast, the $MgSO_4$ in the extraction salt is for purpose of liquid-liquid salted-out separation. The $MgSO_4$ of this table all refers to the anhydrous format. §, for analytes using LC-MS, the ACN extract was directly injected into LC-MS after extraction without sorbent cleanup. ⁋, sample preparation involved EMR cleanup, followed by salting out of the ACN layer, a reverse order of the typical QuEChERS procedure where salting out precedes sorbent cleanup.

Fig. 1. Sample preparation and validation workflow for analysis of raspberry ketone (RK)-related phenolic metabolites in mouse adipose tissue using QuEChERS (quick, easy, cheap, efficient, rugged and safe) and EMR (enhanced matrix removal)-lipid 96-well plate. Abbreviations: ACN, acetonitrile; FMA, formic acid. The black solid lines indicate the workflow for analysis of RK metabolites, and the magenta dashed lines indicate the workflow for assessment of lipid removal efficiency through comparison of lipid content in the ACN extract before and after EMR cleanup. The peak area of analytes and internal standard spiked at various steps are noted as: X, prior to extraction; PX, post-extraction and prior to EMR clean up; PC, post-clean up and prior to SpeedVac drying; PR, post-reconstitution in solvent. ※, this PR step involves reconstitution of the dried residue in 180 μL solvent, followed with the addition of 26-analytes standard mixture (10 μL) and internal standard (10 μL) to make a total of 200 μL. PRneat involves spiking directly in neat solvent without biomatrices. Validation of stepwise recovery, matrix effect and processing efficiency follows formula (1) ~ (6), and terms X, PX, PC, PR and PRneat refers to the absolute peak area. The associated internal standard (IS)-corrected metrics are computed with all terms divided by the peak area of corresponding IS.

Fig. 2. Effect of sorbent and biomatrical coextractives on the recovery of analytes during SpeedVac drying and reconstitution. (**A**), optimization of sorbent formulation consisting normal phase (NP) silica gel and reversed-phase (RP) C18 silica gel. The analytes were in neat solvent of acetonitrile (ACN) with 4% formic acid (FA) when SpeedVac dried, in the absence of biomatrical coextractives. The numbers indicate the blending ratio or percentage, e.g., 25 NP - 75 RP referring to 25:75 blending ratio of NP and RP phase. NP (big size), NP and RP refer to the corresponding normal or reversed phase sorbents with particle size or diameter of 74~640 μm, 15 μm and 40 μm, respectively. The total amount of sorbents used was *ca.* 10 mg per 600 μL ACN with 4% FMA during SpeedVac drying. (**B**), effect of biomatrical coextractives and C18 sorbent (40 μm in diameter) on the recovery of analytes. The C18 sorbent used was 2~5 mg per 700~800 μL ACN extract with 4% FMA. The ACN extract was prepared following the QuEChERS-EMR procedure, spiked with analyte standards after EMR cleanup and prior to drying. The compounds are arranged in decreasing order of overall recovery. The grey bars show the same data as in the first boxplot on the most left side of panel (A).

Fig. 3. Validation parameters of the entire protocol (QuEChERS + EMR-lipid cleanup + UHPLC-MS/MS analysis). (**A**), absolute recovery and IS-corrected recovery at each processing step: extraction (1), EMR sample cleanup (2), SpeedVac drying and reconstitution (3), and matrix effect (ME) at the electrospray ionization (ESI) (4). The overall recovery reflects the accumulative recovery from steps (1) to (3), and overall processing efficiency reflected the accumulative recovery from (1) to (4). The IS-corrected recovery reflects the recovery after IS-correction for signal loss during each processing step. (**B**), streamlined flow chart of the absolute (red) and IS-corrected (blue) recovery for RK and one of its major metabolite rhododendrol (ROH). The figures in the empty rectangles along the vertical lines indicate the analytes' loss at each processing step, and the figures in rectangles with filling along the horizontal lines indicate the accumulated recovery at the given processing step. For flow chart of all analytes, refer to https://yuanbofaith.github.io/RK_adipose_QuEChERS_EMR/Stepwise_Recovery_linePlot.html. (A) and (B) were validated at a spike level of *ca.* 500 ng/mL (actual LC-MS injection concentration; level B). (**C**), validated accuracy at four spike levels, i.e., *ca*. 2000, 500, 100 and 20 ng/mL (levels A, B, C and D, respectively). (**D**), correlations between validated accuracy variance with spike level and background content.

Fig. 4. Assessment of matrix removal efficiency using the EMR 96-well plate. (**A**), total ion chromatogram (TIC) of the lipophilic coextractives in the acetonitrile extract before (red trace) and after (black trace) the EMR cleanup; (**B**) identities of major peaks; (**C**) comparison of total peak area of identified compounds. Lipid abbreviations: PC, glycerophosphocholine; PS, glycerophosphoserine; PE, glycerophosphoethanolamines; PS, glycoerophosphoserines; SM, sphingomyelins; DG, diradylglycerolipids; TG, triradylglycerolipids.

Figure 1

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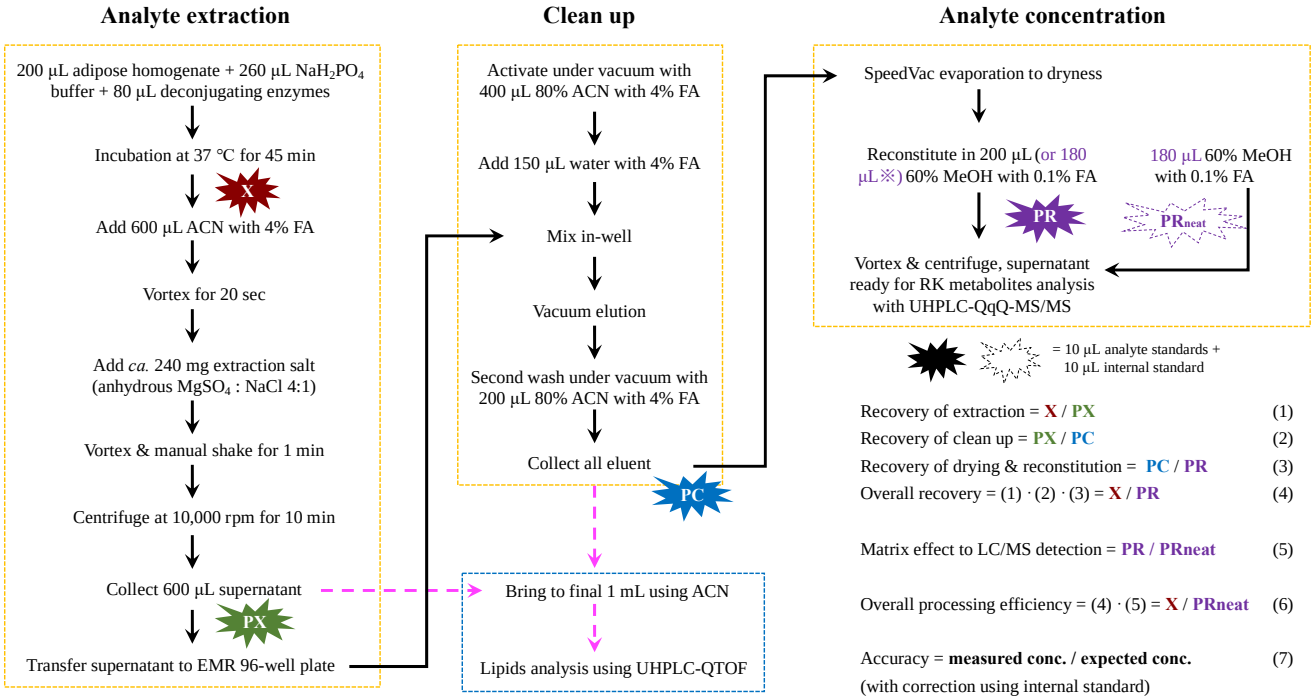

Recovery of extraction = **X** / **PX** (1)

Recovery of clean up = **PX** / **PC** (2)

Recovery of drying & reconstitution = **PC** / **PR** (3)

Overall recovery = (1) · (2) · (3) = **X** / **PR** (4)

Matrix effect to LC/MS detection = **PR** / **PRneat** (5)

Overall processing efficiency = (4) · (5) = **X** / **PRneat** (6)

Accuracy = **measured conc. / expected conc.** (7)
(with correction using internal standard)

Figure 2

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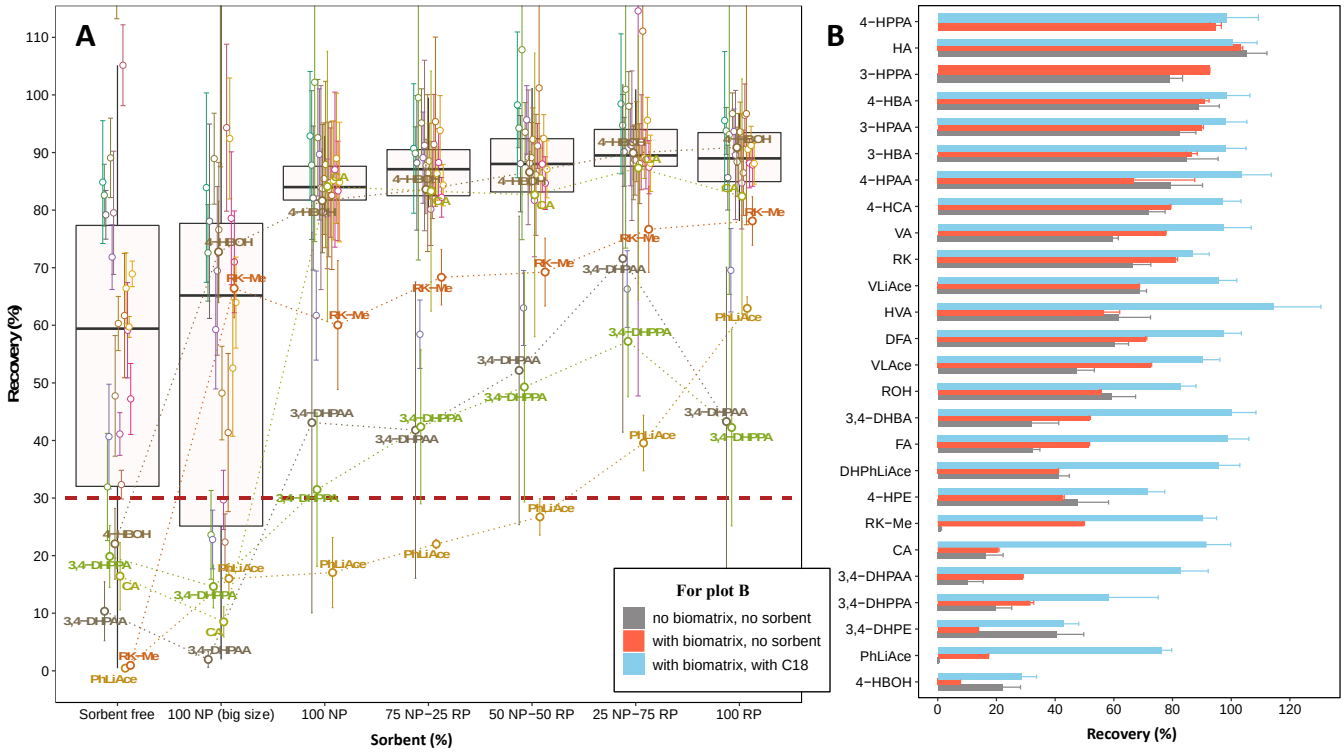

Figure 3

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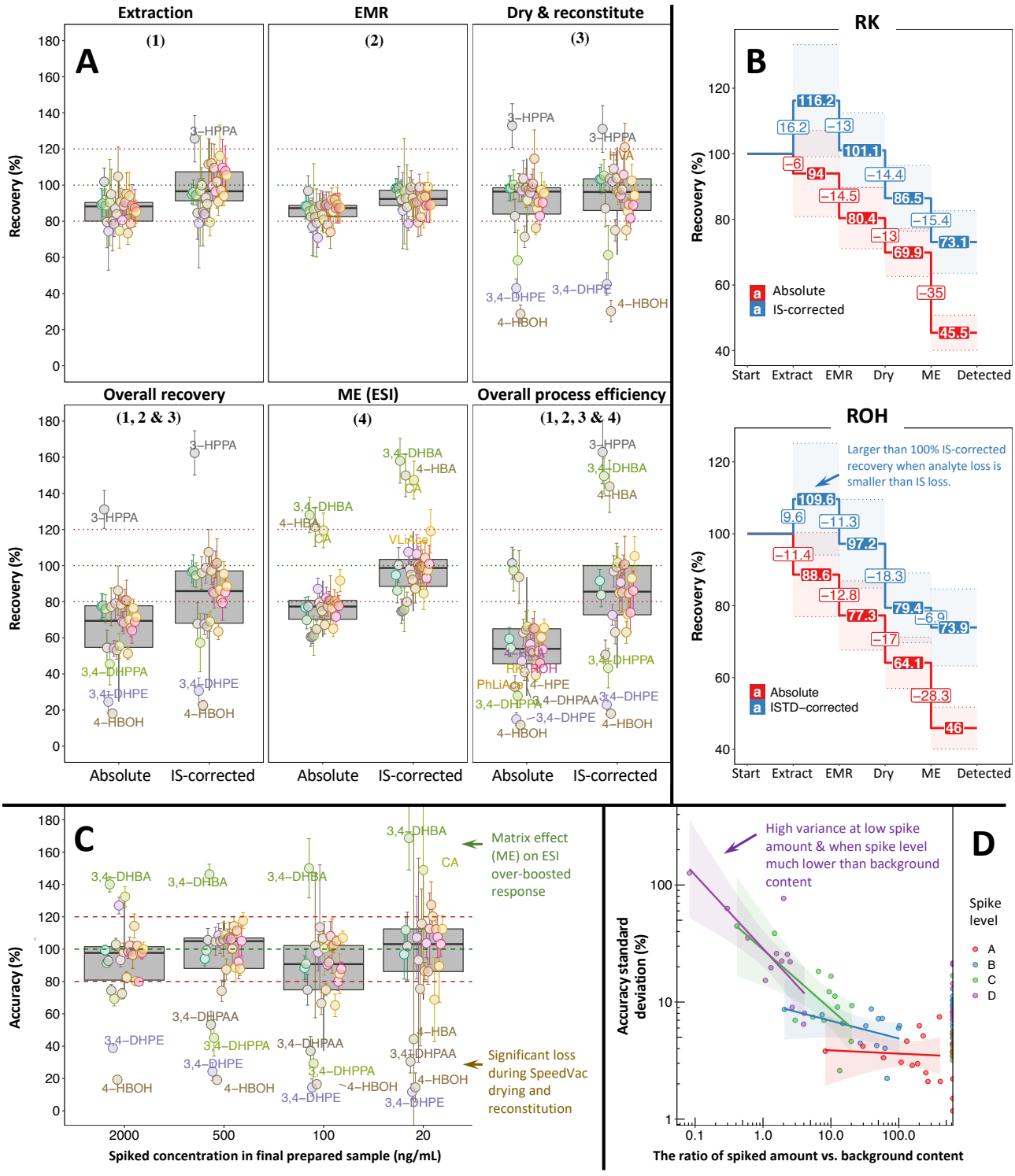

Figure 4

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