

IMPROVED LIPID EXTRACTION**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims benefit of priority to U.S. Provisional Application Serial No. 63/548,338, filed November 13, 2023, the entire contents of which are hereby incorporated by reference.

TECHNICAL FIELD

[0002] The present disclosure provides, inter alia, methods of extracting lipids from samples. The methods comprise a step of precipitating proteins and recovering lipids from the supernatant, as well as washing the protein pellet followed by collecting lipids from the wash. In some embodiments, the methods improve the recovery of certain lipid classes, such as triglycerides (TG, or sometimes called TAG) and cholesterol esters (CE). The methods may optionally further comprise extraction of metabolites, proteins, and/or nucleic acids. After extraction, the extracted analyte(s) can be analyzed, for example with a mass spectrometer or other methods known in the art.

SUMMARY

[0003] Provided herein are methods for extracting lipids from a sample, the methods comprising:

- (a) precipitating proteins from the sample to result in a sample mixture comprising protein precipitates and a supernatant, and separating the protein precipitates from the supernatant;
- (b) passing the supernatant through a solid-phase extraction matrix having an affinity for lipids to result in a first flow-through;
- (c) washing the protein precipitate with a first lipid elution solution to obtain a wash;
- (d) eluting the solid-phase extraction matrix after (b) with a second lipid elution solution to obtain a second flow-through;

wherein the wash and the second flow-through contain lipids.

[0004] The methods may further comprise adding water to the supernatant before passing the supernatant through the solid-phase extraction matrix. The second lipid elution solution may comprise the wash. The first lipid elution solution and the second lipid elution solution may have the same composition. The methods may further comprise additional elution(s) of the solid-phase extraction matrix with lipid elution solution(s) after (d). The methods may further comprise collecting metabolites from the first flow-through.

[0005] The methods may further comprise eluting the solid-phase extraction matrix prior to (d) with a metabolite elution solution to collect metabolites in the resulting eluate. The methods may further comprise combining the resulting eluate with the first flow-through.

[0006] The methods may further comprise collecting proteins from the protein precipitates. The protein precipitates may not be separated from the supernatant by filtration in the methods. The protein precipitates may be separated from the supernatant by centrifugation. The proteins may be precipitated using a solution comprising methanol and ethanol. The proteins may be precipitated using 50:50 methanol/ethanol (v/v). The water content of the sample mixture at the time of said separating is 40% or less, 35% or less, 30% or less, 25% or less, 20% or less, 15% or less, 10% or less, or 5% or less.

[0007] The sample may be plasma, serum, a body fluid besides serum or plasma, or lysed cells. The cells may be from blood, cultured suspension cells, cultured adherent cells, 3D cultured cells (e.g. organoids, spheroids, cell cultures grown in 3D supports), or from tissue.

[0008] The sample may comprise cells that have been treated with a solution comprising a fluoroalcohol. The fluoroalcohol may be one or more of the following: 2,2,2-trifluoroethanol, 2,2-difluoroethanol, 2-fluoroethanol, hexafluoro-2-propanol, nonafluoro-tert-butyl alcohol, 1,1,2,2,2-pentafluoroethanol, and/or 2,2,3,3,3-pentafluoro-1-propanol. The fluoroalcohol may be 2,2,2-trifluoroethanol.

[0009] The method may comprise automated steps.

[0010] At least one of the lipid elution solutions may be 2:1 methanol:dichloromethane.

[0011] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the

invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF DRAWINGS

[0012] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0013] FIG. 1. Mass spectra showing the lipid recovery profiles after the 2in1 (black) and 3in1 (gray) workflows.

[0014] FIG. 2. Mass spectra showing the lipid recovery profiles after the 2in1 (black), 3in1 (without pellet wash, light gray), and the new 3in1 (with pellet wash, dark gray) workflows.

[0015] FIG. 3. Recoveries for various lipid classes when performing the 2in1 (light gray), 3in1 (without pellet wash, dark gray), and the new 3in1 (with pellet wash, gray) workflows.

[0016] FIG. 4. A block diagram for one embodiment of a unified workflow 10 according to the disclosure.

DETAILED DESCRIPTION

Definitions

[0017] In this disclosure, the term “metabolites” refer to one or more compounds which are substrates and/or products of a metabolic process (reaction). Metabolites are usually small molecules (less than 1500 Da), not proteins or nucleic acids, although amino acids, small peptides (such as bi- or tri-peptides), nucleotides, and small oligonucleotides (such as di- or tri-nucleotides) may be metabolites). Metabolites may include substrates and/or products which are produced by metabolic processes (reactions) in a living cell including, but not limited to the reactions of central carbon metabolism, including those involved in glycolysis, tricarboxylic acid cycle (i.e., TCA cycle, Krebs cycle), reductive pentose phosphate cycle (i.e.,

Calvin cycle), glycogen metabolism, pentose phosphate pathway, among other metabolic processes. Accordingly, metabolites may include, but are not limited to, glucose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-phosphate, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, 1,3-bisphosphoglycerate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, acetyl CoA, citrate, cis-aconitate, d-isocitrate, α -ketoglutarate, succinyl CoA, succinate, fumarate, malate, oxaloacetate, ribulose 1,5-bisphosphate, 3-phosphoglycerate, 1,3-bisphosphoglycerate, glyceraldehyde 3-phosphate, ribulose-5-phosphate, ethanol, acetaldehyde, pyruvic acid, 6-phosphogluconolactone, 6-phosphogluconate, ribose-5-phosphate, xylulose-5-phosphate, sedoheptulose 7-phosphate, erythrose 4-phosphate, among other metabolites.

[0018] Metabolites include compounds with various chemical structures from various chemical classes, including, but not limited to, organic acids, sugars, sugar phosphates, amino acids, nucleobases, nucleotides, drug metabolites, steroids, fatty acids and triglycerides. Some metabolites are lipids or lipophilic compounds, including lipids that have been modified with one or more polar groups during various metabolic reactions. These metabolites may be referred in this disclosure as lipid, polar lipid, or lipid-like metabolites.

[0019] Metabolites may include drug compounds and drug metabolites or food compounds and food metabolites. Thus, metabolites in this disclosure include both metabolites naturally produced by a cell and/or an organism and/or xenobiotics.

[0020] A xenobiotic is a chemical compound, e.g., an antibiotic, an inactivated steroid, or any drug metabolite, which is present in a biological sample, e.g. urea or blood, but which is not naturally produced or not expected to be present within this biological sample, or not expected to be present in the amount at which it is present in the biological sample.

[0021] The term “metabolic reaction” means any chemical reaction involved in catabolism and/or anabolism. These are any chemical reactions that occur in living organisms, usually in a living cell (either in vivo or ex vivo).

[0022] The term “metabolism” is the sum of all metabolic reactions.

[0023] The term “biological sample” refers to a whole organism or a subset of its tissues, cells and/or components (e.g. tissue cell culture, body fluids, including but not limited to blood, plasma, serum, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva,

bronchiolar lavage, gastric lavage, amniotic fluid, amniotic cord blood, urine, vaginal fluid, semen and feces). A “biological sample” can also refer to intact cells, a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs. In certain embodiments, the biologic sample has been removed from an animal, plant, and/or fungus.

[0024] Biological samples of the present disclosure may comprise cells. The term “cells” is used in its conventional sense to refer to the basic structural unit of a living eukaryotic or prokaryotic organism. In certain embodiments, cells include prokaryotic cells, such as bacteria or archaea. Cells may include eukaryotic cells. Cells may include plant cells or fungal cells. Cells may include, but are not limited to, cultured cells (e.g., tissue culture cell lines), bacterial cells, recombinant cells which are cells which have been modified in laboratory by for example, gene editing or by any other means known to a person of skill, yeast cells and/or primary cells which may be obtained from an animal, a human, a plant and/or fungus. Biological sample may comprise fungal cells, cells of the domain archaea, cells comprising a larger biological sample (e.g., cells from a biopsy or tissue sample). Cultured cells include, but are not limited to, cells cultured in suspension, cells harvested from adherent cells, or cells from 3D cultures (e.g., organoids, spheroids, cell cultures grown in 3D supports).

[0025] In some of the present methods, a solution comprising a fluoroalcohol is used to lyse a biological sample which comprises cells. By “lyse” cells, it is meant that the cell membranes are at least partially permeabilized such that at least some of the cell content, including at least some of the metabolites, can leak out from the cell. In some instances, the cell membranes may be ruptured or broken open. In some embodiments, a cell lysis may further include a lysis of cellular organelles, for example the nucleus, mitochondria, ribosomes, chloroplasts, lysosomes, vacuoles, Golgi apparatus, such that the contents of the cellular organelles are also released into the surrounding medium.

[0026] In the present disclosure, the term “fluoroalcohol” means an organofluorine compound which comprises a hydroxyl group (-OH) and one or more of fluorine-carbon bonds. Preferably, the fluoroalcohol is an organofluorine compound which comprises a hydroxyl group (-OH) and one or more of fluoroalkyl groups, fluoroalkenyl groups and/or fluoroalkynyl groups. Suitable fluoroalcohols include those with terminal and/or internal fluoroalkyl groups,

fluoroalkenyl groups and/or fluoroalkynyl groups. More detailed descriptions of fluoroalcohols can be found, for example, in US Patent Application Publication No. 20200393342, which is herein incorporated by reference in its entirety, particularly with respect to the various methods and reagents for isolating metabolites, lipids, proteins and/or nucleic acids disclosed therein.

Description of the Invention

[0027] We have developed workflows to isolate both metabolites and lipids (“2in1”), or metabolites, lipids and proteins (“3in1”). The 2in1 methods comprise treating a sample containing metabolites and lipids with a mixture comprising methanol and ethanol, which precipitates proteins, adding water to increase metabolite recoveries, and passing the treated sample through a solid phase extraction (SPE) matrix which has an affinity for lipids. The flow-through from the SPE matrix contains metabolites, and lipids can be eluted from the SPE matrix with a lipid elution solution. The 3in1 methods are similar to the 2in1, but proteins are pelleted prior to SPE extraction. The pellet is then analyzed for proteins.

[0028] We noticed unexpectedly that lipid recovery was affected in the 3in1 workflow. Lipids recovered from plasma samples using the 3in1 and 2in1 methods, respectively, were analyzed by mass spectrometry, and compared to each other in Figure 1.

[0029] As shown in Figure 1, certain lipid classes were selectively reduced in the 3in1 results, particularly lipids eluting later from the LC column in the region where triglycerides and cholesterol esters elute.

[0030] The present invention improves lipid recovery by washing the protein pellet in the 3in1 methods with a lipid elution solution, and recovering lipids from the wash in addition to lipids obtained from eluting the SPE matrix. Figure 2 shows the results of the improved 3in1 vs. 2in1 workflows, and the lipid recoveries were generally comparable. In fact, the recoveries of some lipid classes for the improved 3in1 workflow, such as triglycerides and cholesterol esters, were unexpectedly even higher than those seen in the 2in1 workflow.

[0031] The differences in recoveries of various lipid classes are more clearly shown in the bar graph in Figure 3 below.

[0032] Thus, the present invention provides a method of lipid extraction from a sample, comprising precipitating proteins from the sample and separating the protein precipitates from a supernatant; washing the protein precipitate with a first lipid elution solution to obtain a wash; passing the supernatant that has been separated from the protein precipitates through a solid-phase extraction matrix having an affinity for lipids to result in a first flow-through, and eluting the solid-phase extraction matrix with a second lipid elution solution to obtain a second flow-through, wherein both the wash and the second flow-through contain lipids. Optionally, the solid-phase extraction matrix can be further eluted with additional lipid elution solutions to collect additional lipids in the eluate(s). The wash and the second flow-through (and any other additional eluates) can be combined for lipid analysis and/or recovery. In fact, in some embodiments, the wash is used to elute the solid-phase extraction matrix (i.e., the second lipid elution solution is or comprises the wash). In some other embodiments, the first and second lipid elution solutions are the same. In yet other embodiments, the second lipid elution solution does not comprise the wash, and it is different from the first lipid elution solution in composition. The lipids can be analyzed by any method known in the art, and/or collected for further use. The lipid elution solution can be any solution suitable for the particular solid phase extraction matrix being used, as a person of ordinary skills in the art can decide. For example, a lipid elution solution may comprise dichloromethane and/or chloroform, and optionally also comprise one or more of MTBE, butanol, methanol, ethanol, and isopropanol. In some embodiments, the lipid elution solution is 2:1 methanol:dichloromethane.

[0033] The first flow-through contains polar metabolites. Optionally, the supernatant, after proteins are removed and before being passed through the solid-phase extraction matrix, can be diluted with water to improve metabolite recoveries in the first flow-through. Optionally, after the first flow-through is collected, the solid-phase extraction matrix can be eluted with a metabolite elution solution and the flow-through can be combined with the first flow-through to collect metabolites.

[0034] In some embodiments, organic solvents are used to precipitate proteins from the sample, such as a solution containing methanol and ethanol (for example 50:50 (v/v) of methanol:ethanol). For example, 20 μ L of plasma may be mixed with 112.5 μ L of 1:1 methanol:ethanol, and the resulting protein precipitates are subsequently separated from the supernatant. In this case, the water content of the sample mixture at the time of separating protein precipitates and supernatant is 15%. If, after the precipitation but before separating the

protein precipitates, water is added to the sample mixture, the water content of the sample mixture would be higher than 15%. In some embodiments, the water content at the time of such separation is up to 40%, 35%, 30%, 25%, 20%, 15%, 10% or 5%. As mentioned above, in some embodiments, water is added to the supernatant after protein precipitates are removed, to increase the water content in the supernatant. The protein precipitating solvent can be any solvent or solvent combination known in the art, for example ethanol:acetonitrile (e.g. 1:1), ethanol:methanol (e.g. 1:1), or ethanol.

[0035] The sample may be a biological sample. In some embodiments, the sample comprises cells. In some embodiments, the sample (especially samples that contain cells) is first treated with a fluoroalcohol to lyse cells and/or stop metabolic reactions (i.e., quench metabolism), as described in more detail in US Patent Application Publication No. 20200393342.

[0036] In some embodiments, the present invention is used to detect and analyze lipid profiles, which are helpful in, for example, the diagnosis, monitoring and treatment of lipid disorders. Lipid disorders include, but are not limited to, primary lipid disorders such as hypercholesterolemia and hypertriglyceridemia, and disorders that result in abnormal lipid profiles, such as diabetes mellitus, hypothyroidism, nephrotic syndrome, renal disease, obstructive liver disease, and dysproteinemias. Likewise, lipid profiles are also useful for monitoring impacts of drugs that change lipid profiles.

[0037] In some embodiments, other analytes are also detected and analyzed in addition to lipids, for example metabolites, proteins and/or nucleic acids. In some embodiments, the protein precipitates obtained from the sample are analyzed for proteins. In some embodiments, the first flow-through from the SPE matrix contains metabolites and can be analyzed accordingly. In some embodiments, nucleic acids are isolated (e.g., by filtration) prior to precipitating proteins. Any combination of the analytes can be chosen according to the purpose of the user, such as lipids and metabolites, lipids and proteins, lipids and nucleic acids, and lipids, metabolites and proteins.

[0038] The sample **16** may be a fluid such as plasma or serum, which may proceed directly to block **22**. Or the sample may initially contain cells, such as **12**. The option of using a cell-containing sample is illustrated by dashed lines. The cell-containing sample is contacted with a metabolism-quenching (MQ) solution, **14**. This results in the lysis of the cells and

quenching of metabolism, and produces a mixture comprising metabolites and one or more of the proteins/peptides, lipids and/or nucleic acids, at block 16.

[0039] The metabolites in the mixture are protected from being a substrate for metabolic reactions. By being protected it is meant that at least some enzymes are inactivated at least partially in the mixture and at least some metabolic reactions are inhibited. Accordingly, at least some metabolites are protected (quenched) at least partially for at least a period of time from being a substrate in a metabolic reaction.

[0040] The biological sample can be prepared prior to being contacted with the MQ solution. For example, cells can be pelleted (i.e. via centrifugation) and optionally washed and/or resuspended in a suitable buffer. For example, cells can be filtered via a filter plate to remove cell media and optionally washed with a suitable buffer. For example, the cell culture media can be removed from adherent cells, and a wash buffer, such as PBS or isotonic ammonium bicarbonate can be added and removed (fully or partially) from the cells.

[0041] Optionally, the sample from block 16 can be filtered at block 18 or this step can be omitted. Suitable filtration methods may employ a membrane filter, PVDF (polyvinylidene), nylon, PTFE (polytetrafluoroethylene), PC (polycarbonate), PP (polypropylene), PES (polyether sulfone), PVC (polyvinyl chloride), CA (cellulose acetate), CMF (coated cellulose acetate), HDPE (high density polyethylene), regenerated cellulose and/or glass fiber filter or a stacked combination of filter types with appropriate pore size for the sample being used. Other filter types may be used too. In some applications, the mixture is filtered through a glass fiber filter at block 18. Various glass fiber filters typically used for isolation of nucleic acids can be used. For example, borosilicate glass fiber filter can be used with a various pore size. Typically, a pore size can be in the range from 0.5 μm to 3 μm . The pore size can be adjusted to optimize the recovery from a particular biological sample. For microsamples, the filtration can be accomplished in filter plates, e.g. in a 96-well plate or 384-well plate. Glass fiber filter plates are commercially available from many different suppliers.

[0042] The filtration can be further optimized as needed and may include application of vacuum pressure and/or positive pressure. The filtration can also include wash steps wherein additional fluoroalcohol solution, or other solution, is used to wash the filter. This may increase the recovery of metabolites or other components of the lysate.

[0043] The pore size of the glass fiber filter can be further optimized, depending on whether nucleic acids, such as DNA and/or RNA need to be analyzed. In some embodiments, glass fiber filters are used uncoated. In other embodiments, glass fiber filters may be coated with a binder to improve the binding of nucleic acids (NAs) to the filter. Additives or binders may be also used to repel the binding of certain metabolites or other cell components.

[0044] Nucleic acids such as DNA and/or RNA collected on the glass filter or otherwise at block 18 can be removed and collected at block 20. One method to remove DNA from the glass filter is to add at block 20 a solution with a low (acidic) pH to protonate the DNA and disrupt some of the bonding interactions between the DNA and glass. Another commonly used method is to elute DNA and/or RNA with a low salt buffer. Low salt buffers can be made using high (about 1M) concentrations of EDTA. Purified, deionized water, which has salt ions removed, is another low salt buffer.

[0045] Filtration at block 18 may be set up such that while nucleic acids are captured, the other components of the lysed biological sample (proteins, lipids, and metabolites) flow through.

[0046] After filtration at block 18 or if filtration is omitted, then directly from block 16, the flow through lysate solution or sample comprising proteins, lipids, and metabolites is subjected to protein precipitation at block 22. These protein precipitates are pelleted, and proteins and peptides can be collected from the pellet for further use (block 24). The supernatant is passed through an SPE matrix (block 26), and the pellet is washed with a lipid elution solution (block 36). In some embodiments, the wash is used to elute the SPE matrix as described above in this disclosure and shown in Figure 4. Alternatively, the SPE matrix can be eluted with a lipid elution solution that does not contain the wash, to obtain an eluate. The eluate and the wash, both containing lipids, can be combined or remain separate for further analysis and/or lipid recovery.

[0047] Various SPE-matrixes are known. A matrix that separates lipids from metabolites based at least in part on the affinity of the lipids to the matrix is a preferred matrix in the present methods because it allows for robust separation of polar metabolites from the lipids. Suitable SPE matrix includes solid phase sorbents described in US Patent publication 2018/0080858. Commercially available SPE-cartridges and plates can be also used. One of the commercially available product families for capturing lipids and separating metabolites

from the lipids is the CAPTIVA™ EMR-lipid SPE cartridges and plates available from Agilent Technologies, Santa Clara, CA, USA. Other commercially available sorbents can be also used.

[0048] At block 26, the metabolites flow through the EMR-lipid cartridge or plate, and the sorbent captures the lipids and provides a solution containing predominantly polar metabolites, which can be used for a metabolite analysis. It should be noted under some purification conditions, some lipid compounds such as short-chain fatty acids and sterols can be found in the solution with metabolites, including polar metabolites.

[0049] After the lipids are collected in the flow through solution at block 28, they can be analyzed by liquid chromatography/mass spectrometry (LC/MS) at block 40. If needed, a solvent can be changed prior to the analysis at block 40. This also applies to the metabolite fraction of the sample, and it could apply to the nucleic acid fraction as well. The solvent change also provides an opportunity to make the fractions more concentrated, so low-abundance analytes are more easily detected by LC/MS.

[0050] LC/MS and other methods for analysis can also be used for lipids, metabolites, proteins/ peptides, and nucleic acids. In the diagram of Figure 4, the LC/MS analysis is listed. However, the compounds obtained in the present methods can be analyzed by any other methods typically used in the art.

Example 1

Lipid Identifications Using Various Workflows

[0051] The 2in1, 3in1 (no pellet wash), and new 3in1 (with pellet wash) workflows were performed on plasma samples, and the results are shown in Figures 1-3, as discussed above. Materials and methods are described below.

2in1 (metabolite and lipid) workflow

[0052] Briefly, plasma samples were mixed with 1:1 methanol:ethanol and incubated, followed by adding water. The mixture was passed through a Captiva EMR-Lipid plate (Agilent Technologies). The flow-through was collected, which contained polar metabolites. The plate was eluted with two portions of 2:1:1 water:methanol:ethanol to collect more metabolites in the flow-through. The plate was then eluted with a lipid elution solution, 2:1 methanol:dichloromethane. Lipids were collected in the eluate. The plate was eluted again

with the same lipid elution solution, and the eluate was combined with the first eluate, dried, reconstituted, and analyzed by a mass spectrometer.

[0053] Automation can be added to the 2in1 workflow. For example:

1. 20 μ L aliquots of plasma were transferred to wells of a 96-well plate.
2. Approximately 130-160 μ L 1:1 methanol:ethanol was added to wells of a separate 96-well plate.
 - a. In some iterations, the 1:1 methanol:ethanol solution included a pre-spike of deuterium-labeled lipids (dissolved in 1:1 methanol:ethanol) for assessment of lipid recoveries. A pre-spike of a deuterium-labeled lipid(s) can also be used as an internal standard(s).
 - b. As an alternative, the 1:1 methanol:ethanol can be added to a single well reservoir.
3. 2:1:1 water:methanol:ethanol was poured into a single-well reservoir.
 - a. As an alternative, the 2:1:1 water:methanol:ethanol can be added to wells of a 96-well plate.
4. milliQ water was added to a 96-well plate, with 92.5 μ L (112.5 μ L minus the plasma sample volume) added to wells of the plate.
 - a. As an alternative, the water can be poured into a single-well reservoir.
5. The plasma plate, 1:1 methanol:ethanol plate, water plate, and 2:1:1 water:methanol:ethanol plate were added to an Agilent Bravo Liquid Handler equipped with supplementary protocols for processing plasma samples. All other necessary consumables, e.g. Captiva EMR-Lipid plate, metabolite collection plate, and tips for liquid and sample transfers were also added to the Bravo.
6. Using the supplementary protocols, the Bravo transferred 112.5 μ L 1:1 methanol:ethanol to the plate holding the plasma samples. The plasma and 1:1 methanol:ethanol were mixed using pipet mixing and plate shaking. The 1:1 methanol:ethanol precipitated proteins from the plasma.
7. An optional 10 min incubation was completed to equilibrate the samples.
 - a. Bath sonication is not recommended for these samples, as it can cause significantly slower filtration of the samples through the Captiva EMR-Lipid plate.

8. The Bravo transferred the plasma and 1:1 methanol:ethanol mixture to a plate containing 92.5 μL (112.5 μL minus the plasma sample volume) of water in the plate wells. The samples were mixed using pipet mixing.
 - a. As an alternative, the Bravo can transfer 92.5 μL (112.5 μL minus the plasma sample volume) of water to each well containing the plasma and 1:1 methanol:ethanol mixture. For this option, the samples can be mixed using pipet mixing and plate shaking.
9. A second optional 10 min incubation was completed to equilibrate the samples.
 - a. Bath sonication is not recommended for these samples, as it can cause significantly slower filtration of the samples through the Captiva EMR-Lipid plate.
10. The samples (plasma + 1:1 methanol:ethanol + water) were transferred to a Captiva EMR-Lipid plate. The samples were pulled through the plate using vacuum pressure.
 - a. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional sample solution.
 - b. As an alternative, the samples can be pushed through the plate using positive pressure. After an initial lower vacuum pressure filtration, a higher positive pressure can be used to elute additional sample solution.
11. Polar metabolites were collected in the flow-through.
12. The Captiva EMR-Lipid plate was washed with 250 μL 2:1:1 water:methanol:ethanol, using vacuum pressure to move the solution through the plate. The flow-through was collected with the previous flow-through, adding to the polar metabolite fraction.
 - a. The 250 μL 2:1:1 water:methanol:ethanol was used to wash the plate that previously contained the plasma + 1:1 methanol:ethanol + water samples prior to adding the 250 μL 2:1:1 water:methanol:ethanol to the Captiva EMR-Lipid plate.
 - b. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional wash solution.
 - c. As an alternative, positive pressure can be used to move the 2:1:1 water:methanol:ethanol solution through the plate. After an initial lower positive pressure filtration, a higher positive pressure can be used to elute additional wash solution.

13. The Captiva EMR-Lipid plate was washed a second time with 250 μ L 2:1:1 water:methanol:ethanol, again using vacuum pressure to move the solution through the plate. The flow-through was collected with the previous flow-through, adding to the polar metabolite fraction.
 - a. The 250 μ L 2:1:1 water:methanol:ethanol was used to wash the plate that previously contained the plasma + 1:1 methanol:ethanol + water samples, prior to adding the 250 μ L 2:1:1 water:methanol:ethanol to the Captiva EMR-Lipid plate.
 - b. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional wash solution.
 - c. As an alternative, positive pressure can be used to move the 2:1:1 water:methanol:ethanol solution through the plate. After an initial lower positive pressure filtration, a higher positive pressure can be used to elute additional wash solution.
14. This was the end of the metabolite extraction portion of the workflow.
 - a. The polar metabolite collection plate can be retained and the samples can be dried under the flow of nitrogen with 30-37°C heating applied to the bottom of the plate.
 - i. As an alternative, a vacuum concentrator can be used to dry the samples.
 - b. Dried samples can be stored at -80°C prior to analysis. For analysis, the samples can be re-dissolved in an LC/MS compatible solvent. Samples can be shaken, mixed with a vortex mixer, or bath sonicated to aid in dissolution. Samples can be centrifuged to bring samples to the bottom of the wells or to remove debris. Sample supernatants can be transferred to a new plate or LC/MS vials prior to analysis.
15. The Bravo was reset in order to complete the lipid elution. The Captiva EMR-Lipid plate was retained, as it contained lipids.
16. The lipid elution solution, 2:1 methanol:dichloromethane, was added to a 96-well plate. The plate was glass-lined to prevent leaching of contaminants into the lipid elution solution.
 - a. As an alternative, a glass or glass-lined reservoir can be used to hold the 2:1 methanol:dichloromethane.
17. The Bravo was used to transfer 900 μ L 2:1 methanol:dichloromethane to the retained Captiva EMR-Lipid plate. Vacuum filtration was used to pull the lipid

elution solution through the Captiva EMR-Lipid plate. The target flow rate for lipid elution was 1 drop every 3-5 seconds.

- a. As with the metabolite extraction portion of the workflow, positive pressure can be used to push the lipid elution solution through the Captiva EMR-Lipid plate.
18. When no lipid elution solution remained in the wells of the Captiva EMR-Lipid plate, a higher vacuum pressure was used to elute additional lipid elution solution that was held up in the Captiva EMR-Lipid plate.
 - a. As an alternative, when no lipid elution solution remains in the wells of the Captiva EMR-Lipid plate, a higher positive pressure can be used to elute additional lipid elution solution that is held up in the Captiva EMR-Lipid plate.
19. The lipid elution eluate was collected. This was the lipid-containing fraction of the sample. The lipid eluate collection plate was glass-lined to prevent leaching of contaminants into the lipid-containing sample fraction.
20. An additional lipid elution step was performed, passing 900 μ L 2:1 methanol:dichloromethane through the Captiva EMR-Lipid plate.
 - a. It is recommended to complete at least 2 lipid elution steps; some lipid classes have improved recovery when using up to four lipid elutions.
 - b. When four lipid elutions were completed, the lipid eluate was dried or partially dried after two lipid elutions were completed to provide room in the lipid collection plate for the additional lipid eluate.
 - c. A post-spike of deuterated lipids was optionally added to the lipid collection plate to aid in determination of lipid recoveries. A post-spike of a deuterium-labeled lipid(s) can also be used as an internal standard(s).
 - d. The samples in the retained lipid collection plate were dried under the flow of nitrogen with 30-37°C heating applied to the bottom of the plate.
 - i. As an alternative, a vacuum concentrator can be used to dry the samples.
21. Dried samples were stored at -80°C prior to analysis. For analysis, the samples were re-dissolved in an LC/MS compatible solvent. The plate was sealed, shaken on an orbital shaker, and gently centrifuged to bring the samples to the bottom of the wells. Samples can be mixed with a vortex mixer or bath sonicated to aid in dissolution. Samples can be centrifuged to remove debris. Sample supernatants can be transferred to a new plate or LC/MS vials prior to analysis.

3in1 (protein, metabolite and lipid) workflow, no protein pellet wash

[0054] Briefly, plasma samples were mixed with 1:1 methanol:ethanol and then incubated while also being bath sonicated. Proteins were pelleted by centrifugation, and the supernatant was diluted with water and passed through a Captiva EMR-Lipid plate (Agilent Technologies). The flow-through was collected, which contained polar metabolites. The plate was eluted with two portions of 2:1:1 water:methanol:ethanol to collect more metabolites in the flow-through. The plate was then eluted with a lipid elution solution, 2:1 methanol:dichloromethane. The lipids were collected in the eluate. The plate was eluted again with the same lipid elution solution, and the eluate was combined with the first eluate, dried, reconstituted, and analyzed by a mass spectrometer.

[0055] Automation can be added to the 3in1 workflow. For example:

1. 20 μ L aliquots of plasma were transferred to wells of a 96-well plate.
2. Approximately 130-160 μ L 1:1 methanol:ethanol was added to wells of a separate 96-well plate.
 - a. In some iterations, the 1:1 methanol:ethanol solution included a pre-spike of deuterium-labeled lipids (dissolved in 1:1 methanol:ethanol) for assessment of lipid recoveries. A pre-spike of a deuterium-labeled lipid(s) can also be used as an internal standard(s).
 - b. As an alternative, the 1:1 methanol:ethanol can be added to a single well reservoir.
3. 2:1:1 water:methanol:ethanol was poured into a single-well reservoir.
 - a. As an alternative, the 2:1:1 water:methanol:ethanol can be added to wells of a 96-well plate.
4. milliQ water was added to a 96-well plate, with 92.5 μ L (112.5 μ L minus the plasma sample volume) added to wells of the plate.
5. The plasma plate, 1:1 methanol:ethanol plate, water plate, and 2:1:1 water:methanol:ethanol plate were added to an Agilent Bravo Liquid Handler equipped with supplementary protocols for processing plasma samples. All other necessary consumables, e.g. Captiva EMR-Lipid plate, metabolite collection plate, and tips for liquid and sample transfers were also added to the Bravo.
6. Using the supplementary protocols, the Bravo transferred 112.5 μ L 1:1 methanol:ethanol to the plate holding the plasma samples. The plasma and 1:1

methanol:ethanol were mixed using pipet mixing and plate shaking. The 1:1 methanol:ethanol precipitated proteins from the plasma.

7. An optional 10 min incubation was completed to equilibrate the samples.
 - a. Bath sonication during this incubation period was used to assist with sample equilibration.
8. After bath sonication, the precipitated proteins were pelleted via centrifugation. The sample-containing plate was centrifuged at $2,272 \times g$, 60 minutes, 20°C .
9. After pelleting the proteins, the sample supernatant was transferred to a plate containing $92.5 \mu\text{L}$ ($112.5 \mu\text{L}$ minus the plasma sample volume) of water in the plate wells. The samples were mixed using pipet mixing.
10. A second optional 10 min incubation was completed to equilibrate the samples.
 - a. Bath sonication was not needed during this step, as the protein precipitate was previously removed.
11. The sample supernatant and water solutions were transferred to a Captiva EMR-Lipid plate. The sample supernatant and water solutions were pulled through the plate using vacuum pressure.
 - a. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional sample solution.
 - b. As an alternative, the samples can be pushed through the plate using positive pressure. After an initial lower positive pressure filtration, a higher positive pressure can be used to elute additional sample solution.
12. Polar metabolites were collected in the flow-through.
13. The Captiva EMR-Lipid plate was washed with $250 \mu\text{L}$ 2:1:1 water:methanol:ethanol, using vacuum pressure to move the solution through the plate. The flow-through was collected with the previous flow-through, adding to the polar metabolite fraction.
 - a. The $250 \mu\text{L}$ 2:1:1 water:methanol:ethanol was used to wash the plate that previously contained the sample supernatant and water solutions, prior to adding the $250 \mu\text{L}$ 2:1:1 water:methanol:ethanol to the Captiva EMR-Lipid plate.
 - b. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional wash solution.
 - c. As an alternative, positive pressure can be used to move the 2:1:1 water:methanol:ethanol solution through the plate. After an initial lower

positive pressure filtration, a higher positive pressure can be used to elute additional wash solution.

14. The Captiva EMR-Lipid plate was washed a second time with 250 μ L 2:1:1 water:methanol:ethanol, again using vacuum pressure to move the solution through the plate. The flow-through was collected with the previous flow-through, adding to the polar metabolite fraction.

- a. The 250 μ L 2:1:1 water:methanol:ethanol was used to wash the plate that previously contained the sample supernatant and water solutions, prior to adding the 250 μ L 2:1:1 water:methanol:ethanol to the Captiva EMR-Lipid plate.
- b. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional wash solution.
- c. As an alternative, positive pressure can be used to move the 2:1:1 water:methanol:ethanol solution through the plate. After an initial lower positive pressure filtration, a higher positive pressure can be used to elute additional wash solution.

15. This was the end of the metabolite extraction portion of the workflow.

- a. The polar metabolite collection plate can be retained and the samples can be dried under the flow of nitrogen with 30-37°C heating applied to the bottom of the plate.
 - i. As an alternative, a vacuum concentrator can be used to dry the samples.
- b. Dried samples can be stored at -80°C prior to analysis. For analysis, the samples can be re-dissolved in an LC/MS compatible solvent. Samples can be shaken, mixed with a vortex mixer, or bath sonicated to aid in dissolution. Samples can be centrifuged to bring samples to the bottom of the wells or to remove debris. Sample supernatants can be transferred to a new plate or LC/MS vials prior to analysis.

16. The Bravo was reset to complete the lipid elution. The Captiva EMR-Lipid plate was retained, as it contained lipids.

17. The lipid elution solution, 2:1 methanol:dichloromethane, was added to a 96-well plate. The plate was glass-lined to prevent leaching of contaminants into the lipid elution solution.

- a. Alternatively, a glass or glass-lined reservoir can be used to hold the 2:1 methanol:dichloromethane.

18. The Bravo was used to transfer 900 μ L 2:1 methanol:dichloromethane to the retained Captiva EMR-Lipid plate. Vacuum filtration was used to pull the lipid elution solution through the Captiva EMR-Lipid plate. The target flow rate for lipid elution was 1 drop every 3-5 seconds.
 - a. As with the metabolite extraction portion of the workflow, positive pressure can be used to push the lipid elution solution through the Captiva EMR-Lipid plate.
19. When no lipid elution solution remained in the wells of the Captiva EMR-Lipid plate, a higher vacuum pressure was used to elute additional lipid elution solution that was held up in the Captiva EMR-Lipid plate.
 - a. As an alternative, when no lipid elution solution remains in the wells of the Captiva EMR-Lipid plate, a higher positive pressure can be used to elute additional lipid elution solution that is held up in the Captiva EMR-Lipid plate.
20. The lipid elution eluate was collected. This was the lipid-containing fraction of the sample. The lipid eluate collection plate was glass-lined to prevent leaching of contaminants into the lipid-containing sample fraction.
21. An additional lipid elution step was performed, passing 900 μ L 2:1 methanol:dichloromethane through the Captiva EMR-Lipid plate.
 - a. It is recommended to complete at least 2 lipid elution steps; some lipid classes have improved recovery when using up to four lipid elutions.
 - b. When four lipid elutions were completed, the lipid eluate was dried or partially dried after the first two lipid elutions to provide room in the lipid collection plate for the additional lipid eluate.
 - c. A post-spike of deuterated lipids was optionally added to the lipid collection plate to aid in determination of lipid recoveries. A post-spike of a deuterium-labeled lipid(s) can also be used as an internal standard(s).
 - d. The samples in the retained lipid collection plate were dried under the flow of nitrogen with 30-37°C heating applied to the bottom of the plate.
 - i. As an alternative, a vacuum concentrator can be used to dry the samples.
22. Dried samples were stored at -80°C prior to analysis. For analysis, the samples were re-dissolved in an LC/MS compatible solvent. The plate was sealed, shaken on an orbital shaker, and gently centrifuged to bring the samples to the bottom of the wells. Samples can be mixed with a vortex mixer or bath sonicated to aid in dissolution. Samples can be centrifuged to remove debris. Sample supernatants can be transferred to a new plate or LC/MS vials prior to analysis.

3in1 (protein, metabolite and lipid) workflow, with protein pellet wash

[0056] Briefly, plasma samples were mixed with 1:1 methanol:ethanol and incubated while also being bath sonicated. Proteins were pelleted by centrifugation, and the supernatant was diluted with water and passed through a Captiva EMR-Lipid plate (Agilent Technologies). The flow-through was collected, which contained polar metabolites. The plate was eluted with two portions of 2:1:1 water:methanol:ethanol to collect more metabolites in the flow-through.

[0057] The protein pellet was washed with the lipid elution solution, 2:1 methanol:dichloromethane, bath sonicated, and centrifuged again to collect both the pellet and the wash. The wash, which contained lipids, was used to elute the Captiva EMR-Lipid plate, which had additional lipids bound to it. The lipids were collected in the eluate. The plate was eluted again with the same lipid elution solution, and the eluate was combined with the first eluate, dried, reconstituted, and analyzed by a mass spectrometer.

[0058] Automation can be included in the new 3in1 workflow. For example:

1. 20 μ L aliquots of plasma were transferred to wells of a 96-well plate.
2. Approximately 130-160 μ L 1:1 methanol:ethanol was added to wells of a separate 96-well plate.
 - a. In some iterations, the 1:1 methanol:ethanol solution included a pre-spike of deuterium-labeled lipids (dissolved in 1:1 methanol:ethanol) for assessment of lipid recoveries. A pre-spike of a deuterium-labeled lipid(s) can also be used as an internal standard(s).
 - b. As an alternative, the 1:1 methanol:ethanol can be added to a single well reservoir.
3. 2:1:1 water:methanol:ethanol was poured into a single-well reservoir.
 - a. As an alternative, the 2:1:1 water:methanol:ethanol can be added to wells of a 96-well plate.
4. milliQ water was added to a 96-well plate, with 92.5 μ L (112.5 μ L minus the plasma sample volume) added to wells of the plate.
5. The plasma plate, 1:1 methanol:ethanol plate, water plate, and 2:1:1 water:methanol:ethanol plate were added to an Agilent Bravo Liquid Handler equipped with supplementary protocols for processing plasma samples. All other

- necessary consumables, e.g. Captiva EMR-Lipid plate, metabolite collection plate, and tips for liquid and sample transfers were also added to the Bravo.
6. Using the supplementary protocols, the Bravo transferred 112.5 μ L 1:1 methanol:ethanol to the plate holding the plasma samples. The plasma and 1:1 methanol:ethanol were mixed using pipet mixing and plate shaking. The 1:1 methanol:ethanol precipitated proteins from the plasma.
 7. An optional 10 min incubation was completed to equilibrate the samples.
 - a. Bath sonication during this incubation period was used to assist with sample equilibration.
 8. After bath sonication, the precipitated proteins were pelleted via centrifugation. The sample-containing plate was centrifuged at 2,272 x g, 60 minutes, 20°C.
 9. After pelleting the proteins, the sample supernatant was transferred to a plate containing 92.5 μ L (112.5 μ L minus the plasma sample volume) of water in the plate wells. The samples were mixed using pipet mixing.
 10. A second optional 10 min incubation was completed to equilibrate the samples.
 - a. Bath sonication was not needed during this step, as the protein precipitate was previously removed.
 11. The sample supernatant and water solutions were transferred to a Captiva EMR-Lipid plate. The sample supernatant and water solutions were pulled through the plate using vacuum pressure.
 - a. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional sample solution.
 - b. As an alternative, the samples can be pushed through the plate using positive pressure. After an initial lower positive pressure filtration, a higher positive pressure can be used to elute additional sample solution.
 12. Polar metabolites were collected in the flow-through.
 13. The Captiva EMR-Lipid plate was washed with 250 μ L 2:1:1 water:methanol:ethanol, using vacuum pressure to move the solution through the plate. The flow-through was collected with the previous flow-through, adding to the polar metabolite fraction.
 - a. The 250 μ L 2:1:1 water:methanol:ethanol was used to wash the plate that previously contained the sample supernatant and water solutions, prior to adding the 250 μ L 2:1:1 water:methanol:ethanol to the Captiva EMR-Lipid plate.

- b. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional wash solution.
 - c. As an alternative, positive pressure can be used to move the 2:1:1 water:methanol:ethanol solution through the plate. After an initial lower positive pressure filtration, a higher positive pressure can be used to elute additional wash solution.
14. The Captiva EMR-Lipid plate was washed a second time with 250 μ L 2:1:1 water:methanol:ethanol, again using vacuum pressure to move the solution through the plate. The flow-through was collected with the previous flow-through, adding to the polar metabolite fraction.
- a. The 250 μ L 2:1:1 water:methanol:ethanol was used to wash the plate that previously contained the sample supernatant and water solutions, prior to adding the 250 μ L 2:1:1 water:methanol:ethanol to the Captiva EMR-Lipid plate.
 - b. After an initial lower vacuum pressure filtration, a higher vacuum pressure was used to elute additional wash solution.
 - c. As an alternative, positive pressure can be used to move the 2:1:1 water:methanol:ethanol solution through the plate. After an initial lower positive pressure filtration, a higher positive pressure can be used to elute additional wash solution.
15. This was the end of the metabolite extraction portion of the workflow.
- a. The polar metabolite collection plate can be retained and the samples can be dried under the flow of nitrogen with 30-37°C heating applied to the bottom of the plate.
 - i. As an alternative, a vacuum concentrator can be used to dry the samples.
 - b. Dried samples can be stored at -80°C prior to analysis. For analysis, the samples can be re-dissolved in an LC/MS compatible solvent. Samples can be shaken, mixed with a vortex mixer, or bath sonicated to aid in dissolution. Samples can be centrifuged to bring samples to the bottom of the wells or to remove debris. Sample supernatants can be transferred to a new plate or LC/MS vials prior to analysis.
16. The Bravo was reset in order to complete the lipid elution. The Captiva EMR-Lipid plate was retained, as it contained lipids. The protein pellet containing plate was

- retained for washing of the protein pellet with the lipid elution solution for lipid extraction from the protein pellet.
17. The lipid elution solution, 2:1 methanol:dichloromethane, was added to a 96-well plate. The plate was glass-lined to prevent leaching of contaminants into the lipid elution solution.
 - a. Alternatively, a glass or glass-lined reservoir can be used to hold the 2:1 methanol:dichloromethane.
 18. The Bravo was used to transfer 900 μ L 2:1 methanol:dichloromethane to the retained protein pellets, and the samples were mixed via pipet mixing.
 - a. As an alternative, sample mixing can also include plate shaking.
 19. The protein pellet plate with lipid elution solution was bath sonicated for 10 minutes.
 - a. Some protein pellets did not resuspend during the bath sonication; however, this did not appear to impact lipid recovery compared to wells where the protein pellet was resuspended during bath sonication.
 20. After bath sonication, the protein precipitates were pelleted via centrifugation. The plate was centrifuged at 2,272 $\times$ g, 60 minutes, 20°C.
 21. The Bravo was used to transfer the lipid elution solution supernatant, i.e., wash, to the Captiva EMR-Lipid plate. Vacuum filtration was used to pull the lipid elution solution through the Captiva EMR-Lipid plate. The target flow rate for lipid elution was 1 drop every 3-5 seconds.
 - a. As an alternative, positive pressure can be used to push the lipid elution solution through the Captiva EMR-Lipid plate.
 22. When no lipid elution solution remained in the wells of the Captiva EMR-Lipid plate, a higher vacuum pressure was used to elute additional lipid elution solution that was held up in the Captiva EMR-Lipid plate.
 - a. As an alternative, when no lipid elution solution remains in the wells of the Captiva EMR-Lipid plate, a higher positive pressure can be used to elute additional lipid elution solution that is held up in the Captiva EMR-Lipid plate.
 23. The lipid elution eluate was collected. This was the lipid-containing fraction of the sample. The lipid eluate collection plate was glass-lined to prevent leaching of contaminants into the lipid-containing sample fraction.
 24. An additional lipid elution step was performed, passing 900 μ L 2:1 methanol:dichloromethane through the Captiva EMR-Lipid plate.

- a. It is recommended to complete at least 2 lipid elution steps; some lipid classes have improved recovery when using up to four lipid elutions.
 - b. When four lipid elutions were completed, the lipid eluate was dried or partially dried after two lipid elutions to provide room in the lipid collection plate for the additional lipid eluate.
 - c. A post-spike of deuterated lipids was optionally added to the lipid collection plate to aid in determination of lipid recoveries. A post-spike of a deuterium-labeled lipid(s) can also be used as an internal standard(s).
 - d. The samples in the retained lipid collection plate were dried under the flow of nitrogen with 30-37°C heating applied to the bottom of the plate.
 - i. As an alternative, a vacuum concentrator can be used to dry the samples.
25. Dried samples were stored at -80°C prior to analysis. For analysis, the samples were re-dissolved in an LC/MS compatible solvent. The plate was sealed, shaken on an orbital shaker, and gently centrifuged to bring the samples to the bottom of the wells. Samples can be mixed with a vortex mixer or bath sonicated to aid in dissolution. Samples can be centrifuged to remove debris. Sample supernatants can be transferred to a new plate or LC/MS vials prior to analysis.

* * *

[0059] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

Claims:

1. A method for extracting lipids from a sample, comprising:
 - (a) precipitating proteins from the sample to result in a sample mixture comprising protein precipitates and a supernatant, and separating the protein precipitates from the supernatant;
 - (b) passing the supernatant through a solid-phase extraction matrix having an affinity for lipids to result in a first flow-through;
 - (c) washing the protein precipitate with a first lipid elution solution to obtain a wash;
 - (d) eluting the solid-phase extraction matrix after (b) with a second lipid elution solution to obtain a second flow-through;wherein the wash and the second flow-through contain lipids.
2. The method of claim 1, further comprising adding water to the supernatant before passing the supernatant through the solid-phase extraction matrix.
3. The method of claim 1 or 2, wherein the second lipid elution solution comprises the wash.
4. The method of claim 1 or 2, wherein the first lipid elution solution and the second lipid elution solution have the same composition.
5. The method of any of the preceding claims, further comprising additional elution(s) of the solid-phase extraction matrix with lipid elution solution(s) after (d).
6. The method of any of the preceding claims, further comprising collecting metabolites from the first flow-through.
7. The method of any of the preceding claims, further comprising eluting the solid-phase extraction matrix prior to (d) with a metabolite elution solution to collect metabolites in the resulting eluate.
8. The method of claim 7, further comprising combining the resulting eluate with the first flow-through.

9. The method of any of the preceding claims, further comprising collecting proteins from the protein precipitates.
10. The method of any of the preceding claims, wherein the protein precipitates are not separated from the supernatant by filtration.
11. The method of any of the preceding claims, wherein the protein precipitates are separated from the supernatant by centrifugation.
12. The method of any of the preceding claims, wherein the proteins are precipitated using a solution comprising methanol and ethanol.
13. The method of any of the preceding claims, wherein the proteins are precipitated using 50:50 methanol/ethanol (v/v).
14. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 40% or less.
15. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 35% or less.
16. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 30% or less.
17. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 25% or less.
18. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 20% or less.
19. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 15% or less.
20. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 10% or less.
21. The method of any of the preceding claims, wherein the water content of the sample mixture at the time of said separating is 5% or less.

22. The method of any of the preceding claims, wherein the sample is plasma.
23. The method of any of claims 1-21, wherein the sample is serum.
24. The method of any of claims 1-21, wherein the sample is a body fluid besides serum or plasma.
25. The method of any of claims 1-21, wherein the sample comprises lysed cells.
26. The method of claim 25, wherein the cells are from blood.
27. The method of claim 25, wherein the cells are cultured suspension cells.
28. The method of claim 25, wherein the cells are cultured adherent cells.
29. The method of claim 25, wherein the cells are 3D cultured cells (e.g. organoids, spheroids, cell cultures grown in 3D supports).
30. The method of claim 25, wherein the cells are from tissue.
31. The method of any of claims 25-30, wherein the sample comprises cells that have been treated with a solution comprising a fluoroalcohol.
32. The method of claim 31, wherein the fluoroalcohol is one or more of the following: 2,2,2-trifluoroethanol, 2,2-difluoroethanol, 2-fluoroethanol, hexafluoro-2-propanol, nonafluoro-tert-butyl alcohol, 1,1,2,2,2-pentafluoroethanol, and/or 2,2,3,3,3-pentafluoro-1-propanol.
33. The method of claim 31, wherein the fluoroalcohol is 2,2,2-trifluoroethanol.
34. The method of any of the preceding claims, wherein the method comprises automated steps.
35. The method of any of the preceding claims, wherein at least one of the lipid elution solutions is 2:1 methanol:dichloromethane.