Sorption and Bound Residue Formation of Linuron, Methylparathion, and Metolachlor by Carrot Tissues: Kinetics by On-Line HPLC Microextraction

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · SEPTEMBER 1997

Impact Factor: 2.91 · DOI: 10.1021/jf970037b

3 AUTHORS, INCLUDING:

Luis Eduardo Sojo
Xenon Pharmaceuticals Inc.
25 PUBLICATIONS **224** CITATIONS
SEE PROFILE

Donald Gamble
Saint Mary's University
60 PUBLICATIONS **1,010** CITATIONS
SEE PROFILE

analytical chemistry

Article

pubs.acs.org/ac

Metabolite Profiling of a NIST Standard Reference Material for Human Plasma (SRM 1950): GC-MS, LC-MS, NMR, and Clinical Laboratory Analyses, Libraries, and Web-Based Resources

Yamil Simón-Manso,*[,†] Mark S. Lowenthal,[†] Lisa E. Kilpatrick,[†] Maureen L. Sampson,[‡] Kelly H. Telu,[§] Paul A. Rudnick,[†] W. Gary Mallard,[†] Daniel W. Bearden,[∥] Tracey B. Schock,[∥] Dmitrii V. Tchekhovskoi,[†] Niksa Blonder,[†] Xinjian Yan,[†] Yuxue Liang,[†] Yufang Zheng,[†] William E. Wallace,[§] Pedatsur Neta,[†] Karen W. Phinney,[†] Alan T. Remaley,[‡] and Stephen E. Stein[†]

[†]Biomolecular Measurement Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899-8380, United States

[‡]Department of Laboratory Medicine, National Institutes of Health, Bethesda, Maryland 20892, United States

[§]Chemical Sciences Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899-8380, United States

[∥]Hollings Marine Laboratory, Chemical Sciences Division, National Institute of Standards and Technology, Charleston, South Carolina 29412, United States

Supporting Information

ABSTRACT: Recent progress in metabolomics and the development of increasingly sensitive analytical techniques have renewed interest in global profiling, i.e., semiquantitative monitoring of all chemical constituents of biological fluids. In this work, we have performed global profiling of NIST SRM 1950, "Metabolites in Human Plasma", using GC-MS, LC-MS, and NMR. Metabolome coverage, difficulties, and reproducibility of the experiments on each platform are discussed. A total of 353 metabolites have been identified in this material. GC-MS provides 65 unique identifications, and most of the identifications from NMR overlap with the LC-MS identifications, except for some small sugars that are not directly found by LC-MS. Also, repeatability and intermediate precision analyses show that the SRM 1950 profiling is reproducible enough to consider this material as a good choice to distinguish between analytical and biological variability. Clinical laboratory data shows that most results are within the reference ranges for each assay. In-house computational tools have been developed or modified for MS data processing and interactive web display. All data and programs are freely available online at http://peptide.nist.gov/ and http://srmd.nist.gov/.

Since 1906, the National Institute of Standards and Technology (NIST) has developed and provided reference materials to assist others in making reliable measurements. This has led to the current inventory of over 1300 Standard Reference Materials (SRM).[1] These materials are used to perform calibrations, to verify the measurement accuracy and to support the development of new measurement methods. Because of the degree of effort required to provide "certified" values, a relatively small number of analytes are certified per SRM. To fulfill a demand for a more extensive characterization, a new project to provide access to additional analyte data has been undertaken; the project is known as Standard Reference Material and Data (SRM/D). Here we focused our work on the qualitative analysis of a particular SRM for human plasma (SRM 1950). An accompanying manuscript offers a general overview of the material, basic considerations for its formulation, and the quantitative analysis and certification of 95 chemical components of the material.[2]

Metabolic profiling in human plasma offers unique information concerning metabolism, health, and nutrition but is a complicated task. The physical and chemical complexity of the sample, the variety of organic metabolites, and the wide range of their concentrations pose formidable challenges for most analytical techniques. The range of biomolecule concentrations covers nine or more orders of magnitude.[3] Recently, a number of groups have initiated programs to determine the human plasma metabolome (HPM), i.e., a chemical "parts list" of all identifiable naturally occurring metabolites in human plasma, using a variety of analytical

Received: July 19, 2013
Accepted: October 22, 2013
Published: October 22, 2013

   dx.doi.org/10.1021/ac402503m | *Anal. Chem.* 2013, 85, 11725−11731

techniques.[3−13] Also, small companies validate and perform this kind of analysis in support of clinical, preclinical, and research studies.[14]

For sample preparation and identification of metabolites, researchers have used a variety of approaches that can include one or more of the following steps: sample disinfection, protein precipitation, solid-phase separation, organic extractions, freeze-drying or lyophilization, and spiking of isotopically labeled internal standards. Each of these procedures is relatively simple but can influence the distribution of metabolites in the analysis, restricting the ability to profile metabolites in a reproducible way. Several complicating factors have been mentioned in publications regarding sample preparation and chemical analysis,[6,8−11,15−18] such as the presence of phospholipids, small proteins, and other high molecular weight species, lipids, oligonucleotides, ion suppression, etc.

GC-MS has long been used for identifying components of biological fluids.[19,20] However, the variety of metabolites that can be analyzed with this technique is limited by the volatility or the ability to form appropriate volatile derivatives of the components. Nuclear magnetic resonance (NMR) provides an in situ way of monitoring certain classes of metabolites but is more limited in its sensitivity and dynamic range.[3,13,21,22] LC-MS/MS has also become very effective for metabolite analysis.[4−10] In the past, most GC-MS and LC-MS workflows were dedicated to identifying (or quantifying) individual metabolites, or a group of compounds related by the same metabolic route, and the main differences were focused on the sample preparation procedures.

In this work, we performed the qualitative metabolic profiling of SRM 1950 using GC-MS, LC-MS, and NMR. We also report on the clinical laboratory analysis of this material. In addition, we report on the computational tools for processing of MS data, web-resources for disseminating and visualizing data, and forums for public discussion. This is the first qualitative approach for the global characterization of a NIST SRM and the first interactive website created at NIST for information sharing that complements a Certificate of Analysis.

EXPERIMENTAL DETAILS

All experimental methods include a step of protein and cell removal, adding an organic solvent, and centrifuging the sample, but some procedures are specific to a particular technique. This section describes the experimental procedures that underlie analysis described in later sections.

GC-MS Sample Preparation and Analysis. SRM 1950, initially stored at −80 °C, was allowed to thaw at room temperature. All reagents were HPLC-grade. A 200 μL amount of SRM 1950 was added to 800 μL of methanol and vortexed for 30 s. The mixture was then placed in a freezer at −20 °C for 10 min, vortexed for 3 min, and placed in the freezer again at −20 °C for 10 min. Next the mixture was centrifuged at 19 600 g for 10 min at 20 °C. The supernatant was evaporated to dryness under a nitrogen stream. The residue was directly treated with 30 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) for 3 h at room temperature. The resulting samples are brought to a total volume of 100 μL with the addition of n-hexane. Samples were analyzed in an Agilent 7000 Series Triple Quadrupole GC/MS, using a HP-5MS 5% diphenyl 95% dimethylpolysiloxane with dimension 30 m × 250 μm × 0.25 μm column, split-less, a flow rate of 1.2 mL/min, and a temperature program of 60 °C for 3 min, then 7 °C/min to 140 °C for 0 min, then 5 °C/min to 300 °C for 5 min, with a total run time of 51.429 min.

The NIST/NIH/EPA mass spectral library was used for identifying compounds in GC-MS analyses. NIST-developed software,[23−25] including AMDIS[24] along with available commercial software, were used to align chromatograms and derive peak shapes, intensities, and retention times.

LC-MS/MS Sample Preparation and Analysis. SRM 1950 was handled at Biosafety Level (BSL) 2[26] as recommended in the Certificate of Analysis. SRM 1950 was allowed to thaw at room temperature. Ice-cold ethanol (200 proof, HPLC grade, Sigma-Aldrich, St. Louis, MO) was added to reach a final (optimized) concentration of 80%. The vial was vortexed for 15 s then allowed to sit on ice for 30 min. Other organic solvents have been used for protein precipitation, and a detailed comparison of the solvent effects is reported in ref 8. The samples were then vortexed and stored at −20 °C overnight. The next day, the samples were centrifuged at 4 °C for 15 min at 14 000 g and the supernatant was evaporated to dryness in a CentriVap (Labconco, Kansas City, MO). The samples were reconstituted in water and 2% acetonitrile (ACN) and centrifuged at 4 °C for 15 min at 14 000 g to remove any insoluble material prior to analysis.

Three different platforms were used for the LC-MS/MS analysis: (1) Dionex Ultimate 3000 HPLC coupled to a Thermo Finnigan LTQ, (2) Agilent HPLC 1290 coupled to an Agilent QTOF 6530, and (3) Eksigent NanoLC-2D/Orbitrap Velos. With platform 1 we used a Dionex C18 column (300 μm × 15 cm, 3 μm), at a flow rate of 1 μL/min; in platform 2 a Waters C18 column, 1 mm × 15 cm; in platform 3 an Agilent C18 nanocolumn, 75 μm × 15 cm, packed with Zorbax 300SB, 5 μm. The chromatography conditions were adjusted according to the gradient equation.[27] In most analyses, gradients of water/ACN/formic acid were applied (details of the gradient profiles are given with the chromatograms in the text or in Supporting Information.)

Most MS/MS experiments were recorded in data-dependent mode, using survey scans from 50 Da to 1750 Da (four microscans), followed by MS/MS scans (four microscans) of the four more intense peaks, with a tolerance of 1.3 *m*/*z*, and the precursor ion mass excluded for 18 s after initial MS/MS collection. Also, this study was conducted at different collision energies or different ramps for the collision energies depending on the instrument design. Every experiment has at least three replicates, and two blank runs were included in between to prevent carryover.

We also tested some filtration systems that have been reported for phospholipids removal. We used three different ultrafiltration membranes in 96-well plates, Waters's Ostro, Agilent's Captiva ND^{Lipids}, and Supelco.

In-house software along with available commercial software was used for chromatographic alignment, to derive peak shapes, intensities, and retention times. The NIST tandem mass spectral libraries in conjunction with commercial MS/MS libraries were used for full fragmentation identification of the components of SRM 1950. The NIST pipeline and manual examination were used for quality control purposes. The NIST pipeline is an analysis tool for assessing the repeatability and/or reproducibility of LC-MS/MS runs (see http://peptide.nist.gov/metrics/ for details). Although not always explicitly stated or included in the scoring system, we repeatedly use the concepts of prior probability, spectrum variability, and uniqueness of spectra to assess confidence in compound

identifications. In borderline situations, the compounds were included if they were listed in the Human Metabolome Database (HMDB). We used the NIST MS Search software to search the experimental spectra against NIST's Tandem Mass Spectral reference library. Only compounds with high scores (greater than 600) were selected for manual inspection.

Sample Preparation for NMR Analysis. Plasma samples were received frozen and stored in their original vials at −80 °C until use. Prior to use, an ampule was thawed on ice and vortexed for 30 s. One plasma sample was diluted with D_2O (50:50) for whole plasma analysis. Another sample of plasma was centrifuge filtered through a prewashed (centrifuged twice with 0.1 mol/L NaOH and eight times with water) 3000 Da molecular weight cutoff filter (Millipore Amicon Ultra-4) (7500*g* for 25 min at 4 °C). The filtrate was lyophilized overnight and rehydrated in 600 μL of D_2O containing 1.0 mmol/L sodium 3-(trimethylsilyl)-2,2,3,3-d_4-propionate (TMSP) as an internal NMR chemical shift standard.

All NMR experiments were performed on a Bruker Avance II 700 MHz spectrometer equipped with a cryogenically cooled probe. Spectra were recorded at a temperature of 298 K with a spectral width of 11 ppm, 3 s relaxation delay, with 80 transients and 16 steady-state scans collected into 65 536 data points. One-dimensional spectra of the diluted sample were collected using a Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence. One-dimensional spectra of the rehydrated, filtered sample were collected with water suppression using a three-pulse sequence based on a standard NOESY sequence (noesypr1d). Data were processed by zero filling once (TD = 64k, SI = 64k) and by multiplying the free induction decay by an exponential line broadening function of 0.3 Hz prior to Fourier transformation (FT). Spectra were manually phased, and the chemical shift was calibrated to the standard TMSP peak at 0.000 ppm. Two-dimensional edited heteronuclear single quantum correlation (HSQC) spectra with adiabatic ^{13}C decoupling (hsqcedetgpsisp2.2) were collected for all samples. A relaxation delay equal to 1.5 s was used, and a refocusing delay of 3.45 ms was implemented. In general, 4096 data points with 128 scans per increment were acquired with spectral widths of 11 ppm in F2 and 165 ppm in F1 (^{13}C). The FIDs were weighted using a shifted sine bell function in both dimensions. Manual two-dimensional phasing was applied.

Clinical Laboratory Analysis. SRM 1950 was run in triplicate on all currently available instrumentation in the Clinical Chemistry Laboratory of the NIH Clinical Center. Two main chemistry analyzers were used: the Siemens Vista and the Roche Cobas 6000. Routine clinical chemistry tests were analyzed on Cobas 6000, c501 module. Immunoassays were run with the Siemens Immulite 2000 and Behring Nephlometer.

RESULTS AND DISCUSSION

The main purpose of this work was to provide comprehensive qualitative metabolomic characterization of SRM 1950. We used certain metrics to ensure the quality of the metabolite profiling with GC-MS, LC-MS/MS, and NMR and their different platforms.

Those included, but were not limited to, the following: the total number of identifications, the number of polar, nonpolar, and hydrophobic compounds, the relative intensity of different signals from each identification, influence of sample processing (protein and phospholipids removal, ion suppression, etc.), repeatability, and intermediate precision (based on replicate analyses). Additional data that could be useful for some specific applications of SRM 1950 were also given, e.g., clinical laboratory data. In the following, we discuss the main results obtained using different techniques and compare those results with previous relevant work on the human plasma metabolome.

GC-MS Results. A database search engine has been used to look for the best match to the new version of the NIST/EPA/NIH mass spectral library (NIST 2012), and the resulting data were extracted and analyzed with AMDIS.[24] A detail account of the work with AMDIS is given in ref 28.

A total of 126 compounds with high scores (over 80) were identified in SRM 1950 in all three replicates and three different experiments. Table S.1 of the Supporting Information includes the complete list of identifications, the chemical formula of the parent ions, scan numbers, experimental and library retention times and their differences, relative intensity, and peak area. These are unambiguous, but not "definitive", identifications in the sense of a direct comparison with authentic (known) compounds. (This statement is valid for the rest of the paper.) The identifications include all of the naturally occurring amino acids with the exception of aspartic acid, and 64 carboxylic acids including 10 fatty acids. Previous work on human plasma[5] reported 74 compounds in an untargeted GC-MS experiment, 62 of which were polar and 12 nonpolar. In addition, 25 fatty acids from a separate experiment were reported.[5] Our experiments show that GC-MS is particularly successful in identifying small acids, which makes the technique very useful used in combination with LC-MS/MS. Acids frequently exhibit poor electrospray ionization (particularly in positive mode), limited retention on reversed phase columns, and in-source neutral losses.

LC-MS Analysis. Figure 1 shows a typical base peak chromatogram of SRM 1950 after ethanol protein precipitation, where the largest chromatographic peaks of the chromatogram have been annotated in the figure. The elution gradient was optimized to maximize the number of identifications at the highest possible scan speed. However, many small polar compounds elute early, between 0 and 11 min, and frequently two or more compounds coelute. As expected, compounds with well-balanced hydrophobicity−hydrophilicity moieties elute about the central part of the chromatogram, between 12 and 34 min, but most of them are low abundance components. This part of the chromatogram introduces the highest run-to-run variability (repeatability) in the global profiling of SRM 1950. Also, the MS/MS spectra of low abundance compounds are of a lower quality. The most hydrophobic compounds elute toward the end of the gradient, consisting of mainly fatty acids, steroids, and phospholipids. Results depend strongly on the sample preparation and the later eluters are responsible for the highest batch-to-batch variability (intermediate precision).

The NIST MS Search 2.0 (for a demo: http://peptide.nist.com) was used to look for the best match to the NIST-MS/MS library. More detailed information about the software is given below (see MS/MS libraries and library search software sections.) Table I_S2 of the Supporting Information shows 110 metabolites that were consistently identified in all three replicates and two different experiments on this platform (and usually in more than one ionic form: protonated, sodiated, etc.). The ion formulas in Table I_S2 were automatically generated based on how well they match the experimental mass, abundance pattern of the isotopic cluster, and *m*/*z*-spacing between the monoisotopic ion and the A+1 and A+2 ions. In the table, the library precursor ion masses are given at

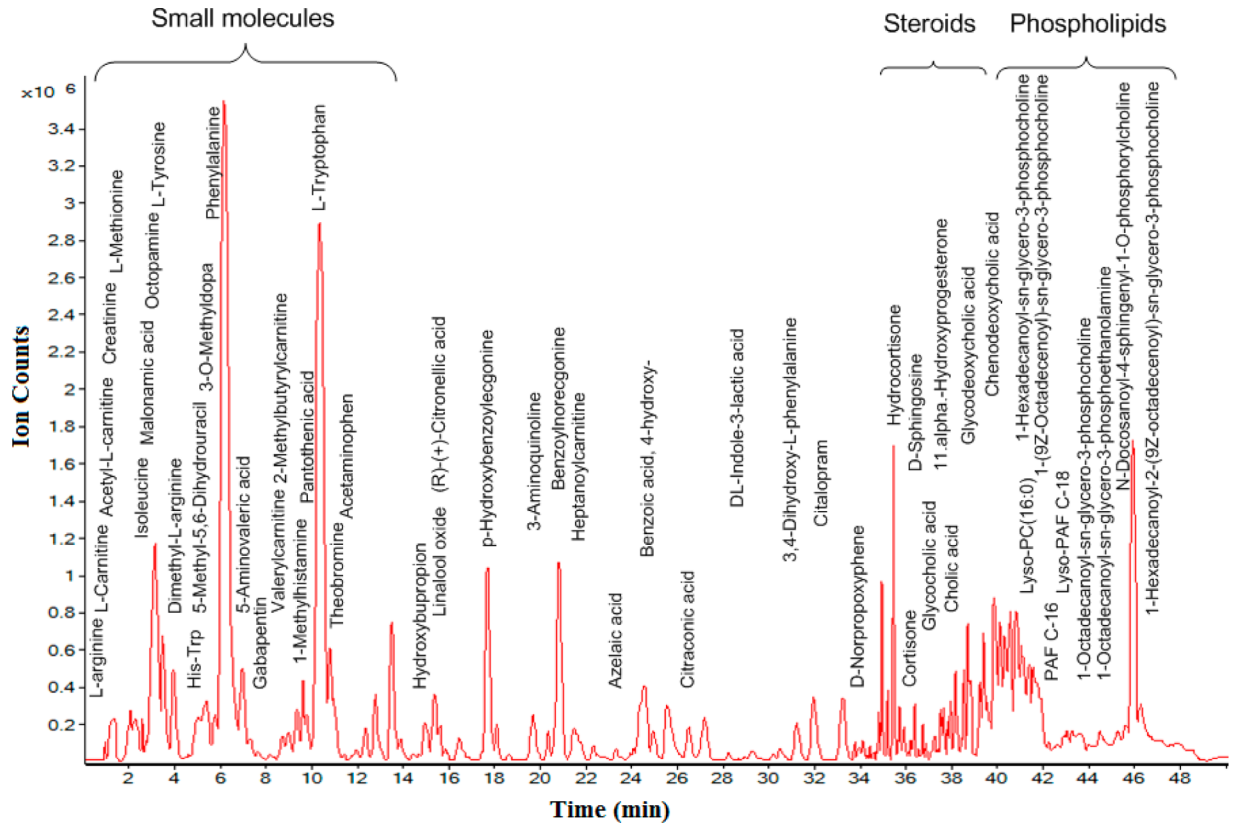

Figure 1. Typical base peak chromatogram of SRM 1950 using the ethanol protein precipitation protocol. Identification of the main peaks. (For this particular run, we used a Waters C18 column (1 mm × 15 cm), mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in acetonitrile, a flow rate of 75 μL/min and the following gradient profile {[t,%B],(0,1),(30,20),(40,90), (45,90), (47,1), (60,1)}. It was run on the Agilent HPLC 1290/QTOF 6530 platform).

the level of precision of the matching library spectrum. The MS2 spectra were also manually inspected to confirm the fragmentation patterns. The table includes compound names, the HMDB ID, the most frequently detected ion of each compound, the formula of the ion, and the library precursor m/z.

The complete list of identifications in all LC-MS platforms is given in Table S2 of the Supporting Information. A total of 322 compounds were identified using four different LC-MS/MS platforms in positive ion mode (see Table S.2 of Supporting Information). It includes 12 out of 20 amino acids, 84 carboxylic acids including 14 heavy fatty acids, 10 phospholipids, 25 hormones and steroids, several dipeptides and tripeptides, and a variety of other small molecules. Previous work[5] reported 96 compounds identified in human plasma by this technique, most of them using targeted experiments and both polarities.

We were able to identify the same prominent precursor ion peaks in most LC-MS platforms but found significant variations for less abundant components. For example, two separate experiments with three replicates performed on an HPLC 1290/Agilent QTOF 6530 system showed excellent repeatability and intermediate precision. The same 110 identified molecular features (with a NIST library match score greater than 600) are found in all replicates with variations of relative intensities for independent components having RSD < 10%. In addition, t test and variance ratio F-test at 95% confidence level on the extracted features revealed no significant difference between the batches. The RSD for the intermediate precision is also estimated to be smaller than 10%. The reported RSD < 10% is an upper bound value representing the maximum variation found among the 110 extracted molecular features. Only identified peaks with more than 5000 counts have been included in this analysis. It is worth mentioning that precision results may vary also depending on the cut-offs used for the analysis, the molecular feature extraction software, and if unidentified features are considered in the analysis. Also, the number of molecular features, MS/MS library identifications, and even the visual appearance of the chromatograms vary significantly when changing the LC/MS platform.

Experiments were repeated in a variety of LC-MS platforms. NanoLC systems allowed us to identify more components but led to increasing the uncertainty of the MS/MS match or the repeatability and intermediate precision. For example, we were able to identify 182 components in an experiment with the Eksigent/Orbitrap Velos platform, but more than 50% of the identifications score between 500 and 600, which represent borderline identifications. Frequently, the experimental spectra were of poor quality to be fully interpretable. NanoLC systems are preferred when most components ionize well or when studies are sample-limited. In most experiments performed with the Dionex/LTQ platform we were able to identify, on average, 89 compounds, but most identifications score on the lower end, ≈600. (Less sensitive instruments, such as the LTQ, usually perform poorer for low abundance compounds and are probably less-recommended for untargeted studies.) In addition, it is worth mentioning that both the LC and the MS introduce significant differences in the metabolite profiling

of SRM 1950 with respect to the detectability of the metabolites and their relative intensities. As we can see, results can vary widely, depending on the LC-MS/MS platform, and it raises a serious question affecting the interpretation of many metabolomics studies. These results suggest that further standardization of the measurement and data processing procedures are required to establish a single protocol to cover all situations, i.e., to find a set of meaningful acceptance criteria for precision of LC/MS patterns that are universally applicable to all platforms. A detailed comparison of NanoLC, conventional, and UPLC-MS for the analysis of plasma is presently ongoing and will be published elsewhere. On the other hand, we found a higher degree of overlap of plasma metabolites detected by LC and GC than reported in a previous work.[5]

In general, results of the global profiling of the material can be only described in statistical terms. Frequently, several low abundance compounds coelute and produce signals of similar intensities, so in untargeted experiments these ions are selected for fragmentation almost randomly. Also, sensitivity and scan speed can vary substantially from one instrument to another and make the comparison across platforms very difficult. To overcome (or minimize) this problem, we are in the process of generating material-oriented libraries that include consensus spectra of all components detectable by MS (identified and unidentified) in the most popular platforms. SRM 1950 samples, when working with the same LC-MS platform, produce reproducible chromatograms in their overall appearance and the number of molecular features that are identified by fragmentation. However, batch-to-batch or interlab comparisons depend strongly on the sample preparation.

Comparison of Sample Preparation for LC-MS Analysis. We have compared several sample preparation procedures available at the moment. Protein precipitation is simple and straightforward, and residual protein and phospholipids are not a major problem if the sample is reconstituted in a low organic solvent mixture and filtered. This is important because depletion of lipid compounds also gives better peak shapes and more reproducible retention times. Protocols using solid phase extraction (SPE) chromatography with the 96-well plates, Ostro, Supelco, and Captiva ND^{Lipids}, were very effective for the phospholipid removals but were found to selectively isolate about 30% of the metabolites from the samples and also introduced a few contaminants. Figure 2 shows the chromatograms obtained under the same conditions after protein precipitation and SPE procedures.

Compared to protein precipitation, all three SPE systems are effective at removing phospholipids and residual proteins. The results in our laboratories suggest that Ostro and Supelco perform better that the Agilent plate but also selectively remove more metabolites from the samples. In general, these products appear to be very useful for cleaning the samples in targeted experiments but are less appropriate for the untargeted global profiling of the material.

MS/MS Libraries and Library Search Software. As part of a NIST program, mass spectral libraries are built and updated continuously from measured spectra of known pure compounds. For this particular work we have increased the number of spectra of small molecules that can be considered as human metabolites. To date, the tandem mass spectral library contains >120 000 spectra for >15 000 ions of >7000 compounds of biological and environmental relevance (including metabolites, bioactive peptides, amino acids and small

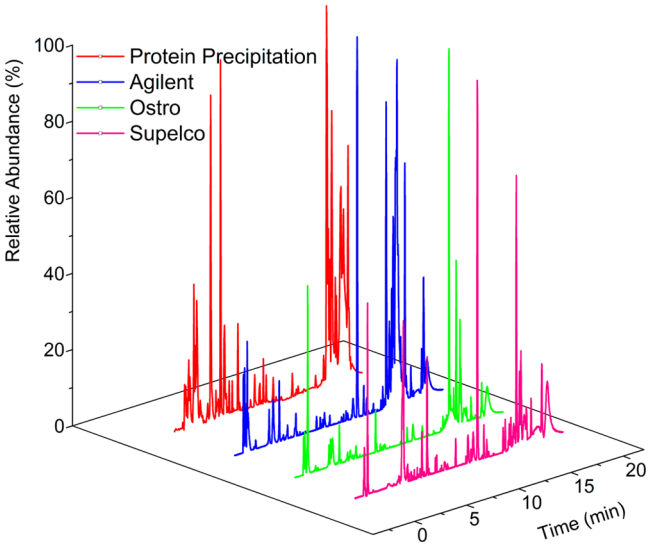

Figure 2. A comparison of the phospholipid removal performance of the filtration systems Ostro, Captiva, and Supelco.

peptides, small sugars and glycans, lipids and phospholipids, drugs, pesticides, surfactants, and various contaminants.[29] The present library contains more than 1500 compounds out of the 4229 compounds listed in the Human Metabolome Database.[3] The spectra in the libraries were validated, critically evaluated, and annotated with detail, enabling the use of sensitive search algorithms. The new library was also tested in some practical applications.[30,31] A new version of NIST MS Search 2.0 [http://peptide.nist.gov/] was developed, specifically for small molecules and it is available online. The basis for the algorithm is found in previous publications.[23−25] However, the software has been adapted to handle spectra with few peaks (see note, ref 32).

NMR Analysis. Human plasma metabolites were identified based on 1D ^{1}H and 2D ^{1}H−^{13}C NMR experiments. Assignments were based on comparison of chemical shifts and spin−spin couplings with reference spectra and tables such as those of Nicholson et al.,[13] the human metabolome database (HMDB),[3] the Madison metabolomics consortium database,[22] the biological magnetic resonance data bank (BMRB),[33] and an in-house compiled database, as well as the SBASE-1-1-2 database included in AMIX (version 3.8; Bruker Biospin, Inc., Billerica, MA).

Table S.3 of the Supporting Information lists the metabolites identified using resonance assignments based on ^{1}H and ^{13}C chemical shifts and spin−spin coupling patterns in diluted and filtered plasma samples. Thirty-five metabolites were identified in the diluted plasma including nine metabolites that were not found in the filtered plasma due to removal of proteins and lipids and lyophilization of volatile compounds such as ethanol and methanol in the filtered sample. Thirty-nine polar metabolites were identified in the filtered plasma with thirteen of the metabolites not observed in the diluted plasma as a result of heavy overlapping shifts of lipids such as cholesterol. The diluted sample did not include a chemical shift reference standard because TMSP binds to proteins, resulting in reduced signal with broad line widths.[7] Thus, the spectrum was referenced to the D_2O peak at 4.7 ppm. Metabolite standards from HMDB are referenced using 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), which caused an average shift in the assignments of metabolites (2.63 ppm in the ^{13}C dimension

dx.doi.org/10.1021/ac402503m | Anal. Chem. 2013, 85, 11725−11731

and 0.06 ppm in the ^{1}H dimension) when comparing database metabolites to metabolites in the acquired diluted spectra. Several peaks remain unidentified in both samples. It is worth mentioning that a quantitative analysis of 28 components of SRM 1950 has been reported previously using NMR approaches.[12]

Clinical Laboratory Results. Although the clinical laboratory analysis, strictly speaking, is not part of the qualitative profiling of SRM 1950, we have decided to report it. It is useful to show how close SRM 1950 is to representing a normal plasma metabolome. Using simplified reference ranges that encompass data from both genders, almost all of the results were within the reference ranges for each assay. Iron, total protein, and albumin were on the low side, and there was some digoxin and lithium present. Calcium was significantly low. Because SRM 1950 is lithium-heparinized these are probably artifacts. Some tests have recommended ranges rather than normal ranges, so some results were out of recommended ranges: HDL cholesterol was low and C Reactive Protein and IgE were high. A complete list of all the clinical tests including the recommended ranges is available in Supporting Information (Table S.4).

Online Resources. A website was developed using a relational database and various web technologies to generate dynamic content (http://srm1950.nist.gov). Results are displayed on a website that provides interactive access to experimental data and tools produced at NIST. The goal of this website is to provide users with "typical" data to browse for descriptions of the methods used to generate the data as well as access to a catalog of quantified and/or putatively identified components of the SRM. Measurements were assigned a confidence type, ranging from certified to identification. A detailed example of the user interaction with the SRM/D webpage is shown in the Supporting Information (see document, NIST_SRMD_Web site.docx). Finally, the data acquisition, processing, and reporting have followed recommendations of refs 34 and 35.

CONCLUSION

A total of 353 metabolites of SRM 1950 were identified using GC-MS, LC-MS/MS, and NMR. GC-MS provides 65 unique identifications, and most of the identifications from NMR overlap with the LC-MS identifications, except for glucose, mannose, and some other small sugars that are not directly found by LC-MS. Results depend on sample preparation, technique, and instruments used for the analysis. The SPE procedures tested in this work, i.e., Agilent's Captiva, Waters's Ostro, and Supelco's HybridSPE, selectively remove metabolites from the sample and are limited for global profiling of plasma. As expected, GC-MS proved to be a very effective choice for identifying compounds of poor ionization in electrospray sources and provided the majority of identifications for this kind of compound, e.g., almost 50% of the GC-MS identifications were small carboxylic acids. Our LC-MS/MS results show a strong dependence on the LC-MS/MS platform but are very reproducible while using the same sample preparation procedure and instruments. As described in Experimental Details, two separate experiments with three replicates showed excellent repeatability and intermediate precision, including variation of the relative intensities of major components with RSD < 10%. The clinical laboratory analysis of SRM 1950 shows no major differences with normal plasma, other than those artifacts derived from the fact that it is a lithium-heparinized product. In addition, similar profiling of either pooled or individual plasma samples (not included in the paper) suggest that SRM 1950 could be a better choice than arbitrary blood pools for studying analytical variability, standardization of methods, calibrations, repeatability (run-to-run), intermediate precision (batch-to-batch), and/or reproducibility through interlaboratory comparisons (by characterizing the same material). Tandem mass spectral libraries and data analysis software have been developed for the qualitative analysis of SRM 1950 and other biological fluids. All data and programs are freely available online at http://peptide.nist.gov/ and http://srmd.nist.gov/.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ysimon@nist.gov and yamil_sm@yahoo.com. Phone: (301) 975-8638. Fax: (301) 975-2643. Address: 100 Bureau Drive, M/S 8362, Gaithersburg, Maryland 20899, United States.

Notes

Certain commercial equipment, instruments, or materials are identified in this document. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.
The authors declare no competing financial interest.

REFERENCES

(1) NIST SRM program, http://www.nist.gov/srm/program_info.cfm.
(2) Phinney, K. W. *Anal. Chem.* **2013**.
(3) Wishart, D. S.; Jewison, T.; Guo, A. C.; Wilson, M.; Knox, C.; Liu, Y.; Djoumbou, Y.Mandal, R.; Aziat, F.Dong, E.; Bouatra, S.; Sinelnikov, I.; Arndt, D.; Xia, J.; Liu, P.; Yallou, F.; Bjorndahl, T.; Perez-Pineiro, R.; Eisner, R.; Allen, F.; Neveu, V.; Greiner, R.; Scalbert, A. *Nucleic Acids Res.* **2013**, *41* (Database issue), D801−7. http://www.hmdb.ca/.
(4) Lawton, K. A.; Berger, A.; Mitchell, M.; Milgram, K. E.; Evans, A. M.; Guo, L.; Hanson, R. W.; Kalhan, S. C.; Ryals, J. A.; Milburn, M. V. *Pharmacogenomics* **2008**, *9* (4), 383−97.
(5) Psychogios, N.; Hau, D. D.; Peng, J.; Guo, A. C.; Mandal, R.; Bouatra, S.; Sinelnikov, I.; Krishnamurthy, R.; Eisner, R.; Gautam, B.; Young, N.; Xia, J.; Knox, C.; Dong, E.; Huang, P.; Hollander, Z.; Pedersen, T. L.; Smith, S. R.; Bamforth, F.; Greiner, R.; McManus, B.; Newman, J. W.; Goodfriend, T.; Wishart, D. S. *PloS One* **2011**, *6* (2), e16957.
(6) Dunn, W. B.; Broadhurst, D.; Begley, P.; Zelena, E.; Francis-McIntyre, S.; Anderson, N.; Brown, M.; Knowles, J. D.; Halsall, A.; Haselden, J. N.; Nicholls, A. W.; Wilson, I. D.; Kell, D. B.; Goodacre, R. *Nat. Protocols* **2011**, *6* (7), 1060−83.
(7) Beckonert, O.; Keun, H. C.; Ebbels, T. M.; Bundy, J.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. *Nat. Protocols* **2007**, *2* (11), 2692−703.
(8) Bruce, S. J.; Tavazzi, I.; Parisod, V.; Rezzi, S.; Kochhar, S.; Guy, P. A. *Anal. Chem.* **2009**, *81* (9), 3285−96.
(9) Want, E. J.; O'Maille, G.; Smith, C. A.; Brandon, T. R.; Uritboonthai, W.; Qin, C.; Trauger, S. A.; Siuzdak, G. *Anal. Chem.* **2006**, *78* (3), 743−52.
(10) Zelena, E.; Dunn, W. B.; Broadhurst, D.; Francis-McIntyre, S.; Carroll, K. M.; Begley, P.; O'Hagan, S.; Knowles, J. D.; Halsall, A.; Wilson, I. D.; Kell, D. B. *Anal. Chem.* **2009**, *81* (4), 1357−64.

(11) Buhrman, D.; Price, P.; Rudewiczcor, P. *J. Am. Soc. Mass Spectrom.* **1996**, *7* (11), 1099−1105.
(12) Gowda, G. A.; Tayyari, F.; Ye, T.; Suryani, Y.; Wei, S.; Shanaiah, N.; Raftery, D. *Anal. Chem.* **2010**, *82* (21), 8983−8990.
(13) Nicholson, J. K.; Foxall, P. J.; Spraul, M.; Farrant, R. D.; Lindon, J. C. *Anal. Chem.* **1995**, *67* (5), 793−811.
(14) For example: Tandem Labs, Salt Lake City, UT, http://www.tandemlabs.com/, and Metabolon, Durham, NC, http://www.metabolon.com/. (This is not intended to be a complete listing of companies, recommendation, or endorsement of any kind.)
(15) Kebarle, P.; Verkerk, U. H. *Mass Spectrom. Rev.* **2009**, *28* (6), 898−917.
(16) King, R.; Bonfiglio, R.; Fernandez-Metzler, C.; Miller-Stein, C.; Olah, T. *J. Am. Soc. Mass Spectrom.* **2000**, *11* (11), 942−50.
(17) Sterner, J. L.; Johnston, M. V.; Nicol, G. R.; Ridge, D. P. *J. Mass Spectrom.* **2000**, *35* (3), 385−91.
(18) Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K. *Rapid Commun. Mass Spectrom.* **1999**, *13* (12), 1175−1185.
(19) Koek, M. M.; Jellema, R. H.; van der Greef, J.; Tas, A. C.; Hankemeier, T. *Metabolomics* **2011**, *7* (3), 307−328.
(20) Fiehn, O.; Kind, T. *Methods Mol. Biol. (Totowa, NJ, U.S.)* **2007**, *358*, 3−17.
(21) Tiziani, S.; Emwas, A. H.; Lodi, A.; Ludwig, C.; Bunce, C. M.; Viant, M. R.; Gunther, U. L. *Anal. Biochem.* **2008**, *377* (1), 16−23.
(22) Cui, Q.; Lewis, I. A.; Hegeman, A. D.; Anderson, M. E.; Li, J.; Schulte, C. F.; Westler, W. M.; Eghbalnia, H. R.; Sussman, M. R.; Markley, J. L. *Nat. Biotechnol.* **2008**, *26* (2), 162−4.
(23) Stein, S. E. *J. Am. Soc. Mass Spectrom.* **1994**, *5* (4), 316−323.
(24) Stein, S. E. *J. Am. Soc. Mass Spectrom.* **1999**, *10* (8), 770−781.
(25) Stein, S. E.; Scott, D. R. *J. Am. Soc. Mass Spectrom.* **1994**, *5* (9), 859−866.
(26) Guideline for Disinfection and Sterilization in Healthcare Facilities, Center for Disease Control and Prevention, 2008 (http://www.cdc.gov/hicpac/Disinfection_Sterilization/acknowledg.html).
(27) Snyder, L. R.; and Dolan, J. W. *High-Performance Gradient Elution*; Wiley-Interscience: New York, 2007 (equation 3.3, p 90) .
(28) Mallard, G. Separation Science, parts I−III, http://www.sepscience.com/Techniques/MS.
(29) NIST/EPA/NIH Mass Spectral Library with Search Program (Data Version: NIST 11, Software Version 2.0g.) .
(30) Lowenthal, M. S.; Phillips, M. M.; Rimmer, C. A.; Rudnick, P. A.; Simón-Manso, Y.; Stein, S. E.; Tchekhovskoi, D.; Phinney, K. W. *Anal. Bioanal. Chem.* **2013**, DOI: 10.1021/ac402689t.
(31) Yang, X.; Neta, P.; Simón-Manso, Y.; Kilpatrick, L.; Liang, Y.; Stein, S. E., *Building a High Quality and Comprehensive Tandem Mass Spectral Library*. Poster presented at the 2012 International Conference of the Metabolomics Society, Washington, DC, June 25−28, 2012.
(32) The scoring algorithm has been slightly changed; the score is increased in the case of matching peak intensity ratios smoothly changing in one direction with an increase of m/z. The score may be decreased if the intensity ratio vs m/z has a maximum or if significant peak ratios do not change in a regular way. These changes are particularly important for metabolites because the fragmentation is usually poor compared, for example, to peptides and also because frequently the major fragmentation channels produce fragments that are not very informative, e.g., neutral losses. Also, the score was made more sensitive to the small peak intensities in the case of one or two dominant peaks. Penalties were added in the case of a small number of peaks in a spectrum.
(33) Ulrich, E. L.; Akutsu, H.; Doreleijers, J. F.; Harano, Y.; Ioannidis, Y. E.; Lin, J.; Livny, M.; Mading, S.; Maziuk, D.; Miller, Z.; Nakatani, E.; Schulte, C. F.; Tolmie, D. E.; Kent Wenger, R.; Yao, H.; Markley, J. L. *Nucleic Acids Res.* **2008**, *36* (Database issue), D402−8.
(34) Sumner, L. W.; Amberg, A.; Barrett, D.; Beger, R.; Beale, M. H.; Daykin, C.; Fan, T. W. M.; Fiehn, O.; Goodacre, R.; Griffin, J. L.; Hankemeier, T.; Hardy, N.; Higashi, R.; Kopka, J.; Lindon, J. C.; Lane, A. N.; Marriott, P.; Nicholls, A. W.; Reilly, M. D.; Viant, M. *Metabolomics* **2007**, *3*, 211−221 24.
(35) Broadhurst, D. I.; Kell, D. B. *Metabolomics* **2006**, *2* (4), 171−196.