Journal of Biomolecular Techniques • Volume 34(2); 2023 Jul

Step-by-Step Approach to Build Multiple Reaction Monitoring (MRM) Profiling Instrument Acquisition Methods for Class-based Lipid Exploratory Analysis by Mass Spectrometry

Leriana G. Reis[1] Theresa M. Casey[2] Tiago J. P. Sobreira[3] Bruce R. Cooper[3] Christina R. Ferreira[3]

[1]University of São Paulo, Department of Animal Science, School of Animal Science and Food Engineering, Rua Duque de Caxias Norte, 225, 13635-900, Pirassununga, SP, Brazil,
[2]Purdue University, Department of Animal Science, West Lafayette, Indiana 47907, USA,
[3]Purdue University, Metabolite Profiling Facility, Bindley Bioscience Center, West Lafayette, Indiana 47907, USA

Association of Biomolecular Resource Facilities

Published on: Jun 15, 2023

DOI: https://doi.org/10.7171/3fc1f5fe.1972c438

License: Copyright © 2023 Association of Biomolecular Resource Facilities. All rights reserved.

ABSTRACT

Multiple reaction monitoring (MRM) profiling is a strategy for the exploratory analysis of small molecules and lipids by direct sample injection, ie, without the use of chromatographic separation. It is based on instrument methods that comprise a list of ion transitions (MRMs), in which the precursor ion is the expected ionized *m/z* of the lipid at its species level, ie, the description of lipid class and number of carbon and double bonds in the fatty acid chain(s), and the product ion is a fragment expected for the lipid class or for the fatty acid neutral loss. The Lipid Maps database is expanding constantly, and therefore the MRM-profiling methods associated with this database need to be continuously updated. Here, we provide a comprehensive overview and the key references for the MRM-profiling methodology and workflow, followed by a step-by-step approach to build MRM-profiling instrument acquisition methods for class-based lipid exploratory analysis based on the Lipid Maps database. The detailed workflow includes (1) importing the list of lipids from the database; (2) for a given class, combining isomeric lipids described at full structural level into 1 entry to obtain the neutral mass at species level; (3) attributing the standard Lipid Maps abbreviated nomenclature for the lipid at its species level; (4) predicting the ionized precursor ions; and (5) adding the expected product ion. We also describe how to simulate the precursor ion for the suspect screening of modified lipids using lipid oxidation and their expected product ions as an example. After determining the MRMs, information about collision energy, dwell time, and other instrument parameters are added to finalize the acquisition method. As an example of final method output, we describe the format for Agilent MassHunter v.B.06 and provide the parameters in which optimization can be performed by lipid class using one or more lipid standards.

Visit the web version of this article to view interactive content.

Video Abstract

ADDRESS CORRESPONDENCE TO: Christina Ramires Ferreira, Purdue University, Metabolite Profiling Facility, Bindley Bioscience Center, 1203 Mitch Daniels Boulevard, West Lafayette, IN 47907, USA (Phone: +1 (765) 494-4905; E-mail: cferrei@purdue.edu).

Conflict of Interest Disclosures: The authors declare no conflicts of interest.

Keywords: MRM profiling, exploratory analysis, lipids, direct injection analysis

BACKGROUND

Lipid exploratory analysis is commonly performed using liquid chromatography (LC) followed by full mass scan data acquisition in high-resolution mass spectrometers (HRMS). Usually, the exploratory lipidomic output is a list of lipids present in the sample at their species or molecular species level.[1] In the current standardized Lipid Maps system, to report lipid structures analyzed, “species level” is considered the lowest hierarchical

level, at which only the lipid class, the sum of carbon atoms, the number of double bond equivalents, and additional oxygen atoms are reported.

In 2016, the multiple reaction monitoring (MRM) profiling strategy for the exploratory analysis of lipids and metabolites was first described by researchers at Purdue University and implemented in the Metabolite Profiling Facility (MPF) as a simplified approach for lipid exploratory studies. The first MRM-profiling publication was focused on identifying potential metabolite biomarkers of Parkinson's disease in cerebrospinal fluid.[2] The analytical concept of MRM profiling is based on direct sample injection and the profiling of chemical functionalities or class-diagnostic fragments. For that, samples are surveyed for lipid classes by precursor ion (Prec) and neutral loss (NL) scans.[2] The ions detected by Prec and NL scan are organized into ion transitions (or MRMs) that are used to screen sample and generate chemical profiles of tentatively attributed lipids at their species level.

Therefore, MRM profiling is usually a 2-step approach. The discovery phase is used to identify molecular functional groups present in pooled samples using NL and Prec scans. The second phase is the screening phase, when information from NL and Prec scans is organized into MRM lists and samples are individually interrogated only for the molecules detected in the discovery phase. Since its initial application, the MRM profiling strategy has been successfully applied to the analysis of lipids in buffy coat for diet compliance[3] and follicular fluid for diagnosis of polycystic ovarian syndrome,[4] the characterization of changes in the epidermis owing to atopic dermatitis in mice,[5] and the lipid and metabolite biomarker search for coronary artery disease, which was validated by LC-HRMS.[6] MRM profiling has also been used to characterize the lipids present in the vaginal mucosa of piglets,[7],[8] bacterial lipids related to antibiotic resistance,[9] and lipids extracted from the surface of nanoparticles after incubation in human serum.[10] Another application of the MRM-profiling strategy is to screen for exogenous compounds (as opposed to metabolites) in biological samples. For example, we confirmed by LC-MS/MS the presence of metformin, acetaminophen, and acrylamide contaminants in human urine after applying the MRM-profiling workflow on the screening of 87 compounds.[11] MRM profiling thus provides a rapid analytical workflow that serves as a first pass analysis to inform and identify the presence, or potential presence, of specific molecules in biofluids. More elaborate LC-MS/MS methods guided by MRM profiling exploratory data can then be used for structural confirmation and quantification of the metabolites or contaminating molecules in the samples after a selected set of MRMs is isolated.

This work specifically describes the steps for the MRM-profiling workflow adapted to more sensitive and fast analytical needs, such as for the analysis of lipids in microscopic (nanogram-size) samples, using bovine oocytes and preimplantation embryos as samples of interest.[12] For that, expected precursor ions for lipids such as phospholipids, triacylglycerides, acyl-carnitines, and cholesteryl esters listed in the LIPID MAPS Structure Database (LMSD) were combined to diagnostic fragments or neutral losses for each lipid class to produce ion transitions or MRMs. Therefore, alternatively to Prec and NL scans, MRM scans were already

used for the discovery step of the method, instead of being used just for the second part (screening) of the workflow. The MRM profiling screening step is focused on interrogating samples only for lipids detected in the discovery phase. In summary, the MRM-profiling method involving the use of MRM scans both for the discovery and screening steps had the disadvantage of being totally targeted, but it is sensitive and practical. As an example, it was successful in profiling almost 400 lipid species in individual, microscopic bovine blastocysts or in small pools (N = 5) of oocytes[11] and was also applied to the analyses of exosomes.[13]

Besides showing more sensitivity due to the use of MRM scans for both discovery and screening steps, the described MRM profiling enabled simpler data analysis workflow and biological interpretation. Although MRM profiling is aimed at exploratory analysis, so far, agreement on up/down regulation of targets was observed between MRM profiling and LC-MS/MS for the instances in which both methods were performed.[5],[6],[14] Therefore, the informative output of MRM profiling for the exploratory analysis of lipids in diverse types of samples translates into a more effective use of researcher funds and enables rapid turnaround times. MRM profiling of small molecules and lipids at Purdue University has been used for analyses described in 34 peer-reviewed publications thus far.[2],[3],[4],[5],[6],[7],[8],[9],[10],[11],[12],[13],[14],[15],[16],[17],[18],[19],[20],[21],[22],[23],[24],[25],[26],[27],[28],[29],[30],[31],[32],[33],[34],[35]

We recently reviewed manuscripts that applied MRM-profiling analysis and described the method development, outcome, and analytical concepts behind it.[31] Since most applications for the method do not include many samples, the percent of agreement between the experimental group and MRM profiling has been used as a metric for the method efficiency. In the review paper about the MRM-profiling concepts,[31] the performance/agreement of the method with reference data is discussed and in the published data listed. Overall, >70% agreement was observed.[31]

Because it is not possible to describe the method foundation in detail in publications and because of the fact that other mass spectrometry cores and research laboratories may be interested in offering such an approach, we share step-by-step instructions to set up MRM-profiling methods. We also added information on how to simulate MRM-profiling methods to screen for modified lipids.

To conduct MRM profiling and attribute data to lipid species, information is obtained from the Lipid Maps Structural Database (LMSD). LMSD is continuously updated, and thus lists of MRMs used need to be modified over time. Here, we share a detailed workflow of generating such MRM-profiling methods for the exploratory analysis of lipids based on the LMSD. A full roadmap is described that results in data acquisition methods for Agilent MassHunter v.B.06 as an example for the finalized acquisition methods. Nonetheless, this workflow can be used for setting up methods for mass spectrometers from other vendors that can perform MRM-profiling experiments. These methods may also include MRMs for deuterated lipid standards for relative quantification.[15],[17],[23],[24]

The discovery step can also be tailored to screen for lipids expected to be modified by enzymatic and nonenzymatic reactions, such as for oxidized and peroxidized lipids. In this tutorial, we describe a workflow and the setup of MRM-profiling methods to screen for lipids for suspected modification by oxidation and peroxidation as a previous step to LC-MS/MS analyses for structural and quantitative validation.

The characterization of oxidized lipids by mass spectrometry has considerably advanced with the contributions of Dr. Maria Fedorova's laboratory, who introduced the concept of epilipidome as a subset of the lipidome formed by lipid modifications via enzymatic and nonenzymatic reactions and required for the regulation of diverse cell functions and in diseases.[36] Over 300 lipids modified through peroxidation were reported by Fedorova's group initially using a short-term nitroxidative stress cell model and LC-MS/MS.[37] Among the analytical limitations for the identification and quantification of lipids modified through oxidation is the lack of internal standards, the large number of lipids possibly modified, and their much lower concentration levels compared with the nonmodified lipids. Because the MRM profiling can be applied as a suspect screening method, we envisage that it can be a fast and simple first-pass strategy to assess the possible presence of lipid modifications.

SETTING UP MRM PROFILING DISCOVERY AND SCREENING METHODS BASED ON THE LIPID MAPS DATABASE

This tutorial overview and how it is associated with the overall MRM-profiling strategy workflow is depicted in Figure 1. MRM profiling is intended to profile lipid classes at the species level. Thus, the information obtained from MRM profiling is aimed at guiding the design of further experiments for which structural confirmation at molecular species level and quantification can be obtained by other approaches such as LC-MS/MS.

Figure 1

Overview of the MRM-profiling workflow and the steps of setting up MRM-profiling discovery methods based on the LMSD information described in this tutorial manuscript (orange-colored boxes). We describe the steps to compose the methods used when the MRM-profiling Discovery step is based on a collection of MRMs derived from the LIPID MAPS database. The Discovery step goal is proportionate to a comprehensive and fast screening for the classes screened and indicates which targets are detectable for a narrow and even faster screening of many samples.

First, the LMSD is downloaded (https://www.lipidmaps.org/data/structure/LMSDSearch.php?Mode=SetupTextOntologySearch) in Excel format with all records of lipids. The lipid classes or subclasses of interest are split into different classes according to the LIPID MAPS Classification System[1] (Table 1).

Table 1

Lipid classes of interest for the current MRM-profiling methods offered at the MPF at Purdue University

Straight chain fatty acids [FA0101]	Glycerophosphoserines [GP03]
Unsaturated fatty acids [FA0103]	Glycerophosphoglyceols [GP04]
Fatty acyl carnitines [FA0707]	Glycerophosphoinositols [GP06]
Diradylglycerols [GL02]	Ceramides [SP02]
Triradylglycerols [GL03]	Ceramide phosphocholines (sphingomyelins) [SP0301]
Glycerophosphocholines [GP01]	Steryl esters [ST0102]
Glycerophosphoethanolamines [GP02]	-
The names within square brackets refer to the Lipid Maps 2-letter lipid category code, such as fatty acids (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), and sterol lipids (ST), followed by the 2-digit lipid class code and, in some cases, the 2-digit subclass code.	

In Supplementary File 1, the first tab lists the 47,602 lipids from the LMSD on May 2, 2022, and the lipids organized by the selected classes in the other tabs. Only lipid classes of interest were selected from the database as listed in Table 1. The steps described below relate to the Supplementary File 1, which is a template file to obtain the precursor and product ions at species level. Refer to the tab “Dictionary” for the description on each tab in the file

1. For each class, filter by “Common_Name.”

2. Create different spreadsheet tabs for each lipid class of interest. Also create spreadsheet tabs to past information on lipids containing alkyl ether and alkenyl ether substituents (see Supplementary File 1 tabs “GP01 – PC-O, GP01 – PC-P” for ester lipids as an example (Note 1).

3. Select each spreadsheet, sort by “Mass,” and remove duplicates from each tab. This step will combine isomeric entries to obtain the species level neutral mass.

4. Insert a new column called “Abbrev.”

5. Look up “LM_ID” in the field of search on LIPID MAPS (https://www.lipidmaps.org) and add the “Synonym” or “Abbrev” into the “Abbrev” column.

6. In the “Abbrev” column, remove all spaces. The total number of carbons and double bonds of all the chains can be placed within parenthesis. Please note that in the updated LIPID MAPS classification, the use of parentheses and brackets is minimized (Note 2).[1]

7. Combine the information about the ester- and ether-linked lipids from the same class again in 1 tab called "FINAL-Step 1."

8. Within each spreadsheet, sort the entries by "Mass."

9. Create a second column named "Mass-2," taking into consideration the value of mass from column "Mass" without the decimals because of the low mass resolution of the quadrupole mass analyzer (Note 3).

10. Create a column named "Conditional" to combine the name of lipids into 1 line based on the "Mass-2" column (Note 4).

11. To exclude the lipids that already had their name combined with another one, add a new column named "Abbrev Concatenate" (Note 5).

12. Duplicate the spreadsheet "FINAL-Step 1" to create another one named "FINAL-Step 2." Change the name of the column "Abbrev Concatenate" to "Abbrev Text" and paste only values in this new column (Note 6). Removes the spaces from the values in the new column.

13. Delete the "Conditional" column.

14. Filter the tab values by "Abbrev Text" and delete all empty/blank cells' rows.

15. Add columns for the precursor ion and for the fragment according to lipid class. For the current methods, we used product ions and neutral losses reported in the literature and mentioned in previous MRM-profiling publications, such as Yannell et al.[6] or de Lima et al.[12] This information is embedded in the product ion calculations used in Supplementary File 1. Clarifications were added as Notes 7, 8, 9, and 10.

16. Remove the columns "Common_Name," "Systematic_Name," and "Sub_class," as these refer to full structure level and subclasses that have been combined to the same entry in the previous steps.

17. Organize the information on the lipid species names, parent, and product ions for each class reported in this tutorial as an instrument method file (Note 11; Supplementary File 2).

18. For setting up MRM-profiling screening methods, compare the maximum ion intensities obtained for the sample(s) and for a blank sample (blank extraction). Establish a threshold of maximum/blank for considering a lipid (MRM) as detected (Note 12).

SUSPECT SCREENING FOR MODIFIED LIPIDS

The MRM-profiling method can also be used to screen for lipid modifications if these still allow the lipids to be ionizable. Usually, modified lipids occur in trace amounts, but some biological systems may accumulate them in short-term fashion.[38],[39],[40] Therefore, the use of MRM profiling for the suspect screening of

modified lipids may be useful in special circumstances, such as when sampling shortly after the cellular stress, and more suitable to in vitro systems instead of as a general method for any type of biological sample. For that, the MRMs detected in the discovery step will include native (ie, nonmodified) and simulated MRMs for the oxidized lipids. Even though most of native and modified MRMs for lipids may present isomeric overlap, the differential MRMs between the control and conditions that are expected to present higher levels of modified lipids, as an example, may guide the selection of MRMs for LC-MS/MS validation.

Here, we describe in detail the calculation for the precursor ion for lipids modified by oxidation, their expected product ion, and how to combine the generated MRMs with the ones of native lipids for the exploratory screening. As mentioned, based on abundance variation and expected behavior in the biological system, the candidate MRMs can then later be confirmed by LC-MS/MS or another appropriate analytical strategy. Figure 2 depicts a schematic overview of the MRM-profiling workflow for the suspect screening on modified lipids. The MRM-profiling discovery method setup for modified lipids is described step by step below.

Figure 2
Overview of the workflow for the modified lipids suspect screening by MRM profiling based on the LIPID MAPS Structure Database (LMDB) information. The mass shifts expected for the modified lipids are simulated. Comparing control and treatment groups, statistical analysis will indicate possibly modified lipids to be confirmed by LC-MS/MS methods. It is important to mention that modified lipids in lower abundance than the instrument's sensitivity threshold will not be detectable.

Supplementary File 3 is a template file for the steps described below. Please refer to the tab "Dictionary" for the description on each tab in the file. Supplementary File 4 contains the final suspect screening method.

Supplementary File 5 describes initial experiments for the screening of oxidized lipids using the proposed approach for a mix of deuterated internal standards, which were partially oxidized in the laboratory just as a proof-of-concept.

1. Add columns for the precursor ion (or parent ion) and the product ion (Note 13). These will be the "Native" tab. We only include calculations for PC and PE lipids in Supplementary File 3, as these are the most abundant phospholipids and mostly susceptible to oxidation in membranes.

2. Copy the "Native" tab information, create a new tab, and rename it as "Oxi." Create another tab, paste the same information, and rename it as "Peroxi" (Note 14).

3. Because oxidation is expected to occur at unsaturation sites, the first step for the screening of oxidized lipids excludes all saturated lipids, and species level (":0"). For fatty acyl chains containing 2 or more degrees of unsaturation, peroxidation is possible. Therefore, for the simulating MRMs of peroxidized lipids, saturated and monounsaturated lipids should be excluded.

4. Create a new column in the tabs listed below to add the monoisotopic mass for the precursor and for the product ion. Add the lipid (per)oxidation modification according to the description below (Note 15).

a. Tab "Oxi": add the mass of 1 oxygen atom (15.99491 amu) to the precursor ion.

b. Tab "Peroxi": add the mass of 2 oxygen atoms (31.98983 amu) to the precursor ion.

5. Duplicate the "Lipid Name" column (column A) to column B and rename it as "New Lipid Name." The names of lipids listed in this column will indicate the presence of (per)oxidation (Note 16).

6. Create a new tab named "Final-1," and within each spreadsheet, sort the entries by "Parent ion." Create a second column named "Mass-2" taking into consideration the value of mass from the column "Parent ion" without the decimals because of the low mass resolution of the quadrupole mass analyzer (Note 3).

7. Create a column named "Conditional" to combine the name of lipids into 1 line based on the "Mass-2" column (Note 17).

8. To exclude the lipids that already had their name combined with another one, add a new column named "Name Concatenated" (Note 18).

9. Duplicate the spreadsheet "Final-1" to create another one named "Final-2." Paste only values in the column "Name Concatenated" (Note 6). Remove the spaces.

10. Delete the "Conditional" column.

11. Filter the tab values by "Name Concatenated" and delete all empty/blank cells' rows (Note 19).

12. Generate the final method with the following columns: "New Lipidname," "Parent ion," "Product Ion," "Mass-2," "Conditional," and "Name Concatenated" (Note 17).

NOTES

Note 1

Splitting ester- and ether-linked lipids is necessary to correctly combine the LMSD entries at species level. For steryl esters, only cholesteryl esters were considered.

Note 2

Modified lipids, such as oxidized lipids, were removed in this example file, as they usually occur in trace amounts and may present distinct fragments. Therefore, dedicated workflows using suspect screening methods or LC-MS/MS are more appropriate for the screening of modified lipids.

Note 3

The following formula was used: =LEFT(F2,3). Observe the number of decimal places present in the integral numbers. If applicable, replace them with another number but do not round, as the mass defect contribution >0.5 will generate a mass shift when calculating the *m/z* values or the precursor ions.

Note 4

The following formula was used: =IF(G2<>G1,A2,H1&","&A2); the condition put the name together if they have the same “Mass-2.”

Note 5

The following formula was used: =IF($G2<>$G3,CONCATENATE(" ", $H2, " ")," "). The concatenation left a blank in place of the name that had already been combined based on “Mass-2.”

Note 6

The following formula was used: (Ctrl + Alt + V, choose “values”).

Note 7

For lipid classes such as the diacylglycerols and triacylglycerols that do not have a diagnostic product ion related to the polar head such as the glycerophospholipids or to the sphingoid base as for the sphingomyelins, NL for the fatty acyl chains can be used as the product ions.[41] In this case, when NL scans are related to saturated fatty acids such as 14:0, 16:0, and 18:0, all precursor ions will be included. When NL for unsaturated fatty acyl chains is used, only unsaturated species-level lipids can be included. As exemplified in the Supplementary File 2, the unsaturated methods for triacylglycerols (triradylglycerols, according to the Lipid Maps nomenclature) were based on the FINAL-Step2 tab (Supplementary File 1). For the NL of a fatty acyl chain containing 1 unsaturation, the species-level lipids (“:0”) without isomers were excluded. For the NL of a fatty acyl chain containing 2 unsaturation degrees, the saturated and monounsaturated species-level lipids were excluded if they were not isomers of lipids with higher degrees of unsaturation, and so on.

Note 8

For acyl-carnitines, 2 product ions are used for each precursor ion. The *m/z* 85.1 is the most abundant product ion. Because of the small size of the molecules in this class and the large number of coincident isomers, the

precursor ion for each acyl-carnitine has also been combined with the product ion of *m/z* 60.1, and this MRM is referred as "QUAL," which means it is supposed to be a qualitative ion transition.

Note 9

For the ceramides, each sphingosine base has a specific fragment, and these have been reported or based on Merrill et al.[42] Please note that deoxy and hydroxy ceramides were not included.

Note 10

For the free fatty acids, both parent and product ion are the same, as they do not fragment in informative ways by collision-induced dissociation without derivatization. Therefore, the results for the screening for this lipid class are not as precise as for the other lipid classes in which a diagnostic fragment for the lipid class or for the fatty acyl chain is used.

Note 11

Currently in our laboratory, Agilent QQQ 6410 is used, and we provide such methods as examples (Supplementary File 2). Collision energy parameters have been optimized using lipid standards.

Note 12

We usually set the control/blank ratio threshold to ≥1.3, but the threshold can be changed by the user. Copy and paste without formulas into another tab. Subsequently, calculate the treatment/blank ratio threshold. The selection criteria of S/N > 0.3 is based on the linear response observed for oocyte lipids reported by Xie et al. [31] Usually, small molecule profiling analysis is based on background subtraction, but the user can also consider only ions above a certain threshold compared to the blank. If performing relative quantification, ie, normalizing data by internal standards, S/N > 3 (LOD) or S/N > 10 (LOQ) is recommended. For the selection of MRMs for the screening step, it is of interest to carry out as much information as possible and sort out noise later using statistical analysis.

Note 13

The precursor or product ion information can be copied from the MRM database or by selecting the MRM column. As it is possible to see in Supplementary File 1, the formula (=LEFT(B2,9)) was used to obtain the precursor ion, and the formula (=RIGHT(B2,9)) was used to obtain the product ion. The data was checked and copied, values only were pasted, and the cells were converted to number formatting when the formula was used.

Note 14

For the oxidized method, the mass of 1 oxygen must be added. For the peroxidized method, the mass of 2 oxygens must be added.

Note 15

For glycerophosphocholines (PC) and phosphatidylethanolamine (PE), the oxidation was simulated only for the precursor ion if the whole molecule had at least 1 unsaturation, and the product ion was readjusted according to the new precursor ion.

Note 16

The bracket was placed at the beginning and at end of the name. The sign “)]” was replaced by “;O)]” for the tab “Oxi” or by “;O_2)]” for the tab “Peroxi.”

Note 17

The following formula was used: =IF(D2<>D1,A2,E1&","&A2). The condition put the name together if they had the same “Mass-2.”

Note 18

The following formula was used: =IF($D3<>$D4,CONCATENATE(" ", $E3, " ")," "). The concatenation left a blank in place of the name that had already been combined, based on “Mass-2.”

Note 19

Only lipid name, parent ion, product ion, and MRM columns should be copied and pasted without the formulas to the Supplementary File 4. This information is then used to generate instrument methods by adding collision energy, dwell time, and other instrument parameters necessary for the data acquisition.

DISCUSSION AND PERSPECTIVES

This tutorial includes a comprehensive overview of the analytical concepts and literature related to the MRM-profiling method. This is followed by the detailed description of the MRM-profiling method setup currently in use at MPF. Providing templates with formulas and detailed step-by-step instructions will support consistency in the regular updates of the LMSD and initiatives to increase the number of lipids screened. Also, the manuscripts reporting these methods can refer to this tutorial for providing methodology details for reproducibility purposes. In this work, statistical analysis and structural confirmation details have not been explored, and this is intended to be detailed in similar tutorial protocols as part of our core effort for reproducibility.

The exploratory analysis of the structural features of small molecule classes is the basis of the MRM-profiling strategy. This capability touches the “dark matter lipidome,” which is believed to represent the largest percentage of the existing small molecules present in cells. Small molecule structural features translate into class-related or functional group–related fragments or product ions, which can be profiled by mass

spectrometry using Prec and NL scans. On its turn, the information recovered by Prec and NL scans can be easily translated into MRM scans, which are the most sensitive scan in mass spectrometry.

One of the main characteristics of the MRM-profiling method is the sample delivery by direct injection. Ion suppression during direct analysis of complex samples clearly occurs in full mass scan, and we have frequently received questions about its impact for the MRM-profiling method. In our experience, the use of MRM lists allows for much lower ion suppression compared with full mass scan mode and more sensitive lipid profiles. MRM is the most sensitive scan in a triple quadrupole mass spectrometer, but in case more abundant ions and contaminations exist, most are filtered out before reaching the detector. We have observed 20% or less signal ion suppression when comparing the ion signal of isotopically labeled internal standards spiked into solvent or into lipid extracts (unpublished data).

We include in this work a template and steps to simulate and include (per)oxidized lipids in the exploratory analysis, but this workflow is being tested in different biological models regarding its usefulness. The implementation of screening for modified lipids is of high interest to the MPF network as well as worldwide for a wider understanding of the biological effects or pathology conditions, as currently the oxidized lipids are recognized as active regulators of multiple cellular and physiological functions. We recognize the limitations of the direct injection approach regarding isotopic overlaps and for the detection of trace lipids, but as a guidance to LC-MS, the method may become informative for specific biological systems.

As the next steps for the MRM-profiling analytical development, we plan on concentrating efforts on expanding the types of lipids that can be surveyed. We also would like to evaluate automated sample preparation and establish a higher throughput for the data acquisition, processing, and analysis, which are currently not integrated. Lastly, we would like to mention the translational aspect of the MRM-profiling method. Because its output is a list of lipids discriminant of experimental groups or disease conditions, the assay can be easily deployed to miniature mass spectrometers for point-of-care analyses.

ACKNOWLEDGMENTS

We are grateful to R. Graham Cooks, who envisioned the MRM-profiling approach, for reviewing this protocol and for all the intellectual and technical support for the implementation and improvement of this method at the MPF over the years. Dr. Cooks has been providing leadership and effort from his students, namely, Fernanda Cordeiro, Karen Yannell, Zhuoer Xie, L. Edwin Gonzalez, and Nicolas Morato, to include the method application and further analytical development in their PhD theses. We are also thankful to the Bindley administration, who recognized very early the usefulness of the MRM-profiling method and provided their users with access and support to use it. We are also thankful for the contribution of the MPF users, who provided the applications and indicated further needs of analytical development over the last 6 years. In addition, we would like to thank the São Paulo Research Foundation (FAPESP) for financial support (Grant number 2021/12819-0).

Supplemental Material

xlsx	S1.xlsx	3 MB
xlsx	S2.xlsx	268 KB
xlsx	S3.xlsx	171 KB
xlsx	S4.xlsx	34 KB
doc	S5.doc	271 KB

References

- Aldrovandi M, Fedorova M, Conrad M. Juggling with lipids, a game of Russian roulette. *Trends Endocrinol Metab*. 2021;32(7):463-473. doi:10.1016/J.TEM.2021.04.012

  ↩
- Antonelo DS, Beline M, Silva SL, et al. Variations in intramuscular fat content and profile in angus × nellore steers under different feeding strategies contribute to color and tenderness development in longissimus thoracis. *Meat Muscle Biol*. 2022;6(1). doi:10.22175/mmb.13043

  ↩
- Antonelo DS, dos Santos-Donado PR, Ferreira CR, et al. Exploratory lipidome and metabolome profiling contributes to understanding differences in high and normal ultimate pH beef. *Meat Sci*. 2022;194. doi:10.1016/J.MEATSCI.2022.108978

  ↩
- Berteli TS, Vireque AA, Da Luz CM, Borges ED, Ferreira CR, Navarro PA. Equilibration solution composition and extended exposure to equilibration phase affect embryo development and lipid profile of mouse oocytes. *Reprod Biomed Online*. Published online January 19, 2022. doi:10.1016/J.RBMO.2022.01.006

  ↩
- Casey T, Harlow KL, Ferreira CR, Sobreira TJP, Schinckel A, Stewart K. The potential of identifying replacement gilts by screening for lipid biomarkers in reproductive tract swabs taken at weaning. *J Appl Anim Res*. 2018;46(1):667-676. doi:10.1080/09712119.2017.1384733

  ↩

↩

- Cordeiro FB, Ferreira CR, Sobreira TJP, et al. Multiple reaction monitoring (MRM)-profiling for biomarker discovery applied to human polycystic ovarian syndrome. *Rapid Commun Mass Spectrom*. 2017;31(17):1462-1470. doi:10.1002/rcm.7927

↩

- de Lima CB, Ferreira CR, Milazzotto MP, Sobreira TJP, Vireque AA, Cooks RG. Comprehensive lipid profiling of early stage oocytes and embryos by MRM profiling. *J Mass Spectrom*. 2018;53(12):1247-1252. doi:10.1002/jms.4301

↩

- Devarshi PP, Pereyra AS, Ellis JM, Henagan TM. A single bout of cycling exercise induces nucleosome repositioning in the skeletal muscle of lean and overweight/obese individuals. *Diabetes, Obes Metab*. 2022;24(1):21-33. doi:10.1111/dom.14541

↩

- Dhillon J, Ferreira CR, Sobreira TJP, Mattes RD. Multiple reaction monitoring profiling to assess compliance with an almond consumption intervention. *Curr Dev Nutr*. 2017;1(9):1-8. doi:10.3945/cdn.117.001545

↩

- Dipali SS, Ferreira CR, Zhou LT, Pritchard MT, Duncan FE. Histologic analysis and lipid profiling reveal reproductive age-associated changes in peri-ovarian adipose tissue. *Reprod Biol Endocrinol*. 2019;17(1):1-13. doi:10.1186/s12958-019-0487-6

↩

- Edwards ME, De Luca T, Ferreira CR, et al. Multiple reaction monitoring profiling as an analytical strategy to investigate lipids in extracellular vesicles. *J Mass Spectrom*. 2021;56(1). doi:10.1002/jms.4681

↩

- Edwards ME, Marasco CA, Schock TB, Sobreira TJP, Ferreira CR, Cooks RG. Exploratory analysis using MRM profiling mass spectrometry of a candidate metabolomics sample for testing system suitability. *Int J Mass Spectrom*. 2021;468. doi:10.1016/j.ijms.2021.116663

↩

- Ferreira CR, Yannell KE, Mollenhauer B, et al. Chemical profiling of cerebrospinal fluid by multiple reaction monitoring mass spectrometry. *Analyst*. 2016;141(18):5252-5255. doi:10.1039/c6an01618a

↩

-

↩

- Franco J, Rajwa B, Ferreira CR, Sundberg JP, Hogenesch H. Lipidomic profiling of the epidermis in a mouse model of dermatitis reveals sexual dimorphism and changes in lipid composition before the onset of clinical disease. *Metabolites*. 2020;10(7):1-18. doi:10.3390/metabo10070299

↩

- Griesser E, Vemula V, Raulien N, et al. Cross-talk between lipid and protein carbonylation in a dynamic cardiomyocyte model of mild nitroxidative stress. *Redox Biol*. 2017;11:438-455. doi:10.1016/J.REDOX.2016.12.028

↩

- Guttenplan KA, Weigel MK, Prakash P, et al. Neurotoxic reactive astrocytes induce cell death via saturated lipids. *Nature*. 2021;599(7883):102-107. doi:10.1038/s41586-021-03960-y

↩

- Harlow KL, Ferreira CR, Sobreira TJP, Casey T, Stewart K. Lipidome profiles of postnatal day 2 vaginal swabs reflect fat composition of gilt's postnatal diet. *PLoS One*. 2019;14(9):1-16. doi:10.1371/journal.pone.0215186

↩

- Jia Z, Yue F, Chen X, et al. Protein arginine methyltransferase PRMT5 regulates fatty acid metabolism and lipid droplet biogenesis in white adipose tissues. *Adv Sci*. 2020;7(23):1-18. doi:10.1002/advs.202002602

↩

- Klopp RN, Ferreira CR, Casey TM, Boerman JP. Relationship of cow and calf circulating lipidomes with colostrum lipid composition and metabolic status of the cow. *J Dairy Sci*. 2022;105(2):1768-1787. doi:10.3168/jds.2021-21008

↩

- Kobos L, Ferreira CR, Sobreira TJP, Rajwa B, Shannahan J. A novel experimental workflow to determine the impact of storage parameters on the mass spectrometric profiling and assessment of representative phosphatidylethanolamine lipids in mouse tissues. *Anal Bioanal Chem*. 2021;413(7):1837-1849. doi:10.1007/s00216-020-03151-0

↩

- Kobos LM, Alqatani S, Ferreira CR, et al. An integrative proteomic/lipidomic analysis of the gold nanoparticle biocorona in healthy and obese conditions. *Appl Vitr Toxicol*. 2019;5(3):150-166. doi:10.1089/aivt.2019.0005

↩

↩

- LIPID MAPS® Lipidomics Gateway. Accessed December 1, 2022. https://www.lipidmaps.org/tools/ms/gp_ox_form.php

↩

- Marasco Júnior CA, Edwards ME, Lamarca RS, et al. Suspect screening of exogenous compounds using multiple reaction screening (MRM) profiling in human urine samples. *J Chromatogr B*. Published online May 12, 2022. doi:10.1016/J.JCHROMB.2022.123290

↩

- Merrill AH, Sullards MC, Allegood JC, Kelly S, Wang E. Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods*. 2005;36(2):207-224. doi:10.1016/J.YMETH.2005.01.009

↩

- Mills KM, Ferreira CR, Stevens JG, Stewart KR, Casey TM. Biomarkers predictive of long-term fertility found in vaginal lipidome of gilts at weaning. *J Anim Sci*. 2021;99(8):1-11. doi:10.1093/jas/skab189

↩

- Morato NM, Hallett JE, Wang WH, et al. Changes in lipid profile and SOX-2 expression in RM-1 cells after co-culture with preimplantation embryos or with deproteinated blastocyst extracts. *Mol Omi*. 2022;18(6):480-489. doi:10.1039/D2MO00071G

↩

- Nakanishi H, Iida Y, Shimizu T, Taguchi R. Analysis of oxidized phosphatidylcholines as markers for oxidative stress, using multiple reaction monitoring with theoretically expanded data sets with reversed-phase liquid chromatography/tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877(13):1366-1374. doi:10.1016/J.JCHROMB.2008.09.041

↩

- O'Neil E V., Burns GW, Ferreira CR, Spencer TE. Characterization and regulation of extracellular vesicles in the lumen of the ovine uterus. *Biol Reprod*. 2020;102(5):1020-1032. doi:10.1093/biolre/ioaa019

↩

- Pereyra AS, Lin C Te, Sanchez DM, et al. Skeletal muscle undergoes fiber type metabolic switch without myosin heavy chain switch in response to defective fatty acid oxidation. *Mol Metab*. 2022;59:101456. doi:10.1016/j.molmet.2022.101456

↩

-

↩

- Reis A, Domingues P, Domingues MRM. Structural motifs in primary oxidation products of palmitoyl-arachidonoyl-phosphatidylcholines by LC-MS/MS. *J Mass Spectrom*. 2013;48(11):1207-1216. doi:10.1002/JMS.3280

↩

- Soundararajan A, Wang T, Sundararajan R, et al. Multiomics analysis reveals the mechanical stress-dependent changes in trabecular meshwork cytoskeletal-extracellular matrix interactions. *Front cell Dev Biol*. 2022;10. doi:10.3389/FCELL.2022.874828

↩

- Suarez-Trujillo A, Huff K, Ramires Ferreira C, Paschoal Sobreira TJ, Buhman KK, Casey T. High-fat-diet induced obesity increases the proportion of linoleic acyl residues in dam serum and milk and in suckling neonate circulation. *Biol Reprod*. 2020;103(4):736-749. doi:10.1093/biolre/ioaa103

↩

- Uchikata T, Matsubara A, Nishiumi S, Yoshida M, Fukusaki E, Bamba T. Development of oxidized phosphatidylcholine isomer profiling method using supercritical fluid chromatography/tandem mass spectrometry. *J Chromatogr A*. 2012;1250:205-211. doi:10.1016/J.CHROMA.2012.05.083

↩

- Xie Z, Ferreira CR, Virequ AA, Cooks RG. Multiple reaction monitoring profiling (MRM profiling): small molecule exploratory analysis guided by chemical functionality. *Chem Phys Lipids*. 2021;235. doi:10.1016/J.CHEMPHYSLIP.2021.105048

↩

- Xie Z, Gonzalez LE, Ferreira CR, et al. Multiple reaction monitoring profiling (MRM-Profiling) of lipids to distinguish strain-level differences in microbial resistance in Escherichia coli. *Anal Chem*. 2019;91(17):11349-11354. doi:10.1021/acs.analchem.9b02465

↩

- Yannell KE, Ferreira CR, Tichy S, Cooks RG. Multiple reaction monitoring (MRM)-profiling with biomarker identification by LC-QTOF to characterize coronary artery disease. *Analyst*. 2018;143(20):5014-5022. doi:10.1039/c8an01017j

↩

- Yue F, Oprescu SN, Qiu J, et al. Lipid droplet dynamics regulate adult muscle stem cell fate. *Cell Rep*. 2022;38(3):110267. doi:10.1016/j.celrep.2021.110267

↩