Multiple Reaction Monitoring Profiling to Assess Compliance with an Almond Consumption Intervention

Jaapna Dhillon,1 Christina R Ferreira,2 Tiago Jose Paschoal Sobreira,2 and Richard D Mattes1

1Department of Nutrition Science, and 2Bindley Bioscience Center, Purdue University, West Lafayette, IN

Abstract

Background: Almonds are extremely rich sources of lipids and flavonoids, and their consumption is associated with several health benefits. However, there are no analytical methods available to document compliance with prescribed or self-reported chronic almond consumption.

Objective: The aim was to use an analytical approach that identifies metabolic profiles associated with long-term almond consumption to ascertain compliance with prescribed consumption.

Methods: A multiple reaction monitoring (MRM)-profiling strategy was designed to isolate metabolic changes in erythrocytes after 12 wk of almond consumption. MRM-profiling data acquisition and analysis involve performing separate discovery and screening steps to detect molecular features related to metabolic changes between experimental groups. Samples used for this research were erythrocytes recovered at baseline, after 12 wk of almond consumption (W12-almond group), and after 12 wk of a nut-free diet (W12-control group). For the MRM-profiling discovery step, representative samples (pools) of erythrocytes from individuals of all groups were interrogated by precursor ion and neutral loss scan experiments on the basis of previous knowledge of chemical functional groups present in the samples. The outputs of the discovery phase were methods used for the MRM-profiling screening phase to interrogate individual samples on the basis of fast-MRM measurements. In addition, we screened the literature for flavonoids identified in almond skins and included them for individual sample screening.

Results: Of the 254 m/z values monitored, 5 ratios and combinations of specific ions with receiver operating characteristic curve AUCs >0.89 provided a sensitivity of 74.2% and a specificity of 90% for blind samples presented in the model. Eight of the 31 participants (25.8%) in the W12-almond group and 3 of the 30 (10%) participants in the W12-control group were misclassified by all 5 ratios. Ratios and combinations of specific transitions were mainly related to membrane lipids.

Conclusion: The misclassifications observed as a result of ratio performance evaluation may indicate noncompliance as supported by the dietary intake data. The parent trial was registered at www.clinicaltrials.gov as NCT02360787. Curr Dev Nutr 2017;1:e001545.

Keywords: almond biomarkers, human erythrocytes, metabolomics, nuts, MRM profiling

Copyright © 2017, Dhillon et al. This is an open access article distributed under the terms of the CCBY-NC License http://creativecommons.org/licenses/by-nc/4.0/, which permits noncommercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited.

Multiple Reaction Monitoring Profiling to Assess Compliance with an Almond Consumption Intervention

Introduction

Almond consumption is associated with several health benefits, such as improvements in lipid profile, glycemic control, and vascular health (1, 2). In addition, despite their high energy content, almond consumption does not promote weight gain (3), and recent evidence indicates that almonds may improve body composition (4). Almonds are good sources of MUFAs, protein, fiber, arginine, vitamin E, polyphenols, and magnesium. These nutrients have been implicated in the aforementioned health benefits (2), but characterization of their benefits requires additional clinical study.

Assessing compliance with dietary interventions in long-term clinical trials is frequently problematic. Although self-reported measures of intake such as 24-h food recalls and FFQs are widely used, these are subjective measures of dietary intake (5) and only

Copyright © 2017, Dhillon et al. This is an open access article distributed under the terms of the CCBY-NC License http://creativecommons.org/licenses/by-nc/4.0/, which permits noncommercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited.

Multiple Reaction Monitoring Profiling to Assess Compliance with an Almond Consumption Intervention

Introduction

Almond consumption is associated with several health benefits, such as improvements in lipid profile, glycemic control, and vascular health (1, 2). In addition, despite their high energy content, almond consumption does not promote weight gain (3), and recent evidence indicates that almonds may improve body composition (4). Almonds are good sources of MUFAs, protein, fiber, arginine, vitamin E, polyphenols, and magnesium. These nutrients have been implicated in the aforementioned health benefits (2), but characterization of their benefits requires additional clinical study.

Assessing compliance with dietary interventions in long-term clinical trials is frequently problematic. Although self-reported measures of intake such as 24-h food recalls and FFQs are widely used, these are subjective measures of dietary intake (5) and only
capture a snapshot of an individual's dietary pattern. Compliance with long-term almond consumption is occasionally documented by using plasma α-tocopherol (4, 6). However, plasma α-tocopherol is more strongly associated with vitamin E supplement intake (7, 8) and might not reflect the intake of vitamin E from dietary sources such as nuts (9). Hence, there is a need to develop methods that can identify metabolic profiles associated with long-term almond consumption in order to ascertain compliance with an almond consumption prescription.

Nutritional metabolomics is a relatively new domain of nutrition that can capture the phenotype of dietary exposure in biological samples (10). Although urine and blood plasma samples are commonly used in the application of nutritional metabolomics, RBCs (erythrocytes) may reflect longer-term dietary intake (11) because the average erythrocyte life span is between 70 and 140 d (12). Because almonds are a rich source of lipids, it is reasonable to screen for diverse lipids in the erythrocytes of individuals who chronically consume almonds.

Recently, we reported the benefits of almond consumption in overweight and obese adults (13, 14). Here, we detail the metabolomics approach, multiple reaction monitoring (MRM) profiling (15, 16), used to support dietary compliance data. This mass spectrometric method for the accelerated discovery of molecular features was initially applied to Parkinson disease diagnostics (16). MRM-profiling development was based on the successful use of lipid profiles for diagnosing diverse types of cancer where chemical profiles acquired in unmodified samples by ambient ionization and data analyzed by multivariate statistics matched histopathologic evaluation (17–19). Another motivation behind the development of the MRM-profiling approach was the use of strategic MS/MS experiments to avoid excessive data acquisition and to allow the effective use of valuable samples (20).

The strategic use of the MS/MS experiments as suggested by Schwartz et al. (20) resulted in a 2-step method. The first step of MRM profiling, or the discovery step, is based on a selection of lipid subclasses, metabolites, and functional groups to be profiled. These experiments are conducted in representative samples, meaning one pool per experimental group, which is put together by mixing individual samples. The goal of the discovery step is to record the informative MS/MS domain data by using representative samples. The second step of the method is performed by screening transitions detected in the discovery step for all individual samples. This is very fast (a few minutes per sample) and allows interrogation only for molecular features detected in the samples.

Multivariate statistical approaches are performed on the resulting data. Transitions recovered at the discovery step and used in the MRM methods of the screening step are here referred to as “molecular features” because they can be related to a chemical characteristic of the sample, but tentative attribution was not pursued until after the statistical analysis, which indicated which molecular features were discriminant.

The objective of the present study was to apply MRM profiling to assess compliance with almond consumption by screening for prospective erythrocyte biomarkers that are discriminatory between individuals who consumed an almond-enriched diet for 12 wk (W12-almond group) and those who did not consume any nuts during that period (W12-control group). Most discriminant molecular features were related to lipids and metabolites in erythrocytes, and we report molecular feature ratios that were highly discriminatory between the W12-almond and W12-control groups. Five ratios were explored in more detail and included 10 transitions, which were mostly related to membrane lipids. It is important to consider that the identified informative ion combinations related to lipids are only prospective biomarkers and the specificity of the method for other types of nuts has not been addressed to our knowledge. Biomarker validation was not in the scope of this study. Nonetheless, these results suggest that long-term almond consumption affects erythrocyte cell membrane composition and that this approach can be useful for assessing compliance with long-term almond consumption.

Methods
Participants
Sixty-one healthy male and female overweight or obese individuals [aged 18–60, BMI (in kg/m²): 25–40] participating in an almond weight-loss study (clinicaltrials.gov; registration number NCT02360787) were recruited (13). Detailed participant and study information can be found in the parent study article (13). This study was conducted according to the guidelines stipulated in the Declaration of Helsinki. All of the procedures involving human participants were approved by the Purdue University Institutional Review Board. Written informed consent was obtained from all individual participants included in the study.

Intervention
The study was a randomized, controlled, parallel-arm, 12-wk clinical trial (13). Participants were randomly assigned to 1 of 2 500-kcal energy-restricted study arms: W12-almond group or W12-control group (Table 1). Weekly energy and nutrient analyses with the use of 24-h food recalls were conducted to determine the participants’ compliance with dietary recommendations. Participants randomly assigned to the almond-enriched energy-restricted diet group (n = 31) were asked to consume dry-roasted, lightly salted almonds providing 15% of energy in their individualized energy-restricted diet. Participants randomly assigned to the nut-free energy-restricted diet group (n = 30) were asked to avoid all nuts, seeds, and nut products during the intervention period.

**Table 1** Baseline characteristics of participants included in the MRM-profiling study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Almond-enriched diet (n = 31)</th>
<th>Nut-free diet (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24 (77.4)</td>
<td>24 (80)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (22.6)</td>
<td>6 (20)</td>
</tr>
<tr>
<td>Age, y</td>
<td>32.06 ± 13.612</td>
<td>33.40 ± 13.21</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.97 ± 3.34</td>
<td>30.93 ± 4.52</td>
</tr>
</tbody>
</table>

1 MRM, multiple reaction monitoring.

2 Mean ± SD (all such values).
Sample extraction and MRM-profiling data acquisition

Fasting blood samples (5 mL) were collected into EDTA-coated evacuated tubes from participants at baseline and at the end of the 12-wk intervention. The erythrocytes were separated from the plasma by centrifugation. They were washed 2 times in saline (0.90% NaCl; wt:vol) followed by centrifugation at 4000 x g for 10 min at 4 °C (Allegra 25R centrifuge; Beckman Coulter). The erythrocyte aliquots were stored at −80 °C until further analysis.

The lipid extracts were prepared by using a procedure by Rose and Oklander (21). Erythrocytes (150 μL) were mixed with 150 μL ultrapure water, and the contents were allowed to stand for 15 min with occasional mixing (vortex). Isopropanol (1.0 mL) was added to the mixture with occasional mixing. After 30 min, 600 μL chloroform was added, mixed, and allowed to stand for another 30 min. The tube was centrifuged at 10,000 x g for 10 min at room temperature (Taylor Scientific Centrifuge). The lipid extract (bottom phase) was subsequently removed and transferred to an ultracentrifuge (Vacufuge; Eppendorf). Dried lipid extracts were resuspended in 270 μL acetonitrile/methanol/ammonium acetate (300 mM) at 3:6.65:0.35 volume ratios. The solvent pumped by the microsampler between injections was acetonitrile + 0.1% formic acid.

The lipid extract from almond seeds was prepared by using a procedure by Bligh and Dyer (22) for comparison purposes. Four almond seeds were individually homogenized in 1 mL water by using a Precellys 24 tissue homogenizer (Bertin Technologies). The homogenate (800 μL) was transferred to a 15-mL Falcon tube and mixed with 1 mL chloroform and 1.6 mL MeOH. After mixing and 15 min of incubation at room temperature, 1 mL chloroform and 1 mL water were added, causing the formation of a 2-phase solution. The lipid extract (bottom phase) was subsequently removed and dried under a nitrogen gas (N2) stream. Aliquots of the 4 almond seeds were then combined for further experiments.

Lipid extracts (6 μL) from individual participants and the almond seeds were delivered to a triple quadrupole mass spectrometer (Agilent QQQ 6460) equipped with a jet stream electrospray (ESI) ion source. The lipid extracts from participants were pooled into 3 groups for conducting the diverse scan modes. The first group comprised lipid extracts for all participants at baseline (n = 52), regardless of intervention group (baseline group); the second group comprised lipid extracts for participants who consumed almonds during the intervention (W12-almond group; n = 31); and the third group comprised lipid extracts for participants who did not consume any nuts or nut products during the intervention (W12-control group; n = 30).

Full scans at the positive and negative ion modes and >80 neutral loss (NL) and precursor ion (Prec) scan modes were carried out on the lipid extracts from the 3 pooled samples and almond seeds (data not shown). Nonetheless, only 20 scans were informative (i.e., presented ions) (Table 2). Initial data processing of the profiles obtained was carried out by MassHunter (B.06.00; Agilent, Santa Clara, California). The molecular features were organized into 2 methods (one for the positive and another for the negative ion mode) for MRM or single reaction monitoring. In addition, we included molecular features compatible with flavonoids present in almonds and nuts (23, 24). With the inclusion of the m/z values related to anthocyanins, 254 molecular features (transitions or single ions: 148 in the positive ion mode and 106 values in the negative ion mode) were monitored in the 113 individual samples of the 3 experimental groups. It is important to consider that some of the structural identification of the molecular features was not pursued until the relevant ones were isolated by univariate and multivariate analyses.

Data analysis

For the informative NL and Prec scans (Table 2) obtained at the discovery step, the mass spectra had the background subtracted, and
The values of \( m/z \) present in mass spectra were exported for organization into fast-MRM methods for the screening step. The files generated by the mass spectrometer were converted to the mzML format by using MSConvert (http://proteowizard.sourceforge.net), and an in-house script was developed to sum the ion intensities from 0.4 to 1.0 min of data acquisition of each of the 254 molecular features monitored. The values of ion intensities were normalized by total ion current to obtain relative ion intensities. The data set was then...
filtered out to remove any ions that did not appear in >50% of the samples. Data were then auto-scaled. The differences in the erythrocyte metabolome due to the diet factors (W12-almond compared with W12-control groups) and time of intervention factors (baseline compared with week 12) were analyzed via univariate (volcano plots) and multivariate [principal components analysis (PCA)] statistics by using Metaboanalyst 3.0 (25). The performance of the identified metabolites in discriminating W12-almond from W12-control groups was evaluated by constructing receiver operating characteristic (ROC) curves on the data set and estimating the AUC. The 50 most informative molecular features (i.e., those discriminating between W12-almond and W12-control groups with an AUC >0.75) were considered for computation of different combinations and ratios by hand and by using the ROC feature of Metaboanalyst. The ratios with AUCs >0.89 were considered as informative and submitted to PCA to visualize the difference between groups. For accessing the discrimination value of specific transitions, transition ratios, or a panel of transitions, samples were randomly divided into testing and validation sets. The testing set was used to build a classification model, and the validation set was presented as new samples. Therefore, classification outcome results mentioned are for blind (presented as unknown to classifier) samples presented to the model. This strategy avoids model overfitting and stronger evidence for the discriminating efficiency of the method in terms of sensitivity and specificity. The 50 informative molecular features were also submitted for MS/MS analysis for lipid class and unsaturation level attribution (data shown only for the values of m/z presented in the 5 selected ratios; Supplemental Figure 1).

### Results

The overall goal of this research was to assess compliance with an almond consumption protocol for weight loss by using an MRM-profiling protocol. The workflow used in the study (Figure 1) was based on fast-sample preparation of erythrocytes, lipid profiling via direct injection including full scan, screening of flavonoids, and NL/Prec scans targeted at specific functional groups.

For the discovery step, lipid extracts were injected without chromatographic separation into the ESI source (flow injection),

---

**TABLE 3** FCs of the 5 selected m/z ratios and combinations between the W12-almond group and the W12-control group at the end of the 12-wk intervention

<table>
<thead>
<tr>
<th>Selected m/z ratios</th>
<th>FC</th>
<th>log₂(FC)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(447 x 930.5)/(703.5 x 1016.5)</td>
<td>2.5901</td>
<td>1.373</td>
<td>0.000000172</td>
</tr>
<tr>
<td>(447 x 930.5)/(703.5 x 876.5)</td>
<td>4.7656</td>
<td>2.2527</td>
<td>0.00000546</td>
</tr>
<tr>
<td>(447 x 930.5 x 782.5)/703.5</td>
<td>8.5938</td>
<td>3.1033</td>
<td>0.0001237</td>
</tr>
<tr>
<td>(447 x 930.5 x 782.5)/813.5 –&gt; 629.5</td>
<td>9.083</td>
<td>3.1832</td>
<td>0.00012746</td>
</tr>
<tr>
<td>(447 x 930.5)/(703.5 x 814.5)</td>
<td>6.8211</td>
<td>2.77</td>
<td>0.0000514</td>
</tr>
</tbody>
</table>

*FC, fold change; W12-almond, group who consumed an almond-enriched diet for 12 wk; W12-control, group who did not consume nuts for 12 wk; Q, quadrupole.*

*Ion intensities have been measured by monitoring just the parent ion both in Q1 and Q3, except for m/z 813.5, which had the fragment of m/z 629.5 monitored. Ion modes of each ion or ion pair are shown in Table 4.*

**FIGURE 3** PC analysis score plot of the 19 m/z value ratios presenting AUCs >0.89. Circles in the graph are intended to facilitate visualization and are not correlated with statistical analysis. PC, principal component; PCA, principal components analysis; W12-almond, group who consumed an almond-enriched diet for 12 wk; W12-control, group who did not consume nuts for 12 wk.
and data on diverse neutral loss (NL and Prec) scan modes were collected for each representative sample (pooled baseline, pooled W12-almond, and pooled W12-control samples). In addition, full mass scan data were considered. For the discovery step, molecular features detected in the discovery phase and m/z values of flavonoids reported in the literature were organized into tailored methods of MRM or single reaction monitoring.

Multivariate analyses of MRM profiling by PCA of all 254 molecular features showed no clear discrimination between the erythrocyte metabolome of the 2 intervention groups at baseline, which was expected (Figure 2A). In addition, we did not see discrimination between the metabolic profiles of the 2 groups over time (baseline compared with week 12 groups) (Figure 2A). This could be a result of an absence of a diet standardization phase before the start of the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention.

Multivariate analyses of MRM profiling by PCA of all 254 molecular features showed no clear discrimination between the erythrocyte metabolome of the 2 intervention groups at baseline, which was expected (Figure 2A). In addition, we did not see discrimination between the metabolic profiles of the 2 groups over time (baseline compared with week 12 groups) (Figure 2A). This could be a result of an absence of a diet standardization phase before the start of the intervention. Hence, participants may have either been consuming nuts or abstaining from nut consumption before the intervention and this could have confused identifying differences over time in each group. However, we did see a relatively distinct clustering of the almond and control groups at the end of the intervention (Figure 2B). This may indicate that some participants were not compliant with their respective intervention diets.

Nevertheless, we evaluated the performance of the most informative molecular features indicated by ROC analysis (n = 50; AUC >0.75) in discriminating between the almond and the control groups at the end of the intervention. In addition, we evaluated the performance of different combinations and ratios of all molecular features, because these relations can capture information lost in univariate and multivariate methods and may provide more relevant information with regard to a biological system than individual metabolites alone (26). Of the 254 molecular features monitored, 9 were observed in 19 ratios, combinations, or both that presented ROC curves with AUCs >0.89. PCA of the 19 ratios showed relatively distinct clustering with little overlap of the almond and control groups at the end of the intervention (Figure 3). Of the 19 ratios, 5 ratios that presented the most unique combinations of ions and those with the most informative fold changes were selected (Table 3).

Structural information obtained from the MS/MS experiments of the molecular features included in the 5 selected ratios is listed in Table 4. Most presented the fragment of m/z 184, which is diagnostic of phosphatidylcholine and sphingomyelin lipids. Values of m/z coincident with flavonoids were also included in the ratios. However, MS/MS was not diagnostic for these ions (Table 4). All 5 ratios had a sensitivity of 74.2% and a specificity of 90% (i.e., 8 of the 31 participants in the almond group and 3 of the 30 participants in the control group were misclassified, indicating the possibility of noncompliance) (Figure 4). To confirm whether these participants were noncompliers in their respective intervention groups, we evaluated their dietary intake data (13). Noncompliers in the almond group showed a trend for either a reduction in or an inconsistent intake of MUFAs, arginine, vitamin E, and phytic acid (nutrients that are high in almonds) over the intervention period, whereas noncompliers in the control group showed a trend for either an increase in or an inconsistent intake of the aforementioned nutrients over the intervention period (data not shown). In contrast, participants who were categorized as compliers in the almond group had greater MUFAs, arginine, and vitamin E intakes over the intervention (data not shown). Although the extremely small number of possible noncompliers precluded a statistical determination of the dietary intake data, it is reasonable to assume that, on the basis of MRM-profiling and dietary intake data, they might not have been compliant with their respective intervention groups.

**Discussion**

To our knowledge, this is the first study that has used MRM profiling to ascertain compliance with an almond intervention. MRM profiling is an informative, rapid, and high-throughput strategy for erythrocyte metabolome screening. The concept of MRM profiling allows accessing more detailed structural information of chemicals present in the sample compared with full mass scan profiling/fingerprinting. MRM profiling also presents a much simpler workflow than traditional metabolomics for biomarker discovery based on LC or GC or capillary electrophoresis. Molecular features can include >1 molecule or isomer. Even though we have acquired MS/MS data of the most relevant ions described in the study (Supplemental Figure 1), they should be further evaluated as biomarkers in subsequent experiments, including chromatographic separation or high mass resolution MS, or even more sophisticated tools such as ion mobility MS.
FIGURE 4  Performance analysis of selected m/z ratios with the use of ROC univariate curves. Graphs for ratios 1–5 show box plots of the values. Individual samples for W12-almond and W12-control groups are depicted as open and solid symbols, respectively. Dotted lines indicate the ROC threshold used to classify samples in 1 of the 2 groups. ROC, receiver operating characteristic; W12-almond, group who consumed an almond-enriched diet for 12 wk; W12-control, group who did not consume nuts for 12 wk.
The exploration of the erythrocyte metabolome in 61 participants (W12-almond and W12-control groups) by MRM profiling identified ratios and combinations of specific molecular features mainly related to membrane lipids that were discriminatory of almond consumption from the nut-free diet at the end of the 12-wk intervention. The main feature of the MRM-profiling method is that it is a chemically supervised profiling method that avoids excessive data acquisition. This is achieved by profiling small molecule classes and functional groups known to be present in the samples by using selected Prec and NL scans. Even though no LC-MS/MS validation was included in this work, it has been shown to be in agreement with MRM-profiling data (H HogenEsch, R Graham Cooks, Purdue University, West Lafayette, Indiana, personal communication, 2017). The main limitation of MRM profiling is the need to know the chemistry of samples because this is based on profiling classes of molecules. Challenges in metabolomics and lipidomics methods, especially related to identifying compounds, are shared by MRM profiling.

Although we cannot ascertain at this point whether the informative lipids detected are biomarkers of almond consumption, the relation between them indicates almond-induced changes in the metabolomics profile, and the misclassifications observed as a result of the ratio performance evaluation could be indications of possible noncompliance, as supported by the intake data. Nevertheless, this approach (MRM profiling + dietary intake data) will have to be further tested in varied studies with larger data sets. In conclusion, an ideal test of compliance with nutritional interventions may involve a combination of biochemical, metabolomics, and subjective dietary recall measures.

Acknowledgments
We thank Bruce R Cooper, Amber Jannasch, and Jackeline M Franco from Purdue's Bindley Bioscience Center for their analytical expertise, and Harm HogenEsch and Graham Cooks from Purdue University for their personal communications. The authors’ responsibilities were as follows—JD, CRF, TJPS, and RDM: conceived the study and shared equal responsibility in writing the manuscript and its final content; and all authors: read and approved the final manuscript.

References