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Method for the Identification of Lipid Classes Based on Referenced Kendrick Mass Analysis

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A rapid method for the determination of lipid classes with high sensitivity is described. The referenced Kendrick mass defect (RKMD) and RKMD plots are novel adaptations of the Kendrick mass defect analysis that allows for the rapid identification of members of a homologous series in addition to identifying the lipid class. Assignment of lipid classes by the RKMD method is accomplished by conversion of the lipid masses to the Kendrick mass scale and then referencing the converted masses to each lipid class. Referencing of the masses to a given lipid class is achieved by first subtracting the heteroatom and lipid backbone contributions to the mass defect, leaving behind the contribution to the mass by the fatty acid constituents. The final step in the referencing makes use of spacing differences in mass defects between members of the same Kendrick class to identify members of the lipid class being referenced. The end result of this is that a lipid belonging to the class being referenced will have an integer RKMD with the value of the integer being the degrees of unsaturation in the lipid. The RKMD method was able to successfully identify the lipids in an idealized data set consisting of 160 lipids drawn from the glyceride and phosphoglyceride classes. As a real world example the lipid extract from bovine milk was analyzed using both accurate mass measurements and the RKMD method.

Traditional methods for analyzing lipids rely on a multistage analytical approach consisting of prefractionation into lipid classes or polar and nonpolar lipids, followed by reversed-phase liquid chromatography to identify individual lipid molecular species based on their retention times.[1−4] Analysis of lipids in this traditional manner is quite problematic, and these problems can be grouped into three primary areas: time requirements, sample integrity, and specificity.[1,2] Lipid analysis using this multistage approach is time intensive, with time needed for the fractionation into lipid classes and often time must be spent in pretreatment of the sample in the form of cleanup or chemical derivatization.[1] Sample integrity issues may arise during the analysis of a lipid sample due to the increased handling required during prefractionation. Lipid oxidation occurring over the time course of the analysis is also of concern and can greatly diminish sample integrity.[2] The final problem encountered is one of specificity. HPLC-based lipid separations suffer from limited resolution and can rarely resolve all lipids in a given fraction. The implication from this practical limitation is that coeluting lipids cannot be distinguished.[1] Gas chromatography (GC) has been successfully employed to overcome the specificity problems to the extent that most lipids in a sample can be resolved and detected, but GC analysis of lipids requires considerable time in sample preparation and also in instrument time, leading to a marked reduction in duty cycle.[1,2]

Aside from chromatography-based platforms, other analytical techniques have been applied to lipid analysis with varying degrees of success, primarily Fourier transform infrared spectroscopy, nuclear magnetic resonance, and mass spectrometry.[1,2] While each of these methods has its own strengths and weaknesses, mass spectrometry (MS) has come to be one of the most powerful platforms for the analysis of lipids, providing an analytical tool that has high sensitivity and specificity while being highly robust and reproducible.[1−3,5] Mass spectrometry based methods for the identification of lipids, and their classes can be divided into two broad areas: identification by tandem mass spectrometry and identification by accurate mass measurements. Identification of lipids and lipid classes by tandem mass spectrometry (MS/MS) relies on the dissociation of lipids into fragments characteristic of the lipid class following ion activation. This is most often accomplished by means of collision induced dissociation (CID).[5−9] While identification of lipids by tandem mass spectrometry is in itself an incredibly powerful tool for identifying the lipid class and also the exact identity of the lipid, this approach often produces complicated fragmentation spectra. These results require careful interpretation that is not easily automated.

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(1) Hu, C.; van der Heijden, R.; Wang, M.; van der Greef, J.; Hankemeier, T.; Xu, G. *J. Chromatogr. B: Anal. Technol. Biomed. Life. Sci.* **2009**, *877* (26), 2836–46.

(2) Carrasco-Pancorbo, A.; Navas-Iglesias, N.; Cuadros-Rodriguez, L. *Trends Anal. Chem.* **2009**, *28* (3), 263–278.

(3) Carrasco-Pancorbo, A.; Navas-Iglesias, N.; Cuadros-Rodriguez, L. *Trends Anal. Chem.* **2009**, *28* (3), 263–278.

(4) Hinrichsen, N. Steinhart, H. Techniques and Applications in Lipid Analysis. In *Lipid Analysis and Lipidomics: New Techniques and Applications*; Mossoba, M. M., Eds.; AOCS Press: Champaign, IL, 2006.

(5) Zehethofer, N.; Pinto, D. M. *Anal. Chim. Acta* **2008**, *627* (1), 62–70.

(6) Hsu, F. F.; Turk, J. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877* (26), 2673–95.

(7) Hein, E. M.; Blank, L. M.; Heyland, J.; Baumbach, J. I.; Schmid, A.; Hayen, H. *Rapid Commun. Mass Spectrom.* **2009**, *23* (11), 1636–46.

(8) McAnoy, A. M.; Wu, C. C.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **2005**, *16* (9), 1498–509.

(9) Pulfer, M.; Murphy, R. C. *Mass Spectrom. Rev.* **2003**, *22* (5), 332–64.

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Two strategies exist for the identification of lipid classes based on accurate mass measurements.[10−12] The first strategy is to determine the molecular formula for the experimental mass and use this formula to assign the lipid to a specific lipid class. Assignment of lipid classes in this manner demands a high degree of mass accuracy, requiring experimental mass errors in the subppm range for unique identification of the molecular formula.[13−15] The mass error required to uniquely determine a molecular formula can be larger, around 1 ppm, if constraints regarding the elemental composition can be enforced.[16] Working in this manner, lipid masses can successfully be assigned to a lipid class using accurate mass measurements and combinatorial data analysis methods. When information regarding the lipid system under study is known, further constraints and biological filters may be applied to reduce the number of possible matches.

Lipid class assignments can also be made by mass defect analysis.[10−12,17] The cornerstone of mass defect analysis is a graphical representation of the mass spectral data in which the measured mass defects are plotted versus the measured nominal masses. The mass defect plot quickly allows trends in a mass spectral data set to be identified, such as the nature of the data (proteomic, carbohydrate, lipidomic, or nucleotide).[12,18] It should be noted that while accurate mass measurements allow for a rapid identification and assignment of lipid classes, the complete structure (i.e., acyl chain placement and location of double bonds) cannot be deduced from the accurate mass measurement, and tandem mass spectrometry experiments must be performed to obtain this information when it is needed.

The aliphatic chemical nature of lipids makes them prime candidates for Kendrick mass defect analysis using a Kendrick plot.[10,11,17,19] The Kendrick plot identifies lipid classes based on trends in the masses, with the primary trend being a constant mass defect difference of 0.0134 for masses having the same type and number of heteroatoms.[10,17] Since the majority of the lipid classes possess a different set of heteroatoms, lipid classes can easily and quickly be identified and assigned from a Kendrick plot by examining differences in mass defect, with the caveat that a lipid of each class is known so that differences in mass defect can be measured from the known lipid. However, the phosphatidylcholine and phosphatidylethanolamine classes do have the same type and number of heteroatoms so extra steps must be taken to differentiate between these two classes, such as intrasource separation.[20]

We report a rapid method for identifying lipid classes with potential for high-throughput analysis based on the Kendrick scale. The referenced Kendrick mass defect, a modification to the Kendrick mass defect method of lipid analysis, is presented here. This unique modification to the Kendrick mass defect analysis allows for the identification of lipid classes without prior knowledge of the lipid class of any lipid masses in the spectrum. The applicability of the method is shown applied to the identification of bovine milk lipids and is contrasted to a combinatorial, accurate mass method of lipid class assignment.

EXPERIMENTAL SECTION

Bovine Milk Lipid Extraction. Whole, unpasteurized Holstein milk pooled from several members of a milking herd was obtained from the dairy at the University of California, Davis. Lipid extraction was performed upon receipt of the milk sample to minimize lipid oxidation. The lipids were isolated from the whole milk using a modified Folch extraction procedure[21,22] in which a 0.5 mL aliquot of milk was added to 9 mL of a chloroform:methanol solution (2:1, v/v). The mixture was thoroughly mixed and allowed to sit for an hour at 4 °C after which the mixture was centrifuged for 15 min also at 4 °C. The organic layer was extracted and stored in chloroform at −20 °C until analysis. The aqueous layer was discarded. All solvents used were purchased from Sigma (St. Louis, MO) and were of HPLC grade.

Mass Spectrometry. Extracted bovine milk lipids were prepared for analysis by nanoelectrospray Fourier transform ion cyclotron resonance mass spectrometry (nESI FT-ICR MS) by performing a one thousand-fold dilution of the lipid extract using a solution of methanol:chloroform (2:1, v/v) plus 5 mM ammonium acetate (Fisher Scientific, Pittsburgh, PA). This diluted sample was then used for mass spectral analysis.

All spectra were acquired on an IonSpec 9.4 T QFT FT-ICR MS instrument (Lake Forest, CA) equipped with the Advion Nanomate nanospray ion source (Ithaca, NY). The Nanomate was controlled using the accompanying ChipSoft software (version 6.4.5). Source voltages and nitrogen gas pressures were adjusted as needed to maintain a stable spray, with these parameters generally in the range of 1.5 kV spray voltage and 0.2 psi nitrogen gas pressure. Experimental pulse sequences for the QFT FT-ICR mass spectrometer were programmed and controlled using the IonSpec Omega software (version 9.1.2) and the OmegaXP data station. The timing and pulse length were optimized for each sample at the time of data acquisition. Briefly, all ions were collected from the source in a hexapole accumulation cell for a given time interval. At the end of the accumulation period, all ions were transferred to the ICR cell by means of an RF-only quadrupole ion guide. Excitation and detection parameters were adjusted to optimum high resolution mass spectra, with experimental resolutions spanning a range of 75 000−100 000 fwhm, with the average resolution being 80 000 fwhm. Mass accuracy was ensured by means of careful external calibration using maltooligosaccharides[23] followed by internal calibration to lipid ions whose identity and composition had been confirmed by MS/MS experiments.

(10) He, H.; Conrad, C. A.; Nilsson, C. L.; Ji, Y.; Schaub, T. M.; Marshall, A. G.; Emmett, M. R. *Anal. Chem.* **2007**, *79* (22), 8423–8430.
(11) Hughey, C. A.; Hendrickson, C. L.; Rodgers, R. P.; Marshall, A. G. *Anal. Chem.* **2001**, *73* (19), 4676–4681.
(12) McFarland, M. A.; Marshall, A. G.; Hendrickson, C. L.; Nilsson, C. L.; Fredman, P.; Mansson, J. E. *J. Am. Soc. Mass Spectrom.* **2005**, *16* (5), 752–62.
(13) Bristow, A. W. *Mass Spectrom Rev* **2006**, *25* (1), 99–111.
(14) Gross, M. T. *J. Am. Soc. Mass Spectrom.*, **1994**, *5*, 57.
(15) Zhang, L. K.; Rempel, D.; Pramanik, B. N.; Gross, M. L. *Mass Spectrom. Rev.* **2005**, *24*, 286–309.
(16) Marshall, A. G. *Anal. Chem.* **2002**, *74* (9), 252A–259A.
(17) Wu, Z.; Rodgers, R. P.; Marshall, A. G. *J. Agric. Food Chem.* **2004**, *52* (17), 5322–8.
(18) Pourshahian, S.; Limbach, P. A. *J Mass Spectrom* **2008**, *43* (8), 1081–8.
(19) Kendrick, E. *Anal. Chem.* **1963**, *25* (13), 2146–2154.
(20) Han, X.; Gross, R. W. *Mass Spectrom Rev* **2005**, *24* (3), 367–412.
(21) Folch, J.; Lees, M.; Sloane Stanley, G. H. *J. Biol. Chem.* **1957**, *226* (1), 497–509.
(22) Clark, R. M.; Ferris, A. M.; Fey, M.; Brown, P. B.; Hundrieser, K. E.; Jensen, R. G. *J. Pediatr. Gastroenterol. Nutr.* **1982**, *1* (3), 311.
(23) Clowers, B. H.; Dodds, E. D.; Seipert, R. R.; Lebrilla, C. B. *Anal. Biochem.* **2008**, *381* (2), 205–13.

Lipid clusters were dissociated within the ICR cell by means of an externally mounted Parallax Technology (Waltham, MA) PLX25s sealed carbon dioxide laser tuned to a fixed wavelength of 10.6 μm. Laser power was adjusted by a potentiometer built into the laser power supply so that the burst length required for dissociation of the clusters was on the time scale of roughly 100 ms, with the exact length of the burst needed for dissociation being adjusted as needed. The laser power was adjusted to a higher setting for performing IRMPD experiments so that fragmentation of the lipid occurred with bursts lengths of 500 ms.

Data Analysis. Acquired mass spectra were calibrated in the Omega software prior to data analysis. Complete data analysis consisted of three steps: accurate mass analysis, Kendrick mass defect analysis, and referenced Kendrick mass defect analysis. All calculations were performed in Microsoft Excel 2007 (Richmond, WA).

Accurate Mass Analysis. Initial lipid class assignment was based on accurate mass measurements and was aided by use of the LIPID MAPS Lipid MS Prediction tool.[24] This tool uses a combinatorial approach to predict the fatty acid composition, determine the number of radyl carbons, the degrees of unsaturation, the molecular formula, and mass error. The lipid class was assigned based on the predicted fatty acid composition returned by the software. The experimental masses were manually entered into the software and were searched for glycerolipid and glycerophospholipid matches. Only lipid matches occurring within a mass error tolerance of less than 10 ppm were considered. Generally all lipid class assignments made in this fashion had a measured mass error less than 5 ppm, with the majority of the assignments being made around 1 ppm or less. In addition to the mass error requirements,all accurate mass based lipid classifications must fit within the confines of the published literature regarding the bovine milk lipidome.[25,26]

Kendrick Mass Defect Analysis. The monoisotopic masses belonging to lipids were converted from IUPAC mass conventions ($^{12}C = 12.0000$) to the Kendrick mass scale in which the methylene unit defines the mass scale and is set to 14.0000.[11,19] Conversion to the Kendrick mass scale was accomplished by the use of eq 1.

$$\text{Kendrick mass} = \text{measured IUPAC mass} \times \frac{14.0000}{14.01565} \qquad (1)$$

Kendrick masses have two distinct parts, the Kendrick nominal mass (KNM) and the Kendrick mass defect (KMD).[10,11,19] As an example of the conversion to the Kendrick scale consider 1,2-distearoyl-*sn*-glycero-3-phosphocholine ($C_{44}H_{88}NO_8P$). The accurate mass for this lipid in the IUPAC mass scale is 789.6248 and 788.7431 Da in the Kendrick mass scale (calculated using eq 1). The KNM is mainly dictated by the number of methylene units in the lipid and for the example lipid is 789 Da (note that the KNM is rounded to the nearest integer),[27] while the KMD is determined by the number and type heteroatoms and degrees of unsaturation in the lipid and is 0.7431 Da for the given example. Kendrick mass defect analysis consists of plotting the KMD versus the KNM for a converted data set to produce a Kendrick plot. This provides a quick and effective method of visualizing lipidomic data. A more detailed explanation of Kendrick mass defect analysis is offered in the results and discussion section.

Referenced Kendrick Mass Defect Analysis. The basic principle behind the referenced Kendrick mass defect (RKMD) is to adjust the KMD for a lipid in a data set so that this lipid is referenced to a given lipid class. This adjustment to the experimental lipid mass is made by first subtracting the KMD for a reference lipid that is representative of the lipid class to which the data set is being referenced. Each referencing lipid has been created so that the lipid contains all of the distinguishing features of that lipid class (i.e., the lipid backbone and polar headgroups) and each fatty acid position being occupied by a two carbon fatty acid (Figure 1, Table 1). In addition to the lipids shown in Figure 1, any other lipid class with a unique set of heteroatoms, such as glycolipids or alkyl substituted lipids, can have a reference lipid created in the manner described and thus used for RKMD analysis. Subtraction of the reference lipid KMD from the KMD of the masses in the experimental data set removes any lipid class specific contributions to the mass, such as the backbone and heteroatoms, leaving behind the contribution of the degrees of unsaturation to the KMDs in the data set. The adjusted experimental KMDs are then divided by 0.0134, which is the difference in mass defect for members of the same Kendrick class (defined as molecules having the same type and number of heteroatoms). This division by 0.0134 effectively reregisters the experimental data set so that it is now terms of the degrees of unsaturation since the contributions to the mass by the heteroatoms in the backbone and polar head groups have been removed. This conversion to the referenced Kendrick mass defect is shown in eq 2.

$$\text{RKMD} = \frac{\text{experimental KMD} - \text{reference KMD}}{0.0134} \qquad (2)$$

Since all lipids of the same lipid class will have the same type and number of heteroatoms, any lipid belonging to the lipid class that is being referenced will have an integer valued RKMD, which directly represents the degrees of unsaturation of the lipid and has implications for referenced Kendrick mass defect analysis. Lipids can then be easily assigned to the lipid class that is being referenced by examining the experimental RKMDs for an integer value.

RESULTS AND DISCUSSION

Accurate Mass Class Assignment by Combinatorial Methods. Lipids can be classified into two primary classes: nonpolar lipids and polar lipids. Lipids belonging to each of these two classes can be further classified according to the chemical makeup of the lipids. The identity of the constituent fatty acids, the degree of unsaturation of the fatty acids, and the identity of any polar groups attached to the lipid are all factors that contribute to the classification of a lipid. The chemical, physical, and bioactive properties of a lipid are often related to the class that the lipid

(24) Fahy, E. *LIPID MAPS MS Prediction Tool*; 2009, LIPID MAPS: San Diego.
(25) Jensen, R. G. *J. Dairy Sci.* **2002**, *85* (2), 295–350.
(26) MacGibbon, A. K. H.; Taylor, M. W. Compositon and Structure of Bovine Milk Lipids. In *Advanced Dairy Chemistry, Vol. 2: Lipids*; Fox, P. F., McSweeney, P. L. H., Eds.; 2006, Springer: New York.
(27) Wu, Z.; Rodgers, R. P.; Marshall, A. G. *Fuel* **2005**, *84*, 1790–1797.

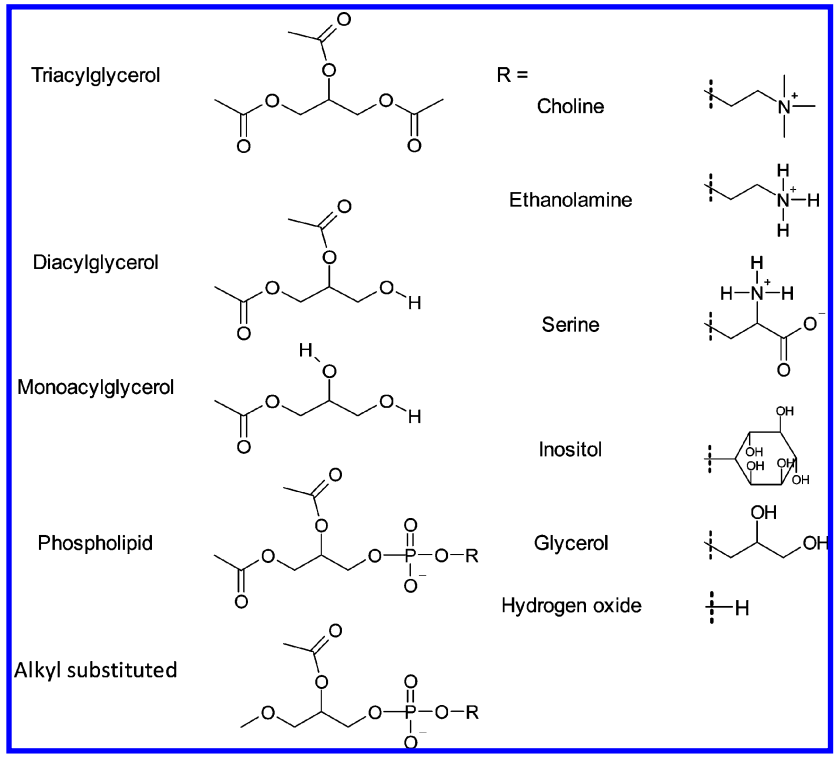

Figure 1. Reference lipids created for use in the referenced Kendrick mass defect method of lipid class identification. Each reference lipid contains all of the heteroatoms that are characteristic of the lipid's class, allowing them to serve as accurate mass references for the respective lipid class.

Table 1. Molecular Formulas and Masses for Reference Lipids[a]

lipid class	molecular formula	exact mass	Kendrick mass
TAG	$C_9H_{14}O_6$	218.0790	217.8355
DAG	$C_7H_{12}O_5$	176.0685	175.8719
MAG	$C_5H_{10}O_4$	134.0579	133.9082
PC	$C_{12}H_{24}O_8NP$	341.1240	340.7430
PE	$C_9H_{18}O_8NP$	299.0770	298.7430
PS	$C_{10}H_{17}O_{10}NP$	342.0590	341.6771
PI	$C_{13}H_{22}O_{13}P$	417.0798	416.6141
PtdG	$C_{10}H_{18}O_{10}P$	329.0638	328.6963
PtdH	$C_7H_{12}O_8P$	255.0270	254.7422

[a] TAG = triacylglycerol, DAG = diacylglycerol, MAG = monoacylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, PtdGro = phosphatidylglycerol, PtdH = phosphatidic acid.

belongs to and the degree of unsaturation of the lipid,[26,28,29] making identification of the lipid class and degree of unsaturation of any lipid of interest imperative.

The LIPID MAPS MS prediction tool (LMPT) employs a combinatorial approach to determine lipid composition (i.e., potential fatty acid compositions, the number of radyl carbons, the degree of unsaturation, and proposed molecular formula) from experimental masses.[24] Although LMPT returns a great deal of information regarding possible lipid assignments, use of the tool to analyze large data sets for class assignment is time and labor intensive. In addition false class assignments are possible in cases where a lipid appears in the spectrum with different charge carriers.

A nano-ESI FT-ICR MS spectrum of bovine milk lipid extract is shown in Figure 2A. This spectrum is representative of the ions and relative abundances that were observed for all collected BML spectra. Cursory examination of the spectrum in Figure 2A reveals the presence of lipid groupings that are almost exclusively separated by 28.031 Da, or the mass of two methylene units. Very low abundance ions that are separated from the other lipid groupings by 14.016 Da can be seen in the region covering m/z 800−900. On the basis of the low abundance of these ions and the separation from neighboring lipid groups being by 14.016 Da rather than 28.031 Da, it can be inferred that these lower abundance lipids have an odd number of radyl carbons and are comprised of either a fatty acid chain of odd number of carbons or a branched fatty acid derived from phytanic acid.[26,30,31] Accurate mass analysis of these ions using LMPT confirmed them as possessing an odd number of radyl carbons. Heterogeneity of the lipids can also be seen in this spectrum with each lipid grouping progressing from some degree of unsaturation to being fully saturated (Figure 2B). This progression toward saturation is seen as a progressive increase in mass of the monoisotopic ions by 2 Da, which is the addition of two hydrogen atoms across a single carbon−carbon double bond.

Initial analysis of the lipid data was performed using the glycerolipid and glycerophospholipid prediction portions of LMPT. Since LMPT uses a combinatorial approach to determine lipid assignments the mass spectral data must be carefully calibrated to ensure the highest degree of mass accuracy and therefore high confidence in the lipid assignments.[14,32] The acquired bovine milk lipid spectra were initially calibrated using maltooligosaccharide

(28) Molkentin, J. *Br. J. Nutr.* **2000**, *84* (Suppl 1), S47–53.
(29) Shah, N. P. *Br. J. Nutr.* **2000**, *84* (Suppl 1), S3–10.
(30) Lough, A. K. *Lipids* **1977**, *12* (1), 115–9.
(31) Verhoeven, N. M.; Jakobs, C. *Prog. Lipid Res.* **2001**, *40* (6), 453–66.

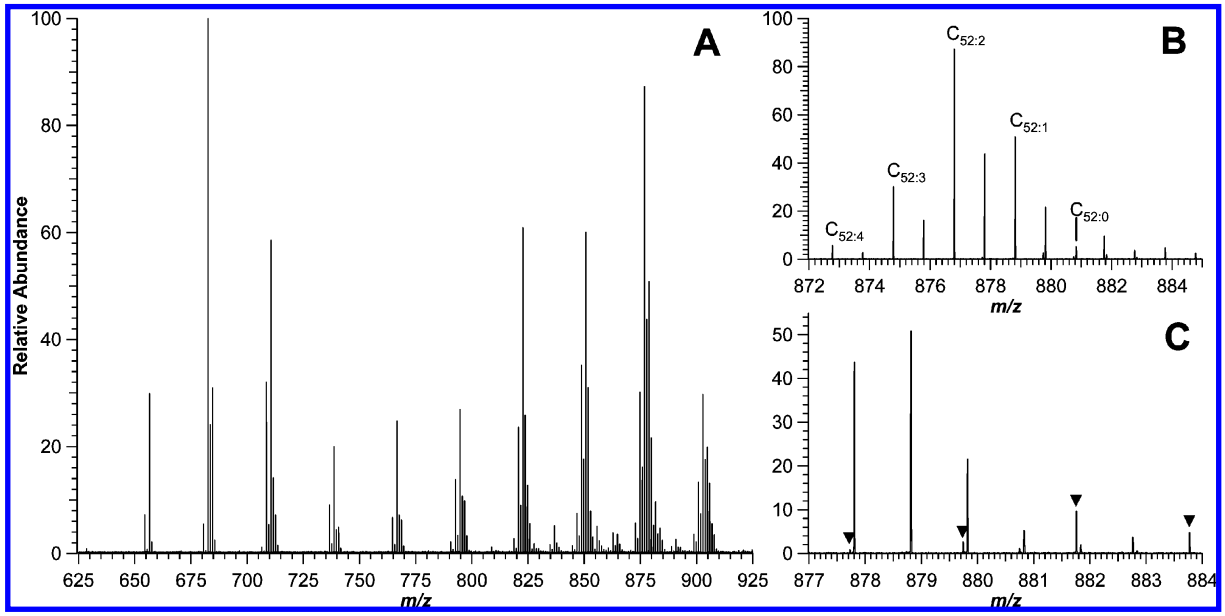

Figure 2. (A) Bovine milk lipid spectrum acquired on a 9.4 T nESI FT-ICR MS. Accurate mass analysis results show that the lipids in the spectrum are primarily ammoniated triacylglycerides with an even number of radyl carbons. (B) Enlargement of the spectrum in 2A between *m*/*z* 872−885 showing the lipid heterogeneity and varying degrees of unsaturation for an ammoniated triacylglyceride having 52 radyl carbons. (C) Enlargement of the spectrum in 2A between *m*/*z* 877−884. The ions marked with a triangle are the monoisotopic peaks for sodium coordinated triacylglycerols, which can be mistaken for ammoniated phospholipids when lipid class assignment is made on accurate mass assignments. The mass error associated with these assignments, greater than 10 ppm, reveals that these peaks are not ammoniated triacylgycerols because the mass error is much too large for FT-ICR MS.

spectra of similar total intensity and mass range as the lipid spectra[23] and were then internally calibrated to the most abundant lipid ion in each grouping to improve the mass accuracy and therefore confidence in lipid class assignment. Internally calibrating the spectra in this manner decreased the experimental mass error to 1.3 ppm (rms) when all lipid peaks were taken into consideration. The overlap of the saturated lipid peaks with the isotopic envelope of the unsaturated lipid peaks in each cluster introduces a larger mass error for the saturated lipid masses. Determination of a single, unique molecular formula based solely on accurate mass measurements requires measured mass accuracies of less than 1 ppm for masses occurring in the lower range of bovine milk lipid masses to less than 0.5 ppm for higher mass lipids.[13,16,32,33] Assignment of a lipid to a lipid class based on accurate mass measurements is essentially the determination of a molecular formula for the lipid since each lipid class has a distinct group of heteroatoms. Assignment of lipid classes within the confines of the bovine milk lipidome lessens the requirements for mass accuracy to uniquely identify a molecular formula.[16]

All of the lipids in the spectra were identified as belonging to the triacylglycerol class and the majority of the ions were detected as singly charged ammoniated ions. The radyl carbon count for the detected triacylglycerols ranged from 36 to 54 carbons (Table 2). The majority of the lipids possessed an even number of radyl carbons while a small fraction of the detected ions belonged to lipids with an odd number of radyl carbons. As can be seen in Figure 2A, a range of degrees of unsaturation exists for each grouping of lipids. The degrees of unsaturation range from being fully saturated to up to a detected maximum of five double bonds within some lipid species. These findings are in agreement with previously published data regarding the triacylglycerols present in bovine milk.[25,26]

Care had to be taken in assigning lipid classes using LMPT because of the occasional ion bearing a charge carrier other than NH_4^+, such as Na^+ or H^+. These lipids failed to be classified as triacylglycerols and instead matched to phospholipid classes when LMPT was set for ammoniated ions. Given that these ions were much less abundant than the ammoniated ions (Figure 2C), the assignment as ammoniated phospholipids is a reasonable conclusion considering the low abundance of phospholipids in bovine milk. Analysis of the mass errors for the assignment of these low abundance ions as ammoniated phospholipids revealed that this assignment was a poor choice since the mass errors were around 100 ppm. Further analysis of the mass spectra revealed that each grouping of these ions had an isotopic envelope that was similar in distributions and intensities to lipid groupings appearing at masses that were 17 Da lighter or 6 Da heavier. These differences correspond to the replacement of NH_4^+ by H^+ and the replacement of NH_4^+ by Na^+, respectively. Final confirmation that these lipids were not phospholipids but rather triacylglycerols with varying charge carriers was made when the mass error of the assignments as being either sodiated or ammoniated triacylglycerols was examined and found to be a few parts per million.

Kendrick Mass Defect Analysis. Kendrick mass defect analysis has gained considerable interest for lipid analysis because of the separation of carbon chain and heteroatom contributions

(32) Bristow, A. W. T.; Webb, K. S. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 1086–1098.
(33) Marshall, A. G. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. In *Fourier Transform Spectroscopy: 11th International Conference*; de Haseth, J. A., Ed.; The American Institute of Physics: Athens, GA, 1998; p 3−13.

Table 2. Lipids Identified in Bovine Milk Using Accurate Mass Measurements[a]

exptl m/z	calcd m/z	mass error (ppm)	radyl carbons	degrees of unsaturation	exptl m/z	calcd m/z	mass error (ppm)	radyl carbons	degrees of unsaturation
654.5658	654.56672	1.4	36	2	820.7402	820.73887	1.6	48	2
656.5807	656.58237	2.5	36	1	822.7542	822.75452	0.4	48	1
680.5811	680.58237	1.9	38	2	824.7703	824.77017	0.2	48	0
682.5965	682.59802	2.2	38	1	836.7732	836.77017	3.6	49	1
684.6119	684.61367	2.6	38	0	846.7574	846.75452	3.4	50	3
708.6123	708.61367	1.9	40	2	848.7715	848.77017	1.6	50	2
710.6273	710.62932	2.8	40	1	850.7858	850.78582	0.0	50	1
712.6423	712.64497	3.7	40	0	852.8004	852.80147	1.3	50	0
736.6451	736.64497	0.2	42	2	862.7892	862.78582	3.9	51	2
738.6604	738.66062	0.3	42	1	864.8046	864.80147	3.6	51	1
740.6758	740.67627	0.6	42	0	872.7739	872.77017	4.3	52	4
764.6769	764.67627	0.8	44	2	874.7881	874.78582	2.6	52	3
766.6919	766.69192	0.0	44	1	876.8015	876.80147	0.0	52	2
768.7081	768.70757	0.7	44	0	878.8163	878.81712	0.9	52	1
790.6933	790.69192	1.7	46	3	890.8207	890.81712	4.0	52	0
792.7091	792.70757	1.9	46	2	898.7898	898.78582	4.4	54	5
794.7232	794.72322	0.0	46	1	900.8052	900.80147	4.1	54	4
796.7397	796.73887	1.0	46	0	902.8199	902.81712	3.1	54	3
818.7248	818.72322	1.9	48	3	904.8358	904.83277	3.3	54	2
					906.8496	906.84842	1.3	54	1

[a] The m/z values listed for both the experimental and theoretical m/z are for the ammoniated ions.

to the mass of the lipid and also the clarity that it provides for large data sets and complicated spectra.[10,11] In performing a Kendrick mass defect analysis, one plots the Kendrick mass defects versus the Kendrick nominal masses for a given spectrum to obtain a Kendrick plot[10,11,19] following conversion to the Kendrick mass scale (data not shown).[19] The guiding principle of Kendrick plot analysis is that the contribution to the mass of any methylene units is represented in the Kendrick nominal mass (KNM) so that an increase in Kendrick nominal mass represents an increase in the number of methylene units in the lipid, while the contribution to the mass from any heteroatoms and degrees of unsaturation in the lipid is represented in the Kendrick mass defect (KMD).[11]

The implication of this separation of the mass into two distinct parts, the KNM and the KMD, is that specific relationships become more apparent in the data set.[10,11] One such relationship is the class, or the type and number of heteroatoms in the lipid, hereafter referred to as the Kendrick class to avoid confusion with the lipid class (i.e., type of glycerol or phospholipid).[10,11] When analyzing a Kendrick plot, the Kendrick class of any lipid is represented in the vertical spacing on the plot so that all lipids with the same Kendrick class will be spaced by a difference of 0.0134 in their KMD.[11] It then follows that once the lipid class of one member of a Kendrick class is known all other members of that class can be identified as belonging to the same lipid class based on spacing differences in the KMD. Kendrick plot analysis of the bovine milk lipids revealed that they belonged to the same class because they were all spaced equally from each other in the vertical axis, and this spacing was calculated to be about 0.0134. Without the prior knowledge regarding lipid class assignment from the accurate mass analysis in LMPT, it would not be possible to assign the lipids to the triacylglycerol lipid class based on the Kendrick plot alone. To accomplish this assignment tandem mass spectrometry must be performed on an ion to determine the lipid class of that ion. Once the lipid class of one ion was known, the difference in KMD of that ion from every other ion would have to be calculated and any other ion having a KMD of 0.0134 from the lipid ion of known lipid class could then be assigned to the same class. Any ion that was found to be at a difference other than 0.0134 would have to be analyzed by tandem mass spectrometry to determine the lipid class, thus extending the time needed for the analysis, both on the instrument and also in data analysis. This can be problematic for large sample sets since the multiple tandem experiments can lead to a drastically decreased duty cycle because of the increase in time needed for each ICR experiment so that the collision gas from the CID event can be evacuated from the ICR cell before initiation of the detection event. In addition to quickly identifying members of the same Kendrick class, the Kendrick plot also allows the members of a homologous series to be quickly identified.

Referenced Kendrick Mass Defect. Mass defect analysis has previously been used as a powerful tool for assigning lipid classes to lipids in mass spectral data sets.[10,11,34] However, if the mass defect spacing between members of the same class is exploited then it is possible to identify lipid classes without prior knowledge of the lipids in the mass spectrum by referencing the mass spectral data to a reference lipid from each lipid class. The process of referencing mass spectral lipid data to the different lipid classes has three parts: conversion of the data to the Kendrick mass scale, removal of lipid class specific contributions to the KMD by subtraction of the KMD for a reference lipid, and finally reduction of the data to multiples of the mass defect spacing for each lipid class. This process is shown in Figure 3A for a model monounsaturated TAG, 1,3-dihexadecanoyl-2-hexadecenoyl-glycerol. It should be noted that while the traditional Kendrick mass analysis rounds the KNM to the nearest integer this rounding step is not used in the RKMD. The functional basis of the RKMD method is the KMD, and the chemical information contained in it therefore not rounding the KNM has no effect on the RKMD results.

The conversion of a mass to the Kendrick mass scale (Figure 3A, step 1) has previously been discussed and can be found in the Experimental Section of this paper with the implications and

(34) Jones, J. J.; Stump, M. J.; Fleming, R. C.; Lay, J. O., Jr.; Wilkins, C. L. *J. Am. Soc. Mass Spectrom.* **2004**, *15* (11), 1665–74.

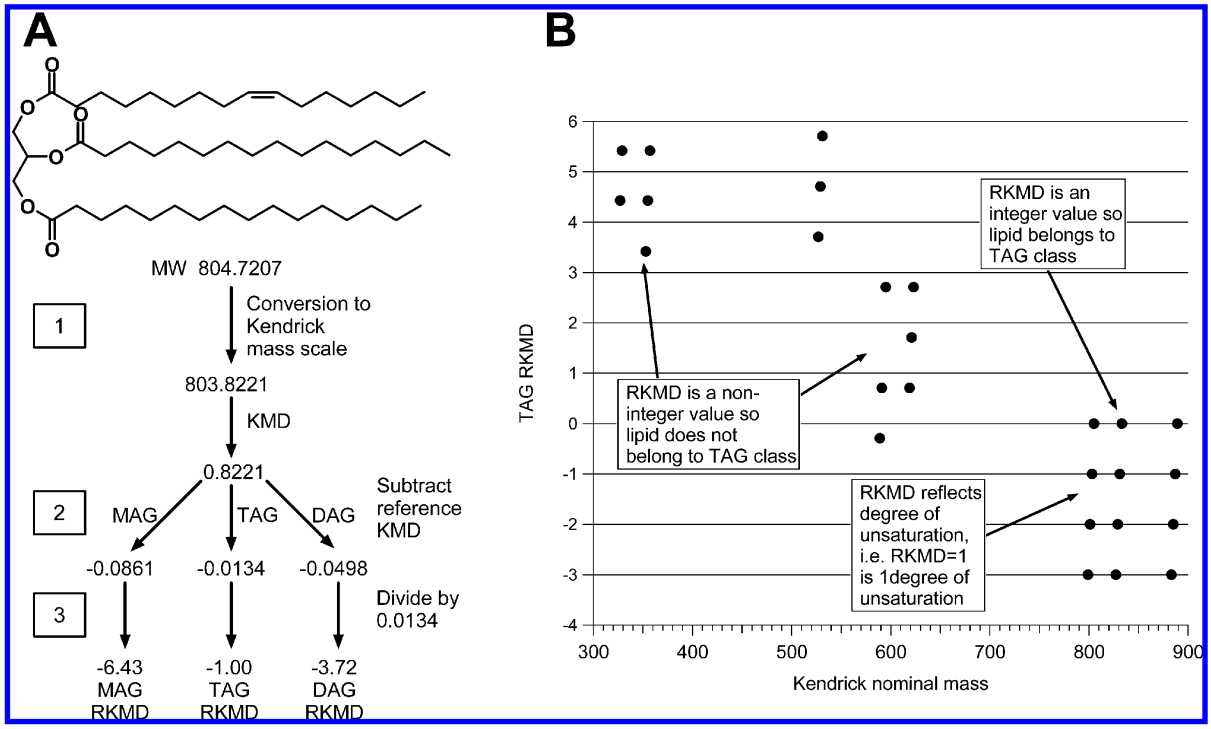

Figure 3. Referenced Kendrick mass defect analysis is a modification of Kendrick mass defect analysis in which masses in the Kendrick scale are reregistered in terms of mass defect spacing from a given reference lipid. (A) The conversion of an accurate mass to a referenced Kendrick mass defect occurs in three steps: conversion to the Kendrick mass scale (step 1), subtraction of the mass defect of the reference lipid from the KMD of the lipid of interest (step 2), and finally division of the difference by 0.0134 (step 3). If a lipid belongs to the class being referenced then the RKMD will be an integer value. (B) A referenced Kendrick plot can be created by plotting the RKMD versus the KNM. Any lipids belonging to the lipid class that is being referenced will lie at integer values while lipids not belonging to the referenced class will fall at noninteger values. In addition, the RKMD also reflects the degrees of unsaturation, allowing members of a homologous series and false assignments to be quickly identified.

uses of the Kendrick mass scale discussed in the section preceding this. The reference lipid for each lipid class was created such that the lipid was composed of the glycerol backbone, the specific polar headgroup, and a two carbon fatty acid at each fatty acid position (Figure 1). Subtraction of the reference lipid KMD from the KMD for a lipid in the mass spectral data (Figure 3A, step 2 and Table 1) removes any contributions to the experimental KMD arising from the heteroatoms in the lipid backbone and the polar headgroup. Once the contributions of the backbone and polar group heteroatoms to the mass defect have been removed the remaining mass defect is divided by 0.0134 (Figure 3A, step 3), which is the spacing between members of the same class differing in degrees of unsaturation in the Kendrick mass scale. By adjusting the KMD in this manner it has been referenced to a particular lipid classification and has now been converted to a "referenced Kendrick mass defect" (RKMD). Since all members of the same lipid classification have the same number and type of heteroatoms they will all belong to the same class and will therefore have an integer valued RKMD reflecting the number of mass defect spacings from the reference lipid, that is, the degree of unsaturation. It can be seen in Figure 3A that the example lipid has a TAG RKMD of −1.00, the integer value indicating that the lipid belongs to the tricacylglyceride class and the numeric value of the integer indicating one degree of unsaturation. Referencing the example lipid to either the mono- or diacylglyceride class results respective RKMDs of −6.43 and −3.72, indicating that the lipid does not belong to either of these two classes. By referencing the masses in a mass spectral data set through the different lipid classes one can quickly identify members of the lipid class being referenced by examining the RKMDs for integer values.

The robustness of the RKMD method was tested against an idealized data set of 160 lipids based on the reported bovine milk lipidome.[25,26] The exact mass of each lipid was calculated, converted to the Kendrick mass scale, and referenced to the lipid classes of the idealized data set. The end result of this processing was a matrix containing 1280 RKMD for all lipids in the idealized data set. In addition the RKMD for phosphatidylglycerol was also calculated for each idealized lipid giving a matrix of 1,431 RKMD (Table S-1 in Supporting Information). This calculation was performed to check for any false assignments to a lipid class that was not in the data set. Examination of these matrices showed that each lipid was assigned to the correct lipid class based on the RKMD, as shown in Table 3. It can be seen that integer RKMD are only found when a lipid of given class is being referenced to that class, that is, a triacylglycerol KMD is being referenced to the triacylglycerol class.

An important benefit of the conversion to RKMD is that information regarding the degrees of unsaturation of a lipid is retained and is represented as the numerical value of the integer RKMD. The subtraction of the KMD of the reference lipid from that of the experimental lipid removes the contributions of heteroatoms to the mass defect, leaving only the contribution from the degrees of unsaturation. This is demonstrated in the idealized triacylglycerols included in Table 3. It can clearly be seen that the RKMD is the same as the degree of unsaturation of the lipid

Table 3. Matrix of Referenced Kendrick Mass Defects for Twenty of the Sixty-Four Idealized TAGs[a]

					referenced Kendrick Mass defects								
Sn1	Sn2	Sn3	AccMass	Kendrick Mass	TAG	DAG	MAG	PC	PE	PS	PI	PtdGro	PtdH
18:0	18:0	18:0	890.8302	889.835491	0.00	−2.71	−5.43	6.90	6.90	11.82	16.52	10.39	6.96
18:0	18:0	18:1	888.8146	887.8221417	−1.00	−3.71	−6.42	5.90	5.90	10.83	15.53	9.39	5.96
18:0	18:0	18:2	886.7989	885.8086924	−2.00	−4.71	−7.43	4.90	4.90	9.82	14.52	8.39	4.96
18:0	18:0	18:3	884.7833	883.7953431	−3.00	−5.71	−8.42	3.90	3.90	8.83	13.53	7.39	3.96
18:0	18:1	18:1	886.7989	885.8086924	−2.00	−4.71	−7.43	4.90	4.90	9.82	14.52	8.39	4.96
18:0	18:1	18:2	884.7833	883.7953431	−3.00	−5.71	−8.42	3.90	3.90	8.83	13.53	7.39	3.96
18:0	18:1	18:3	882.7676	881.7818938	−4.00	−6.71	−9.43	2.90	2.90	7.82	12.52	6.39	2.96
18:0	18:2	18:1	884.7833	883.7953431	−3.00	−5.71	−8.42	3.90	3.90	8.83	13.53	7.39	3.96
18:0	18:2	18:2	882.7676	881.7818938	−4.00	−6.71	−9.43	2.90	2.90	7.82	12.52	6.39	2.96
18:0	18:2	18:3	880.752	879.7685444	−5.00	−7.71	−10.42	1.90	1.90	6.83	11.53	5.39	1.96
18:0	18:3	18:1	882.7676	881.7818938	−4.00	−6.71	−9.43	2.90	2.90	7.82	12.52	6.39	2.96
18:0	18:3	18:2	880.752	879.7685444	−5.00	−7.71	−10.42	1.90	1.90	6.83	11.53	5.39	1.96
18:0	18:3	18:3	878.7363	877.7550952	−6.00	−8.71	−11.43	0.90	0.90	5.82	10.52	4.39	0.96
18:1	18:1	18:1	884.7833	883.7953431	−3.00	−5.71	−8.42	3.90	3.90	8.83	13.53	7.39	3.96
18:1	18:1	18:2	882.7676	881.7818938	−4.00	−6.71	−9.43	2.90	2.90	7.82	12.52	6.39	2.96
18:1	18:1	18:3	880.752	879.7685444	−5.00	−7.71	−10.42	1.90	1.90	6.83	11.53	5.39	1.96
18:1	18:2	18:1	882.7676	881.7818938	−4.00	−6.71	−9.43	2.90	2.90	7.82	12.52	6.39	2.96
18:1	18:2	18:2	880.752	879.7685444	−5.00	−7.71	−10.42	1.90	1.90	6.83	11.53	5.39	1.96

[a] TAG = triacylglycerol, DAG = diacylglycerol, MAG = monoacylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, PtdGro = phosphotidylglycerol, PtdH = phosphatidic acid.

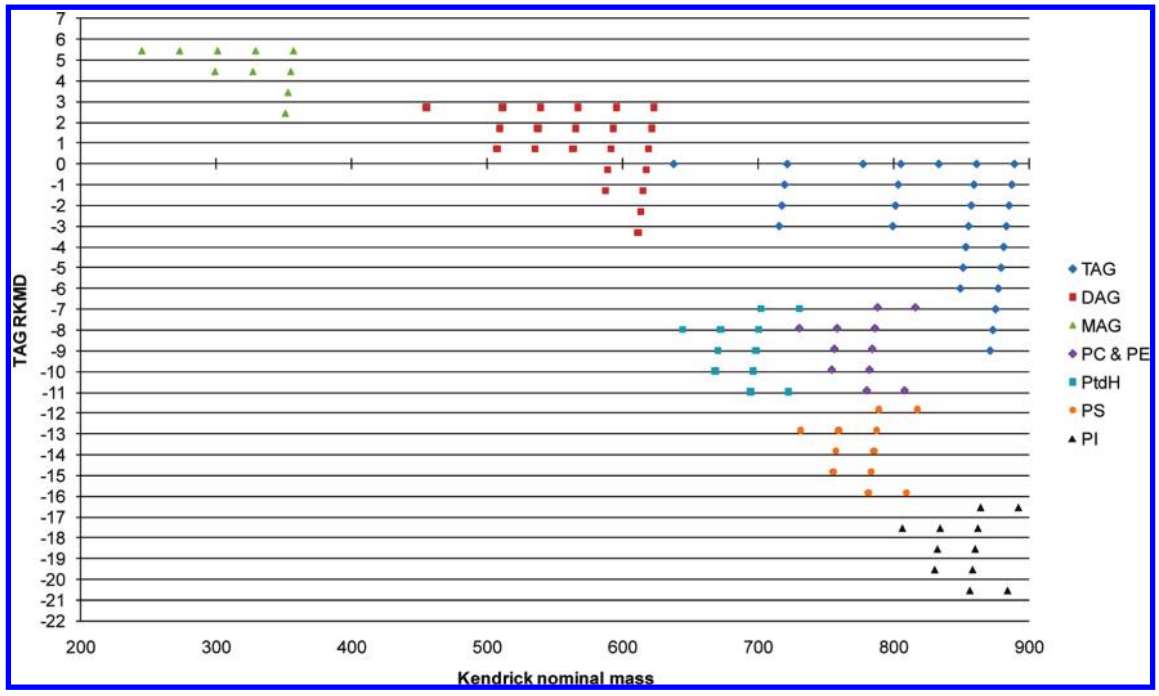

Figure 4. TAG RKMD for all lipids in the idealized data set showing the distribution in RKMD values for the lipids in the data set. It can be seen that in addition to the TAGs several of the phospholipids in the data set also fall at integer values. The assignment of these lipids as TAGs can be recognized as being false assignments because the degrees of unsaturation are too great (7−16).

and that this holds for all of the lipids in Table 3 and also the entire idealized data set. The decision was made to not use an absolute value for the RKMD to maintain the fidelity of the unsaturation information. Any lipid belonging to the referencing lipid class must have a Kendrick mass defect that is either the same as the referencing lipid (having the same degree of unsaturation) or less than the referencing lipid (having a greater degree of unsaturation). Since each of the referencing lipids is fully saturated any lipid that belongs to the lipid class that is being referenced will have a negative RKMD.

In addition to the numerical method for assigning lipid classes based on the RKMD that has been described above, a graphical procedure may also be utilized by plotting the RKMD for a given lipid class versus the KNM to create a "referenced Kendrick plot", which is demonstrated in Figure 3B. This "referenced Kendrick plot" allows a data set to quickly be visualized in terms of the lipid class being referenced. It can be seen in Figure 3B that any lipid belonging to the referencing class will plot to integer valued RKMDs and that the RKMD represents the degrees of unsaturation, while lipids not belong-

ing to the class will plot to noninteger values. A referenced Kendrick plot for the entire idealized data set is shown in Figure 4, in which lipids belonging to the triacylglycerol class have integer values for the RKMD while lipids belonging to other classes do not. Careful examination of the referenced Kendrick plot in Figure 4 shows that there are nontriacylglycerol lipids that are very close to possessing integer value RKMDs and that these lipids could falsely be assigned to the triacylglycerol class. However two filters exist that can be applied to help eliminate false assignments.

The first filter uses the fact that all lipids belonging to the lipid class being referenced will have a negative RKMD. This filter removes the di- and monoacylglycerol points that lie in the positive portion of the plot. The diacylglycerol data points falling in the negative portion of the graph can also be removed with those lying in the positive portion since these are members of homologous series and must belong to the same class. The second filter relies on knowledge of the biological source of the lipids and the lipid biochemistry of that source. From the plot in Figure 4 it appears that the lipids belonging to the phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, and phosphatidylserine classes have integer value RKMD and would therefore introduce a large false positive error in the lipid class assignment if this were an actual data set. Closer examination reveals that these data points have RKMDs falling between −7.00 to −16.00. As has previously been discussed the RKMD maintains information regarding the degrees of unsaturation and therefore these lipids would have degrees of unsaturation ranging from seven to sixteen. Keeping in mind that this idealized data set was created within the limits of the bovine milk lipidome it is possible, but highly unlikely, for a triacylglycerol to have up to nine degrees of unsaturation; it would not be possible for there to be ten or eleven degrees of unsaturation.[25,26] On the basis of this argument these data points can be ruled out as not belonging to the triacylglycerol class. By similar arguments the data points belonging to the idealized phosphatidyl serines can also be ruled out as not being triacylglycerides.

Referenced Kendrick Mass Defect Analysis of Bovine Milk Lipids. The referenced Kendrick mass defect method was applied to the data in Figure 2A. Figure 5 shows the monoisotopic peaks of the experimental data from Figure 2A as tri-, di-, and monoacylglycerol referenced Kendrick plots. It can be seen that at lower masses the RKMD method works well for correctly assigning the data to the triacylglycerol class rather than the diacyl- or monoacylglycerol classes. It is believed that the deviation from the ideal integer RKMD at higher masses is caused by an increase in measured mass error arising from two primary sources. The first source of this increase in measured mass error arises from ions of low abundance being detected with ions of much greater abundance. The cyclotron frequency of the low abundance ion cloud is affected by Coulombic repulsions from the high abundance ion cloud with the result being broadened peak shape of the low abundance ion and an increase in the measured mass error.[15,35] This effect is one possible cause of the deviation from ideal RKMD values for the lipids with an odd number of radyl carbons. The second source of measured mass error is the contribution of heavier isotopes from the highly unsaturated lipids

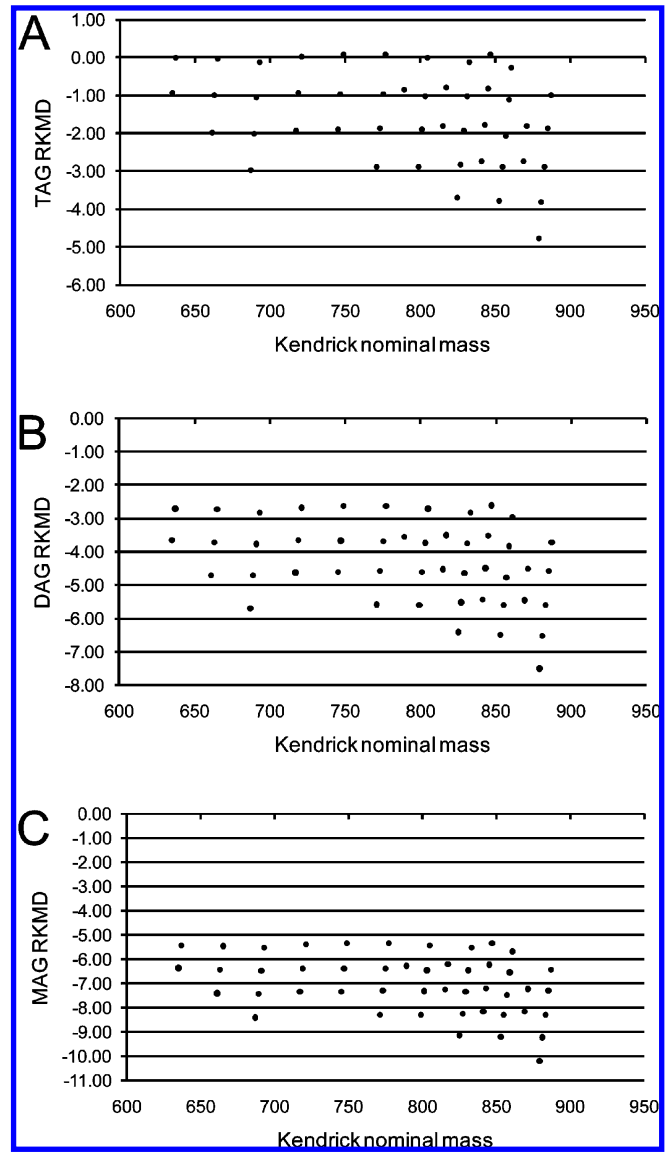

Figure 5. TAG, DAG, and MAG reference Kendrick plots for the bovine milk lipids shown in Figure 2A. Assignment of the experimental data to a lipid class can be made by plotting the data as it is referenced to different lipid classes. (A) TAG referenced Kendrick plot for the milk lipids showing that the majority of the data has integer valued RKMD. The deviation from integer values for the higher mass lipids is thought to be the result of isotopic overlap with lipids having the same number of radyl carbons and varying in degrees of unsaturation. (B) DAG referenced Kendrick plot in which the DAG RKMDs for the bovine milk lipids are clearly shown to be noninteger values. (C) The MAG referenced Kendrick plot for the bovine milk lipids shows that none of the milk lipids belong to the MAG class. It should be noted that while the MAG RKMDs in this plot are close to integer values the degree of unsaturation is too great for a MAG, and therefore, these lipids do not belong to the MAG class.

overlapping with the monoisotopic peaks of the less unsaturated lipids. Even with these deviations from ideality, the RKMD and referenced Kendrick plot can still be used to successfully identify the lipid classes of the bovine milk lipids in Figure 2A when the homologous series are identified and one member of the homologous series is successfully identified by the RKMD.

The effect of experimental mass error on the assignment of lipids to lipid classes by referenced Kendrick mass defect analysis was studied by adjusting the mass of each TAG in the idealized

(35) Williams, D. K., Jr.; Chadwick, M. A.; Williams, T. I.; Muddiman, D. C. *J Mass Spectrom* **2008**, *43* (12), 1659–63.

lipid data set by the appropriate amount so that new data sets at 0.5, 1, 2.5, and 5 ppm mass error were created. From these data sets, it was determined that a mass error of 2.5 ppm or less was sufficient for identification of a lipid as belonging to the class being referenced. This decision was reached by determining the number of molecular formula at each level of mass error within the confines of the referencing class. As an example there is only one possible molecular formula for a mass of 806.7363, corresponding to a tripalmitic TAG, at a mass error of 2.5 ppm or less when search constraints are limited to $C_{0-100}H_{0-200}O_{0-6}$. Limiting the search constraints to the heteroatoms in each lipid class is valid when determining the total number of possible molecular formulas since the referenced Kendrick mass defect is in essence a similar formula filter. Combined with the biological filters given above and relationships within homologous series a mass error of 2.5 ppm or less is sufficient for assignment of a lipid to a given lipid class using the referenced Kendrick mass defect and plot. Mass errors of 2.5 ppm or less are perfectly feasible with FT-ICR MS. Application of the filters previously described also help to eliminate any false lipid class assignments.

It should be noted that while the experimental data in this paper was acquired using a FT-ICR MS, any mass spectrometer capable of accurate mass measurements will generate data that can be processed and analyzed using the RKMD and referenced Kendrick plot.

CONCLUSION

The referenced Kendrick mass defect, a novel modification to the Kendrick mass defect, has been presented and has been shown to be highly useful in rapidly identifying lipid classes in mass spectral data. While the referenced Kendrick mass defect was developed to analyze bovine milk lipids, this approach should also prove to be valuable for the study of other lipidomes.

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SUPPORTING INFORMATION AVAILABLE

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

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