Fatty acid sentinels as covalently bound randomization standards for triacylglycerol (TAG) quantitative analysis

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Abstract

RATIONALE. Quantitative analysis of triacylglycerols (TAG) is impeded by a lack of standards and the huge number of potential TAG molecular species that may be present due to the combinatorial nature of glycerolipids. Randomization of acyl groups yields TAG mixtures with profiles predictable from fatty acid profiles, however their use as calibration mixtures has been limited.

METHODS. We introduce here the principle of fatty acid isotopic sentinels that are quantitatively added prior to randomization to enable verification that randomization is complete, and that can be used as internal standards. A mixture of two or more isotopically labeled fatty acid methyl esters (FAME) are prepared in quantitative proportions and randomized covalently into the acyl groups of TAG mixtures.

RESULTS. Reaction with catalytic amounts of NaOCH₃ yields complete randomization, such that the product FAME and TAG have the same fatty acid profile. TAG mixture analysis reveals that the isotopic sentinels have been covalently incorporated into TAG molecular species and <1% of the expected proportions thus verifying randomization within experimental error.

CONCLUSIONS. The sentinel principle demonstrated here as covalently incorporated internal standards verifies that randomization chemistry went to completion. It applies in general to use of combinatorial chemistry for quantitative standards.

Keywords: fatty acid sentinels; randomization (interesterification); triacylglycerol calibration standards, quantitative analysis
1. Introduction

The physicochemical and biological properties of glycerolipids (GL) depend on arrangement and structures of fatty acids (FA) attached to the glycerol backbone. Traditional regiospecific analysis (stereospecific numbering, sn-1,2,3) of GL requires conversion of acyl groups to fatty acid methyl esters (FAME) with analysis by gas chromatography. These methods have shown that native GL in most biological samples have different FA profiles at the different sn positions due to acyl specificity of the many biosynthetic enzymes catalyzing lipolysis and re-esterification. A well-established example is the uniquely high concentration of palmitic acid (16:0) at the sn-2 position of human and pig milk triacylglycerols (TAG) \(^1,\,2\) when most natural TAGs place it in the sn-1/3 positions, including vegetable oils\(^2,\,3\), fish oils\(^4\) and ruminant milks\(^2\). TAG with palmitic acid in the sn-2 position are functional because they survive digestion.\(^2\)

Viewed in one modern chemical way, GL resemble a mixture made by combinatorial chemistry (cc), albeit imperfectly randomized. Decades before cc came to prominence, randomization was developed as a method to modulate the physicochemical properties of food fats and oils without resort to methods such as hydrogenation that alter the character of the specific FA. Interestereified fats, produced at the multi-ton scale,\(^5\) is a major component of processed foods. Interestereification by chemical means causes acyl groups in TAG to be redistributed among the sn-1,2,3 positions to approach a random distribution.

Molecular species analysis of TAG is a formidable task due to their inherent combinatorial nature. Consider the FA pool as the mixture of FA liberated from a TAG mixture by hydrolysis. When derived from a fat native to a biological tissue, the number of FA is rarely less than 10, and ranges to over 400 in retail cow’s milk with more being discovered.\(^6\) The number of possible combinatorial TAG species from any of 3 FA goes as the third power of the FA in the pool, thus for 10, 50 and 400 FA in the pool, the number of TAG molecular species will be 1,000, 125,000, and 64,000,000, respectively. Table S1 summarizes the possible number of TAG with up to 50 FA substituents. More sobering with respect to these daunting numbers is that the most bioactive FA are those at concentrations in the FA pool below 1%, for instance the 20 and 22 carbon polyunsaturates, FA with non-methylene interrupted conjugated double bond systems, and branched chain FA.

Important strides have been made recently in structural elucidation of GL.\(^7-\,10\) Analyses of GL has lagged behind that of other lipids because of the large variety of isomeric double bond and chain branched hydrocarbons which, in general, are not amenable to differentiation by high resolution or exact mass measurement. As a result, methods requiring specialized chemistry and fragmentation are generally required, and are in development to differentiate structures. Unlike ionization by simple attachment of small ions, e.g., H\(^+\), NH\(_4^+\), chemistry and fragmentation are typically highly dependent on the native structure, and thus response factors for characteristic fragments must be measured empirically. Standards are required.

Quantitative GL analysis remains a problem without a generally accepted approach for the combinatorial number of molecular species. Pioneering developments for TAG
based on interesterification have been developed and exploited for TAG mixture
analysis, however adoption of these methods appears to be limited. The basis of
this approach is to establish a chemical reference point such that the quantitative FA
profile, routinely measured by gas chromatography (GC), can be used to calculate the
concentration of any particular TAG species. Randomized TAG mixtures can then
be analyzed to obtain response factors that can then be applied to convert instrument
signal into concentration. Measures of the thoroughness of randomization based on
careful separations and calibrations of individual TAG species yield accuracies around
15-25% for major TAG.

We report here on proof of principle of a strategy to establish the degree of
randomization by quantitatively adding exogenous FAME to a native TAG mixture prior
to interesterification. Measurement of the relative levels of these “sentinel” FA in the
final TAG mixture then form a convenient index of the extent of randomization in the
particular reaction mixture to verify the reaction goes to completeness and possibly to
serve as internal standards.

2. Experimental

2.1 Materials and reagents
All solvents, NaOCH₃, dimethyl sulfoxide (DMSO), chloroform and ammonium acetate
were purchased from Fisher Scientific (Waltham, MA, USA). BF₃ (14%) in methanol
was purchased from MilliporeSigma (St. Louis, MO, USA), Triglyceride standards
(>99%) and GLC 462 equal weight FAME mixture were obtained from Nu-Chek Prep
(Elysian, MN, USA). Isotopic labeled 2,2-d₂-16:0 and U-¹³C₁₈:₂ fatty acids (>98%) were
purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Silica gel GF
TLC plates (20 cm × 20 cm, 250 µm) were purchased from Miles Scientific (Gardena,
CA, USA).

2.2 FA Sentinels and Randomization
A ratio of about 2:1 d₂-palmitic acid : U-¹³C₁₈ linoleic acid sentinel mixture was
prepared and methylated by treatment with 14% BF₃ in methanol. The isotopic labeled
standards 2:1 mixture (12 mg) were mixed with 90 mg of triolein in a glass tube (16 ×
100 mm) and put on a magnetic heater and preheated to 90°C, with a gentle stream of
N₂ flushing the tube and mixed with spinbar (7 × 2 mm). Six mg NaOCH₃ were added as
catalyst; the tube was incubated 2 hours and then cooled. Two mL CHCl₃ were added
and centrifuged at 3000 rpm for 10min. The CHCl₃ bottom layer (~0.6mL) was aspirated
into a 1.5 mL glass vial. Product (10µL) was diluted to 1/100 in 7:3 chloroform: methanol
with 10 mM ammonium acetate and then another 1/10 (total 1/1000) with the same
solvents for ESI-MS direct infusion analysis. Other FAME and triglyceride standards
were used directly for randomization without the methylation step following the same
protocol as isotopic labeled FAME and triolein randomization.

2.3 Thin layer chromatography
TLC plates were activated in a 100 °C oven for at least one day. Plates were developed in 80: 20 : 1.8 heptane : ethyl ether : formic acid (v:v:v) with 0.1% BHT. Samples were streaked onto the TLC plates and developed until 1 cm from the top. Migration of the bands was visualized with iodine staining or under short-band UV light. The bands corresponding to FAME and TAG were scraped and extracted for analyses.

2.4 Instrumental, and calibration

An LTQ linear ion trap mass spectrometer (Thermo Scientific (Waltham, MA, USA)) in direct infusion mode was used for TAG relative quantification. Sheath gas flow rate was at 2 (arbitrary units), spray voltage at 3.8 kV, capillary temperature at 275 °C, capillary voltage at 35 V and tube lens at 110 V. A GC-FID GC-2010 Plus (Shimadzu Corporation (Kyoto, Japan)) equipped with a BPX-70 column (25m x 0.22mm i.d. x 0.25um film; Trajan Scientific and Medical, Victoria, Australia) was used to quantify FAME, which were calibrated by GLC 462 standards mixture (Nu-chek prep, Elysian, MN). The GC temperature profile was 80 °C to 170 °C at 30 °C /min, hold for 2 min, increase at 10 °C /min to 240 °C and hold for 8 min. The injector operated in splitless mode was at 250 °C.

All quantitative GC-FID analyses were calibrated using response factors (RF) measured by analysis of an equal weight standards mixture (GLC 462). These calibrations included measurement of the precise and accurate ratio of isotopic sentinels.

The relative response of ammoniated TAG in ESI-MS1 was put in a quantitative basis by observing that the NH₄⁺ affinity of any particular TAG molecular species is related to the number of double bonds in its acyl components. We prepared quantitative solutions of monoacetyl TAG: PPP, OOO, LLL, LnLnLn (defined immediately below) and determined their relative RF to be 1, 0.92, 0.87, 0.81, respectively under our conditions. For mixed acyl TAG, we interpolated based on the relative acyl content, e.g., the RF for OOP was calculated as RF(OOP) = 0.67×RF(OOO) + 0.33×RF(PPP). This approach is similar to that adopted by others studying TAG with different ionization processes.¹¹,²⁰

2.5 Nomenclature

When convenient, fatty acyl chains esterified to the three positions in glycerol backbone or as methyl esters are designed using a simplified nomenclature: P = palmitate (16:0), O = oleate (18:1n-9), L = linolate (18:2n-6), Ln = linolenate (18:3n-3); thus a TAG referred to as “POL” would have one of each fatty acyl chain. We also designate 8:0, 10:0 and 12:0 by 8,10,12, respectively. The current TAG measurements do not distinguish TAG stereoisomers, and thus a TAG consisting of, for instance, P, O, and L is the sum of POL, PLO, OPL, OLP, LPO, and LOP. For consistency, we refer to Me-X for FAME where X = P,O,L or Ln.

We define the extent of randomization as a percent of mixing of FA groups from FAME into TAG and vice versa. If completely randomized, a mixture of 25% Me-O and 75% LLL, where the percentages refer to molar proportions of acyl groups, then both FAME and TAG would be 100% randomized when they both contain 25% O and 75% L.
3. Results and discussion

3.1 Development of randomization protocol

Preliminary experiments. Initially, randomization was attempted in compatible aprotic solvents to avoid the viscosity of the native oils. We found that products resulting from TAG × TAG reaction in DMSO yielded products with high deviations from prediction (not shown). Others report approximately random distributions with mostly 10-20% deviations for TAG × TAG dissolved in hexane. Trials with chloroform resulted in unpredictable TAG distributions, and attempts to run the reaction with any solvent in our FAME × TAG trials were unsuccessful.

We therefore adapted interesterification chemistry similar to that used by the food industry on the multi-ton scale, employing catalytic amounts (1% or less) of NaOCH$_3$ and no solvent. We found that 1% NaOCH$_3$ led to unacceptably slow reaction, and that excess NaOCH$_3$ (>10 mg) led to excess hydrolysis and very low FAME/TAG yields. Ramping the amount of NaOCH$_3$ in the mixture led to a compromise level of about 6 mg (6%) (Fig. 1). At this level diacylglyceride (DAG) and monoacylglyceride (MAG) accumulation were several percent of the final TAG level (data not shown).

We performed a test randomization with a simple commercially-available food grade oil, fractionated coconut oil (fCO), and methyl oleate (Me18:1). Fig. 2a shows an infusion-ESI-MS of the oil prior to treatment. The FA in this oil are the saturated 8:0, 10:0, and 12:0 with a smaller amount of 14:0 and very little else. Analyses of the post-randomized sample produced the appearance of several newly synthesized TAG that include 18:1 (Fig. 2b).

Final test method. The final method was similar to that for TAG interesterification reported by Byrdwell, on our smaller scale. A pale yellow solid formed within a few minutes and the reaction proceeded to completion. This method was used for all reactions in the following.

The randomization of kinetics 1.5% Me-L into OOO is presented in Fig. 3. In three minutes, FAME reached its randomized composition while TAG incorporated only <10% L. The asynchronous interesterification of FAME and TAG arises from the formation of DAG, MAG and free fatty acids as by-products. Two hour incubations successfully achieved TAG complete randomization.

3.2 Quantitative demonstration of randomized sentinel incorporation in TAG

Randomization of a mixture of FAME and TAG with initially different fatty acid profiles would produce FAME and TAG with identical fatty acid profiles which would in turn be identical to the fatty acid profiles of the initial mixture. As a first demonstration, methyl 17:0 (~6%, w/w) and OOO were mixed and treated. FAME and TAG were purified by TLC separation, bands collected, and methylated for GC-FID analyses. Table 1 shows the results of four independent trials. The relative differences between FAME and TAG profiles, FAME and total FA profiles, TAG and total FA profiles are all well below 0.25% which can be taken as within experimental error.
To test randomization using sentinels, a mixture of two isotopically labeled FAME, d$_2$-16:0 and U-$^{13}$C18:2 were mixed gravimetrically in about 2:1 ratio, added to the TAG triolein, an aliquot of total fatty acids analyzed precisely, and an aliquot treated. Fig. 3a presents the ESI single stage mass spectrum of the randomized TAG. A total of 10 combinatorial TAG, ignoring regiospecificity, are expected (Table S1), and were detected after the randomization. Here we focus on the strong signals from the main, newly synthesized, isotopically labelled TAG, (d$_2$-16:0)OO and (U-$^{13}$C18:2)OO. Since two of the fatty acyl substitutions, OO are identical, the ratio of these two TAG should correspond to isotopic sentinel ratio in the original d$_2$-16:0 and U-$^{13}$C18:2 FAME mixture.

A total of four independent trials conducted on separate days are summarized in Table 2. Expected (d$_2$-16:0)OO/(U-$^{13}$C18:2)OO ratios were calculated from GC-FID quantification of d$_2$-16:0 and U-$^{13}$C18:2. The deviation $\Delta$ of the sentinel ratio acylated to TAG vs sentinel ratio is at most 0.61% with a mean of 0.064%, all within experimental error. We conclude that our procedure effectively randomized the acyl groups with no significant steric effects obvious between TAG of differing acyl composition.

4. Conclusion

The close correspondence between predicted and measured FA sentinels indicates that for saturates and dienes, randomization into TAG is complete. However, our measurements do not distinguish regioisomers and thus some structure could be present among the various isobaric TAG. Strictly speaking, the key feature of this approach is to achieve a chemical reference point corresponding to maximum combinatorial entropy, rather than a thoroughly random distribution. Sentinels appear to be a simple and convenient way to ensure that such a point is reached in any particular experiment. With judicious choice of sentinels, they would also enable correction factors to be measured when random distributions are not observed.

We also note that combinatorial chemistry approaches, even with imperfectly randomized TAG, will be of value for GL structure elucidation studies. Adding in FAME of specific unusual structures, for instance non-methylene interrupted PUFA or branched chain FA, would result in all possible combinations of GL at predictable concentration ranges. Novel structure elucidation methods would then be able to access the relevant structures without resort to expensive stereospecific syntheses.

Notes

The authors declare no competing interests.

Author Contributions

J.T.B. and D.H.W. designed the study; D.H.W., Z.W. did the experiments; D.H.W. and Z.W. analyzed the data; D.H.W., J.T.B. wrote the manuscript. All authors contributed and approved the final submission.


Table 1. FAME and TAG exhibit the same FA profile as the original pool after randomization of methyl 17:0 and triolein (Tri-18:1n-9). Analysis is by GC of TLC separated lipid classes.

<table>
<thead>
<tr>
<th>Replicate Reaction</th>
<th>% 18:1n-9 in FAME</th>
<th>% 18:1n-9 in TAG</th>
<th>Relative Δ (%)</th>
<th>Total FA profile</th>
<th>FAME vs Total FA Relative Δ (%)</th>
<th>TAG vs Total FA Relative Δ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 1</td>
<td>93.39</td>
<td>93.35</td>
<td>0.04</td>
<td>93.57</td>
<td>-0.19</td>
<td>-0.23</td>
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<td>No 2</td>
<td>93.82</td>
<td>93.75</td>
<td>0.08</td>
<td>93.89</td>
<td>-0.07</td>
<td>-0.15</td>
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<tr>
<td>No 3</td>
<td>94.26</td>
<td>94.19</td>
<td>0.07</td>
<td>94.31</td>
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<td>-0.12</td>
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<tr>
<td>No 4</td>
<td>94.26</td>
<td>94.02</td>
<td>0.25</td>
<td>94.17</td>
<td>0.09</td>
<td>-0.16</td>
</tr>
</tbody>
</table>
Table 2. TAG synthesized by addition of isotopically labeled FAME have relative abundance strictly corresponding to theoretical calculations. This demonstrates that the FAME are incorporated randomly into the TAG.

<table>
<thead>
<tr>
<th>Replicate Reaction</th>
<th>TAG</th>
<th>m/z</th>
<th>Area (x10^6)</th>
<th>RF*</th>
<th>Corrected area</th>
<th>P^OO/L^OO (by ESI-MS)</th>
<th>FA pool (by GC)</th>
<th>Weight % of total FA</th>
<th>Expected P^OO/L^OO</th>
<th>Relative Δ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 1</td>
<td>P^OO</td>
<td>878</td>
<td>115</td>
<td>0.95</td>
<td>109</td>
<td>2.36</td>
<td>2,2-d2-16:0</td>
<td>8.10</td>
<td>2.36</td>
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<td>L^OO</td>
<td>918</td>
<td>51</td>
<td>0.91</td>
<td>46</td>
<td></td>
<td>U-13C18:2</td>
<td>3.43</td>
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<td>0.90</td>
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<td>U-13C18:2</td>
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</table>

Mean Δ 0.064%

Note:

*RF (Response factor) is calculated from mono-acyl TAG standards triolein (OOO), trilinolein (LLL) and tripalmitin (PPP) measured with the samples. For example, RF[(d2-16:0)OO]=1/3RF(PPP)+2/3RF(OOO).

^Here P is 2,2-d2 16:0 and L is U-13C 18:2.

Detailed calculations are provided as an excel file in supplement.
Figure 1. Kinetics of FAME & TAG randomization by various catalyst amounts. About 5-10mg of methyl 14:0 was randomized into 90mg triolein.
Figure 2. Incorporation of Me18:1 into fCO. a) Infusion ESI-MS of the ammoniated adducts of TAG of fCO show the predominate TA made of 8:0, 10:0, and 12:0; small sodiated TAG peaks also appear. b) After addition of methyl oleate (Me18:1) and treatment with NaOCH₃, newly synthesized TAG appear with esterified 18:1.
Figure 3. Kinetics of FAME (methyl linoleate, Me-L) & TAG (triolein, OOO) randomization by time. FAME composition was randomized completely within minutes while TAG needs 1 hour to reach >98% randomized (not shown). A total of 2 hours were subsequently implemented for complete randomization. For TAG, % Randomization = (% L in TAG) / (% L in total FA); for FAME, % Randomization = (% O in FAME) / (% O in total FA). 100% randomized FAME or TAG were defined as having the same fatty acid composition as the originally pooled FAME and triolein (total FA).
(d$_2$-16:0)OO

(U-$^{13}$C$_{18}$:2)OO

18:1 from Triolein

d$_2$-16:0 from FAME addition

U-$^{13}$C$_{18}$:2 from FAME addition
Figure 4. The isotopically labeled FAME covalently incorporated into TAG in the proportions added as FAME. a) Isotopically labeled (deuterated and $^{13}$C) FAME synthesized triglyceride (TAG) of known concentration: $(d_2\text{-}16:0)\text{OO} / (U\text{-}^{13}C18:2)\text{OO}=2.36$ measured by ESI/MS. b) GC-FID of TAG+FAME mixture. FAME for analysis prepared from the combined TAG-FAME mixture, reflecting the ratio of isotopically added FAMEs, $d_2\text{-}16:0 / U^{13}C18:2 = 2.36$. 
Fatty acid sentinels as covalently bound randomization standards for triacylglycerol (TAG) quantitative analysis

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Table S1. The number of combinatorial triglycerides (without/with sn-1, sn-2 and sn-3 regiospecificity distinction) for a given number of fatty acids.

<table>
<thead>
<tr>
<th>FA number</th>
<th>TAG number (no regiospecificity)</th>
<th>TAG number (all isomers)</th>
<th>FA number</th>
<th>TAG number (no regiospecificity)</th>
<th>TAG number (all isomers)</th>
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