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Analysis

Critical Ratios for structural analysis of triacylglycerols using mass spectrometry

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Summary

Recent developments have finally allowed fragment behaviors using APCI-MS to be elucidated after twenty years of literature reports. Critical Ratios have been defined that correspond to various aspects of triacylglycerol (TAG) analysis, from overall degree of unsaturation to localization of fatty acids on the glycerol backbone (regioisomers), to grouping of unsaturated fatty acids. The same ratios also constitute a compact library of TAG mass spectra.

Introduction

It has been twenty years since the first report of HPLC coupled to atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) for triacylglycerol (TAG) analysis [1]. One trend reported in that first report that has held true until now is that the protonated molecule, $[M+H]^+$, is the base peak for TAGs with many sites of unsaturation, and it decreases with decreasing unsaturation until a diacylglycerol-like ion, $[DAG]^+$, becomes the base peak. This trend follows through to the point that saturated TAGs give little or no $[M+H]^+$, but instead give virtually only $[DAG]^+$ fragments by APCI-MS.

In 1996, Laakso and Voutilainen reported another trend that documented the fact that the loss of the middle fatty acid (FA), designated in the stereospecific numbering (sn) system as *sn*-2, was the smallest $[DAG]^+$ fragment for ABA/AAB/BAA-type TAGs [2]. Mottram and Evershed reported a similar trend for ABC/CBA/BAC/CAB/ACB/BCA-type TAGs [3], and concluded that loss of the *sn*-2 FA was energetically disfavored. For the sake of brevity, I refer to 'AAA' TAGs as Type I TAGs, since they have only one FA, and refer to 'ABA/AAB/BAA' TAGs Type II TAGS, since they contain two FAs, and 'ABC/CBA/BAC/CAB/ACB/BCA' TAGs as Type III TAGs, since they contain three FAs. The trends regarding loss of the *sn*-2 FA allowed identification, and later quantification, of specific TAG regioisomers (TAGs with FAs located at specific *sn*-locations on the glycerol backbone). This has important implications for nutrition, since enzymes in the gut (e.g. pancreatic lipase) break down TAGs with regiospecificity, i.e., the FAs at the *sn*-1 and *sn*-3 positions are preferentially removed. Also, plants produce TAGs with regiospecificity, i.e., polyunsaturated FAs are preferentially located at the *sn*-2 position. When you combine the trends observed for regioisomers by APCI-MS with the regioisomeric trends for TAG synthesis and metabolism it is obvious that APCI-MS represents a powerful tool to assess the regiospecificity of biological systems. Jakab et al. demonstrated how calibration curves could be constructed to perform quantification of regioisomers in vegetable oils [4].

Critical Ratios

Based on the trends above, I reported "The Bottom Up Solution (BUS) to the Triacylglycerol Lipidome" in 2005, which described three "Critical Ratios" that could be used describe the structural characteristics of TAGs [5]. I recently made a few minor changes to the BUS nomenclature, resulting in the Updated Bottom Up Solution (UBUS) [6]. In the past, I had seen articles that presented tables of mass spectral abundances that the reader could take and transcribe into a spreadsheet to calculate the ratio used to perform quantification of regioisomers (actually different authors used slightly different ratios for the same purpose). What I proposed was to provide the Critical Ratios that give the desired structural information at face value, instead of giving raw abundances that had to be converted to useful ratios. The use of the Critical Ratios had several important benefits: they provide all of the desired information in fewer values than the raw abundances (they take up less space); they provide more information at face value than the raw abundances; and they still allow the complete mass spectrum to be reproduced, if desired. In short, they are a compressed data set that provides more information in fewer values than raw abundances, yet still constitutes a library of mass spectra.

The first Critical Ratio provides information about the first trend described above: the degree of unsaturation. Critical Ration 1 is the ratio of the $[M+H]^+$ (or just $[MH]^+$ to save space) to all of the $[DAG]^+$ fragments, or $[MH]^+/\Sigma[DAG]^+$. This ratio is high for polyunsaturated TAGs, and goes toward zero for TAGs with only saturated FAs. Critical Ratio 1 exhibited a sigmoid behavior that was modelled with a new, simple sigmoid function, which is shown in **Figure 1**. The sigmoid model provided a good approximation to the average $[MH]^+/\Sigma[DAG]^+$ associated with varying degrees of unsaturation, meaning that from just the $[MH]^+/\Sigma[DAG]^+$ the average number of sites of unsaturation could be estimated. The first Critical Ratio allowed the relationship between unsaturation and the $[MH]^+$ and $[DAG]^+$ to be understood better than any other approach to date.

The second Critical Ratio is the one that is most useful, and allows the amount of regioisomers to be quantified. It takes two forms, depending on whether it is for a Type II or Type III TAG. For Type II TAGs, if all else were equal, the amount of the $[AA]^+$

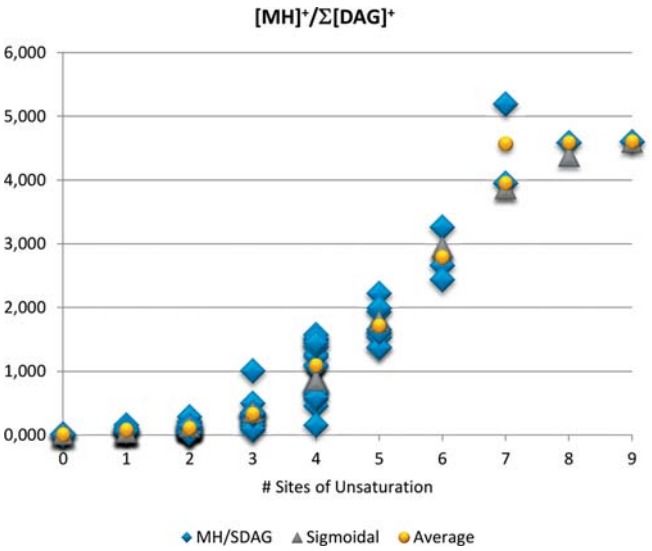

Figure 1. Plot of $[MH]^+/\Sigma[DAG]^+$ ratios and averages from APCI-MS data for soybean oil triacylglycerols and sigmoidal fit for 0 to 9 sites of unsaturation. Minimum value = 0, maximum value set to the $[MH]^+/\Sigma[DAG]^+$ ratio for LnLnLn (= 4.6048), and inflection point set to 5.5 for model function. The average for 7 sites is shown with (upper) and without (lower) LnLnO (0.47% comp.) due to interference from LLLn. Figure adapted from Ref. [6].

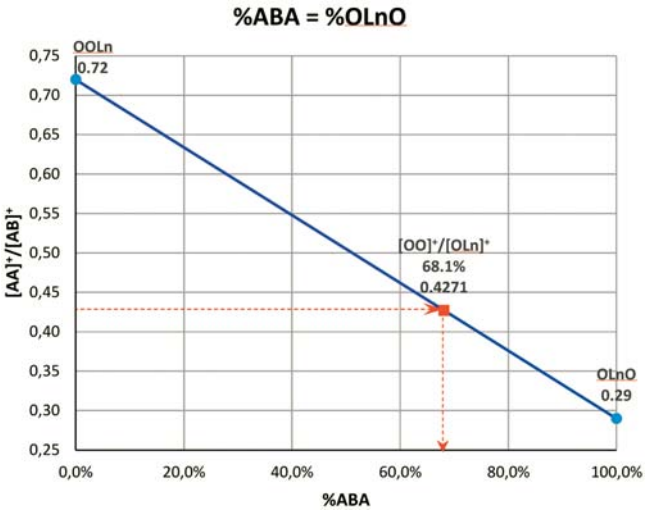

Figure 2. Two-point calibration curve for calculating the percentage of *sn*-1,3 ABA = *sn*-1,3 OLnO in soybean oil by APCI-MS using Critical Ratio 2, $[AA]^+/[AB]^+$ (i.e., $[OO]^+/[OLn]^+$), compared to pure regioisomer standards for a Type II TAG. Critical Ratio observed = 0.4271, compared to tabulated values of 0.29 for pure OLnO and 0.75 for pure OOLn, gives a calculated amount of OLnO of 68.1% (and so 31.9% OOLn).

fragment should be exactly one-half the $[AB]^+$, since there are two ways to get an $[AB]^+$ fragment from ABA, but only one way to get an $[AA]^+$ fragment. Thus, the ratio of $[AA]^+/[AB]^+$ should theoretically always be 1 : 2, or 0.5. However, since loss of the FA in the *sn*-2 position is energetically disfavored, if the $[AA]^+$ happens to correspond to $[(sn\text{-}1,3)AA]^+$, then its abundance will be less than statistically expected. By comparing the $[AA]^+/[AB]^+$ ratio of a real sample to that of pure regioisomer standards, the amount of regioisomer can be quantified, as follows:

$$\%\mathrm{ABA} = \left(\frac{\left(\frac{[\mathrm{AA}]^+}{[\mathrm{AB}]^+}\right)_{\mathrm{AAB}} - \left(\frac{[\mathrm{AA}]^+}{[\mathrm{AB}]^+}\right)_{\mathrm{Obs}}}{\left(\frac{[\mathrm{AA}]^+}{[\mathrm{AB}]^+}\right)_{\mathrm{AAB}} - \left(\frac{[\mathrm{AA}]^+}{[\mathrm{AB}]^+}\right)_{\mathrm{ABA}}} \right) \times 100$$

where $([AA]^+/[AB]^+)_{Obs}$ is the observed Critical Ratio from a real sample, and $([AA]^+/[AB]^+)_{AAB}$ and $([AA]^+/[AB]^+)_{ABA}$ are the Critical Ratios from pure regioisomers (noting that enantiomers AAB and BAA cannot be distinguished).

Analogously for Type III TAGs, the $[AC]^+/([AB]^+ + [BC]^+)$ ratio should be 1 : 2 if all else were equal and all $[DAG]^+$ ions gave the same abundances. But since loss of the *sn*-2 FA is disfavored, the smallest of the three $[DAG]^+$ fragments can be assigned to $[(sn\text{-}1,3)AC]^+$, allowing that regioisomer to be identified, using the observation first pointed out by Mottram and Evershed [3]. A variety of Type III TAG regioisomers were recently analyzed by Holcapek et al. [7], and this trend held true without exception. Thus, Critical Ratio 2, $[AA]^+/[AB]^+$ for Type II TAGs and $[AC]^+/([AB]^+ + [BC]^+)$ for Type III TAGs allows identification and/or quantification of regioisomers.

The third Critical Ratio is the $[BC]^+/[AB]^+$ ratio, and applies to Type III TAGs. For twenty years, no trends have been reported to distinguish $[BC]^+$ from $[AB]^+$. Because of this, Byrdwell proposed [5] to assign $[BC]^+$ and $[AB]^+$ such that Critical Ratio 3 was less than 1, so that perhaps trends might become apparent in which fragments ended up in the numerator versus the denominator. Recently, when Byrdwell analyzed the data of Holcapek et al. [7] for a range of regioisomers using Critical Ratios, new trends were identified. It turned out that the abundances of $[AB]^+$ and $[BC]^+$ were dictated by not only the degree of unsaturation in the FAs, but also the way they were arranged on the glycerol backbone relative to each other, not their absolute *sn*-positions. Specifically, for TAG that contained "L" (linoleic acid, 18 : 2) and "Ln" (linolenic acid, 18 : 3), if L and Ln were adjacent to each other, the $[LLn]^+$ fragment was larger, but when L and Ln were separated by a saturated or monounsaturated fatty acid as the $[(sn\text{-}1,3)LLn]^+$ fragment, then loss of Ln as a more stable leaving group became the predominant mechanism, and the $[DAG]^+$ formed by loss of Ln gave the larger peak [6]. The same principle applied to Type III TAGs containing "O" (oleic acid, 18 : 1) and "L". When O and L were adjacent, the $[DAG]^+$ was larger, but when they were separated by a saturated FA, then loss of the L as a more stable leaving group became the predominant mechanism. These and other trends described by using Critical Ratio 3 allowed 25 of 27 Type III TAG regioisomers to be characterized for the first time using APCI-MS.

The reason why Critical Ratios are more effective at elucidating trends than raw abundances is that often as one abundance goes up, another goes down, such as the case of the $[MH]^+$ versus $[DAG]^+$. Therefore, the ratio of abundances exhibits more change than the raw abundances, accentuating the amount of change associated with structural differences. Thus, when Critical Ratios are judiciously constructed, they reveal trends that are more subtle than may be evident in raw abundances.

Multiple parallel mass spectrometry and other lipids

The raw abundances reported by Holcapek et al. [7], which were converted to Critical Ratios by Byrdwell [6], provided valuable and unprecedented information for TAG regioisomers. Over the

years, Byrdwell had shown in several book chapters that tabulated values for Critical Ratio 2, for the few commercially available TAG regioisomers analyzed by multiple research groups using different instruments over numerous years, agreed pretty well. Holcapek et al. [7] showed even more thoroughly and definitively, for many synthesized regioisomers compared using five different instruments, that all those instruments gave similar abundances, which then gave similar Critical Ratios. This fairly consistent behavior across most instruments by APCI-MS allowed Byrdwell to use the tabulated values for quantification of regioisomers by APCI-MS [6]. It also pointed to the real need for the exact same kind of data for atmospheric pressure photoionization (APPI) MS and electrospray ionization (ESI) MS of TAG regioisomers. In the absence of data like that of Holcapek et al. [7] for APPI-MS and ESI-MS, Byrdwell tested the effect of using data from APCI-MS for quantification of regioisomers by APPI-MS, ESI-MS, and ESI-MS/MS [8]. It turned out that the regioisomer assignments made by APPI-MS agreed quite well with those by APCI-MS, as shown in **Table 1**, though quantification varied slightly. There was only one Type II TAG, LLnL, that APPI-MS indicated as a different regioisomer than indicated by APCI-MS. ESI-MS and ESI-MS/MS, on the other hand, showed more disagreement with APCI-MS than did APPI-MS. This is not surprising, given the radically different ratio of $[DAG]^+$ fragments to the $[MH]^+$ by ESI-MS, reflected in higher values for Critical Ratio 1. This simply points to the need for data like that of Holcapek et al. [7]. Their data proved very valuable for structural analysis of TAGs using Critical Ratios by APCI-MS, and needs to be replicated for the other API techniques.

Critical Ratio 1 by APPI-MS followed similar overall trends to APCI-MS (higher ratio for polyunsaturates, values approaching zero for saturates), but exhibited a higher range of values and did not show the distinctly sigmoidal relationship. Critical Ratio 1 for polyunsaturated TAGs was as high as 11.85 by APPI-MS [8], versus a high of 5.20 by APCI-MS [6]. This indicated that APPI-MS showed a greater response to polyunsaturated TAGs than APCI-MS. ESI-MS gave higher values for Critical Ratio 1 in general, but due to the low levels of $[DAG]^+$ fragments, the effect of chromatographically unresolved neighboring isobaric TAGs skewed the $[MH]^+/\Sigma[DAG]^+$ more than the other API-MS techniques. Other Critical Ratios can also be affected to varying degrees by chromatographically unresolved neighboring TAGs that share common ions. Because of this, Critical Ratios from TAGs in real samples (having TAG isomers) can differ from Critical Ratios for pure isolated TAG standards.

While the previous reports and the above discussion has been focused on TAGs, there are analogous reports for phospholipids that indicate that the ratios of fragments formed by the loss of one or other FA indicate the locations of the FAs on the phosphoglycerol backbone. For instance, Hsu and Turk [9] showed that the loss of acyl fragments from phosphatidylethanolamine (PE) provided information about their positions: "The preferential formations of $R_2CO_2^- > R_1CO_2^-$, and of $[M\text{-}H\text{-}R'_2CH{=}C{=}O]^- > [M\text{-}H\text{-}R'_1CH{=}C{=}O]^-$ are attributed to the findings that charge-driven processes are sterically more favorable at *sn*-2.", and that "These features of tandem spectra readily identify and locate the fatty acid substituents of GPE in the glycerol backbone." Thus, it would be logical to construct the Critical Ratios $[R_1CO_2]^-/[R_2CO_2]^-$ and $[M\text{-}H\text{-}R'_1CH{=}C{=}O]^-/[M\text{-}H\text{-}R'_2CH{=}C{=}O]^-$, as well as $[M\text{-}H\text{-}R_1CO_2]^-/[M\text{-}H\text{-}R_2CO_2]^-$ and $[M\text{-}H]^-/\Sigma[Frag]^-$ to describe the regioisomers and other structural characteristics of PE.

Table 1. Percentage of 'ABA' regioisomers of Type II TAGs based on calibration curves from literature values [7] and observed $[AA]^+/[AB]^+$ ratios for soybean oil TAGs. Table reproduced from Ref. [8].

TAG[a]	APCI-MS[b]	APPI-MS	ESI-MS	ESI-MS/MS
LnLLn	90.5%	100.0%	92.2%	45.6%
LLnL	70.5%	4.1%	49.0%	79.4%
LnOLn	60.7%	93.7%	100.0%	39.2%
LnLnP	0.0%	6.8%	49.1%	0.0%
[c]LLM_{LLP}	0.0%	0.0%	0.0%	0.0%
LLO	3.2%	0.0%	74.2%	0.0%
OLnO	68.1%	79.4%	71.7%	82.4%
LLP	0.0%	0.0%	59.2%	0.0%
LLG_{LLO}	0.0%	0.0%	0.0%	89.6%
PLnP	100.0%	100.0%	100.0%	100.0%
OLO	100.0%	73.1%	100.0%	100.0%
MOM_{POP}	100.0%	70.4%	94.2%	–
$OOPo_{OOP}$	0.0%	0.0%	0.0%	0.0%
LLS	0.0%	0.0%	46.4%	0.0%
OOM_{OOP}	12.4%	0.0%	0.0%	0.0%
PLP	100.0%	100.0%	100.0%	100.0%
LLA	0.0%	0.0%	48.5%	0.0%
OOP	0.0%	0.0%	0.0%	0.0%
$LL\text{-}21:0_{LLA}$	0.0%	0.0%	0.0%	–
OOG_{OOA}	0.0%	0.0%	0.0%	0.0%
POP	100.0%	100.0%	74.9%	96.7%
LLB_{LLA}	0.0%	0.0%	51.7%	0.0%
$LL\text{-}23:0_{LLA}$	0.0%	0.0%	12.5%	–
OOS	23.1%	0.0%	14.2%	0.9%
SLS	60.8%	100.0%	74.0%	0.0%
OOE_{OOA}	0.0%	0.0%	0.0%	12.9%
$LLLg_{LLA}$	0.0%	0.0%	41.8%	–
$LL\text{-}25:0_{LLA}$	0.0%	0.0%	0.0%	–
OOA	7.6%	0.0%	0.0%	–
PPS_{PPO}	15.4%	0.0%	0.0%	–
$LLCe_{LLA}$	0.0%	0.0%	0.0%	–
$OO\text{-}21:0_{OOA}$	0.0%	0.0%	0.0%	19.6%
SOS	100.0%	95.8%	57.1%	87.8%
OOB_{OOA}	19.4%	0.0%	0.0%	15.2%
$OO\text{-}23{:}0_{OOA}$	0.0%	0.0%	0.0%	0.0%
$OOLg_{OOA}$	23.5%	0.0%	0.0%	–
$OO\text{-}25:0_{OOA}$	0.0%	0.0%	0.0%	–
$OOCe_{OOA}$	10.5%	0.0%	0.0%	–
$OOMo_{OOA}$	0.0%	0.0%	0.0%	–

[a] TAG names assigned by APCI-MS
[b] From: Ref. [6], DOI:10.1007/s00216-015-8590-9
[c] When no standard was available, a TAG standard with similar chain length or degree of unsaturation was used

Conclusion

We have conclusively demonstrated multiple times that Critical Ratios provide more structural information for TAGs than raw abundances, and also constitute a reduced data set from which the raw abundances can be reproduced, if desired. But Critical Ratios have the benefit of providing more information in those fewer values than the raw data. The Critical Ratios, and specifically Critical Ratio 3 for Type III TAGs, allowed new trends to be identified that were sufficiently subtle that they had previously defied description. Now that the utility of Critical Ratios has been shown,

we hope that the gaps in knowledge regarding the Critical Ratios for TAG regioisomers determined by APPI-MS and ESI-MS will be filled by others, using the report by Holcapek et al. [7] as a template.

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