

The Bottom-Up Solution to the Triacylglycerol Lipidome Using Atmospheric Pressure Chemical Ionization Mass Spectrometry

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1 **Abstract**

2 Presented here is an approach to determining the identities of triacylglycerols (TAG) by
3 constructing ‘Critical Ratios’ from the abundances of the protonated molecule, $[M+H]^+$, and the
4 diacylglycerol fragment ions, $[DAG]^+$, obtained by atmospheric pressure chemical ionization
5 (APCI) mass spectrometry (MS). These Critical Ratios provide direct information regarding the
6 structural characteristics of the TAG. For instance, one of the two Critical Ratios constructed for
7 an ABA/AAB TAG can be used to determine the identities and relative amounts of the
8 regioisomers of the TAG. An ‘AAA’-type TAG has only one Critical Ratio, an ‘ABA/AAB’-
9 type TAG has two Critical Ratios, and an ‘ABC’-type TAG has three Critical Ratios. It is shown
10 that, in addition to providing direct structural information, the Critical Ratios actually constitute
11 a ‘reduced data set’ from which the mass spectrum of any individual TAG can be reconstructed
12 in its entirety. To do this, the Critical Ratios are processed through a construct that contains a
13 series of conditional tests leading to final equations. The construct contains a series of Critical
14 Values and Critical Limits that allow the TAG to be categorized into Cases. The Cases define
15 the exact form of the equations to be used to calculate the specific abundances of the protonated
16 molecule, $[M+H]^+$, and all $[DAG]^+$ fragment ions. Since the mass spectrum of any TAG can be
17 reproduced using this construct, it is referred to as a solution to the TAG lipidome (the set of all
18 possible structures of TAG). It is demonstrated that the equations may be automated into a
19 spreadsheet, such that given only the Critical Ratio(s), the complete mass spectrum of any TAG
20 can be reproduced.

21

1 **Introduction**

2 During the preparation of a recent book chapter (1), quantifiable numerical values were
3 being sought that could be related to observed mass spectroscopic fragmentation characteristics.
4 We wanted to use some empirically determined 'Critical Ratios', to describe, in a more
5 quantitative way, the characteristics that were ascribed to APCI-MS mass spectra in that chapter.
6 Among the fragmentation characteristics was the dependence of the abundance of the protonated
7 molecule, $[M+H]^+$ or $[MH]^+$, on the amount of unsaturation in the triacylglycerol (TAG) fatty
8 acyl chains. TAG containing numerous sites of unsaturation had a protonated molecule, $[M+H]^+$
9 as the base peak, whereas TAG with fewer sites of unsaturation gave a $[DAG]^+$ fragment as base
10 peak. Saturated TAG gave a $[DAG]^+$ fragment as base peak, with no $[M+H]^+$ whatsoever. This
11 dependence of $[M+H]^+$ abundance on the amount of unsaturation has been observed and reported
12 ever since our first report on the HPLC/APCI-MS analysis of TAG (2). This dependence is
13 reflected in the ratio of the protonated molecule, $[MH]^+$, to the sum of the diacylglycerol
14 fragment ion abundances, $\Sigma[DAG]^+$, which is shown as: $[MH]^+ / (\Sigma[DAG]^+)$. This ratio can be
15 used as a 'Critical Ratio' to provide information regarding the number of sites of unsaturation in
16 the molecule, which determines whether the $[M+H]^+$ is the base peak, or if a $[DAG]^+$ is the base
17 peak. TAG containing numerous sites of unsaturation have an $[MH]^+ / (\Sigma[DAG]^+)$ larger than 1,
18 and so have a $[M+H]^+$ base peak. TAG with few sites of unsaturation have a low
19 $[MH]^+ / (\Sigma[DAG]^+)$ ratio, and a $[DAG]^+$ base peak. Saturated TAG usually have a
20 $[MH]^+ / (\Sigma[DAG]^+)$ ratio of '0', because they give no $[M+H]^+$ whatsoever. Thus, the
21 $[MH]^+ / (\Sigma[DAG]^+)$ ratio can be constructed for any TAG to provide information regarding the
22 amount of unsaturation, and the identity of the base peak.

1 For ABA/AAB TAG, the ratio $[AA]^+/[AB]^+$ can be used to assess the amount of
 2 positional isomer preference. Table 2 in Chapter 7 (1) showed a tabulation of the $[AA]^+/[AB]^+$
 3 ratios of pure positional isomers, as reported in the literature. Equation 1 in that chapter (1) and
 4 given below was derived from the recent report by Jakab *et al.* (3) that described how the ratio of
 5 the abundances of $[AA]^+$ and $[AB]^+$ fragment ions changed with the percentage of LOL versus
 6 LLO TAG isomers.

$$7 \quad \frac{[AA]^+}{[AB]^+} = \left[\frac{\% AAB}{100} \times \left(\left(\frac{[AA]^+}{[AB]^+} \right)_{AAB} - \left(\frac{[AA]^+}{[AB]^+} \right)_{ABA} \right) \right] + \left(\frac{[AA]^+}{[AB]^+} \right)_{ABA} \quad \text{Eq. 1}$$

9
 10 While equation 1 allows the $[AA]^+/[AB]^+$ fragment ratio to be calculated if the % of the
 11 positional isomers is known, it is more desirable to be able to determine the percentage of each
 12 positional isomer in a mixture from the fragment ratios in an average mass spectrum (or from
 13 integrated fragment areas). Equation 1 may be rearranged to yield an equation to calculate the
 14 percentage of the *sn*-1,2 and 2,3 isomer that is present, based on the observed fragment ion ratio:

$$15 \quad \% AAB = \left[\frac{\left(\frac{[AA]^+}{[AB]^+} \right)_{Obs} - \left(\frac{[AA]^+}{[AB]^+} \right)_{ABA}}{\left(\frac{[AA]^+}{[AB]^+} \right)_{AAB} - \left(\frac{[AA]^+}{[AB]^+} \right)_{ABA}} \right] \times 100 \quad \text{Eq. 2}$$

$$16 \quad \% ABA = \left[\frac{\left(\frac{[AA]^+}{[AB]^+} \right)_{AAB} - \left(\frac{[AA]^+}{[AB]^+} \right)_{Obs}}{\left(\frac{[AA]^+}{[AB]^+} \right)_{AAB} - \left(\frac{[AA]^+}{[AB]^+} \right)_{ABA}} \right] \times 100 \quad \text{Eq. 3}$$

17 Equations 2 and 3 show that the $[AA]^+/[AB]^+$ ratio can be used to calculate the amount of each
 18 isomer from any TAG, when compared with $[AA]^+/[AB]^+$ ratios of pure isomers of that TAG.

1 In these equations, the $([AA]^+/[AB]^+)_{ABA}$ ratio and $([AA]^+/[AB]^+)_{AAB}$ ratio are the
2 $([AA]^+/[AB]^+)$ ratios from the pure isomers, while $([AA]^+/[AB]^+)_{obs}$ is the observed ratio for the
3 TAG which composition is sought.

4 Thus, it is useful to construct the $([AA]^+/[AB]^+)$ ratio for any ABA/AAB-type TAG, to
5 provide information regarding the degree of positional isomer preference. This ratio constitutes
6 another 'Critical Ratio' that can be used to visualize structural trends of TAG.

7 For 'ABC' TAG, the ratio of the *sn*-1,3 TAG versus the *sn*-1,2 plus *sn*-2,3 TAG allows
8 the preference for the *sn*-1,3 isomer, $[AC]^+$, isomer to be assessed. The Critical Ratio to specify
9 the positional isomer preference in 'ABC' TAG is $[AC]^+ / ([AB]^+ + [BC]^+)$. If all fatty acids in a
10 TAG were distributed statistically, and there was no influence due to different levels of
11 unsaturation or preferential loss, then the $[AC]^+$, $[AB]^+$ and $[BC]^+$ fragment abundances should
12 all be equal, and the $[AC]^+ / ([AB]^+ + [BC]^+)$ ratio would be 0.5. An $[AC]^+ / ([AB]^+ + [BC]^+)$ ratio
13 greater than 1 would certainly indicate a preference for $[AC]^+$.

14 If there were any *sn*-1,2 versus *sn*-2,3 preference, this could be described using the ratio
15 $[BC]^+ / [AB]^+$, where $[BC]^+$ is the abundance of the *sn*-2,3 isomer, and $[AB]^+$ is the abundance of
16 the *sn*-1,2 isomer. The ratio $[BC]^+ / [AB]^+$ would constitute the Critical Ratio necessary to specify
17 any $[AB]^+$ versus $[BC]^+$ preference (or *vice versa*).

18 The ratios mentioned above can be constructed and can be directly interpreted to provide
19 information regarding structural trends. These ratios provide a method for visualizing trends that
20 is more direct than using the 'raw' abundances alone. The trends are not as apparent in the raw
21 data as they are in the Critical Ratios. Therefore, is it useful to construct these Critical Ratios as
22 an aid to qualitative analysis of TAG.

23 We will demonstrate herein that, not only do these Critical Ratios highlight structural
24 trends, but also they constitute a 'reduced data set' from which the mass spectrum of any TAG

1 can be reconstructed in its entirety. The Critical Ratios can be used with inherent Critical Values
2 and calculated Critical Limits to classify all TAG into Cases. The Case into which a TAG is
3 classified provides all of the equations necessary to calculate the abundances of every ion in the
4 mass spectrum, as well as the sum of all ions. This report will be present the basic equations that
5 encompass every structural possibility within the Triacylglycerol Lipidome. The solution to the
6 ‘Triacylglycerol Lipidome’ comes in the form of the Critical Ratios applied with an
7 ‘Interpretation Matrix’ to accomplish the classification of the TAG into the proper Cases, based
8 on the Critical Values and Critical Limits. Once a TAG is properly classified by its Critical
9 Ratios, the Case indicates the correct equations used for the solution of all ion abundances. This
10 combination of the Critical Ratios with the Interpretation Matrix that contains the Critical Values
11 and Critical Limits used to define the Cases, and the Equations constitutes one solution to the
12 Triacylglycerol Lipidome. This solution to the Triacylglycerol Lipidome is one approach to the
13 goal of finding a quantitative description for the set of structural possibilities exhibited by TAG
14 during APCI-MS.

15 **The Bottom-Up Solution (BUS)**

16 During the routine processing of data from LC/APCI-MS using the quantitative method
17 of Byrdwell *et al.* (4,5,6) the area under every peak is integrated in every extracted ion
18 chromatogram (EIC) of each m/z value for every $[DAG]^+$ fragment ion and protonated molecule,
19 $[M+H]^+$. The areas of $[DAG]^+$ fragment ions and protonated molecules, $[M+H]^+$, that came from
20 a particular TAG had to elute at the same specific retention time, since they came from the same
21 TAG. Therefore, the areas under the fragment ion and protonated molecule chromatographic
22 peaks constitute an average mass spectrum across the retention time window used for integration.
23 The average mass spectrum is calculated as the normalized percentage composition of the
24 fragment ions and the protonated molecule across the integrated chromatographic retention time

1 window for a particular TAG. Small differences in retention times at the beginning and end of
2 the fragment peaks has no substantial impact on the integrated area of each peak. The integrated
3 areas provide a good representation of the mass spectrum averaged across a chromatographic
4 peak. All solutions provided herein work equally well on an average mass spectrum produced
5 from integrated peak areas, or an average mass spectrum obtained from manual qualitative
6 analysis, or a single mass spectrum manually acquired.

7 From an average mass spectrum across a TAG peak, one has all of the information
8 necessary to construct a set of Critical Ratios for each TAG. The values that were used to
9 constitute the Critical Ratios were mentioned above. The first Critical Ratio is the ratio of the %
10 abundance of the protonated molecule as a ratio to the sum of all [DAG]⁺ fragment ions:

$$\text{Critical Ratio 1} = \left(\frac{[\text{MH}]^+}{\sum [\text{DAG}]^+} \right) \quad \text{Eq. 4}$$

11
12 Table 1 lists the Critical Ratios for a 35-TAG mixture of synthetic TAG. The critical ratios are
13 listed as a percentage simply for the most convenient display of four significant figures, with up
14 to two decimal places. This is the same method of representation that Jakab *et al.* (3) used in
15 their use of the [AA]⁺/[AB]⁺ ratio for characterization of mixtures of LOL/LLO. It is important
16 to realize that the Critical Ratios as percentages must be converted to pure ratios (fractions), by
17 dividing by 100, before they are used in the equations below. Throughout all of the discussion
18 below, all ratios are expressed in pure fraction form, not percentage form. As a fraction, 100 %
19 equals 1, so 1 is the upper limit of the axes in Figure 1, and in the equations below.

20 The first Critical Ratio can be used to define the overall unsaturation of a molecule. For
21 instance, it is well known that saturated TAG have either a very low ([MH]⁺/Σ[DAG]⁺), or = 0.
22 The polyunsaturated TAG have large values of ([MH]⁺/Σ[DAG]⁺). The ([MH]⁺/Σ[DAG]⁺) ratio
23 for 'OOO' = 3.24 %, and so for this Type I TAG (mono-acid TAG), the ([MH]⁺/Σ[DAG]⁺) IS

1 the protonated molecule abundance. For ‘LLL’ and ‘LnLnLn’, the large $([MH]^+/\Sigma[DAG]^+)$ ratio
2 indicates that the $[M+H]^+$ is the base peak, and the $[DAG]^+$ is easily calculated.

3 The second Critical Ratio depends on whether it is a Type II or Type III TAG. For a
4 Type II TAG, the second Critical Ratio is $[AA]^+/[AB]^+$. For a Type III TAG, the second Critical
5 Ratio is $([AC]^+/([AB]^+ + [BC]^+))$:

Type II:

$$\text{Critical Ratio 2} = \left(\frac{[AA]^+}{[AB]^+} \right) \quad \text{Eq. 5a}$$

Type III:

$$\text{Critical Ratio 2} = \left(\frac{[AC]^+}{([AB]^+ + [BC]^+)} \right) \quad \text{Eq. 5b}$$

7 This second Critical Ratio has an obvious relationship to the positional preference of a TAG. It
8 is expected that the $([AA]^+/[AB]^+)$ ratio should always be less than 1, unless there is a strong
9 $[AA]^+$ positional preference. Likewise, the ratio $([AC]^+/([AB]^+ + [BC]^+))$ should be less than 1,
10 unless there is a strong $[AC]^+$ positional preference. But, the degree of unsaturation located in
11 the $[AC]^+$ fragment also has an effect on this ratio. Deconstruction of the second Critical Ratio
12 of an ‘ABC’ TAG into its two components of positional preference and degree of unsaturation
13 remains to be finalized. For now, this ratio can simply be compared to positional isomer
14 standards.

15 The third Critical Ratio is only observed from Type III TAG. It is the ratio of
16 $[BC]^+/[AB]^+$, or the ratio of the *sn*-2,3 $[DAG]^+$ to the *sn*-1,2 $[DAG]^+$. For now, it is assumed that
17 there is no particular preference between these two, but evidence at a future date may allow
18 quantification of a preference for the *sn*-1,2 $[DAG]^+$ peak over the *sn*-2,3 $[DAG]^+$ peak. All else
19 being equal, we use a $[BC]^+/[AB]^+$ of < 1 as the default assumption (Assume $[AB]^+ \geq [BC]^+$).

20 Now that the critical ratios have been defined, it is useful to examine the visualization
21 approach that led to the definition of the **Cases**. Figure 1 shows the appearance of typical APCI-
22 MS mass spectra of TAG. As mentioned, the axes in these figures have a maximum value of ‘1’,
23 representing 100 % as a pure ratio. ‘AAA’ TAG are shown first in this figure.

1 Since an ‘AAA’ TAG has only one $[DAG]^+$ and the $[M+H]^+$, it is obvious that the
2 Critical Value occurs when both the $[AA]^+$ and the $[MH]^+$ have their maximum values, and the
3 $([MH]^+/\Sigma[DAG]^+)$ is equal to 1. The Critical Value for an ‘AAA’ TAG is depicted in the upper
4 right panel of Figure 1. From this Critical Value of $([MH]^+/\Sigma[DAG]^+) = 1$, if the $\Sigma[DAG]^+$ were
5 to diminish by the least amount, the $([MH]^+/\Sigma[DAG]^+)$ ratio would be larger than 1 and, the
6 $[M+H]^+$ would be the base peak. This is referred to as ‘Case 1’, and the solution would use the
7 equation shown in Scheme 1, Part I for Case 1. On the other hand, if the $[MH]^+$ were to diminish
8 by the least amount, the $([MH]^+/\Sigma[DAG]^+)$ ratio would be less than 1 and, the $[AA]^+$ would be
9 the base peak. This is referred to as ‘Case 2’, and the solution would use the equation listed in
10 Scheme 1, Part I for Case 2. No other Critical Limit is necessary to specify the Case for an
11 ‘AAA’ TAG. Once the case has been specified, the actual percentages of the $[M+H]^+$ and the
12 $[AA]^+$ are given by the equations in Scheme 1, Part I, for either the Case 1 solution ($[M+H]^+$
13 base peak) or the Case 2 solution ($[AA]^+$ base peak). The value ‘1’ serves as a Critical Value for
14 all Type I, II and III TAG. If the $([MH]^+/\Sigma[DAG]^+)$ ratio is greater than 1 for any TAG, this
15 automatically means that the $[M+H]^+$ is the base peak, and specifies a Case 1 solution. More
16 generally, if any Critical Ratio has a value greater than 1, the numerator is automatically the
17 largest peak of the peaks used to construct the ratio.
18

Table 1. The TAG composition and Critical Ratios from the synthetic 35-TAG mixture, determined by APCI-MS (3 replicates).

E C N	TAG	Statistical % Comp.	Adjusted APCI-MS	[M+H]⁺ / (Σ[DAG]⁺)	± SD	[AA]⁺ / [AB]⁺	± SD	[BC]⁺ / [AB]⁺	± SD
48	PPP	0.84	0.75	0.00	0.00				
54	SSS	0.94	0.82	0.00	0.00				
48	OOO	0.85	0.88	3.24	0.39				
42	LLL	0.77	0.78	120.9	12.2				
36	LnLnLn	0.62	0.78	224.2	21.3				
50	PPS/PSP	2.62	2.40	0.00	0.00	49.26	0.84		
48	PPO/POP	2.54	2.49	0.55	0.12	65.28	2.35		
46	PPL/PLP	2.45	2.87	0.40	0.28	84.11	2.45		
44	PPLn/PLnP	2.28	2.53	12.82	2.37	69.94	3.11		
52	SSP/SPS	2.72	2.44	0.00	0.00	40.91	3.31		
52	SSO/SOS	2.73	2.48	0.45	0.03	75.14	2.78		
50	SSL/SLS	2.64	2.96	0.42	0.11	82.89	2.08		
48	SSLn/SLnS	2.45	2.75	9.60	1.19	60.98	3.16		
48	OOP/POP	2.55	2.72	1.89	0.17	36.12	1.42		
50	OOS/SOS	2.65	2.76	1.12	0.31	39.82	1.73		
46	OOL/OLO	2.47	2.54	11.93	0.95	50.10	2.20		
44	OOLn/OLnO	2.30	2.27	36.92	1.86	41.62	2.52		
44	LLP/LPL	2.38	2.00	27.41	6.91	88.84	9.62		
46	LLS/LSL	2.47	2.29	43.53	7.23	76.07	8.61		
44	LLO/LOL	2.39	2.33	44.44	7.74	74.62	7.01		
40	LLLn/LLnL	2.15	2.21	104.7	21.9	51.53	3.70		
40	LnLnP/LnPLn	2.06	1.94	102.6	13.3	75.00	13.63		
42	LnLnS/LnSLn	2.13	1.96	62.07	8.13	96.04	10.10		
40	LnLnO/LnOLn	2.07	2.26	106.1	25.6	82.36	9.97		
38	LnLnL/LnLLn	2.00	2.15	129.8	18.9	64.57	5.77		
50	OPS	5.27	5.11	0.47	0.08	37.65	1.96	69.79	2.12
48	SPL	5.09	5.89	0.46	0.11	35.39	1.68	59.43	2.95
46	SPLn	4.73	5.42	9.29	1.46	39.78	1.27	76.70	3.46
46	LOP	4.93	4.57	6.46	1.40	40.38	1.34	98.27	2.51
44	LnOP	4.59	4.60	27.37	8.06	41.97	2.53	91.76	5.68
42	LnLP	4.43	3.78	50.09	9.53	37.00	2.15	72.45	9.92
48	LOS	5.11	5.72	7.47	0.86	43.86	1.28	85.70	4.63
46	LnOS	4.75	4.38	19.91	4.77	42.48	1.82	89.53	2.14
44	LnLS	4.59	4.00	46.69	12.12	38.98	2.65	79.07	3.92
42	LLnO	4.45	4.20	58.63	12.69	40.08	1.55	77.19	3.47
	Sum	100.01	100.03			=([AC] ⁺ /([AB] ⁺ + [BC] ⁺))			

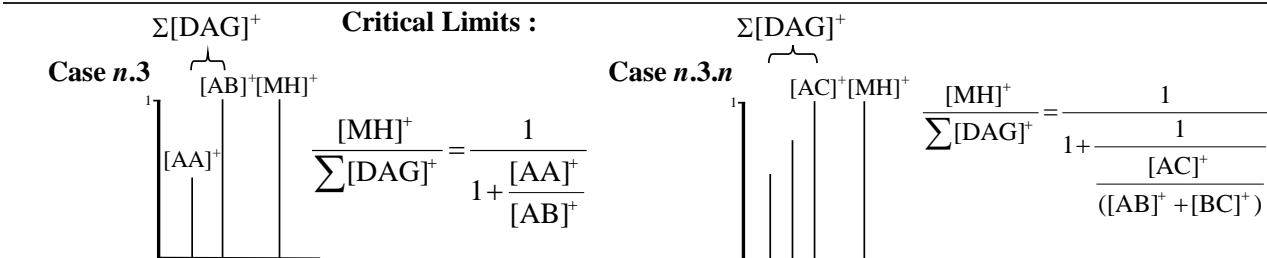
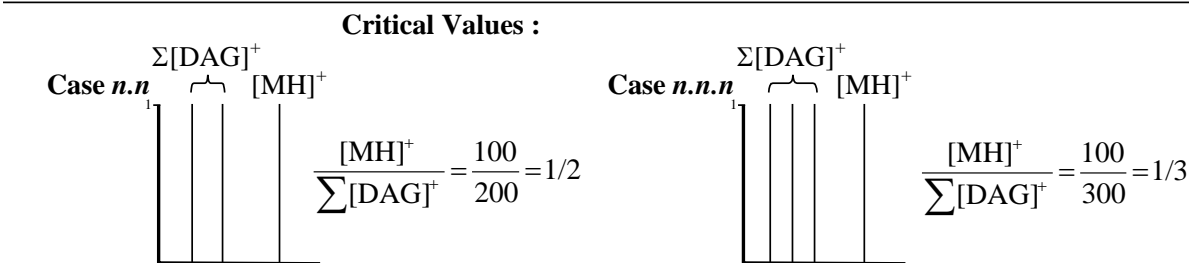
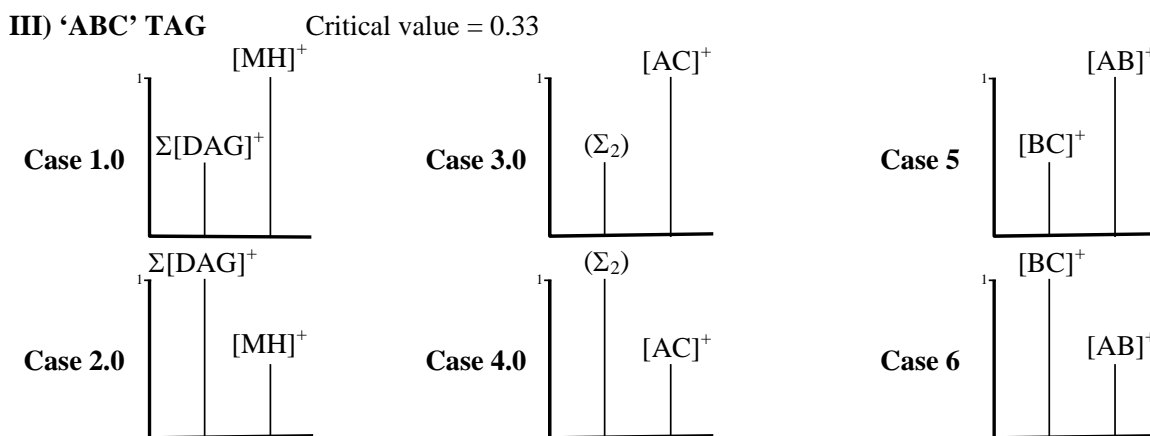
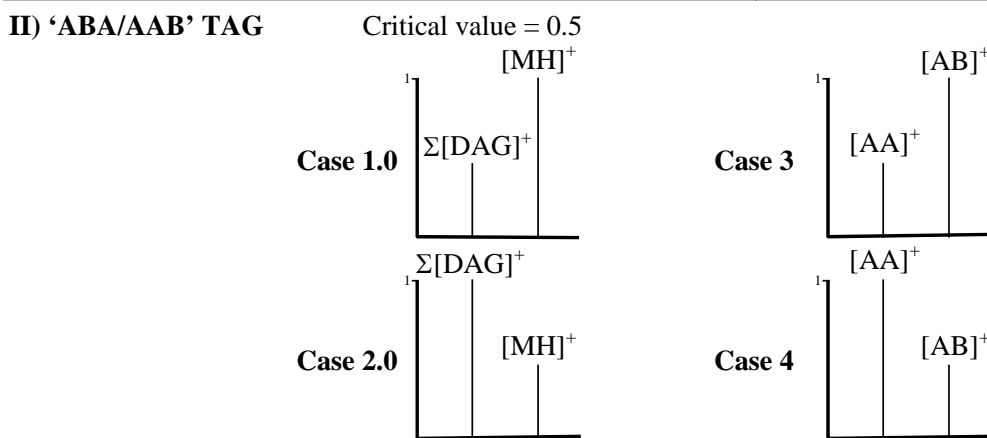
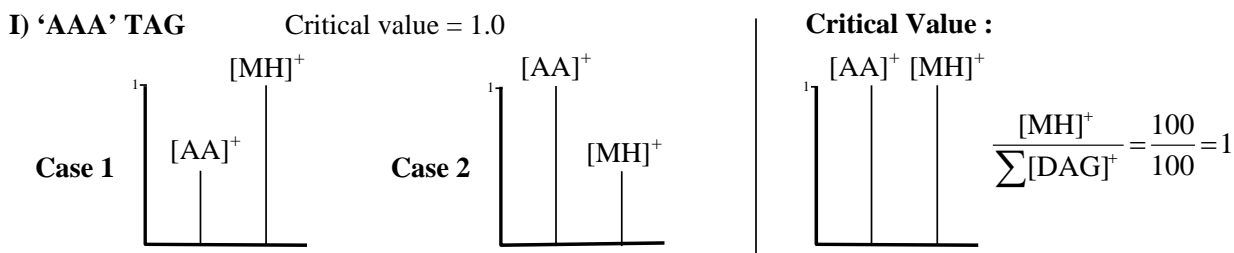
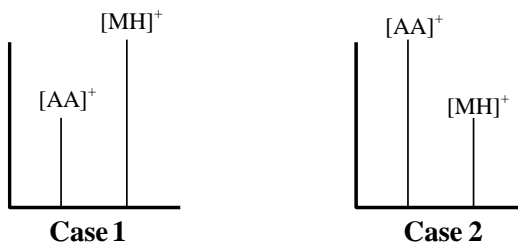


Figure 1. Generalized representations of mass spectra used to calculate Critical Ratios. Also, generalized mass spectra are shown that demonstrate Critical Values and Critical Limits.

1 For Type II and Type III TAG, a $([MH]^+/\Sigma[DAG]^+)$ ratio greater than 1 certainly
2 indicates that the $[M+H]^+$ is the base peak, but a $([MH]^+/\Sigma[DAG]^+)$ ratio less than one does not,
3 by itself, indicate that the $[M+H]^+$ is not the base peak. Thus, for Type II and III TAG, a
4 $([MH]^+/\Sigma[DAG]^+)$ ratio greater than 1 is sufficient, but not necessary to define the $[M+H]^+$ as the
5 base peak for any TAG. Type II and III TAG have other Critical Values inherent in the
6 construction of the ratios. The same logic demonstrated for 'AAA' TAG of setting all peaks
7 equal to 1 at the Critical Value, and comparing the observed Critical Ratio to this Critical Value
8 to determine the identity of the base peak, is used for Type II and Type III TAG.

9 At the Critical Value for Type II TAG, the $[M+H]^+$ and both $[DAG]^+$ have their
10 maximum values of 1. The Critical Value for a Type II TAG is depicted in the fourth panel
11 down in Figure 1. The Critical Value of the $([MH]^+/\Sigma[DAG]^+)$ ratio for an 'ABA/AAB' TAG is
12 $\frac{1}{2}$, or 0.5, as shown in Figure 1. A mass spectrum with a $([MH]^+/\Sigma[DAG]^+)$ ratio less than the
13 Critical Value cannot have a protonated molecule base peak. But a $([MH]^+/\Sigma[DAG]^+)$ ratio
14 greater than the Critical Value does not immediately indicate a protonated molecule base peak.
15 The Critical Ratio must be further tested with another limit to determine whether the $[M+H]^+$ is
16 the base peak. The Critical Value serves as the lower limit for the possibility of having a
17 protonated molecule base peak. The Critical Limit, which is the upper limit of the
18 $([MH]^+/\Sigma[DAG]^+)$ ratio that defines whether the $[M+H]^+$ is the base peak, is discussed below.

D) 'AAA' TAG



$$\sum(I^+) = \sum([DAG]^+ + [MH]^+) = \left(1 + \frac{1}{\left(\frac{[MH]^+}{\sum[DAG]^+}\right)^*}\right) \quad \left(1 + \left(\frac{[MH]^+}{\sum[DAG]^+}\right)_*\right)$$

$$\left(\frac{[MH]^+}{\sum[DAG]^+}\right)^* \geq 1 \xrightarrow{\mathbf{Y}} \mathbf{Case\ 1} \quad \% [M+H]^+ = 100 \% \text{ (base peak)}$$

$$\downarrow \mathbf{N} \quad \% [DAG]^+ = \frac{1}{\left(\frac{[MH]^+}{\sum[DAG]^+}\right)} \times 100$$

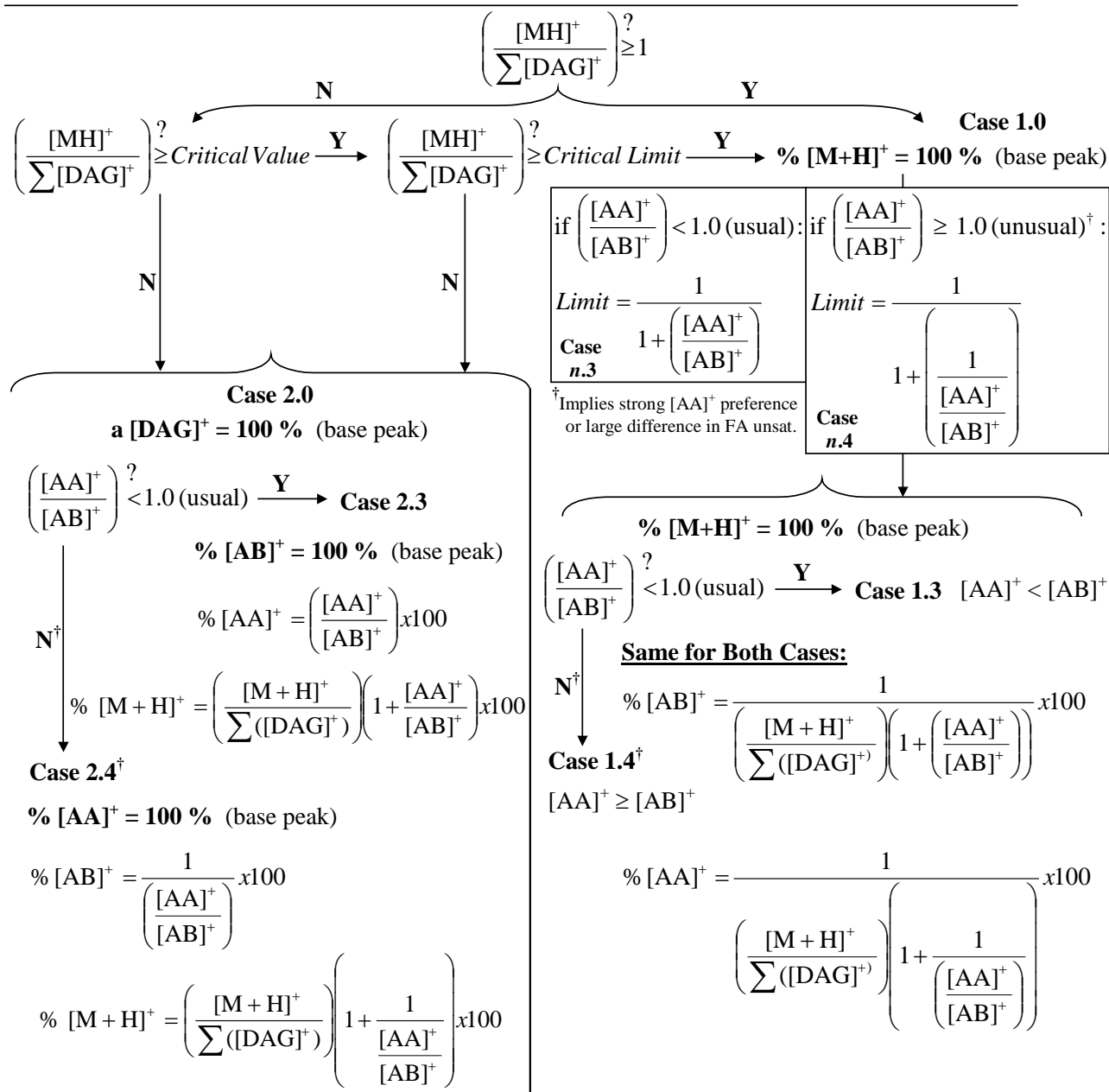
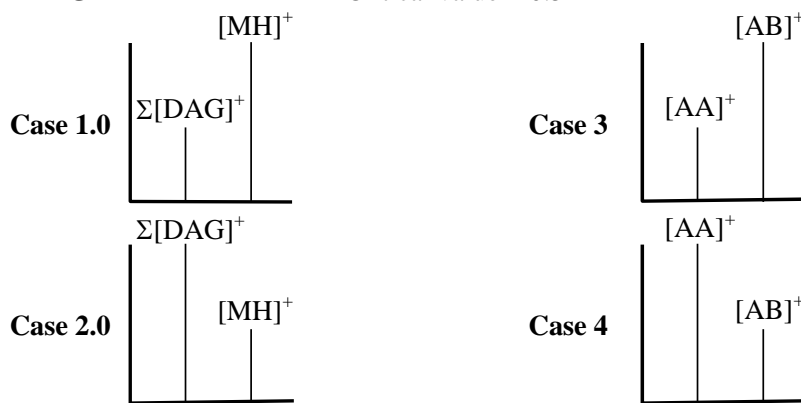
Case 2
the [DAG]⁺ = 100 % (base peak)

$$\% [M+H]^+ = \left(\frac{[MH]^+}{\sum[DAG]^+}\right) \times 100$$

Scheme 1, Part I. Equations to calculate the relative abundances of the [M+H]⁺ and the [DAG]⁺ fragment ion for an 'AAA' TAG using the 'Critical Ratio' from APCI-MS data (Table 1).

II) 'AAB/ABA' TAG

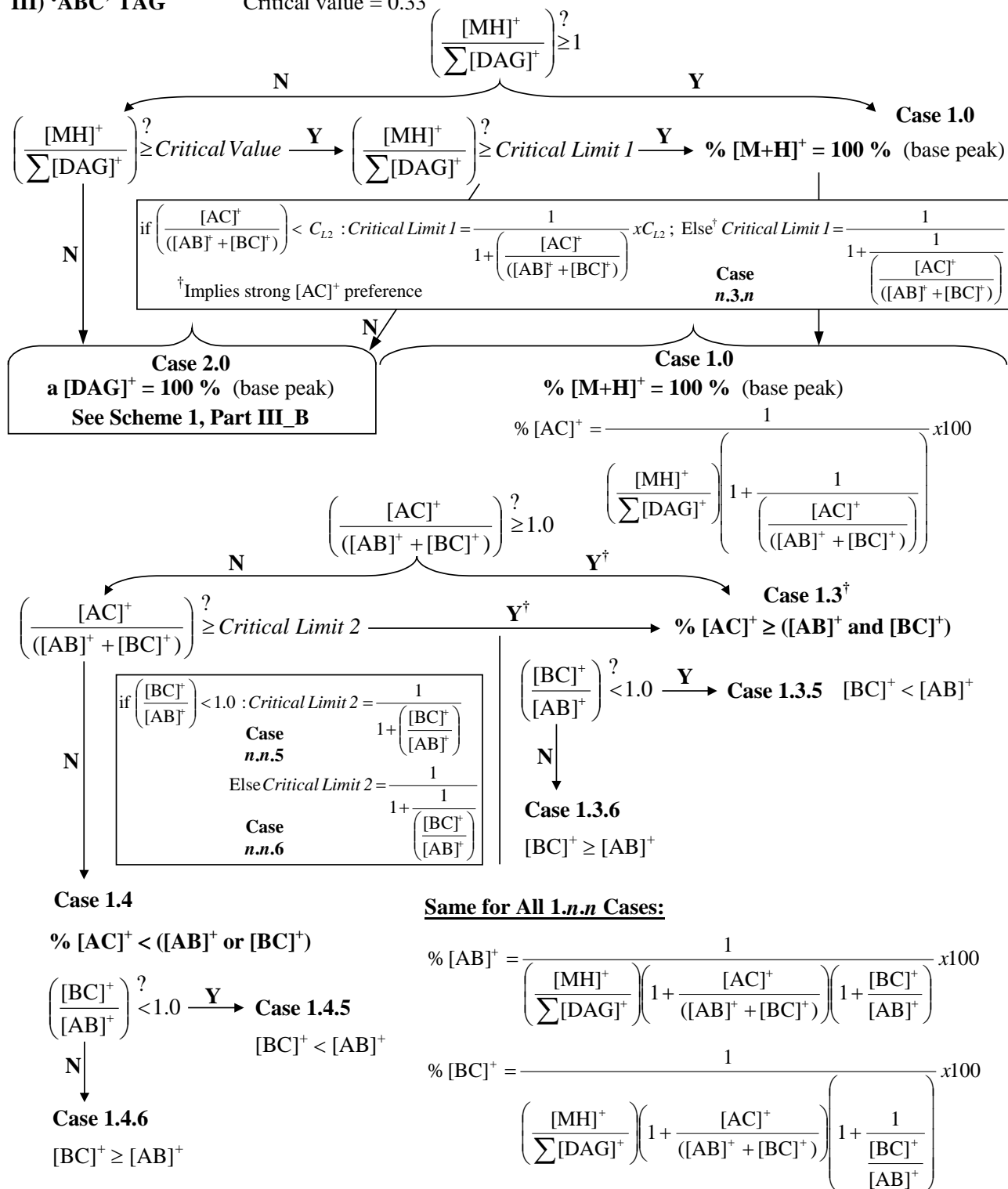
Critical value = 0.5



Scheme 1, Part II. Equations to calculate the relative abundances of the [M+H]⁺ and [DAG]⁺ fragment ions for 'ABA/AAB' TAG using 'Critical Ratios' from APCI-MS data (Table 1).

III) 'ABC' TAG

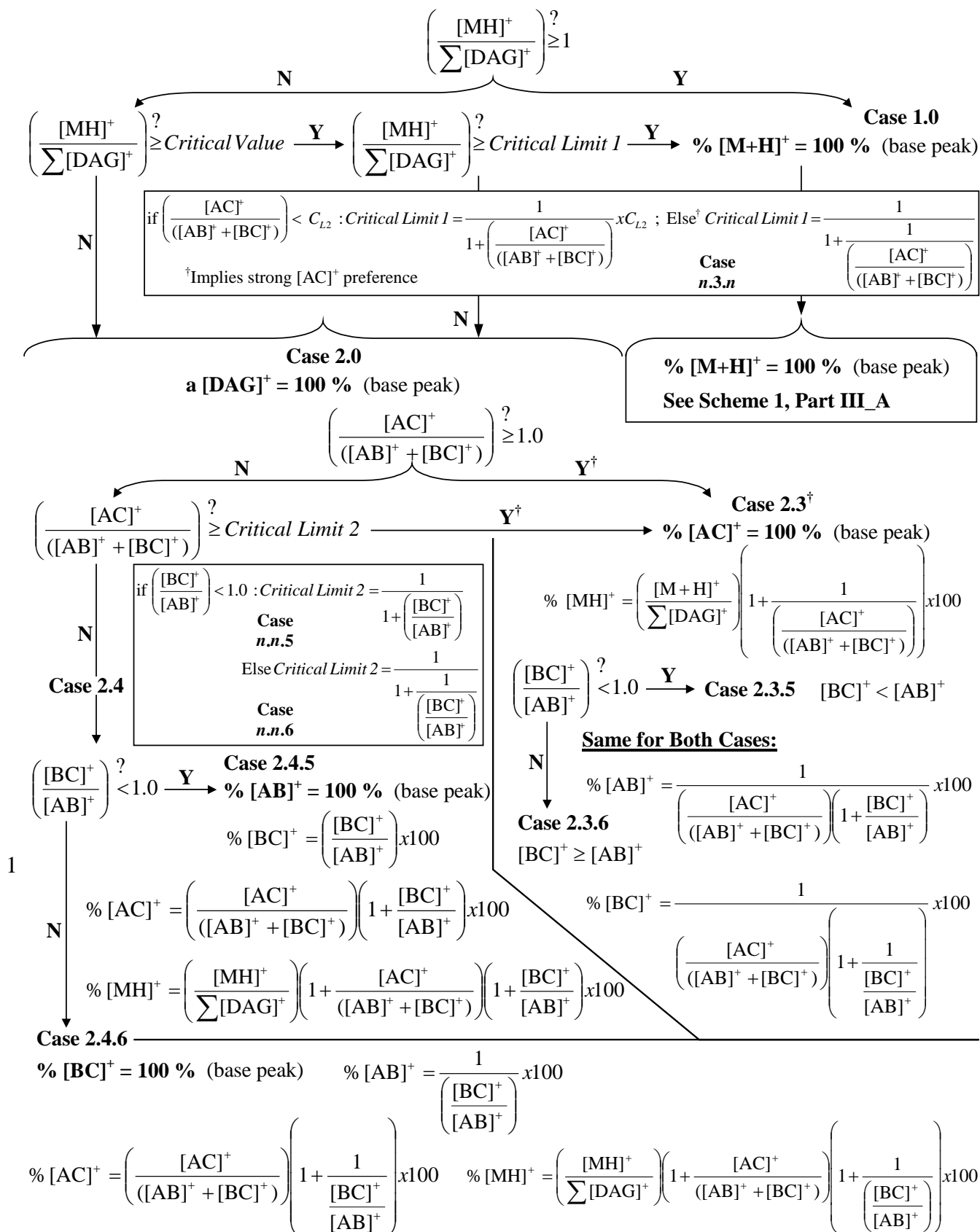
Critical value = 0.33



Scheme 1, Part IIIA. Equations to calculate the relative abundances of $[M+H]^+$ and $[DAG]^+$ fragment ions for 'ABC' TAG, Case 1.n.n, using 'Critical Ratios' from APCI-MS data (Table 1).

III 'ABC' TAG

Critical value = 0.33



Scheme 1, Part IIIB. Equations to calculate the relative abundances of the [M+H]⁺ and [DAG]⁺ fragment ions for 'ABC' TAG, Case 2.n.n, using 'Critical Ratios' from APCI-MS data (Table 1).

1 Type III TAG also have a Critical Value for the $([MH]^+/\Sigma[DAG]^+)$ ratio, based on all ions
2 being equal to 1. The Critical Value for a Type III TAG is depicted in the right side of the fourth
3 panel down in Figure 1. The Critical Value of the $([MH]^+/\Sigma[DAG]^+)$ ratio for an ‘ABC’ TAG is
4 $1/3$, or 0.33, as shown in Figure 1. A mass spectrum of a TAG with a $([MH]^+/\Sigma[DAG]^+)$ ratio
5 less than the Critical Value cannot have a protonated molecule base peak. But a
6 $([MH]^+/\Sigma[DAG]^+)$ ratio greater than the Critical Value does not immediately indicate a
7 protonated molecule base peak. The Critical Ratio must be further tested with other limits to
8 determine whether the $[M+H]^+$ is the base peak. As with Type II TAG, the Critical Value serves
9 as the lower limit for the possibility of having a protonated molecule base peak. The
10 $([MH]^+/\Sigma[DAG]^+)$ ratio may be above the Critical Value, yet still not be the base peak, since the
11 Critical Value is based on all $[DAG]^+$ being at their maximum values, which is not usually the
12 case. Critical Limits used for Type III TAG, including the limit of the $([MH]^+/\Sigma[DAG]^+)$ ratio
13 that defines whether the $[M+H]^+$ is the base peak, are discussed below.

14 We have shown that if the $([MH]^+/\Sigma[DAG]^+)$ ratio is lower than the Critical Value of a
15 TAG, the $[M+H]^+$ cannot be the base peak. Above the Critical Value, the $[M+H]^+$ may still be
16 the base peak, but not all of the $[DAG]^+$ are equal to 1. In such case, the $\Sigma[DAG]^+$ would depend
17 on the ratios of the other ions, as expressed in the other Critical Ratios. If the $([MH]^+/\Sigma[DAG]^+)$
18 is greater than the Critical Value, then the $[M+H]^+$ may be the base peak, but the
19 $([MH]^+/\Sigma[DAG]^+)$ needs to be further tested. The limit that determines whether the $[M+H]^+$
20 actually is the base peak or not is referred to as a ‘Critical Limit’, and is calculated from the
21 Critical Ratios for a given TAG type. The Critical Values of TAG are inherent values based on
22 the definition of the scale (= 100 %), and do not depend on a particular Critical Ratio. A Critical
23 Limit, on the other hand, is based on observed Critical Ratios from actual data. A Type II TAG
24 has only one Critical Limit; a Type III TAG has two Critical Limits.

1 For Type II TAG with a $([MH]^+/\Sigma[DAG]^+)$ ratio below the Critical Value, the identity of
 2 the base peak depends on the Critical Ratio $[AA]^+/[AB]^+$. The $[AA]^+/[AB]^+$ ratio is used to
 3 construct the Critical Limit for the $([MH]^+/\Sigma[DAG]^+)$ ratio for Type II TAG. At exactly the
 4 Critical Limit, the $[M+H]^+$ is 1 and one of the $[DAG]^+$ is 1. If one of the $[DAG]^+$ fragments is 1,
 5 and $[AA]^+/[AB]^+ < 1$ (Case 3), then $[AB]^+ = 1$ and the sum of the $[DAG]^+$ fragments would be
 6 equal to $(1 + ([AA]^+/[AB]^+))$. On the other hand, if one of the $[DAG]^+$ fragments is 1, and
 7 $[AA]^+/[AB]^+ \geq 1$ (Case 4), then $[AA]^+ = 1$ and the sum of the $[DAG]^+$ fragments would be equal
 8 to $(1 + (1/([AA]^+/[AB]^+)))$. The ratio of the $[M+H]^+ (= 1)$ to the $\Sigma[DAG]^+ (= 1 + ([AA]^+/[AB]^+))$
 9 or $= 1 + (1/([AA]^+/[AB]^+))$ equals :

$$\frac{[MH]^+}{\Sigma[DAG]^+} = \frac{1}{1 + \frac{[AA]^+}{[AB]^+}} \quad \text{or} \quad \frac{[MH]^+}{\Sigma[DAG]^+} = \frac{1}{1 + \frac{1}{\frac{[AA]^+}{[AB]^+}}} \quad \text{Eq. 6}$$

10 .

11 In the example of the Type II Critical Limit in the fifth panel down in Figure 1, it can be seen
 12 that i) $[AA]^+/[AB]^+ < 1$ (Case 3), so the sum of the $[DAG]^+$ is equal to $1 + ([AA]^+/[AB]^+)$, and ii)
 13 if the $\Sigma[DAG]^+ (= 1 + ([AA]^+/[AB]^+))$ diminishes by the least amount, then the $[M+H]^+$ will be
 14 the base peak, but if the $[M+H]^+$ diminishes by the least amount, then the $[AB]^+$ is the base peak.
 15 Thus, it is the ‘Critical Limit’, which is based on the second Critical Ratio, that defines which
 16 peak is the base peak of Type II TAG, and therefore whether a Case 1 or Case 2 solution is used.
 17 Note that if $[AA]^+/[AB]^+ \geq 1$ (Case 4), the $\Sigma[DAG]^+$ is given by $(1 + (1/([AA]^+/[AB]^+)))$, and
 18 that if the $[M+H]^+$ diminishes by the least amount, then the $[AA]^+$ is the base peak.

19 The easiest way to confirm the truth of the above equations is to construct hypothetical
 20 mass spectra with specific percentages (easiest if constructed as multiples of 5 %), and then to
 21 construct the critical ratios from the spectra and insert actual ratios into the equations. For

1 example, assume that a typical mass spectrum of 'LSL/LLS' has a $([MH]^+/\Sigma[DAG]^+)$ ratio of 1/2
 2 (= 0.5) and an $[AA]^+/[AB]^+$ ratio of 4/5 (= 0.8). The Bottom-Up Solution is implemented as
 3 follows: First, the $([MH]^+/\Sigma[DAG]^+)$ ratio is < 1 , so the $[M+H]^+$ is not automatically the base
 4 peak. To determine the identity of the base peak, the $([MH]^+/\Sigma[DAG]^+)$ ratio must first be
 5 compared to the Critical Value. In this example, the $([MH]^+/\Sigma[DAG]^+)$ ratio is exactly equal to
 6 the Critical Value, so the $[M+H]^+$ MAY be the base peak. Next, the $([MH]^+/\Sigma[DAG]^+)$ ratio
 7 must be compared to the Critical Limit. Since the ratio of $[AA]^+/[AB]^+$ is < 1 , this specifies that
 8 $[AA]^+/[AB]^+$ obeys Case 3, as shown in the second panel down in Figure 1. Since $[AA]^+/[AB]^+$
 9 obeys Case 3, the Critical Limit for the $([MH]^+/\Sigma[DAG]^+)$ ratio is given as:

10 $Critical\ Limit\ 1 = \frac{1}{1 + \frac{[AA]^+}{[AB]^+}} = \frac{1}{1 + \frac{4}{5}} = \frac{1}{\frac{9}{5}} = \frac{5}{9}$. This equation for the calculation of the

11 Critical Limit is given in Scheme 1, Part II. This calculation tells us that if the
 12 $([MH]^+/\Sigma[DAG]^+)$ ratio is $\geq 5/9$, then $[M+H]^+$ is the base peak. In our example, the
 13 $([MH]^+/\Sigma[DAG]^+)$ ratio is 1/2, which is less than 5/9, so the $[M+H]^+$ is NOT the base peak in the
 14 example mass spectrum. Thus, the $([MH]^+/\Sigma[DAG]^+)$ ratio defines this as a Case 2, while the
 15 $[AA]^+/[AB]^+$ obeys Case 3. This is classified as a Case 2.3 TAG. Just knowing the
 16 classification of the Case is enough to know that the $[M+H]^+$ is not the base peak (Case 2) and
 17 that $[AB]^+$ is larger than $[AA]^+$ (Case *n.3*), and so $[AB]^+$ is the base peak. The exact values of all
 18 ions in the mass spectrum are given by the Case 2.3 solution in Scheme 1, Part II. The values
 19 that result from the application of the equations from Scheme 1, Part II for Case 2.3 are:

$$[AB]^+ = 100\% ; [AA]^+ = \frac{4}{5} \times 100 = 80\% ; [M + H]^+ = \left(\frac{1}{2}\right) \left(1 + \frac{4}{5}\right) \times 100$$

$$= \left(\frac{1}{2}\right) \left(\frac{9}{5}\right) \times 100 = \frac{9}{10} \times 100 = 90\%$$

20

1 Examination of these abundances shows that they would, indeed, give the two Critical Ratios of
2 1/2 and 4/5 used for the example, confirming that the abundances of every ion in the mass
3 spectrum, plus the sum of all ions (= 270 %) can be calculated using only the two Critical Ratios.

4 The Critical Values, Critical Limits, and the Cases of the solutions constitute what we
5 refer to as the 'Interpretation Matrix' used to interpret the meaning of the Critical Ratios. Once
6 the Case is known from the Interpretation Matrix, the base peak and the relative sizes of all peaks
7 are immediately known, and the specific percentage abundances can be calculated using the
8 equations given for that Case. The Interpretation Matrix also contains additional information,
9 such as indicating that an $[AC]^+ / ([AB]^+ + [BC]^+)$ ratio greater than Critical Limit 2 implies a
10 strong $[AC]^+$ preference.

11 For Type II TAG, once the Case has been specified as Case 1 or Case 2 for the base peak,
12 then the $[DAG]^+$ fragments follow either Case 3, where $[AA]^+ < [AB]^+$, or Case 4, where
13 $[AA]^+ \geq [AB]^+$. As Scheme 1, Part II shows, the equations used to calculate the % $[AA]^+$ and the
14 % $[AB]^+$ are the same for both the Case 1.3 and Case 1.4 solutions. In Case 2 solutions, either
15 $[AA]^+$ or $[AB]^+$ is the base peak, depending on the $[AA]^+ / [AB]^+$ ratio. In Case 2 solutions, the
16 $[M+H]^+$ abundance can only be calculated after the complete Case has been specified.

17 For an 'ABC' TAG, the Critical Value is calculated in a way similar to that for
18 'ABA/AAB' TAG. The Critical Value for an 'ABC' TAG is represented in the fourth panel
19 down in Figure 1. It can be demonstrated that the minimum possible value that the
20 $([MH]^+ / \Sigma[DAG]^+)$ ratio can have, and still have a $[M+H]^+$ base peak, is 1/3 ,or 0.33. If the
21 $([MH]^+ / \Sigma[DAG]^+)$ ratio is less than the Critical Value of 0.33, then the $[M+H]^+$ cannot be the
22 base peak, but if the $([MH]^+ / \Sigma[DAG]^+)$ ratio is greater than the Critical Value of 0.33, but is less
23 than 1.0, then the $[M+H]^+$ may be the base peak. If the $([MH]^+ / \Sigma[DAG]^+)$ ratio is greater than
24 0.33, then the $([MH]^+ / \Sigma[DAG]^+)$ ratio must be compared to Critical Limit 1 for this TAG.

1 *Critical Limit 1* is used to determine whether $[M+H]^+$ is the base peak, which for a Type III TAG
 2 is constructed as follows: if the ratio $[AC]^+ / ([AB]^+ + [BC]^+)$ is greater than 1, then $[AC]^+$ is larger
 3 than $[AB]^+$ and $[BC]^+$, and *Critical Limit 1* can be constructed based only on
 4 $[AC]^+ / ([AB]^+ + [BC]^+)$. Furthermore, if $[AC]^+ / ([AB]^+ + [BC]^+)$ is greater than 1, this indicates a
 5 Case *n.3.n* solution. For a Case *n.3.n* solution, the value of *Critical Limit 1* is given as: **Eq. 7**
 6
 7 On the other hand, if $[AC]^+ / ([AB]^+ + [BC]^+)$ is less than 1, then the $[AC]^+ / ([AB]^+ + [BC]^+)$ must be
 8 compared to its *Critical Limit*, *Critical Limit 2*, to see if $[AC]^+$ is larger than $[AB]^+$ and $[BC]^+$.
 9 The third panel down in Figure 1 shows Case 3.0, in which $[AC]^+$ is larger than both $[AB]^+$ and

$$\text{Critical Limit 1} = \frac{1}{1 + \frac{1}{\left(\frac{[AC]^+}{([AB]^+ + [BC]^+)} \right)}}$$

10 $[BC]^+$ (labeled as Σ_2 in this Figure), and Case 4.0 in which $[AC]^+$ is less than $[AB]^+$ or $[BC]^+$.
 11 $[AC]^+ / ([AB]^+ + [BC]^+)$ must be compared to *Critical Limit 2* to determine whether $[AC]^+$ is larger
 12 than both $[AB]^+$ and $[BC]^+$, and therefore whether $[AC]^+ / ([AB]^+ + [BC]^+)$ obeys a Case 3.0 or a
 13 Case 4.0 solution. *Critical Limit 2*, in turn, depends on whether $[AA]^+$ is greater than $[AB]^+$.
 14 *Critical Limit 2* is calculated as follows:

$$15 \text{ if } \left(\frac{[BC]^+}{[AB]^+} \right) < 1.0, \text{ then } \text{Critical Limit 2} = \frac{1}{1 + \frac{[BC]^+}{[AB]^+}}, \text{ Else } \text{Critical Limit 2} = \frac{1}{1 + \frac{1}{\left(\frac{[BC]^+}{[AB]^+} \right)}} \quad \text{Eq. 8}$$

18 When this *Critical Limit 2* is calculated, it is then used to calculate *Critical Limit 1*:

$$\text{if } \left(\frac{[AC]^+}{([AB]^+ + [BC]^+)} \right) < \text{Critical Limit 2}, \text{ then } \text{Critical Limit 1} = \frac{1}{1 + \left(\frac{[AC]^+}{([AB]^+ + [BC]^+)} \right)} \times \text{Critical Limit 2};$$

$$\text{Else } \text{Critical Limit 1} = \frac{1}{1 + \frac{1}{\left(\frac{[AC]^+}{([AB]^+ + [BC]^+)} \right)}}$$

1
2 *Critical Limit 1* is used to specify whether $[M+H]^+$ is the base peak or not, which determines
3 either Case 1.0 or Case 2.0. *Critical Limit 2* is used to specify whether $[AC]^+$ is larger than **Eq. 9**
4 $[AB]^+$ and $[BC]^+$ or not, which specifies either Case 3.0 or 4.0. The $[BC]^+/[AB]^+$ ratio alone (< 1
5 or ≥ 1) specifies either Case 5 or 6. Thus, the complete Case classification can be given by
6 comparison of the three Critical Ratios to *Critical Limit 1*, *Critical Limit 2*, and 1. Once the Case
7 has been classified, the complete set of equations necessary to calculate the abundance of every
8 ion is given in Scheme 1, Part III A or B.

9 Up to five specific values can be calculated from three Critical Ratios: 1. the abundance
10 of the protonated molecule, 2. the abundances of each of the three $[DAG]^+$ fragment ions, and 3.
11 the sum of all ions. Three critical ratios are required to specify the five specific values that
12 describe the primary ions in an APCI-MS mass spectrum of an 'ABC'-type TAG. Only two
13 critical ratios are required to fully describe the primary fragment ions in the mass spectrum of an
14 'ABA/AAB'-type TAG. Four specific values may be calculated for the mass spectrum of a Type
15 II TAG: 1. the abundance of the protonated molecule, 2. the abundances of each of the two
16 $[DAG]^+$, and 3. the sum of all ions. Only one single Critical Ratio is necessary to describe an
17 'AAA', or Type I TAG. That one ratio specifies that one peak is the base peak and the other is a
18 ratio to the first, and the sum of the ions is then given.

19 There is no easy way to demonstrate or reproduce the difficult process of arriving at all of
20 the above equations and producing at the final equations for the Cases. Each Case had to be
21 solved and tested individually. It is not practical to reproduce a complete proof of every
22 equation given above. The easiest way for a reader to become convinced of the truth of the
23 equations is by testing with actual values. Upon sufficient testing with numerous mass spectra,
24 the reader will become convinced that the mass spectrum of every possible TAG can be
25 reconstructed from only the Critical Ratios. Thus, the Critical Ratios constitute a 'reduced data

1 set' from which the mass spectrum of any TAG can be reproduced. The set of Critical Ratios
2 renders the original data obsolete and unnecessary, since all information can be obtained solely
3 from the ratios. Additionally, the Critical Ratios, when examined at face value, provide
4 information regarding trends in the structures (number of sites of unsaturation and acyl chain
5 positional preference) of the TAG.

6 The calculation of Critical Limits and the decision-making process to perform the Case
7 classification process are readily incorporated into an Excel spreadsheet. We have now included
8 a page in our data processing spreadsheet that automatically calculates the Critical Ratios and
9 Critical Limits, then automatically classifies the TAG according to case. Once the TAG is
10 classified by case, the spreadsheet then calculates the abundances of all fragments in the mass
11 spectrum, along with the sum of all ions. The results from these automated spreadsheet
12 calculations are demonstrated below. The Critical Limits calculated from the Critical Ratios (as
13 given in Table 1) are shown in Table 2. These were then programmed into the spreadsheet using
14 the conditional statement: 'if($x \geq C_L$, then y , else z)' and related commands to compare the
15 Critical Ratios, ' x ', to the Critical Limits. The Case for each TAG was determined based on the
16 comparison of the Critical Ratios to the Critical Values and Critical Limits. As mentioned
17 above, by specifying nothing more than the Case classification, the identity of the base peak and
18 the relative sizes of all peaks can be identified, and the equations used to calculate all ions are
19 then automatically specified. For example, all Case $1.n.n$ TAG in Table 2 have a protonated
20 molecule as the base peak, while all Case $2.n.n$ TAG in Table 2 have a $[DAG]^+$ base peak. All
21 Case $n.3.n$ Tag in Table 2 have an $[AC]^+$ peak larger than the $[AB]^+$ or $[BC]^+$ peak, whereas all
22 Case $n.4.n$ TAG have either the $[AB]^+$ or $[BC]^+$ peak larger than the $[AC]^+$ peak. All Case $n.n.5$
23 TAG in Table 2 have a $[BC]^+$ peak smaller than the $[AB]^+$ peak, whereas all Case $n.n.6$ TAG
24 have a $[AB]^+$ peak larger than the $[BC]^+$.

- 1 As shown above, the Critical Ratios can be used to classify the Case, which can then be
- 2 used to specify the equations necessary to calculate the abundance of every ion. Also, the
- 3 Critical Ratios can be used to make structural assignments.

Table 2. Calculated Critical Values and Critical Limits used with the Interpretation Matrix to calculate ion abundances from Critical Ratios determined by APCI-MS.

TAG	Critical Value	Critical Limit 1	Critical Limit 2	Case	
PPP	1.00			2	
SSS	1.00			2	
OOO	1.00			2	
LLL	1.00			1	
LnLnLn	1.00			1	
PPS/PSP	0.50	67.00		2	3
PPO/POP	0.50	60.50		2	3
PPL/PLP	0.50	54.31		2	3
PPLn/PLnP	0.50	58.85		2	3
SSP/SPS	0.50	70.97		2	3
SSO/SOS	0.50	57.10		2	3
SSL/SLS	0.50	54.68		2	3
SSLn/SLnS	0.50	62.12		2	3
OOP/POP	0.50	73.47		2	3
OOS/SOS	0.50	71.52		2	3
OOL/OLO	0.50	66.62		2	3
OOLn/OLnO	0.50	70.61		2	3
LLP/LPL	0.50	52.96		2	3
LLS/LSL	0.50	56.79		2	3
LLO/LOL	0.50	57.27		2	3
LLLn/LLnL	0.50	65.66		1	3
LnLnP/LnPLn	0.50	57.14		1	3
LnLnS/LnSLn	0.50	51.01		1	3
LnLnO/LnOLn	0.50	54.84		1	3
LnLnL/LnLLn	0.50	60.77		1	3
OPS	0.33	42.79	58.90	2	4 5
SPL	0.33	46.33	62.72	2	4 5
SPLn	0.33	40.49	56.59	2	4 5
LOP	0.33	35.93	50.44	2	4 5
LnOP	0.33	36.73	52.15	2	4 5
LnLP	0.33	42.33	57.99	1	4 5
LOS	0.33	37.43	53.85	2	4 5
OLnS	0.33	37.03	52.76	2	4 5
LnLS	0.33	40.18	55.84	1	4 5
LLnO	0.33	40.29	56.44	1	4 5

Table 3. Percent Relative Ion Abundances calculated from the Critical Ratios in Table 1, using the Interpretation Matrix in Scheme 1.

TAG	[MH]⁺	[AA]⁺ or [AC]⁺	[AB]⁺	[BC]⁺	ΣI %
PPP	0.00	100.00			100.00
SSS	0.00	100.00			100.00
OOO	3.24	100.00			103.24
LLL	100.00	82.71			182.71
LnLnLn	100.00	44.60			144.60
PPS/PSP	0.00	49.26	100.00		149.26
PPO/POP	0.91	65.28	100.00		166.19
PPL/PLP	0.74	84.11	100.00		184.85
PPLn/PLnP	21.79	69.94	100.00		191.72
SSP/SPS	0.00	40.91	100.00		140.91
SSO/SOS	0.78	75.14	100.00		175.92
SSL/SLS	0.76	82.89	100.00		183.65
SSLn/SLnS	15.46	60.98	100.00		176.44
OOP/POP	2.58	36.12	100.00		138.69
OOS/SOS	1.56	39.82	100.00		141.38
OOL/OLO	17.91	50.10	100.00		168.01
OOLn/OLnO	52.28	41.62	100.00		193.90
LLP/LPL	51.75	88.84	100.00		240.59
LLS/LSL	76.65	76.07	100.00		252.72
LLO/LOL	77.60	74.62	100.00		252.22
LLLn/LLnL	100.00	32.47	63.02		195.49
LnLnP/LnP Ln	100.00	41.79	55.72		197.51
LnLnS/LnSLn	100.00	78.92	82.17		261.10
LnLnO/LnOLn	100.00	42.56	51.67		194.23
LnLnL/LnLLn	100.00	30.24	46.83		177.06
OPS	1.09	63.92	100.00	69.79	234.80
SPL	0.99	56.43	100.00	59.43	216.86
SPLn	22.94	70.29	100.00	76.70	269.93
LOP	17.98	80.06	100.00	98.27	296.32
LnOP	74.52	80.48	100.00	91.76	346.76
LnLP	100.00	53.92	84.49	61.22	299.63
LOS	19.95	81.44	100.00	85.70	287.09
OLnS	53.76	80.50	100.00	89.53	323.79
LnLS	100.00	60.07	86.07	68.06	314.19
LLnO	100.00	48.80	68.72	53.05	270.57

1 For instance, in Chapter 7 (1), it was reported that an ‘ABC’ TAG produced the lowest
2 abundance from the 1,3-DAG. Thus, the identity of the $[AC]^+$ peak can be immediately
3 identified as the $[DAG]^+$ peak having the lowest abundance. Thus, an $[AC]^+ / ([AB]^+ + [BC]^+)$
4 ratio greater than one indicates either a strong preference for that ion (*e.g.*- if the *sn*-1,3 TAG
5 contains substantial unsaturation), or it may indicate mis-assignment of the $[AC]^+$ label. In this
6 way, the Critical Ratios themselves can guide one to the proper assignment. The TAG
7 assignments herein were made such that the $[AC]^+ / ([AB]^+ + [BC]^+)$ ratio in Table 1 was always
8 less than 1, and the $[AC]^+$ abundances in Table 3 are smaller than all $[BC]^+$ and $[AB]^+$ fragments.

9 There is, as yet, no unambiguous evidence to show that there is reproducible
10 discrimination between the $[AB]^+$ and the $[BC]^+$ positions in APCI-MS mass spectra. However,
11 as a matter of convention, it may be useful to label the $[BC]^+$ peak as the peak having a smaller
12 abundance than the $[AB]^+$ peak. It is important to note that, while the Critical Ratios may be
13 useful for structural assignments (*e.g.*- assigning positional isomers), the Equations for each Case
14 are accurate and yield the correct abundances of all ions even if they were not assigned correctly.

15 In a way analogous to proteomics, the sum of all possible species and structures of lipid
16 molecules may be referred to as the ‘Lipidome’, and the study of the lipidome is referred to as
17 ‘Lipidomics’ (7). The sum of all possible structures of triacylglycerols may be referred to as the
18 Triacylglycerol Lipidome. There is not a single TAG that cannot be represented by its Critical
19 Ratios, and from these Critical Ratios, all abundances of ions may be reproduced. Thus, Scheme
20 1 represents the complete boundary to the solution of the “Triacylglycerol Lipidome”. There is
21 no TAG that cannot be represented within this construct. In the future, further information may
22 be added to the Interpretation Matrix to allow a preference for $[BC]^+$ versus $[AB]^+$ to be further
23 described, or other information may be added to allow more information to be interpreted from
24 the Critical Ratios. But, while this may provide incremental additional information, the essential
25 construct of the Triacylglycerol Lipidome is complete in its entirety as given in Scheme 1, and

1 the mass spectrum of any TAG may be reproduced from its equations. The bottom-up solution
2 (BUS) constitutes the first full solution to the Triacylglycerol Lipidome.

3 This first solution to the TAG lipidome is referred to as the Bottom-Up Solution because
4 it was accomplished through arduous manual solutions to every possible case. Once the
5 complete solution was accomplished, trends within the equations were observed, and equations
6 were developed to describe these equations. Once the patterns within the equations were
7 recognized and the equations behind the equations were found, we were able to produce two
8 more mathematically equivalent solutions to the TAG lipidome. Since these other solutions were
9 arrived at by using the equations behind the equations, it may be said that these solutions were
10 developed using a “top-down” approach. These other solutions will be presented in due time.
11 One last note, the masses of the fragments have not been discussed, since they are constant and
12 are dictated by the identity of the TAG. For instance, it is automatically known that the TAG
13 ‘POL’ will produce a [PO]⁺ fragment having m/z 577.5, a [PL]⁺ fragment having m/z 575.5, an
14 [OL]⁺ fragment having m/z 601.5, and [POL+H]⁺ ion having m/z 856.8. These m/z values do not
15 change and so do not need to be reiterated. It is the abundances of the protonated molecule and
16 the [DAG]⁺ fragment ions that change, and which are affected by unsaturation and by positional
17 placement of the fatty acid on the glycerol backbone. It is these structural features that the
18 Critical Ratios elucidate. As demonstrated, these Critical Ratios can then be used to reconstruct
19 the appearance of the APCI-MS mass spectrum of any TAG.

20 **Conclusion**

21 The Bottom-Up Solution represents the first complete solution to the triacylglycerol lipidome.
22 Critical Ratios have been defined which, used by themselves, provide direct information
23 regarding the structural characteristics (*e.g.*-amount of unsaturation and positional isomer
24 distribution) of TAG. Furthermore, these Critical Ratios constitute a ‘reduced data set’ from

1 which the mass spectrum of any TAG can be reproduced in its entirety. This reduced data set
2 has potential advantages for storage of library spectra. Normally, the abundances of all four ions
3 in the mass spectrum of a TAG would be presented in tabular form to represent a mass spectrum.
4 Using the Bottom-Up Solution, only three pieces of data are required (the three Critical Ratios).
5 This represents a saving of 25 % (3 values needed instead of 4) for ABC TAG. For ABA/AAB
6 TAG, only two critical ratios are necessary to provide all of the information necessary to
7 reproduce the mass spectrum, compared to three abundance values that would be used to tabulate
8 the spectrum. This constitutes a data storage reduction by 33 % (2 values needed instead of 3)
9 for ABA/AAB TAG. Since only one critical ratio is necessary to completely describe the mass
10 spectral abundances of an AAA TAG, the BUS provides a 50 % saving in data storage space
11 required (1 value needed instead of 2). The benefit of reduced data storage requirements is in
12 addition to the benefits of being able to use the Critical Ratios directly to determine the
13 distribution of regioisomers and the degree of unsaturation.

14 In the past, articles often presented tabulated abundances of mass spectral ions (8,9),
15 which could later be used to calculate the ratio of $[AA]^+/[AB]^+$ abundances to allow the
16 positional isomer preference to be assessed (1). We have now demonstrated that it is possible to
17 list Critical Ratios that can be used directly to provide structural information (*e.g.*- assessing the
18 degree of unsaturation or assigning positional isomers), and which constitute a reduced data set
19 that uses fewer values, and therefore takes less space, than conventional tabulated mass spectra.
20 We have demonstrated that the mass spectrum of any TAG can be reproduced from the Critical
21 Ratios using the Bottom-Up Solution, which used Critical Values and Critical Limits in an
22 Interpretation Matrix to define the Case of a TAG, which then defined the exact equations used
23 to calculate the abundance of every ion in the mass spectrum.

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