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Identification of the geometrical isomers of α-linolenic acid using gas chromatography/mass spectrometry with a binary decision tree

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ABSTRACT

Gas chromatography, using a highly polar column, low energy (30 eV) electron ionization mass spectrometry and multivariate curve resolution, are combined to obtain the mass spectra of all eight geometrical isomers of α-linolenic acid. A step by step Student's *t*-test is performed on the *m*/*z* 50–294 to identify the *m*/*z* by which the geometries of the double bonds could be discriminated. The most intense peak discriminates between *cis* (*m*/*z* 79) and *trans* (*m*/*z* 95) at the central (carbon 12) position. The configuration at carbon 15 is then distinguished by *m*/*z* 68 and 236, and finally the geometry at carbon 9 is determined by *m*/*z* 93, 173, 191 and 236. A three-question binary tree is developed based on the normalized intensities of these ions by which the identity of any given isomer of α-linolenic is accurately determined. Application of Bayes theorem to data from independent samples shows that the complete configuration is determined correctly with a minimum probability of 87%.

1. Introduction

The double bonds in unsaturated fatty acids (FAs) can exhibit either "*cis*" or "*trans*" geometry. The former appears in the naturally occurring fats and the latter is mainly produced by heating and hydrogenation of vegetable and marine oils or by bacteria in the rumen of ruminants [1,2]. Trans FAs are associated with several health issues including cardiovascular disease [3,4], obesity [5] and inflammation [6,7]. This in turn implies the need for accurate discrimination between FAs with *cis* and *trans* double bonds. The separation and identification of polyunsaturated fatty acid (PUFA) isomers is a challenging problem [1,8,9]. For a fatty acid with n double bonds there are 2^n possible geometrical isomers. The extent of the overlap of chromatographic peaks of the isomers increases with n. We and others have shown that separation of ω-3 fatty acids is challenging even using long and highly polar gas chromatography columns [1,8]. FAs are usually separated, in the form of fatty acid methyl esters (FAMEs), by gas chromatography (GC) and are identified using mass spectrometry (MS). However the geometrical isomers are both difficult to separate, and then to identify by MS. As an example the 70 eV electron ionization (EI) MS spectra of α-linolenic acid (9,12,15-octadecatrienoic acid) methyl ester isomers differing in the geometry of the side double bonds are almost identical [1,8]. In a recent paper [1] we attributed this to the high energy of the ionizing electrons in the MS which break up the original geometry of the double bonds, thus losing the information required to distinguish among them. Consequently there is no diagnostic MS peak by which the double bond geometry could be identified. EI-MS using low energy electrons (30 eV) was proposed by us to overcome this problem [1]. Although the amount of information was increased, no single *m*/*z* was shown to be adequate for differentiation of all eight geometrical isomers of α-linolenic acid. Principal component analysis (PCA) was successfully applied to a range of *m*/*z* to discriminate among the isomers, and all the eight isomers were clustered into separate groups by the scores of three principal components. This implies that there is information in the peak heights of other *m*/*z* which are indicative of the geometry of the double bonds of α-linolenic acid methyl ester. However, to use PCA, standard mixtures of the isomers need to be run to construct the score space in which an unknown compound would be located. In the present paper we have considered relative ratios of particular peaks that can be used in an absolute test to discriminate among pairs of isomers of α-linolenic acid. Bayes theorem, the approach used here to assign probability density functions to the decision alternatives, has been applied to assignment of mass spectra since the earliest examples of computer-aided analysis. Karrer et al. in 1983 gave code for a Bayesian deconvolution of, and measurement of mole fractions of compounds from, mass spectra [10]. For complex systems such as peptides and proteins, Bayes theorem has been shown to be a powerful tool for identification from mass spectra [11], and LC–MS data [12]. For a brief review of Bayesian methods in mass spectrometry, see Section 6 in [13]. Decision tree

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Table 1
Composition of the mixture of α-linolenic acid methyl ester isomers (Supelco 47792) and the naming convention of each isomer.

Abbreviation	Compound	Approximate mass fraction (%) in solution
ccc	cis-9, cis-12, cis-15-octadecatrienoic acid methyl ester	3
cct	cis-9, cis-12, trans-15-octadecatrienoic acid methyl ester	7
ctc	cis-9, trans-12, cis-15-octadecatrienoic acid methyl ester	7
ctt	cis-9, trans-12, trans-15-octadecatrienoic acid methyl ester	15
tcc	trans-9, cis-12, cis-15-octadecatrienoic acid methyl ester	7
tct	trans-9, cis-12, trans-15-octadecatrienoic acid methyl ester	15
ttc	trans-9, trans-12, cis-15-octadecatrienoic acid methyl ester	15
ttt	trans-9, trans-12, trans-15-octadecatrienoic acid methyl ester	30

methods applied to mass spectrometric data are becoming popular in biological and medical research [14–16], with Bayesian methods also being applied [17].

1.1. Terminology

In describing the geometry of double bonds in different molecules we use the symbols *t* and *c* for *trans* and *cis* respectively and *x* for either, and the convention of writing the order of bonds from the carboxylic acid end of the FA. Thus *ctt* in relation to α-linolenic acid refers to *cis*-9, *trans*-12, *trans*-15-octadecatrienoic acid (Table 1).

Statistical probabilities are written Pr(A | B, C. . .) which is read as "the probability of the truth of A given the truth of B, C. . .". When A is data and B an hypothesis (e.g. geometry = *ccc*), Pr(A|B) also symbolises the likelihood function. Pr(A), where A is an hypothesis, symbolises the prior probability of A, and is the probability of the truth of A before any data is gathered.

1.2. Proposed approach

For all the isomers with a *cis* middle double bond (written *xcx*, where *x* is *c* or *t*) the base peak is at *m*/*z* 79 and for those with a *trans* middle double bond (*xtx*) the base peak is at *m*/*z* 95 [1]. This indicates that a rule based on the *m*/*z* of the most intense peak would enable the identification of the geometry of the double bond at this position along the FAME hydrocarbon chain.

Having determined the geometry of the middle bond, the next to be considered was the bond at carbon 15 (the right hand bond). The interest is to distinguish *xcc* from *xct*, and *xtc* from *xtt*. Assuming a Student's *t*-distribution, for each set of *m*/*z* data it is possible to conduct a series of *t*-tests on the intensity ratios (peak of interest/base peak) of the different geometries. The *m*/*z* ratio that has the smallest probability associated with the test (H_0 being there is no difference in ratios between isomers) for a particular geometry will be used to decide on the geometry to be assigned. Given estimates (from measurements) of the normal probability density functions for the discriminating ratios for each geometry, Bayes theorem can then be used to obtain the probability that the molecule is in one or other geometry [13,18] from the appropriate measured intensity ratios.

2. Experimental

A Supelco 47792 α-linolenic acid methyl ester (C18:3 ω3) isomer mixture containing all eight geometrical isomers was purchased from Sigma–Aldrich (Castle Hill, Australia). It contained a total FAME weight of 10 mg mL^{-1} in methylene chloride. The mixture composition and compound naming system are given in Table 1.

Three different dilutions of this mixture were prepared in hexane with dilution factors 10, 100 and 200 in 2 mL GC vials. This was done to investigate the robustness of the approach with concentration-dependent variations in the relative intensity of the ions in mass spectra of the same isomer. Data were obtained with a GCT (Micromass, Waters Co.) mass spectrometer equipped with an Agilent HP6890 GC. The GC was fitted with a highly polar column (BPX-70, 70% cyanopropyl polysilphenylene-siloxane, 60 m, 0.25 mm i.d., 0.25 μm film thickness from SGE, Ringwood, Australia). 1.0 μL of each sample was injected via a splitless injector at 240 °C at constant pressure of helium with column flow of 0.4 mL min^{-1} at an oven temperature of 140 °C. A starting oven temperature of 140 °C was used and was held for 2 min. The temperature was then increased by 2 °C min^{-1} to 180 °C where it was held for 30 min. The total run time was 50 min.

The electron ionisation (EI) mass spectra were acquired at 30 eV. The trap current was regulated at 250 μA and the ion source temperature was 180 °C. Ion detection and TOF measurement were facilitated by a time to digital converter (TDC) with a sampling rate of 3.6 GHz. With a 30 kHz pusher pulse, a full spectrum is generated every 33 μs. Data acquisition was performed for *m*/*z* 50–300 Da with 30,000 full spectra being accumulated per second yielding the continuum GC–MS spectra with a mass resolution of 7000 FWHM. Masslynx 4.0 software was used for data acquisition and manipulation.

2.1. Data analysis

As has been described previously [1], only six out of eight isomers were separated using the chromatographic method applied. The mass spectra of the separated isomers were recorded for different replicates of each concentration. To obtain the mass spectra of the two co-eluting isomers *ttc* and *cct*, multivariate curve resolution (MCR) was performed. The data set used for MCR was of size (34 × 245) which consisted of the retention time interval embracing the co-eluting isomers *ttc* and *cct* (36.85–37.41 min, increments of 0.017 min and *m*/*z* 50–294). Separate MCRs were performed on the data obtained for each dilution and each replicate of the standard mixture (Supelco 47792) described above. No preprocessing was done on the data before performing MCR. (The analysis was performed using Matlab R11. For details refer to [1].)

The mass spectra of all eight isomers at three different concentrations with two replicates in each were scaled individually so that the intensity of the base peak was 100. An exception was the isomer *ccc* which was studied only at two concentrations with two replicates in each. The concentration of *ccc* in the most dilute mixture was too close to its detection limit to provide useful data and was not used in the analysis. For each of the 46 spectra a total of 245 ion intensities (*m*/*z* 50–294) were recorded. A series of Student's *t*-tests were performed on each *m*/*z* to discover those *m*/*z* by which the geometry of the double bonds could be determined. The null hypothesis (H_0) was that the population mean intensities of a given *m*/*z* for molecules differing by the geometry of a particular bond are equal. As an example, for the geometry of the double bond on carbon 15, given a *cis* configuration at carbon 12, the *t*-parameter

calculated was,

$$t_{\text{expt}} = \frac{|\bar{R}_{236}(xcc) - \bar{R}_{236}(xct)|}{s_p\sqrt{(1/n_{xcc} + 1/n_{xct})}} \quad (1)$$

where $\bar{R}_{236}(xcc)$ is the measured mean ratio of m/z 236 to m/z 79 of the $n_{xcc} = 10$ measurements of the two isomers *ccc* and *tcc*, and $\bar{R}_{236}(xct)$ is the measured mean ratio for *cct* and *tct*. s_p is the pooled standard deviation. In this case $t_{\text{expt}} = 8.66$ and $P(T > t_{\text{expt}})$ (i.e. the probability of finding the experimental t or a greater value in a repeated experiment for which H_0 is true) $= 3 \times 10^{-8}$. The smaller the value of the probability is, the greater the discriminating power of the m/z.

3. Results and discussion

It is already known that there is a distinct difference between the mass spectra of the isomers with *cis* and *trans* middle double bonds (at carbon 12) and the difference is regardless of the geometry of the side double bonds (see Fig. 1 for representative 30 eV EI MS spectra for each of these two groups).

In this study there are eight different isomers and for each there are several samples (replicates at different concentrations). Therefore the ideal analysis method would seem to be a one-way ANOVA with the replicated peak heights of the eight isomers being the instances of the single factor "geometrical isomer". The outcome of such analysis would be a list of m/z whose normalized intensities differ significantly among at least two of the isomers. A least significant difference (LSD) [19] could then be used to identify those m/z by which all eight isomers could be distinguished. Unfortunately the degree of similarity among the geometrical isomers is high and by this procedure there is no single m/z that could be used to discriminate among all eight isomers.

3.1. Hierarchical tests

In considering the mass spectra within each group defined by the geometry of the double bond, carbon 15 was determined to be the next bond after carbon 12 that was most amenable to the proposed method. The peak ratio of m/z 236 over base peak (here m/z 79) can distinguish between *cis* and *trans* geometry of the double bond at carbon 15 given the geometry of the double bond at carbon 12 is *cis*; and the peak ratio of m/z 68 over the base peak (here m/z 95) can distinguish between *cis* and *trans* geometry of the double bond at carbon 15 given the geometry of the double bond at carbon 12 is *trans*. Once the geometry of the double bonds at carbons 12 and 15 had been determined m/z 236 could be used to separate *ccc* and *tcc*, m/z 93 to separate *tct* and *cct*, m/z 173 to separate *ttc* and *ctc*, and m/z 191 to separate *ctt* and *ttt*. An example of the significant difference between the values of an m/z ratio for two different isomers is shown in Fig. 2 for six samples (two replicates of three different concentrations) of *cct* and *tct* for m/z 93.

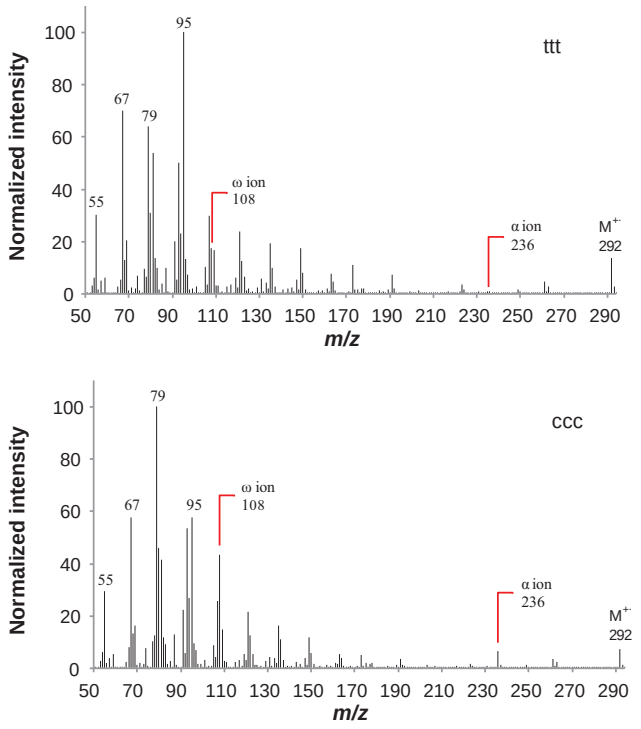

Fig. 1. 30 eV EI mass spectra of *ttt* (top) and *ccc* (bottom), two of the geometrical isomers of α-linolenic acid methyl ester.

The results of the Student-t tests are given in Tables 2–4. As H_0 is that there is no difference between the peak intensities of the two isomers, the smaller the probability the greater the significance of the difference between the peak intensities.

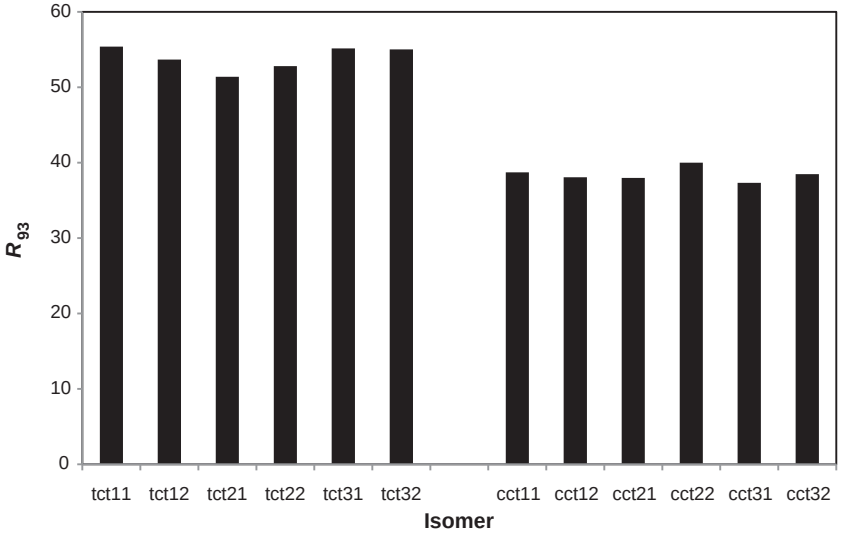

Fig. 2. Plot of the peak ratios I_{93}/I_{79} in the mass spectra of *tct* and *cct* obtained from the duplicate analysis of solutions with three different concentrations.

Table 2
Top five diagnostic m/z for the double bond at carbon 12 (X). The probability $\Pr(T > t_{expt}|H_0)$ is for the observed t value (Eq. (1)) given the null hypothesis that the population mean ratios for the alternative geometries are the same.

xXx	
m/z	$\Pr(T > t_{expt}\|H_0)$
79	0[a]
95	0
96	0
108	0
292	1.11E−16

[a] The probability is zero to the limit of the spreadsheet calculation.

Table 3
Top five diagnostic m/z for the double bond at carbon 15 (X), given the geometry of the double bond at carbon 12. The probability $\Pr(T > t_{expt}|H_0)$ is for the observed t value (Eq. (1)) given the null hypothesis that the population mean ratios for the alternative geometries are the same.

xcX		*xtX*	
m/z	$\Pr(T > t_{expt}\|H_0)$	m/z	$\Pr(T > t_{expt}\|H_0)$
236	3.35E−08	68	8.99E−09
68	5.01E−07	121	1.70E−08
99	9.12E−05	81	4.79E−07
237	1.50E−04	164	1.24E−06
59	1.50E−04	149	1.82E−06

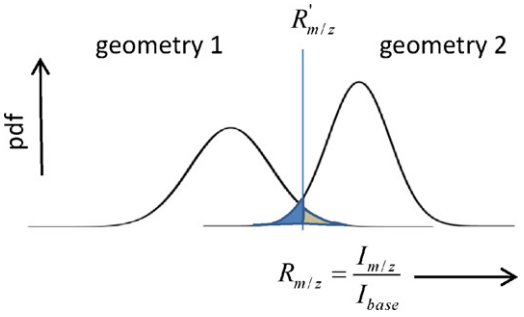

Fig. 3. Schematic of t-distributions corresponding to the variation of a diagnostic peak ratio for two geometries. The line at $R'_{m/z}$ corresponds to the ratio for which the likelihoods for each geometry are equal.

To provide useful rules that can be applied to any mass spectrum, for a discriminating m/z we chose the value of the ratio that exactly divides the two distributions (see Fig. 3).

The dividing ratio ($R'_{m/z}$) for which the likelihood functions are the same for each binary comparison is calculated for a single measurement. Thus the t value for a given geometry at the dividing ratio is:

$$t_{m/z}(\text{geom}) = \frac{|\bar{R}_{m/z}(\text{geom}) - R'_{m/z}|}{s} \quad (2)$$

s is the sample standard deviation and $\bar{R}_{m/z}(\text{geom})$ is the mean of the ratios for the particular geometry. $R'_{m/z}$ is chosen so that,

$$\Pr(T > t_{m/z}(\text{geom1})|H_0) = \Pr(T > t_{m/z}(\text{geom2})|H_0) \quad (3)$$

with due regard to the degrees of freedom. Table 5 gives the values of the ratios and the corresponding likelihood values for the geometry at carbon 15 and then at carbon 9.

When the ratio moves towards one side or the other, the probability for the favoured geometry rapidly increases. Fig. 4 shows the comparison scheme with dividing ratios and likelihoods.

To summarise, this approach provides step by step rules, based on the comparison of the intensities (normalized to the corresponding base peak) of three selected m/z, to determine the geometry of all double bonds and so the exact identity of an unknown isomer of α-linolenic acid methyl ester using 30 eV EI MS data.

3.2. Application of Bayes theorem

To obtain a decision on the complete geometry, two probabilities are involved once the middle bond is assigned. The following analysis gives the example of identifying a geometry as *cct*. The base peak for this compound is m/z 79 which implies the middle bond is *cis*, i.e. *xcx*. The Student-t tests gave m/z 236 as the best discriminator between *xcc* and *xct*, and then m/z 93 to discriminate between *tct* and *cct*. For the measured ratios $R_{236} = I_{236}/I_{79}$ and $R_{93} = I_{93}/I_{79}$ we can formulate the required probability as $\Pr(cct|R_{93}, R_{236})$ which reads "the probability of the geometry being *cct* given the value of the ratios R_{93}, and R_{236}". The decision *xct* is made on the value of the ratio R_{236} with probability $\Pr(xct|R_{236})$. Therefore,

$$\Pr(cct|R_{93}, R_{236}) = \Pr(cct|R_{93}, xct) \times \Pr(xct|R_{236}) \quad (4)$$

for each position there are two, mutually exclusive, geometries (*cis* or *trans*) and so, if the central position is decided, the discrete form of Bayes theorem gives:

$$\Pr(cct|R_{93}, R_{236}) = \frac{\Pr(R_{93}|cct)\Pr(cct)}{\Pr(R_{93}|cct)\Pr(cct) + \Pr(R_{93}|tct)\Pr(tct)} \times \frac{\Pr(R_{236}|xct)\Pr(xct)}{\Pr(R_{236}|xct)\Pr(xct) + \Pr(R_{236}|xcc)\Pr(xcc)} \quad (5)$$

$\Pr(R_{93}|cct)$ is known as the likelihood of R_{93} given that the geometry is *cct*. For normal distributions of ratios this likelihood is the one-tailed Student's-t distribution for $t = |R_{93} - \bar{R}_{93}|/s$, where $\bar{R}_{93}$ is the mean intensity ratio of n independent measurements on samples with the known geometry *cct*, and s is the sample standard deviation with $n-1$ degrees of freedom. In Bayesian analysis it is important to appreciate the difference between the probabilities of an hypothesis (e.g. geometry = *cct*) given the data (values of R), and the probability of finding the data given the prior knowledge of the geometry (the likelihood). All the likelihoods are known from calculations on the mass spectra recorded from samples of known geometry. $\Pr(cct)$ is the prior probability of the geometry

Table 4
Top five diagnostic m/z for the double bond at carbon 9 (X), given the geometry of the double bonds at carbons 12 and 15. The probability $\Pr(T > t_{expt}|H_0)$ is for the observed t value (Eq. (1)) given the null hypothesis that the population mean ratios for the alternative geometries are the same.

Xcc		*Xct*		*Xtc*		*Xtt*	
m/z	$\Pr(T > t_{expt}\|H_0)$	m/z	$\Pr(T > t_{expt}\|H_0)$	m/z	$\Pr(T > t_{expt}\|H_0)$	m/z	$\Pr(T > t_{expt}\|H_0)$
236	6.62E−04	93	1.46E−09	173	8.18E−07	191	2.64E−07
57	8.92E−04	67	1.55E−07	108	7.39E−06	223	1.27E−06
93	2.91E−03	81	1.66E−07	174	2.25E−04	173	2.51E−05
173	3.23E−02	107	5.91E−07	131	2.95E−04	174	2.58E−04
223	3.59E−02	173	8.48E−07	57	3.13E−04	172	4.32E−04

Table 5
Dividing ratios and associated likelihood values for comparisons between geometries.

Comparison (1/2)	*m/z*	$\bar{R}_{m/z}(1) < R'_{m/z} < \bar{R}_{m/z}(2)$	$\Pr(\bar{R}_{m/z}(1) < R'_{m/z}\|H_0) = \Pr(\bar{R}_{m/z}(2) > R'_{m/z}\|H_0)$
xct/*xcc*	236	0.038 < 0.048 < 0.070	0.045
xtt/*xtc*	68	0.113 < 0.134 < 0.187	0.030
ccc/*tcc*	236	0.058 < 0.067 < 0.078	0.078
cct/*tct*	93	0.384 < 0.441 < 0.539	0.001
ctc/*ttc*	173	0.096 < 0.119 < 0.144	0.013
ctt/*ttt*	191	0.043 < 0.056 < 0.068	0.009

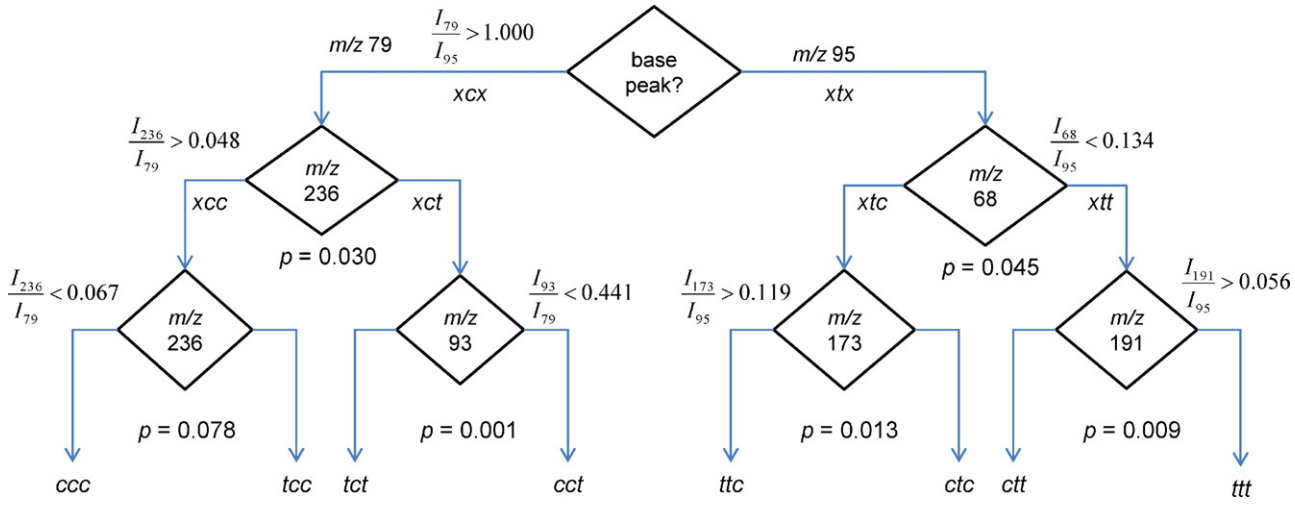

Fig. 4. A diagram of the identification steps of an unknown isomer of α-linolenic acid methyl ester using a series of *m/z*, each being diagnostic for one of the three double bonds. The ratio inequalities show the dividing ratios for the particular pairs of geometries. Likelihood values of H_0 (no discrimination) are given for each comparison.

cct, and answers the question for an unknown test sample is there any prior knowledge about the sample that would favour any particular geometry? When the method is used for real samples, the knowledge that the *cis* geometry is often favoured over the *trans* geometry may be incorporated here. For the analysis used in this paper, we adopt a 'flat prior', that is the prior probability of each geometry is the same (and therefore equal to 1/8 for a sample that could be any of the possible eight isomers). In this case, in Eq. (5), the equal prior probabilities all cancel and the equation for the probability of a particular geometry has only likelihoods for which the values are known.

The rules provided in Fig. 4 were applied to several test samples with different concentrations of known α-linolenic acid isomers. In all of the cases a correct assignment was made. Table 6 shows the

Table 6
Results of assignments of FAME samples containing α-linolenic acid methyl ester from single GC–MS analyses. Underlined ratios are greater than the threshold. Ratios not underlined are less than the threshold. Probabilities of the assignment are the product of the probabilities of the two outer positions once the central geometry is decided.

	Known geom.	$\frac{I_{79}}{I_{95}}$	$\frac{I_{236}}{I_{79}}$	$\frac{I_{68}}{I_{79}}$	$\frac{I_{236}}{I_{79}}$	$\frac{I_{93}}{I_{79}}$	$\frac{I_{191}}{I_{95}}$	$\frac{I_{173}}{I_{95}}$	Assigned geom.	Pr(geom.|data)
Assigned geom. if ratio < $R'_{m/z}$		*XtX*	*Xct*	*Xtt*	*ccc*	*cct*	*ctt*	*ctc*		
Threshold ratio $R'_{m/z}$		1.000	0.048	0.134	0.067	0.441	0.056	0.119		
Assigned geom. if ratio > $R'_{m/z}$		*XcX*	*Xcc*	*Xtc*	*tcc*	tct	*ttt*	*ttc*		
Sample (dilution)										
Canola oil (1000:1)	*ccc*	1.789	0.059		0.059				*ccc*	97.8%
Canola oil (1000:1)	*ccc*	1.707	0.060		0.060				*ccc*	97.3%
FAME standard (10:1)	*ccc*	1.779	0.059		0.059				*ccc*	97.8%
FAME standard (10:1)	*ccc*	1.748	0.060		0.060				*ccc*	97.5%
Linolenic standard (200:1)	*ttc*	0.455		0.170				0.128	*ttc*	98.2%
Linolenic standard (400:1)	*ttt*	0.595		0.109			0.059		*ttt*	94.2%
Linolenic standard (400:1)	*ctt*	0.578		0.084			0.041		*ctt*	88.3%
Linolenic standard (400:1)	*tct*	1.396	0.032			0.550			*tct*	98.3%
Linolenic standard (400:1)	*ctc*	0.902		0.143				0.107	*ctc*	87.1%
Linolenic standard (400:1)	*ccc*	1.648	0.056		0.0562				*ccc*	96.1%
Linolenic standard (400:1)	*tcc*	1.500	0.077		0.0769				*tcc*	99.7%
Linolenic standard (200:1)	*cct*	3.433	0.030			0.373			*cct*	98.0%
Linolenic standard (100:1)	*ttc*	0.685		0.197				0.144	*ttc*	100.0%
Linolenic standard (100:1)	*cct*	2.285	0.038			0.400			*cct*	98.2%

peak ratios and assignments with calculated posterior probabilities by Eq. (5).

It is worth noting that this paper only addresses the difficulty associated with the identification of geometrical isomers, which is the hardest challenge in fatty acid analysis. Differentiation of positional isomers, which is much easier, has been described elsewhere and is not the focus of the present research.

4. Conclusion

Electron ionization mass spectrometry is a popular technique for identification of fatty acids. However, it is believed that in most of the cases there is no ion in the mass spectrum by which one could identify *cis* from *trans* isomers. This paper presents a hierarchical set of comparisons of peak height ratios of low energy electron ionization mass spectrometry data to discover ions which are diagnostic for the geometry of the double bonds of α-linolenic acid. It allows accurate identification of an unknown isomer of α-linolenic acid methyl ester.

The assignment of different diagnostic m/z to each of the three double bonds is also of interest which suggests the approach could be used in determining the fragmentation mechanism of PUFAs and in general polyenes, which are known to have complex fragmentation patterns [20].

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