Journal of Chromatography A, 1217 (2010) 5986–5994

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

The vector of calibration ratios: A simple transfer method for mass spectra

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ARTICLE INFO

Article history:
Received 22 April 2010
Received in revised form 6 July 2010
Accepted 14 July 2010
Available online 22 July 2010

Keywords:
Calibration ratios
Mass spectral differences
Fatty acid methyl esters
Gas chromatography–mass spectrometry
Standardization

ABSTRACT

A robust method for reduction of instrument differences, the vector of calibration ratios, was developed to eliminate differences in unit resolution mass spectra of fatty acid methyl esters (FAME) caused by experimental conditions. Mass spectra of FAMEs were analyzed by two different mass spectrometers and after application of different tune procedures. The proposed method could remove 51–95% of the systematic difference in spectra caused by instrumental conditions. Highly similar spectra, which were incorrectly identified because of the contribution from experimental conditions, could be correctly identified after application of the calibration vector. The proposed method is simple, easy to implement and shows robustness when applied on spectra that is outside the range spanned by the calibration sample.

1. Introduction

Gas chromatography–mass spectrometry is among the most versatile and widely applied technology platforms in modern metabolomics, crude oil analysis, environmental monitoring and medicine [1–6]. A major challenge in the processing of mass spectral data is the identification of components in complex samples [7]. In addition to the use of retention times and retention indices [8,9], compounds are identified by mass spectral library searching, utilizing various methods such as the dot product function and probability based matching [10–14]. A much overlooked problem in this respect is the effects that different instrument conditions may have on the mass spectra.

Fatty acids are widely analyzed by gas chromatography–mass spectrometry (GC–MS) and are routinely identified by comparing retention times with those of the authentic fatty acid methyl ester standards, the use of retention indices, or by similarity search in mass spectral libraries [15–18]. With electron impact ionization, FAMEs and other fatty acid derivatives fragment extensively, giving rise to a 'fingerprint area' with abundant ions in the m/z region from approximately 40 to 110. This fingerprint area is usually regarded as unsuitable for compound identification by visual interpretation, except for the identification of the main classes of fatty acids [19]. However, in most cases, the spectra are still different enough to be distinguished by mass spectral search algorithms. Multivariate regression and classification methods have also been applied to extract information about the number, position, and geometry of double bonds [20–24].

Mass spectra are not only a result of the compound structure, but the relative abundances of the different ions are also highly dependent on acquisition conditions, such as ionization energy and the geometry of the mass spectrometer. These may lead to significant differences between mass spectra acquired on different instruments, and also between spectra acquired under different conditions on the same instrument. Since correct identification of fatty acids may depend on rather small differences in the relative abundances of ions, these instrumental effects may contribute to errors in the identification of FAMEs. In cases where spectra acquired at different conditions are compared, it may therefore be advantageous to reduce the effect of the instrument differences by transforming spectra from one of the conditions to better match spectra acquired under the other condition. In the field of calibration this is commonly referred to as standardization of instruments [25]. Standardization and calibration transfers have been applied to correct for instrument differences in near infrared spectroscopy [25–27], nuclear magnetic resonance spectroscopy [28,29] pyrolysis mass spectra [30,31] and gas sensor arrays [32]. Because of different noise patterns, standardization and calibration methods are transferable only to a limited degree between these techniques.

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doi:10.1016/j.chroma.2010.07.039

Table 1
Average Euclidean distance between target (Condition A) and original and standardized mass spectra of Conditions B and C for the calibration set. The number of replicates of each compound is 9. Internal variation is the average Euclidean distance of the original spectra acquired at Condition B or C to the average spectrum of the same groups.

FAME	Condition B	Tuning difference		Condition C	Instrument difference	
	Original	Standardized	Internal variation	Original	Standardized	Internal variation
10:0	4.58	1.32	0.13	14.75	4.27	0.61
12:0	5.29	1.61	0.16	16.05	3.83	0.53
14:0	5.51	1.50	0.19	16.94	3.41	0.60
14:1 *n*–5	19.13	3.57	0.54	32.91	8.11	1.27
15:0	5.55	1.39	0.15	17.03	3.16	0.53
16:0	5.63	1.34	0.16	17.11	2.74	0.62
16:1 *n*–7	22.93	3.25	0.51	43.21	8.97	1.48
17:0	5.70	1.27	0.17	17.41	2.58	0.55
17:1 *n*–7	21.22	2.58	0.48	42.39	7.03	1.02
18:0	5.75	1.19	0.18	17.32	2.28	0.64
18:1 *n*–9	23.40	2.54	0.51	49.17	6.49	1.25
18:2 *n*–6	10.51	2.02	0.53	36.15	3.23	1.36
18:3 *n*–6	8.31	1.33	0.62	31.79	3.72	1.47
18:3 *n*–3	7.03	1.47	0.61	26.24	3.83	1.30
20:0	5.74	1.00	0.20	17.71	2.14	0.58
20:1 *n*–9	20.65	1.42	0.44	49.22	4.38	1.09
20:2 *n*–6	10.64	2.26	0.55	41.46	3.97	1.29
20:3 *n*–6	9.41	1.54	0.74	34.85	3.62	1.60
20:4 *n*–6	6.40	2.08	0.58	28.98	4.46	1.70
20:3 *n*–3	7.63	2.00	0.80	28.88	5.23	1.36
22:0	5.93	1.18	0.21	18.25	2.56	0.69
22:1 *n*–9	19.51	1.59	0.46	50.91	5.33	1.22
20:5 *n*–3	5.23	1.86	0.62	24.21	5.77	1.08
22:2 *n*–6	10.42	2.95	0.57	45.82	6.71	1.52
22:4 *n*–6	7.06	1.53	0.61	29.85	3.70	1.46
24:0	6.02	1.46	0.25	18.74	3.75	0.70
24:1 *n*–9	18.43	2.73	0.63	53.05	7.15	1.26
22:5 *n*–3	5.82	1.37	0.47	24.71	4.56	1.32
22:6 *n*–3	4.45	1.39	0.46	23.28	5.89	1.32

In this study, mass spectra acquired on different instruments and with different tune procedures were compared and analyzed. After comparison, a standardization method was developed and employed to reduce the systematic spectral differences caused by instrument effects. Examples related to the use of this transfer method are presented.

2. Experimental

2.1. Samples

Different mixtures of fatty acid methyl esters (FAME) were applied in this study. The calibration sample was GLC-461 from Nu-Chek Prep (Elysian, MN, USA), which contains 29 common saturated and unsaturated FAMEs from C10 to C24. The composition of the mixture is given in Table 1. The calibration sample was analyzed by GC–MS at three different levels, corresponding to approximately 6, 11 and 16 ng of each fatty acid on the GC-column.

Compounds in the test sets where from the following sources: Methyl esters of branched fatty acids (i-15:0, ai-15:0, i-16:0), hydroxy fatty acids (2-hydroxy-decanoate, '10:0-2OH', 2-hydroxy-dodecanoate, '12:0-2OH'), cyclopropane fatty acid (*cis*-9,10-methyleneoctadecanoate, '9,10-cyclo-19:0′) and two odd-numbered saturated fatty acids (11:0, 13:0) were from the bacterial fatty acid methyl ester mixture 47080-U (Sigma–Aldrich). *Trans* isomers of tri- and tetra-unsaturated fatty acids were prepared by isomerization and isolation by silver ion chromatography as explained in [20]. All other FAMEs in the test sets were purchased in pure form (>99%) from Nu-Chek Prep and dissolved in HPLC grade isooctane.

2.2. GC–MS analysis

Two different GC–MS instruments were applied in this study. Instrument 1 was an Agilent 5975 mass spectrometer equipped with an Agilent 6890 gas chromatograph and version D.03.00.552 of the Chemstation software for GC–MS (Agilent). Two different autotune procedures in the software were applied with this instrument: 'Standard spectra autotune' and 'Autotune'. These are referred to as 'Condition A' and 'Condition B', respectively. Instrument two was an HP 5972 mass spectrometer equipped with an HP 5890 gas chromatograph and version C.03.00 of the Chemstation software for GC–MS (Agilent). The instrument was tuned using the 'Autotune' procedure in the software. This is referred to as 'Condition C'. The instruments have single quadrupole mass filters and the ionization energy is approximately 70 eV in electron impact mode.

Both instruments were applied with split/splitless injector and a BPX-70 capillary column (L = 60 m, i.d. = 0.25 mm, d_f = 0.25 μm). One microliter was injected splitless and the following temperature program was applied: isothermal at 60 °C for 4 min, thereafter 30 °C/min to 160 °C followed by a gradient of 2 °C/min to 265 °C. Helium was used as carrier gas with a constant average velocity of 26 cm/s. Electron impact ionization was applied for both instruments, and the mass spectrometers scanned from m/z 50 to m/z 109 with a scan rate of 2.8 scans/s for the 5975 instrument and 1.9 scans/s for the 5972 instrument.

Chromatographic peak widths (defined as 4σ) were approximately 6.1 s (measured for 18:3 *n*–3); this gave 17 and 12 scans per peak for the 5975 and 5972 instrument, respectively. All compounds were chromatographically resolved. After baseline subtraction, the spectra were extracted as the sum of ion intensities where the total ion current was above 1% of peak maximum (from approximately -3σ to $+3\sigma$).

2.3. Datasets

All FAME mixtures were analyzed in triplicates. The analytical sequence was designed so that any instrument drift would equally affect calibration and test set compounds. Baseline removal, peak integration and export of spectra were performed in an in-house

program, 'Q (12-09)', running under Matlab (The Mathworks, Natick, MA).

The calibration set used in this study consisted of the 29 FAMEs in the GLC-461 mixture with chain lengths of 10 or more carbons. There are a total of nine mass spectra of each FAME in the calibration set, which are three replicates of the GLC-461 mixture analyzed at the three different concentrations given in Section 2.1.

The test set consisted of FAMEs that are structurally different from the compounds in the calibration set, *i.e.* different in chain lengths or functional groups, or positional or geometrical isomers of compounds in the calibration set. In the case of geometrical isomers, the compounds in the test set are restricted to polyunsaturated fatty acids with *trans* geometry in the central double bonds that have been shown to be dominated by other fragmentation mechanisms than the corresponding all-*cis* isomers in the calibration set [20,21]. In addition, a second test set containing only geometrical isomers of di-unsaturated and tri-unsaturated FAMEs was used for comparison of highly similar mass spectra. The close similarity of these spectra has been shown previously [20]. There are three replicates of each FAME in the test sets.

2.4. Calculations and software

2.4.1. The vector of calibration ratios

In the proposed method we assume that all spectra can be standardized by elementwise division by a single vector of calibration ratios with the same number of entries as the mass spectra, *i.e.* there is a single correction factor for each mass. The calculation of the vector is explained below and application is described in Section 2.4.2.

The applied standardization vector is based on the average of ratios between the mass spectra. However, precautions must be taken to avoid influence of weak signals that contain relatively large proportions of noise. The spectra must also be normalized to the same ion before the vectors are calculated. This means that there must be at least one ion that is abundant in all calibration spectra. If there are several ions that fulfill this requirement, the best choice may be the ion that has the highest minimum abundance in all the calibration spectra. In this case, this was m/z 55 that was above 18% of the base peak in all spectra. The influence of weak signals was avoided by setting a threshold value and deleting ions with lower abundance from the calculations.

In the following description there are a *target* condition and also a *test* condition, and the purpose is to fit spectra acquired at the test condition to spectra acquired at the target condition. In this study, the target is always acquired at the conditions described as 'Condition A' in Section 2.2. A simplified algorithm for calculating the calibration vector is described below:

1. Normalize all spectra to m/z 55 (=100%)
2. For each compound, k, calculate the average spectrum, $\boldsymbol{s}$, acquired at the test condition and the target condition, respectively:

$$\boldsymbol{s}_{\text{test}(k)} = \frac{1}{r} \cdot \sum_{j=1}^{r} \boldsymbol{s}_{\text{test}(i,j)} \quad \text{and} \quad \boldsymbol{s}_{\text{target}(k)} = \frac{1}{r} \cdot \sum_{j=1}^{r} \boldsymbol{s}_{\text{target}(i,j)} \tag{1}$$

   where $s_{(i,j)}$ means the relative abundance of mass number i in the jth replicate spectrum, and r is the number of replicates of each compound (nine in this case).
3. For each compound, k, calculate the standardization vector, $\boldsymbol{v}$, by dividing the abundance of each mass in $\boldsymbol{s}_{\text{test}}$ by $\boldsymbol{s}_{\text{target}}$:

$$\boldsymbol{v}_{(i,k)} = \frac{\boldsymbol{s}_{\text{test}(i,k)}}{\boldsymbol{s}_{\text{target}(i,k)}} \tag{2}$$

   where $\boldsymbol{v}_{(i,k)}$ represents calibration ratio for mass i for the kth compound. Masses with abundance in $\boldsymbol{s}_{\text{test}}$ below the specified threshold level are excluded from the calculation.
4. Calculate the final standardization vector ($\boldsymbol{v}_{\text{final}}$) by taking the average of the standardization vectors of all m compounds. Missing values in the final standardization vector (where the masses were below the specified threshold in all spectra) are set to 1.

$$\boldsymbol{v}_{\text{final}(i)} = \frac{1}{m} \cdot \sum_{k=1}^{m} \boldsymbol{v}_{(i,k)} \tag{3}$$

   where $\boldsymbol{v}_{\text{final}(i)}$ signifies the general calibration ratio for mass i.

2.4.2. Application of the calibration vector

To correct any spectrum acquired at the test conditions to fit the target conditions, corrected spectra, say $\boldsymbol{s}_{\text{corrected}}$, are calculated by elementwise division of the test spectra, say $\boldsymbol{s}_{\text{test}}$, by the standardization vector, $\boldsymbol{v}_{\text{final}}$:

$$\boldsymbol{s}_{\text{corrected}(i)} = \frac{\boldsymbol{s}_{\text{test}(i)}}{\boldsymbol{v}_{\text{final}(i)}} \tag{4}$$

It should be emphasized that the normalization of $\boldsymbol{s}_{\text{test}}$ does not affect the accuracy of the standardization. However, the standardization in Eq. (4) changes the abundance of the base peak to be different from 100 (unless the standardization factor for the base peak is 1) and it also influences the total sum of ions in a spectrum. Depending on the method used for spectral comparisons, it may therefore be necessary to re-normalize the spectra after standardization. In this work Eq. (4) was applied on spectra that was normalized to base peak (*i.e.* most abundant ion = 100%) and the spectra were re-normalized since the Euclidean distance (see Section 2.4.3) used for spectral comparisons is sensitive to the absolute size of the objects.

2.4.3. Metrics for comparison of spectra

In library search algorithms, scale invariant methods like the normalized dot-product and the correlation coefficient are usually preferred for measuring spectral similarity. Because standardization of mass spectrometers may have a broader range of applications than just pretreatment of spectra prior to library matching, we have chosen to use a metrics that is not scale invariant for comparison of spectra. The Euclidean distance (ED) was therefore used for comparison of spectra. In this case the ED is the Euclidean norm of the difference spectrum of the two spectra that are compared, and is calculated according to Eq. (5)

$$\text{ED}_{(p,q)} = \sqrt{(\boldsymbol{p}-\boldsymbol{q})^T \cdot (\boldsymbol{p}-\boldsymbol{q})} = \sqrt{\sum_{i=1}^{n} (p_i - q_i)^2} \tag{5}$$

The vectors $\boldsymbol{p}$ and $\boldsymbol{q}$ of length n contain the two spectra, p_i and q_i are abundances of the individual masses, i is the individual mass numbers and n is the total number of masses in each spectrum.

2.4.4. Software

Data handling was performed on a Pentium 4 personal computer. All programs were coded in Matlab 6.5 for Windows (The Mathworks, Natick, MA).

3. Results and discussion

3.1. Spectral differences caused by different tunes and instruments

A principal component analysis (PCA) scores plot of the spectra of a single FAME (18:3 n–3) is shown in Fig. 1. The plot

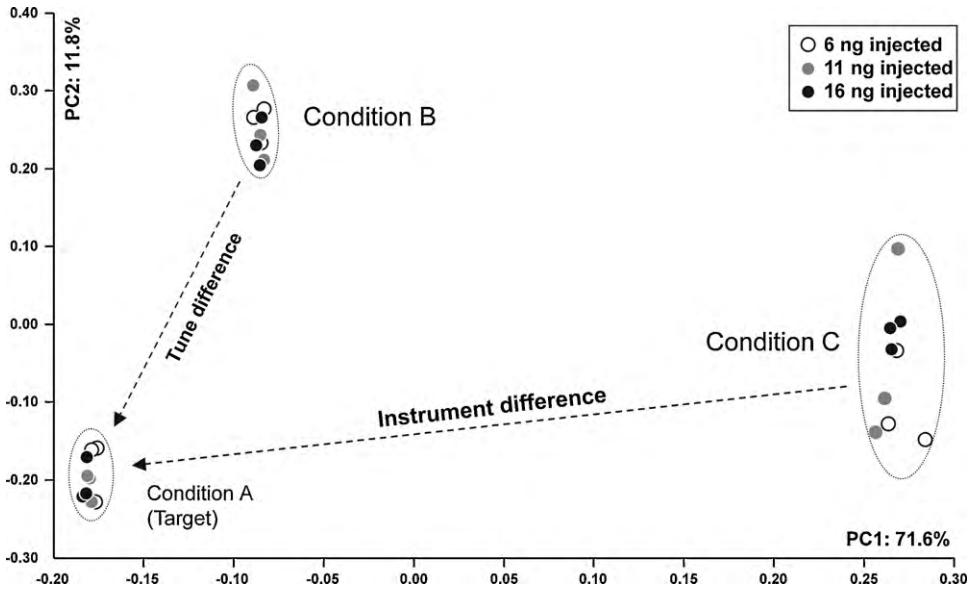

Fig. 1. Principal component score plot of three sets of base peak normalized mass spectra of 18:3 *n*–3 at Conditions A, B and C.

shows that the systematic differences between the three acquisition conditions are much larger than the random variation within each class. It can also be seen that the amounts applied on the column (6, 11 or 16 ng) seem to have no effect. All nine analyses acquired at the same conditions will therefore be treated as replicates. The difference between Condition C and Condition A (different instruments) is explained basically by the first principal component (PC1), which explains 72% of the variance, while the difference between Conditions B and A is found basically along the second principal component (PC2), which explains 12% of the variance. This means that the effect of using different instruments is much larger than the effect of different tuning on the same instrument.

The spectra are normalized to the base peak, which is *m*/*z* 79 in all spectra of this compound. The magnitudes of differences on other abundant ions are shown in Fig. 2. A clear difference between Conditions A and C can be seen for all ions, but Conditions A and B differ basically in the abundance of *m*/*z* 55.

Based on visual inspection, the mass spectra of FAME can be divided into four sub-classes according to the number of double bonds: saturated, monounsaturated, di-unsaturated and polyunsaturated, with base peaks of 74, 55, 67 and 79, respectively. The degree of fragmentation (*i.e.* the number of fragments above noise level) generally increases with the number of double bonds. It should be emphasized that natural fatty acids show numerous examples of deviations from these patterns. Further details about

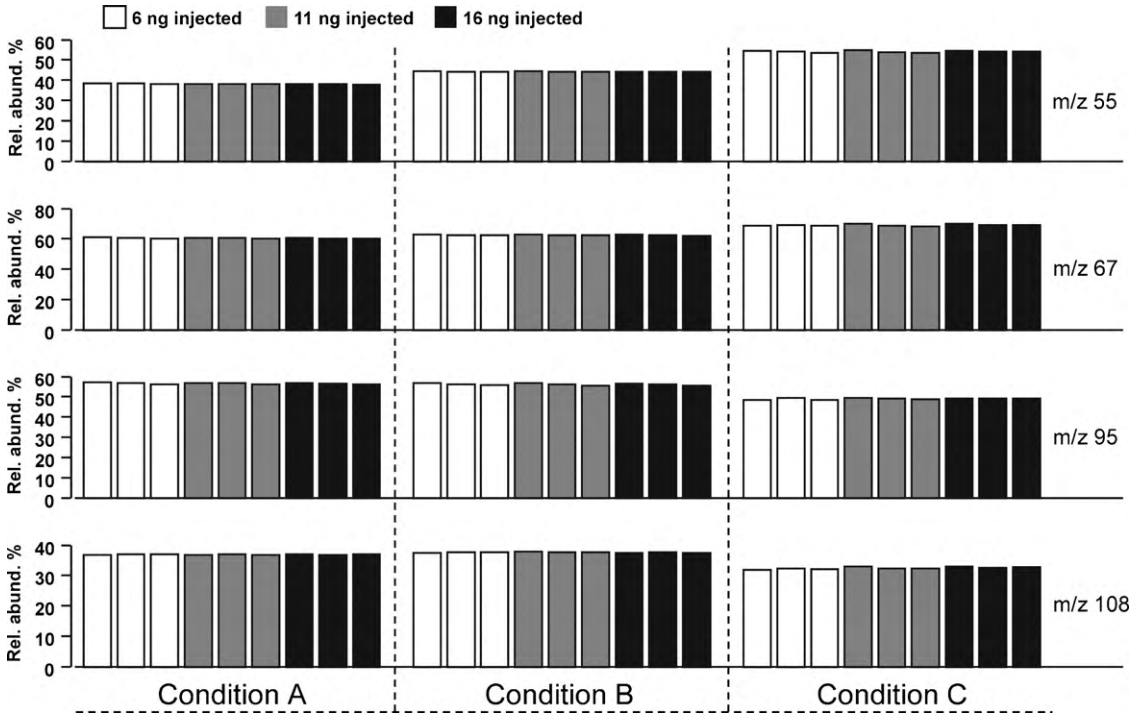

Fig. 2. Relative abundances of peaks at *m*/*z* 55, 67, 95 and 108 in the 27 mass spectra of 18:3 *n*–3. The mass spectra were normalized to the base peak, *m*/*z* 79.

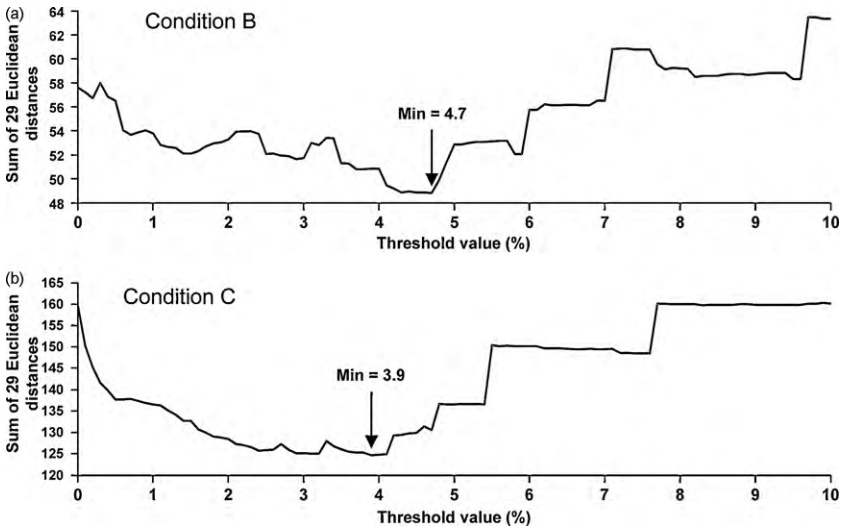

Fig. 3. the sum of Euclidean distances of 29 mass spectra in calibration set from their mass spectra ((a) Condition B; (b) Condition C) to their corresponding target under different threshold values.

fragmentation patterns in FAME can be found in Refs. [19–23]. The calibration mixture includes several fatty acids of the most common sub-classes. In general, they show the same instrument differences as observed for 18:3 *n*–3.

3.2. Establishment of the vector of calibration ratios

As described above, the pattern varies significantly among the four sub-classes of FAMEs. Since there is high degree of spectral similarity within each class, it is trivial to find standardization vectors that work well for a single class only (local models). Finding a common vector that works well with all spectra requires balancing the influence of the different spectra.

The only parameter in the method proposed in Section 2.4.1 that has to be decided by the user is the threshold level for which ions that should be excluded from the calculation of $\boldsymbol{v}$ in Eq. (2). This threshold level has two functions. The primary function is to avoid influence of signals that are dominated by noise. Since $\boldsymbol{v}$ is a vector of ratios, extreme values in $\boldsymbol{v}$ that are basically based on noise will be taken for masses close to zero in $\boldsymbol{s}_{target}$. Depending on the method of baseline subtraction, one may also have negative values in $\boldsymbol{s}_{test}$ or $\boldsymbol{s}_{target}$. This situation will lead to negative values in $\boldsymbol{v}$, which should be avoided. With spectra of poor quality (high in random noise and contributions from baseline) it is necessary to set the threshold level high for these reasons. In the current study, the compounds analyzed are in the upper range of typical amounts on the applied column, so spectra are expected to be of relatively good quality.

The second function of the threshold level is to decide the minimal relative abundance used in calculation of the final vector. By setting the value high, ions of low abundance are excluded in the calculation of $\boldsymbol{v}$ in Eq. (2), and the correction factors for the respective ions in the resulting $\boldsymbol{v}_{total}$ calculated in Eq. (3) will be based only on spectra where the ions are abundant. This may lead to a $\boldsymbol{v}_{total}$ that works well for the most abundant ions, but is less accurate for ions of low abundance. With spectral search algorithms that depend largely on ions of high abundance, this may be a good strategy. A drawback of high threshold values is that the number of missing values in $\boldsymbol{v}_{total}$ that must be set to 1, increases. A higher threshold level also means that each value in $\boldsymbol{v}_{final}$ is based on fewer spectra, which makes $\boldsymbol{v}_{final}$ more influenced by fragmentation patterns that are unique for single compounds or groups of compounds, but leads to poorer performance for a broader range of structures. This weighting problem may also be solved by using a weighted mean

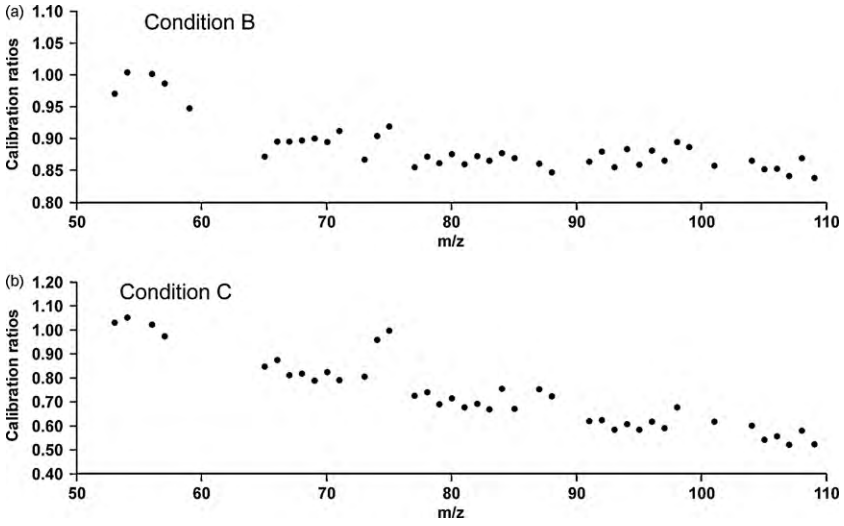

Fig. 4. The two vectors of calibration ratios calculated as described in Section 2.5.1 and with threshold values of 4.7% (Condition B) and 3.9% (Condition C).

Table 2
Average Euclidean distance between target (Condition A) and original and standardized mass spectra of Conditions B and C for the test set. The number of replicates of each compound is 3. Internal variation is the average Euclidean distance of the original spectra acquired at Condition B or C to the average spectrum of the same groups.

FAME	Condition B	Tuning difference		Condition C	Instrument difference	
	Original	Standardized	Internal variation	Original	Standardized	Internal variation
11:0	5.10	1.78	0.15	16.35	4.46	0.48
13:0	5.65	1.96	0.22	17.33	3.71	0.48
i-15:0	5.99	1.77	0.21	18.52	2.85	0.56
ai-15:0	6.94	2.00	0.16	20.99	3.62	0.56
i-16:0	5.92	1.54	0.14	18.28	2.84	0.53
11:1 *n*–1	11.76	2.52	0.27	22.92	7.25	0.70
12:1 *n*–1	11.64	2.51	0.48	27.31	7.55	1.10
13:1 *n*–1	11.71	2.19	0.55	27.40	6.64	1.16
17:1 *n*–7 *t*	22.46	3.03	0.41	45.16	5.34	1.13
18:1 *n*–7	21.41	2.41	0.41	44.50	4.59	1.01
18:1 *n*–12	25.76	5.57	0.56	64.07	13.29	1.15
19:1 *n*–9	21.80	1.79	0.33	48.42	4.30	0.61
19:2 *n*–6	11.54	2.12	0.32	39.54	3.87	1.06
18:3 *n*–3 *ttt*	7.49	1.04	0.39	32.05	13.36	1.02
18:3 *n*–6 *ttt*	10.48	3.81	0.60	38.75	8.37	0.81
20:3 *n*–3 *ttt*	7.34	0.98	0.34	33.13	9.46	0.68
20:3 *n*–6 *ttt*	8.85	1.82	0.38	35.29	5.35	0.94
20:4 *n*–6 *tttt*	8.53	2.68	0.69	38.94	4.79	1.48
22:3 *n*–3 *ttt*	6.83	1.12	0.36	35.16	8.06	1.18
10:0-2OH	6.94	3.44	0.45	14.87	7.86	1.01
12:0-2OH	16.50	5.91	1.83	39.34	9.67	2.43
9,10-cyclo-19:0	21.86	1.55	0.50	49.22	4.42	1.32

in the calculation of $\boldsymbol{v}_{\text{final}}$ where the abundance of the ions in each spectrum is taken into account. However, it produces a more complicated algorithm and it will still be necessary with a threshold value to exclude noise.

The ability of the algorithm to correct for the systematic differences between the three conditions was tested using different threshold levels on the spectra from the calibration sample. The targets, $\boldsymbol{s}_{\text{target}}$, were the average spectrum of the nine replicates analyzed at Condition A. To determine the optimum threshold values, the Euclidean distances (EDs) between the target spectra and the corrected spectra of Conditions A and B were summarized. The EDs for the nine replicates of the same spectra were averaged before the sum of EDs for all compounds was calculated.

Both Conditions B and C show a trend were the sums of EDs show a minimum when the threshold is approximately 4–5% of the base peak (Fig. 3). Below this region, too many masses with low abundance are included. Above this value, the final standardization vector is based on too few ions to be accurate. The optimal threshold values of 4.7% for Condition B and 3.9% for Condition C were used for calculation of the final standardization vectors.

The standardization vectors, $\boldsymbol{v}_{\text{final}}$, are shown in Fig. 4. As can be expected, higher values ranging from 0.52 to 1.10, are found in the correction factor for the instrument difference (Condition C to A) than for the differences in tuning (Condition B to A) where the values range from 0.84 to 1.00. In the case of the instrument difference (Fig. 4b) it can be seen that there is a trend from high to low values as the masses increase. This means that there is a smaller change in sensitivity with increasing mass for the 5975 instrument than for the 5972 instrument.

The effects of applying the two vectors given in Fig. 4 are shown in Table 1. In addition to the average ED between the target and the nine replicates of uncorrected and corrected spectra acquired at Conditions B and C, a measure for internal variation of each of the groups is given. This is the ED between the nine uncorrected spectra at Conditions B and C and the average spectrum calculated from these spectra. These values are representative for the random variation in the system, and they provide a measure for a lower limit for ED if all systematic difference between the instruments was removed. The influence on random error on the average of nine spectra (the target spectrum) is regarded negligible compared to the influence of random variation on single spectra.

The reduction in ED is calculated after the ED representing internal variation is subtracted from ED of standardized and non-standardized spectra as shown in Eq. (6).

$$\text{Distance reduction } (\%) = \frac{\text{ED}_{\text{original}} - \text{ED}_{\text{corrected}}}{\text{ED}_{\text{original}} - \text{ED}_{\text{internal}}} \cdot 100\% \quad (6)$$

These values range from 72% to 95% (average 83%) for Condition B and from 74% to 95% (average 87%) for Condition C. These figures represent the amount of the systematic error in the spectra that is removed by the standardization procedure.

Prior to calculation of the vector of calibration ratios, the spectra were in this case normalized to a single peak instead of to the sum of intensities which is a common way of normalizing spectra. This was done because the closure effect [33] when ions are normalized to total intensity introduces noise if combined with the use of the threshold in step 4 of the algorithm. Normalization to total intensity may be a good alternative in cases where the use of a threshold is not relevant, for instance for selected ion monitoring spectra. Normalization to total intensity may also work well in cases where the spectra are more similar than in this case (because closure will then have a similar effect on all spectra).

3.3. Application on fatty acids in the test set

The effect of the standardization method was evaluated on a test set consisting of spectra which are expected to be representative for spectra of FAME, but still significantly different from any single spectrum in the calibration set. The test set consists of 22 FAMEs which are homologs or isomers of compounds in the calibration set, and that are known to have different spectra from those in the calibration set. In addition, there are compounds belonging to other fatty acid classes than those in the calibration set. These are iso- and ante-iso branched FAMEs, two hydroxy FAMEs, and one cyclopropane FAME.

The EDs between the spectra in the test set and the respective target spectra are given in Table 2. The reduction in ED range from 54% to 95% (average 80%) for Condition B and from 51% to 94% for Condition C (average 81%). This is just slightly worse performance

Table 3
Euclidean distances between spectra of geometrical isomers. Values are average ($n = 3$) distance of spectra acquired at Conditions B and C to target spectra. The target spectra are averages of three spectra acquired at Condition A. Bold face denotes the lowest value in each column. *Cis*/*trans* geometry of double bonds is given from the carbonyl group (left) to the methyl end (right) of the carbon chain.

a) 18:3 *n*–6	Condition B, original spectra							Condition B, standardized spectra						
Targets	*cct*	*ctc+tcc**	*ctt*	*tct*	*ttc*	*ttt*	*ccc*	*cct*	*ctc+tcc**	*ctt*	*tct*	*ttc*	*ttt*	*ccc*
cct	**8.8**	28.7	41.7	9.3	39.2	46.4	10.4	**2.3**	28.8	39.5	3.8	37.7	44.4	10.8
*ctc+tcc**	27.6	**11.1**	38.7	26.6	30.1	43.4	24.2	29.7	**3.0**	34.7	28.8	25.9	39.8	23.8
ctt	38.4	28.0	**10.2**	39.2	**10.7**	13.0	44.1	38.4	30.5	**3.3**	39.2	9.9	8.2	41.8
tct	10.2	28.4	42.9	**8.6**	39.8	47.5	8.9	5.7	28.3	40.6	**2.1**	38.1	45.5	9.4
ttc	37.3	21.6	20.0	37.4	**10.7**	22.5	39.8	37.9	22.8	15.0	37.9	**3.3**	18.0	38.1
ttt	43.1	32.9	11.1	43.8	12.6	**10.5**	49.2	42.8	35.3	6.2	43.6	12.4	**3.8**	47.2
ccc	15.8	23.3	41.5	15.2	38.3	46.6	**8.2**	9.8	24.4	40.5	8.8	37.8	45.5	**1.0**

b) 18:3 *n*–6	Condition C, original spectra							Condition C, standardized spectra						
Targets	*cct*	*ctc+tcc**	*ctt*	*tct*	*ttc*	*ttt*	*ccc*	*cct*	*ctc+tcc**	*ctt*	*tct*	*ttc*	*ttt*	*ccc*
cct	**32.2**	42.0	58.4	**31.0**	55.2	63.0	30.7	**4.4**	29.3	41.3	5.6	41.1	47.7	9.9
*ctc+tcc**	37.9	44.0	63.1	37.4	57.3	67.3	42.7	28.2	**8.8**	37.6	28.8	31.1	44.1	25.6
Ctt	46.0	**32.0**	38.1	46.5	33.7	40.4	61.9	38.3	25.1	**6.8**	40.0	**8.5**	12.5	43.8
tct	33.5	43.2	59.9	31.8	56.5	64.5	**29.2**	6.7	29.1	42.5	**4.3**	41.6	48.8	9.6
ttc	46.0	36.2	47.3	46.0	41.1	49.6	57.9	37.5	17.8	18.1	38.5	**8.5**	22.2	41.9
ttt	50.5	34.6	**37.5**	50.9	**33.1**	**38.7**	66.8	42.9	29.8	7.6	44.4	9.1	**8.4**	50.7
ccc	35.6	37.1	48.5	36.3	47.2	53.2	31.5	13.1	22.4	41.1	12.1	37.8	46.2	**4.3**

c) 20:3 *n*–3	Condition C, original spectra							Condition C, standardized spectra						
Targets	*cct*	*ctt*	*tcc+ctc**	*tct*	*ttc*	*ttt*	*ccc*	*cct*	*ctt*	*tcc+ctc**	*tct*	*ttc*	*ttt*	*ccc*
cct	27.7	71.6	66.6	34.0	82.2	72.0	38.3	**4.8**	76.5	49.8	10.9	62.9	72.7	13.5
ctt	75.8	29.7	93.7	78.2	78.7	39.4	84.4	69.8	**9.9**	79.4	69.9	40.2	**5.6**	75.7
*tcc+ctc**	58.6	71.9	**38.2**	49.5	51.2	64.8	56.8	56.0	90.3	**4.1**	43.2	48.7	82.2	50.1
tct	31.2	69.9	56.6	31.0	73.2	68.3	37.0	18.4	78.4	36.7	**4.3**	56.3	73.1	14.3
ttc	65.3	38.9	57.7	60.0	**42.5**	**32.2**	67.3	63.4	56.1	42.0	55.1	**7.8**	46.4	61.9
ttt	72.9	**26.2**	86.6	74.0	70.6	33.1	80.5	67.6	19.1	72.1	66.1	30.8	9.5	72.2
ccc	**26.8**	74.7	58.2	**27.7**	77.0	73.3	**30.0**	19.8	83.0	44.2	12.3	62.5	78.2	**4.4**

d) 19:2 *n*–6	Condition B, original spectra				Condition B, standardized spectra			
Targets	*ct*	*tc*	*tt*	*cc*	*ct*	*tc*	*tt*	*cc*
ct	12.19	12.65	10.72	15.29	**2.71**	4.02	3.45	6.64
tc	12.23	11.88	10.48	14.95	4.07	**2.28**	3.84	6.47
tt	13.89	14.02	11.53	17.72	5.00	5.09	**2.12**	9.78
cc	**10.03**	**9.84**	**9.94**	**10.85**	5.47	4.87	8.02	**1.25**

e) 19:2 *n*–6	Condition C, original spectra				Condition C, standardized spectra			
Targets	*ct*	*tc*	*tt*	*cc*	*ct*	*tc*	*tt*	*cc*
ct	39.83	40.69	39.69	42.35	**3.32**	5.36	4.82	7.68
tc	38.82	39.46	38.57	41.21	4.21	**3.45**	4.32	6.87
tt	40.09	40.82	39.53	42.95	5.90	6.36	**3.87**	10.40
cc	**36.89**	**37.64**	**37.19**	**38.72**	4.74	4.90	7.90	**2.59**

* Spectra are of two isomers that were not chromatographically separated.

than observed for the calibration set. For both Conditions B and C the lowest reduction was observed for the hydroxy fatty acid 10:0-2OH (2-hydroxy-decanoate). This compound and the homologous 12:0-2OH differ in base peaks from all compounds in the calibration set, and they also have a generally different fragmentation pattern. Details about mass spectra of hydroxy fatty acids can be found in Ref. [34]. A significant reduction in the ED also for these FAMEs indicates that the method is robust towards spectra that are not represented by the calibration set. However, a decrease in performance can be expected, especially for compounds with abundant ions that are absent or of low abundance in the calibration spectra.

3.4. Application to highly similar spectra

The effects of the standardization procedure on highly similar spectra were studied using geometrical isomers of di- and tri-unsaturated FAMEs. Except for the central double bonds in polyunsaturated FAMEs, which influence fragmentation mechanisms [20,21], the geometry of double bonds in FAMEs have limited impact on the fragmentation, and mass spectra of geometrical isomers are therefore highly similar.

The results are reported in Table 3, which shows the ED between target spectra acquired at Condition A, and uncorrected and corrected spectra acquired at Conditions B and C. The lowest value in each column is shown in boldface. Ideally, these values should be found in the diagonal elements in the matrices and deviations from this pattern mean incorrect identifications.

For isomers of 18:3 *n*–6 acquired at Condition B (Table 3a) it can be seen that the lowest values are found in the diagonal elements, but the *ttc* isomer have equal distance to the *ctt* and *ttc* target spectra. After standardization of the spectra, the average value of the diagonals drops from 9.7 to 2.7, while the effects on the other elements are less profound. In the corrected spectra the *ttc* isomer is clearly closer to the *ttc* than to the *ctt* target spectrum, and the risk for incorrect identifications is consequently reduced. For the same isomers analyzed at Condition C (Table 3b), there are only two isomers which are correctly identified by the EDs. After standardization, the lowest values are found in the diagonal elements.

However, the *ttc* isomer has equal distance to the *ctt* and to the *ttc* target spectrum.

For isomers of 20:3 *n*–3 analyzed at Condition C (Table 3c) there is a similar situation where only three compounds are correctly identified before standardization. After standardization, the number of errors drops from four to one, and for most spectra there are good margins to the nearest incorrect spectrum. The *ttt* isomer is misclassified as the *ctt* isomer. For spectra of 20:3 *n*–3 isomers acquired at Condition B, all compounds were correctly identified both for uncorrected and corrected spectra. The margins to incorrect identification were generally good, except for the distance between the *ttt* and the *ctt* isomers. Before standardization the distances between the *ttt* spectra acquired at Condition B, and the *ctt* and *ttt* target spectra were 9.7 and 7.3. After standardization the corresponding distances were 9.8 and 1.0.

Spectra of 20:3 *n*–6 and 22:3 *n*–3 showed the same trends as the spectra of 18:3 *n*–6 and 20:3 *n*–3. In general, the spectra acquired at Condition B are correctly identified even without standardization, but there are small margins between some of the fatty acids with similar geometry in the central double bond. This margin clearly increases after standardization. For Condition C the standardization also has a clear effect on the number of incorrect identifications, which drops from 3 to 1 in the case of 20:3 *n*–6 and from 6 to 1 in the case of 22:3 *n*–3. As with the 20:3 *n*–3 isomers, the incorrect identifications after standardization were caused by confusion of the *ctt* and *ttt* isomers.

The spectra of *trans* isomers of di-unsaturated FAME are even more similar than spectra of tri-unsaturated FAMEs [20], and a clear separation of all geometrical isomers have not been previously reported. The results for 19:2 *n*–6 are shown in Table 3d and e. For both conditions there are three incorrect identifications before the standardization and none after. However, because of the general similarity between the spectra, the margins are in this case very small. Isomers of other *n*–6 dienes showed similar patterns. When all isomers of 19:2, 20:2, and 22:2 are considered together, standardization leads to a reduction in the number of incorrect identifications from five to zero at Condition B and from seven to one for Condition C.

The results for the geometrical isomers show that the proposed standardization procedure leads to significant improvements of the number of correctly identified compounds evaluated by ED, particularly in the case of Condition C. It should be emphasized that several of the geometrical isomers considered here have not been distinguished in previous work on the same compounds, even when analyzed under identical conditions [20]. Except for the difference caused by the geometry in the central double bonds in tri-unsaturated fatty acids, the differences between geometrical isomers are in many cases too small to have any practical significance for identification of unknown spectra. It seems therefore that the proposed standardization procedure in most practical situations will remove the instrument differences that lead to incorrect identifications.

3.5. Implementation

In the above description, we have assumed that each test spectrum is corrected for instrumental effects before it is tested against spectra acquired with the target conditions (for which we assume there exist a mass spectral library). This may be necessary if frequent calibrations are required, *e.g.* in cases of poor instrumental stability. The drawback of this strategy is that each test spectrum must be corrected before the spectral comparisons, and the standardization method must therefore be implemented in the software applied for compound recognition.

In many cases, it a better strategy is to do the reverse calibration, *i.e.* the entire library is standardized to fit the instrument. This requires modification of each spectrum in the library, but these calculations may need to be done only once. Since no modification of the test spectra is done with this strategy, the new library can be applied with conventional software for mass spectral recognition.

In cases where libraries already exist for both instruments or tuning conditions, the use of a calibration sample may not be necessary. As long as high quality spectra of reasonable large number of unique compounds are found in both libraries, these can be used for calibration and validation of the standardization model.

3.6. Alternative methodology

The use of a single vector for standardizing the spectra was chosen in this work for reasons of robustness. The application of a single vector is only able to correct for instrumental differences that affect all spectra equally, such as the sensitivity to high masses and to some degree effects on fragmentation mechanisms that is common for the compounds considered. However, instrumental effects, such as ionization energy, and the time span from ionization to detection (*i.e.* time available for fragmentation) also have effects that are dependent on the structure of the compounds, which are not easily corrected by a single vector.

There are many alternative standardization procedures that may be applied. The use of local models, *i.e.* models for the different classes of fatty acids (*i.e.* saturated, monounsaturated, polyunsaturated) is one possibility that performs better than the common vector. However, local models require the application of a classification algorithm prior to the standardization procedure, and there are numerous 'rare' fatty acids that are not easily classified into the main classes present in the calibration mixture.

Alternatives to local models may be the use of flexible multivariate regression models such as partial least squared regression (PLSR) [32], direct and piecewise direct standardization [28,35,36], neural networks [30], orthogonal least squares [27] and slope/bias corrections [26,27]. Multivariate calibration strategies offer more flexibility than the use of a single vector, because individual calibration ratios are calculated for each spectrum. However, the more a model depends on the spectra, the more vulnerable it is to failures by unexpected fragmentation patterns of compounds that are poorly covered by the calibration set. The hydroxy fatty acids present in the test set are typical examples of fatty acids that have radically different fragmentation patterns from the ones in the calibration set. It can be seen from Table 2 that the common standardization vector performs well also in these cases. The use of alternative calibration strategies will be pursued further with comparative studies on the same dataset as used in this study.

4. Conclusions

The differences between spectra of identical compounds analyzed at different conditions are often neglected in the processing of mass spectral data. The effects of instrument types and tuning procedures on the spectra of fatty acid methyl esters were studied. A method for reduction of the differences caused by acquisition parameters is proposed. The method focuses on finding a single vector for standardization of the instruments that represents a suitable compromise of individual vectors for a set of calibration compounds. Robustness is achieved by excluding weak ions in the calculation of the common vector.

The mass spectral differences caused by differences in acquisition parameters were significantly reduced by the proposed method. Furthermore, it was shown that the standardization had a profound positive effect on the identification of highly similar

structures when spectra acquired under different conditions were compared.

Acknowledgements

The first author is grateful to the scholarship under the State Scholarship Fund (No. 2009637060), the Graduate degree thesis Innovation Foundation of Central South University (Grant No. 1960-71131100015) and Special Funds of Central South University for Fostering Outstanding Doctoral Degree Thesis (Grant No. 2008yb033) for financial support. Also, this article is supported financially by the National Nature Foundation Committee of P.R. China (Grants No. 20875104), the International Cooperation Project on Traditional Chinese Medicines of Ministry of Science and Technology of China (Grant No. 2007DFA40680).

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