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Characterization and determination of fatty acids in fish oil using gas chromatography–mass spectrometry coupled with chemometric resolution techniques

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Abstract

Characterization and determination of a complex mixture of fatty acid methyl esters was performed for commercial fish oil using two-dimensional GC–MS data coupled with resolution techniques. Various principle component analysis methods such as significant factor analysis and fixed size moving window evolving factor analysis were used for the number of factors, zero concentration and selective regions. Then, the convoluted chromatograms were resolved into pure chromatograms and mass spectra using heuristic evolving latent projections (HELP) method. Fatty acids of $C_{16:1\omega7}$, $C_{18:4\omega3}$, $C_{18:1\omega11}$, $C_{18:1\omega9}$, $C_{18:0}$, $C_{20:2\omega6}$, $C_{20:1\omega9}$, $C_{22:1\omega11}$, $C_{22:1\omega9}$ and $C_{24:1\omega9}$ were resolved and identified by using similarity searches between deconvoluted mass spectra and MS database, in different parts of total ion current chromatogram. Window target testing factor analysis is also applied for confirming the presence or absence of target analytes. The results of the present work show that combination of hyphenated chromatographic methods and resolution techniques provide a complementary method for accurate analysis of real multi-component systems such as fish oil.

Keywords: Oils; Chemometrics; Curve resolution; Target testing; Fatty acid methyl esters

1. Introduction

Fatty acids (FAs) in foods and biological samples are commonly analyzed by gas chromatography (GC) of their fatty acid methyl esters (FAMEs), for half a century [1]. Within a few years after the first separation of individual volatile fatty acids [2], GC had become widely adopted as a highly applicable tool in a number of research areas of fatty acids.

Characterization and determination of the various kinds of saturated, mono and polyunsaturated fatty acids (PUFAs) together with their positional and geometrical isomers have been studied many times using GC and GC–MS methods in different samples [3–6]. Tandem mass spectrometry [7] and HPLC are the other techniques, which are applied for the study of the free and bound FAs and PUFAs [6,8,9]. Hyphenation of the chromatographic techniques with mass spectrometry provides a very selective tool for the study of FAs structures. Comprehensive two-dimensional gas chromatography (GC × GC) has also been applied for unraveling complex samples such as petrochemical and biological oil samples [10].

FAs play important roles in biological tissues and as constituents of lipids in biological membranes influence their properties such as fluidity, integrity and the activities of membrane-bound enzymes. One of the most significant advances that have been made is in understanding the importance of dietary FAs for human health. For example, C_{20} and C_{22} FAMEs such as arachidonic acid (20:1), eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA), which play major roles in the cardiovascular systems, are the most ω3 fatty acids in fish oils. On the other hand, high level of erucic acid ($C_{22:1\omega9}$) shows high accumulation of fat in the heart muscles and erucic acid is assumed to be the factor responsible for cardiac lesions [10].

Due to the importance of the location of the double bonds in unsaturated long-chain fatty acids (positional isomers), they have been studied by using fast atom bombardment (FAB) MS–MS and electrospray ionization (ESI) MS techniques in different fatty acids [11]. These points illustrate the importance of study of the fatty acid composition of oils.

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In general, the procedure for the GC analysis of FAMEs consists of several steps including, FA esterification and the injection, separation, identification and determination of them. Modern commercially available fused silica capillary columns offer excellent separation of FAMEs, even from biological samples. Special polar stationary phases such as Sil-88 (100% cyano-propylpolysiloxane) are used with high resolution for the analysis of FAMEs. However, the disadvantage for such columns is that, due to low thermal stability and resulting in long retention times, they are restricted to a maximum operation temperature of 500 K. On the other hand, non-polar stationary phases have a much greater thermal stability, wide range of operating temperature and chemical inertness, but inferior resolution. With respect to these advantages, non-polar phases can be applied effectively in the analysis of fatty acids with higher molecular masses.

In recent years, two-way chemical methods such as HPLC–diode array detection (DAD) and GC–MS have been applied for the analysis of multi-component systems [12]. Within these methods an important group of chemometric techniques have been proposed for determination of peak purity, detection of interferences, resolution and quantitative estimation improvement. Current non-iterative approaches in curve resolution based on evolutionary nature of data consist of evolving factor analysis (EFA) [13,14], heuristic evolving latent projections (HELP) [15,16], window factor analysis (WFA) [17], orthogonal projection resolution (OPR) [18] and subwindow factor analysis (SFA) [19]. The iterative approaches consist of iterative target transformation factor analysis (ITTFA) [20] and alternating regression (AR) [21] that have also been developed rapidly. The HELP method, that was reported in 1992 by Kvalheim and Liang [15] was applied extensively in the resolution of many complex matrixes such as herbal drugs [22,23]. In many studies of curve resolution, the selectivity has been used as a fundamental factor for acquiring the recovery of true solution [24]. Window target testing factor analysis (WTTFA) method that was introduced by Lohnes et al. was applied to the chromatographic analysis of polycyclic aromatic compounds in a complex petroleum sample using fluorescence detection [25]. As a result, the chemometric methods can be coupled with the hyphenated chromatographic systems in order to increase the separation ability and making possible to quantify the complicated systems.

The aim of the present work was characterization and determination of a complex matrix of FAMEs in a commercial fish oil by using the PCA local rank analysis methods and then a curve resolution method (HELP) on two-dimensional data obtained by GC–MS techniques. Mass spectrum of each component becomes accessible through unique resolution. Then WTTFA was applied for the investigation of relative assessment of potential matches that have been done with curve resolution technique, by using the mass spectra of a few pure analytes as the targets. After confirming this qualitative analysis of the analytes, the quantitative analyses were performed using overall volume integration (OVI) method [22].

2. Experimental

2.1. Materials and reagents

Boron trifluoride–methanol complex for synthesis, sodium hydroxide, HPLC-grade methanol and sodium chloride, used for sample preparation and *n*-hexane for extraction step, were of analytical reagent quality and were purchased from Merck (Darmstadt, Germany). Two methyl ester standard mixtures in which the identity and concentration of several major peaks is known, PUFA, No.1 which has a marine source and Grain fatty acid methyl ester were purchased from Supelco (Sigma–Aldrich, Zwijndrecht, The Netherlands). The fish oil sample was a commercial fish oil and was purchased from a local market in Iran.

2.2. Sample preparation

FAMEs were obtained according to the AOCS official method [26]. This method can be explained briefly as follows: the fatty acids were split off by saponification with methanolic sodium hydroxide, and then methylated with boron trifluoride–methanol reagent (containing BHT (butylated hydroxytoluene) as an antioxidant) under nitrogen atmosphere. The corresponding FAMEs were extracted with hexane by adding salt solution for complete recovery [3]. For determination of percent of each component in the real sample, the prepared sample was directly injected in the GC column.

2.3. Equipment

The analyses were performed with the use of a Hewlett-Packared 6890 series gas chromatograph interfaced to a Hewlett-Packared 5973 mass spectrometer. A HP ChemStation G1701 CA version C.00.0021 was used for the data collection and conversion to ASCII format.

2.4. GC–MS analysis

In the gas chromatographic system, a DB-5 capillary column (30 m × 0.25 mm i.d.) was used. Column temperature was first set at 130 °C and then programmed from 130 to 250 °C at a rate of 5 °C min^{-1}. This is the optimum temperature programming for the best conditions of both run time and resolution quality. Inlet temperature was kept at 260 °C. Split injections were performed with a 20:1 split ratio. Helium carrier gas was used at a constant flow rate of 1 ml min^{-1}. In the mass spectrometer, impact with ionization (EI^+) mass spectra were recorded at 70 eV ionization energy in full scan mode (20–350 unit mass range) with 0.2 s $scan^{-1}$ velocity. The ionization source temperature was set at 230 °C.

2.5. Theory

The aim of curve resolution techniques is the recovery of response profiles of each component from a bilinear data matrix that is produced by GC–MS analysis:

$$\boldsymbol{X}_{m\times n}=\sum_{k=1}^{A}\boldsymbol{X}_k=\sum_{k=1}^{A}c_k s_K^{\mathrm{T}}+\boldsymbol{D}+\boldsymbol{E}=\boldsymbol{C}\boldsymbol{S}^{\mathrm{T}}+\boldsymbol{D}+\boldsymbol{E} \quad (1)$$

$$\boldsymbol{X}_{m\times n}=\boldsymbol{T}\boldsymbol{P}^{\mathrm{T}}=(\boldsymbol{T}\boldsymbol{R})\,(\boldsymbol{R}^{-1}\boldsymbol{P}) \quad (2)$$

Here, $\boldsymbol{X}_{m\times n}$ denotes an ion current matrix expressing the chromatographic profiles of m retention time points measured at n different m/z value (Eq. (1)). The matrix $\boldsymbol{X}_{m\times n}$ can be represented as the product of a score matrix $\boldsymbol{T}$ and loading matrix $\boldsymbol{P}$ (Eq. (2)). By multiplying these abstract factors with rotation matrix $\boldsymbol{R}$, the concentration matrix $\boldsymbol{C}$ and the spectral matrix $\boldsymbol{S}$ will be obtained. The matrix $\boldsymbol{D}$ is the matrix of background ion current. The matrix $\boldsymbol{E}$ denotes the measurement noise (Eq. (1)). The HELP method is based on the local full rank analysis of the data set and focuses on finding selective regions (concentration or spectral) by a sophisticated graphical tool (data scope). The presence of selective zone for all species in a data matrix eliminates the $\boldsymbol{R}$ ambiguities, reduces the feasible solutions and ensures the recovery of the real response profiles of the chemical system by matrix multiplication (Eq. (3)).

$$\boldsymbol{C}=\boldsymbol{X}\boldsymbol{S}(\boldsymbol{S}^{\mathrm{T}}\boldsymbol{S})^{-1} \quad (3)$$

A thorough description of the theory of the method is given in [12], and has not been repeated here for the sake of brevity. The following procedure is carried out for every cluster in the total ion current (TIC) chromatogram:

(1) Background correction and the other pretreatments of data.
(2) Application of various PCA base methods for obtaining the correct number of factors, zero concentration and selective regions, such as: significant factor analysis, fixed size moving window evolving factor analysis (FSMWEFA) [27] or so called eigenstructure tracking analysis (ETA) [28].
(3) The system is resolved into pure chromatogram and mass spectra by means of the HELP technique. The method is earlier shown to perform better than AR and ITTFA [29].
(4) Confirming the reliability and quality of the resolved profiles by spectral similarity match with MS database and using the WTTFA method.
(5) Determination of the relative components by obtaining the sum of peak area integrations of mass chromatograms at every m/z value of the mass spectra. The value of $c_K s_K^{\mathrm{T}}$ (Eq. (1)) is known as OVI value of each component [22,23]. This value is directly proportional to the mass of its component and therefore, it is determined.

All calculations were performed on an ATLON (AMD) 1200 MHz processor and 256 M RAM personal computer. All programs were coded in MATLAB (version 6.0.0.88 R12, The Mathworks, Natick, MA, USA). The programs for the HELP and WTTFA methods and data preprocessing were written in our laboratory and the chemometrics toolbox (the Mathworks) was used for the other chemometric methods. The library searches and spectral matching of resolved pure components were conducted on the US National Institute of Standard and Technology (NIST) MS database together with another database on the web [30].

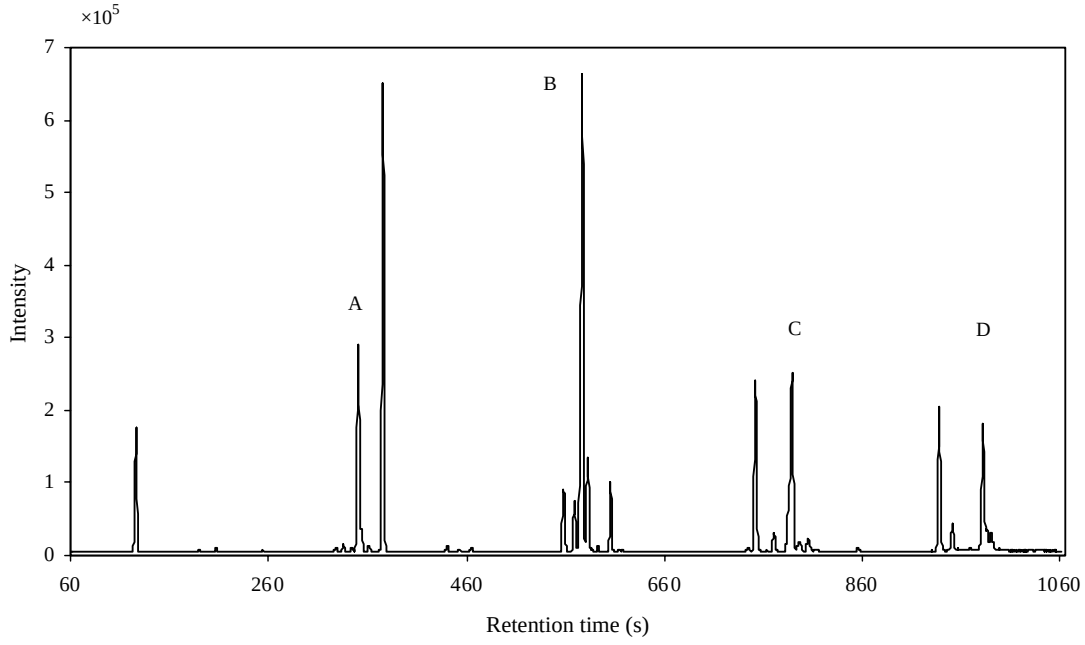

Fig. 1. TIC curve of fatty acid methyl esters of the commercial fish oil. The A–D peak clusters are defined in the text.

3. Results and discussion

3.1. Qualitative analysis of FAMEs in the fish oil

Fig. 1 shows the TIC of fatty acid methyl esters in the commercial fish oil. This chromatogram has been obtained after optimizing the heating rate. This trace has almost a similar pattern to the PUFA-1 trace, but they must have been different in relative content of fatty acids or even the kinds of unsaturated fatty acids. As can be seen from this profile, almost twenty chromatographic peaks have appeared and some of them overlapped with one another. Due to the overlapping peaks, similarity indices (SIs) obtained from direct searching with the MS database is quite low for many of these peaks. Another problem is the probability of finding the same components at different chromatographic scan points, especially in the case of positional isomers. Because of the similarity between the mass spectrum patterns of unsaturated fatty acids, it is not possible to use mass chromatograms for reducing co-elution. These facts indicate the complexity of the matrix and clearly reveal the need for using curve resolution techniques that can resolve the co-eluted peaks. The two problems that are always involved in processing of two-dimensional data obtained by GC–MS measurements are background shifting and heteroscedastic noise. Because of background up warding, both resolution of the overlapped peaks and accurate identification of the components with low concentrations are impossible. The background subtraction in this work has been performed using Liang procedure [15]. In this way, the local rank analysis of zero component regions can provide sufficient information for performing linear regression with respect to the retention time and finally correcting the base line. By using this

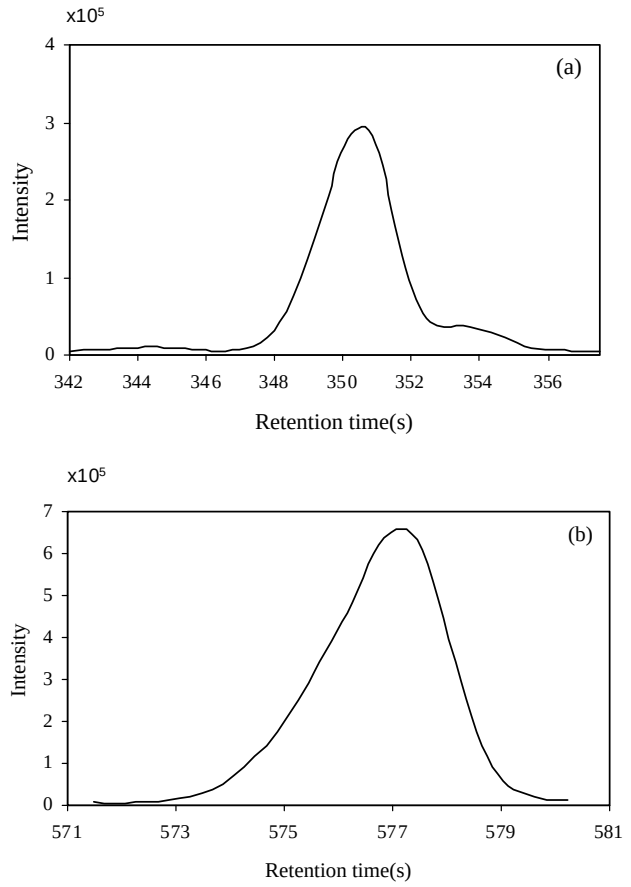

Fig. 2. TIC curves of peak cluster A (341–358 s) (a) and B (571–581 s) (b).

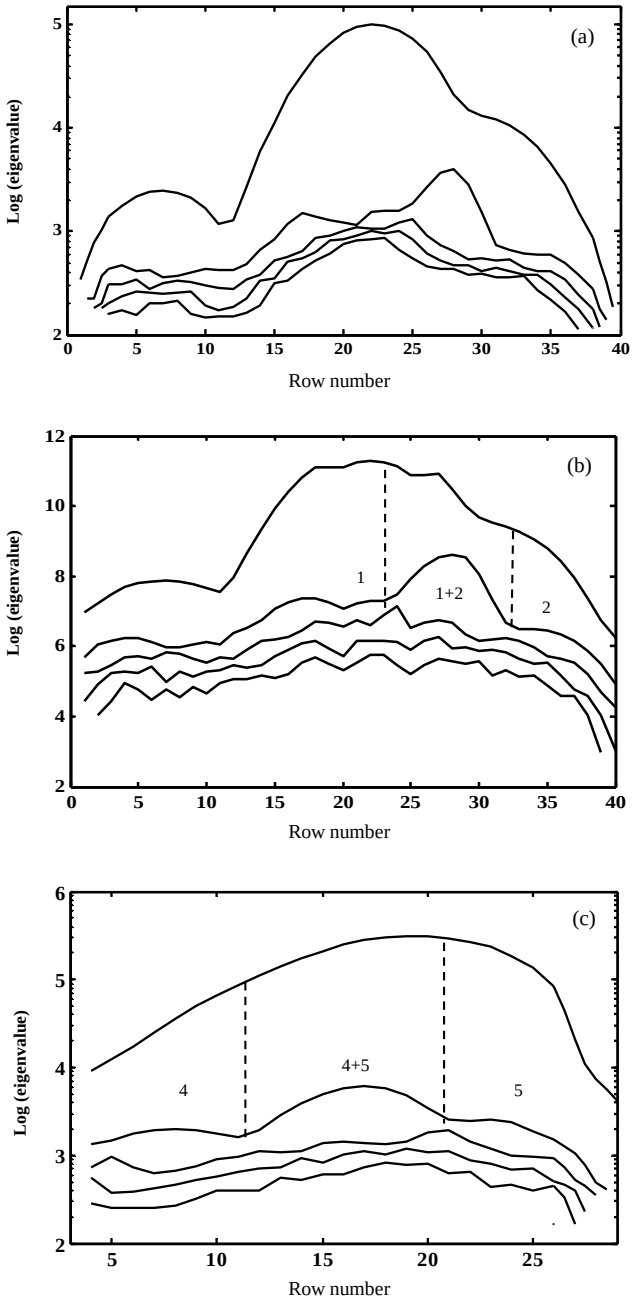

Fig. 3. FSWM plots of peak clusters A, before correcting heteroscedastic noise (a), after correcting in Fig. 2a (b), and after correcting in Fig. 2b for peak cluster B (c). The horizontal lines represent the noise level. Different regions are defined in the text.

technique a much better background subtraction could be obtained.

In the resolution step, two peak clusters named A (40 × 330 matrix), within 342–358 s (or between 711 and 750 scan points) and B (23 × 330 matrix), within 571–581 s (or between 1287 and 1309 scan points) (Fig. 1) are taken to illustrate the data analysis process.

Fig. 2 shows the TIC for the two peak clusters A and B. Cluster A clearly shows the co-elution of two major and minor peaks. Without curve resolution, similarity search indicates (with M.I. 99) that the major peak is 9-hexadecenoic acid methyl ester, but in the case of minor peak the similarity search reveals the presence of 6,9-hexadecadienoic acid methyl ester (M.I. 90) and 6,9-heptadecadiene (M.I. 83).

Since searching of different compounds results in low matching indices on the minor peak, using the curve resolution seems to be necessary for analysis of this cluster. However, the HELP, SFA and EFA resolution methods were applied to the two-dimensional data matrix of peak cluster A and two components of 9-hexadecenoic acid methyl ester ($C_{16:1\omega7}$) and 9,12,15,18-octadecatetraenoic acid methyl ester($C_{18:4\omega3}$) were resolved. Cluster B looks like a one component chromatographic peak in a classical analytical way. However, using the library search reveals that many fatty acid isomers of *x*-octadecenoic acid methyl esters with match indexes of 95–90 are present in this cluster. Using the above mentioned chemometric methods show that three components of *cis*-7-octadecenoic acid ($C_{18:1\omega11c}$), *cis*-9-octadecenoic acid ($C_{18:1\omega9c}$) and 9-octadecenoic acid ($C_{18:1\omega7}$) methyl ester can be resolved. This result is obtained by using the following peak purity techniques.

Peak purity and also co-eluted parts of chromatograms are identified using FSMWEFA plots. In these plots, the logarithmic curve of eigenvalues higher than the noise level represents the presence of a new component. If a system contains only one species, only one curve is higher than the noise level in its FSWM plot, otherwise there are at least two curves higher than the noise levels.

Fig. 3 shows the FSMWEFA plots for the clusters A and B. The FSMW plot depicted in Fig. 3a shows the heteroscedastic nature of the noise, because all curves are higher than the noise level. In order to identify the number of significant factors in these situations, it is necessary to correct the heteroscedasticity. There are many methods in the literature for doing this correction such as data transformation for reduction of data amplitude and also different smoothing techniques [12,31–33]. The procedure for noise correction applied in the present work is taken from Savitzky–Golay

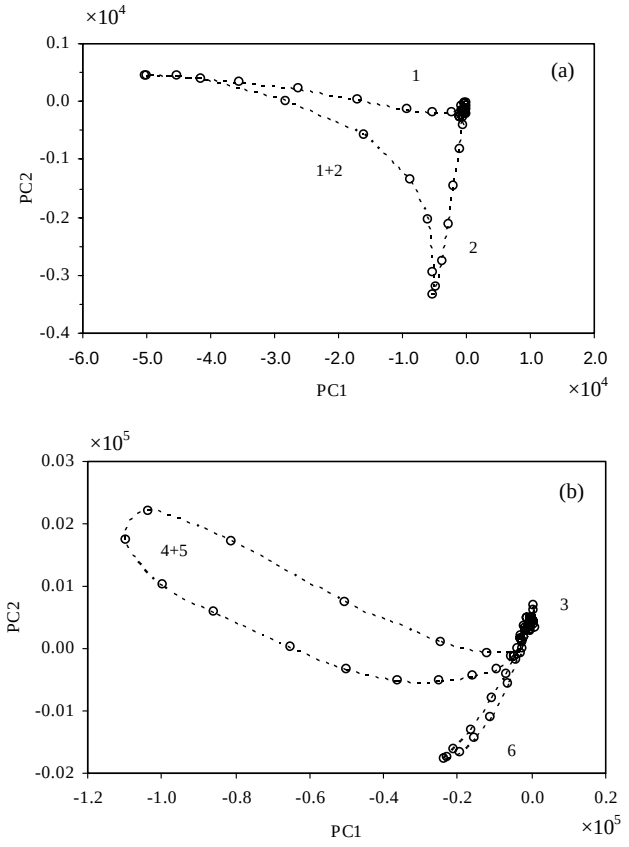

Fig. 4. ELPGs of peak cluster A (a) and B (b) in Fig. 2. The straight lines 1 and 2 in (a) denote the pure regions of components 1 and 2 while curve 1 + 2 represents the overlapping region of the components 1 and 2. Fig. 4b shows two straight lines 3 and 6 with some noise fluctuations related to the presence of two pure peaks (before and after peak cluster B) and a middle region with a little straight line segment, denotes to high degree of co-elution of two components of 4 and 5.

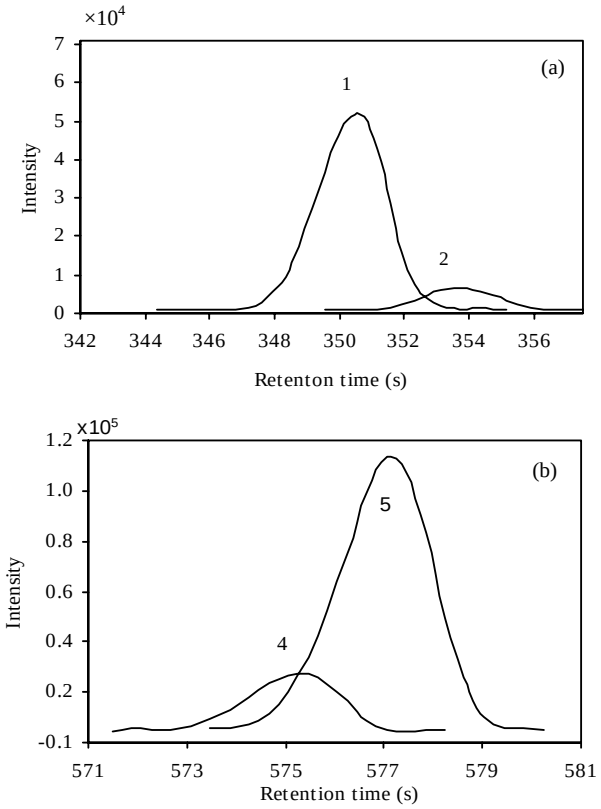

Fig. 5. Resolved chromatograms of peak clusters A (a) and B (b) given in Fig. 2.

filter [12]. Then, the Keller method of selective normalization is applied on the smoothed chromatograms. From the final results of these corrections (Fig. 3b), one may conclude that the region of 1 + 2 is the overlapping region of the first and second components. The regions of 1 and 2 are possibly the regions of the pure first and second components, respectively. The similar pattern for the cluster B is shown in Fig. 3c. It should be mentioned that due to the high amount

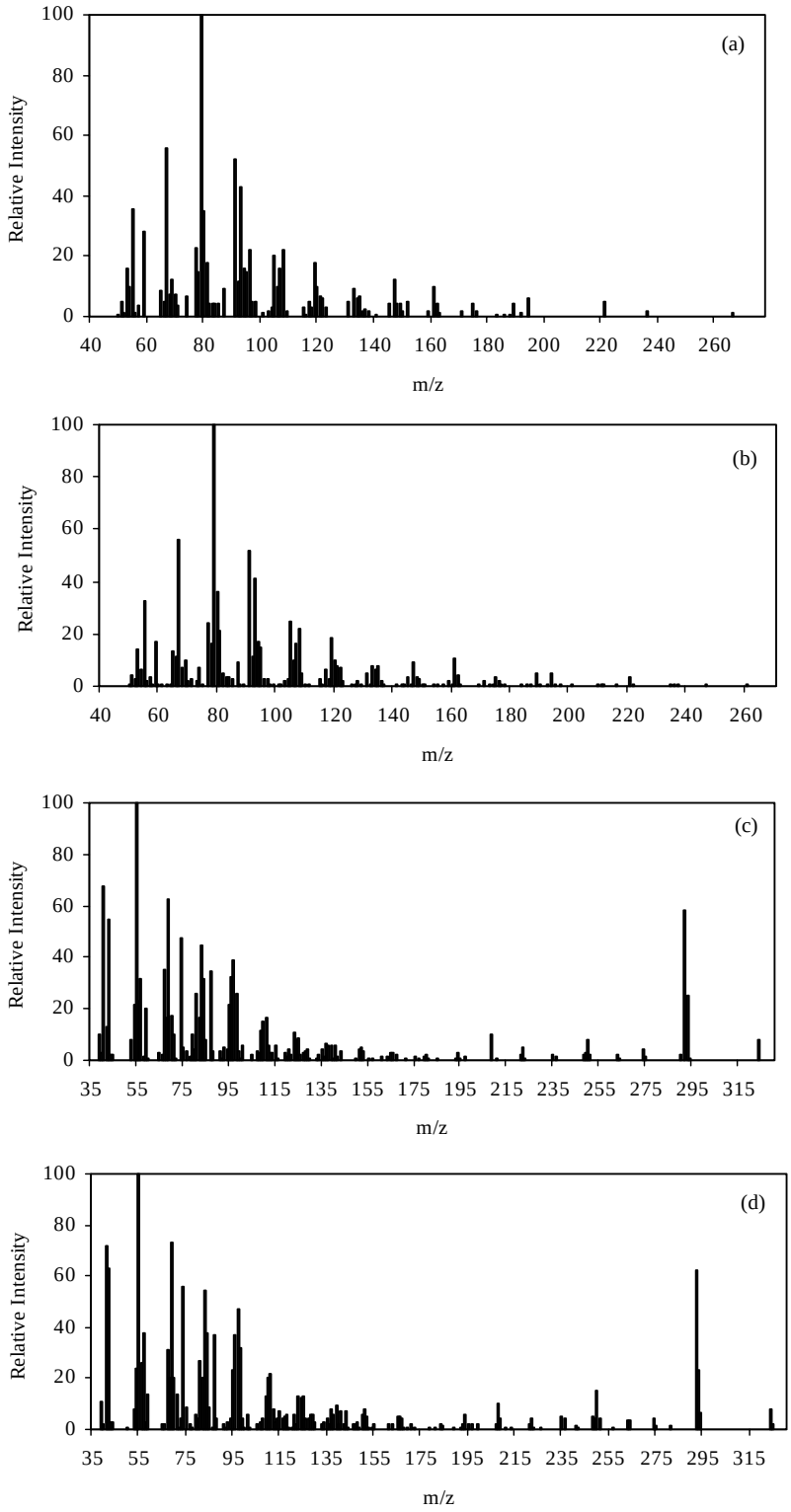

Fig. 6. Recovered and their standard mass spectra of some components of peak clusters A and B in Fig. 2. Resolved (a) and standard (b) mass spectra of $C_{18:4\omega3}$. Resolved (c) and standard (d) mass spectra of $C_{18:1\omega9c}$.

of current in the cluster B and probability of overloading that may leads to incorrect results, two excess tests were carried out for confirming the reliability of the results. First, the peak purity analysis was performed on methyl ester standard sample with almost the same amount of signal, and second, a diluted sample was used, and the same numbers of significant eigenvalues (two eigenvalues) were obtained for the cluster.

In order to further confirm the results obtained from FSMWEFA, the evolving latent projection graphs (ELPGs) are also plotted [15]. The ELPG is actually a principal component projective plot that is based on the use of evolutionary nature of the hyphenated data. In the ELPG from the chromatographic direction, the straight-line section represents the pure selective region of one component, while the curving section denotes the overlapping region containing at least two components. The ELPGs of the peak clusters A and B are shown in Fig. 4. From Fig. 4a one can see that the peak cluster A is a two-component system. The two straight lines (marked by 1 and 2) clearly indicate the selective information from two different components, which strongly supports the results obtained from the FSWM plots. Assessment of peak purity by using ELPG method in the cluster B was performed along with two other peaks, one before and another after the peak B. This test was carried out due to susceptibility of these peaks for having more than one components. In Fig. 4b one can easily see the four-component system for this set of peaks. The middle part of this plot clearly shows the co-elution region of two components (4 + 5) that is related to the cluster B. The first and third peaks surrounding the cluster B show the straight lines (marked by 3 and 6) with some noise fluctuations that suggest there must be only one component in each peak. Therefore, the results obtained from FSWM and ELPG methods are consistent with each other.

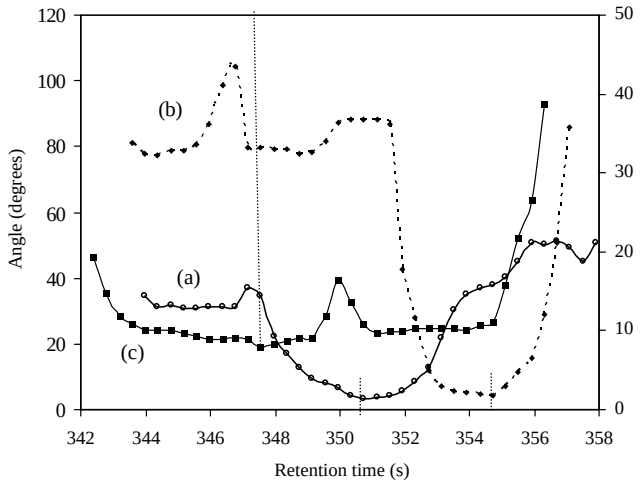

Fig. 7. Results of WTTFA applied to GC–MS data for $C_{16:1\omega7c}$ (curve a), $C_{18:4\omega3}$ (curve b) and $C_{18:1\omega9c}$ (curve c). The retention time values corresponding to minimum angle for each target are shown by dashed lines. For curve c retention time is shown on the upper axis and its minimum corresponds to 577 s on the cluster B.

The information obtained from the above techniques can be used in determination of chromatographic eluting order, number of components in the system, selective region and also zero concentration regions of all the constituents [12,15]. Finally, the two-dimensional data matrix can be uniquely resolved into pure chromatographic profiles and mass spectra of related components. As the chromatographic curve and mass spectrum of each component have been resolved, the qualitative analysis can then be directly carried out by means of the similarity searches in the standard mass library (NIST and a library on web [30]). The high match index obtained in all cases indicates the high quality of resolution process. It shows that the components in the peak cluster A are $C_{16:1\omega9}$ and $C_{18:4\omega3}$. In the case of the cluster B, because of the high similarity between the mass spectra of different positional isomers deciding on the kind of the isomers just by matching index values is not easy. By comparing the elution order of $C_{18:1}$ isomers of standard PUFA-1 with the above sample, one may conclude that the components corresponding to the peaks 3, 5 and 6 are certainly $C_{18:2\omega6}$, $C_{18:1\omega9}$ and $C_{18:1\omega7}$, respectively. Although it has been found that $C_{18:2\omega6}$ co-eluted with $C_{18:3\omega3}$ on non-polar columns [5], we obtained only one significant factor for this peak. Therefore, we believe that the peak 3 is pure. It

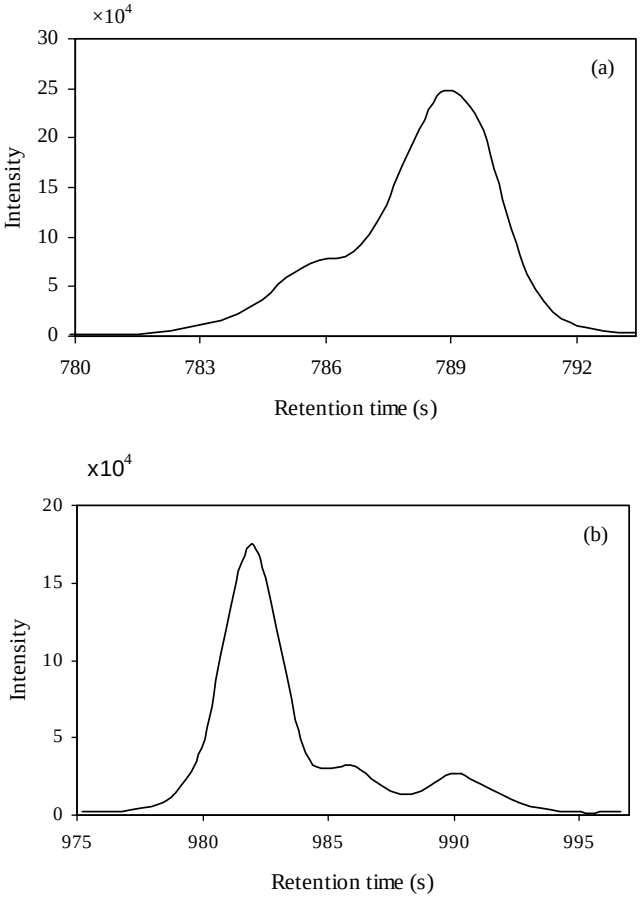

Fig. 8. TIC curves of peak clusters C (780–793 s) (a) and D (975–996 s) (b).

should be noted that the quality of this peak has been also confirmed by using the standard mass spectrum from the library. Identification of the peak 4 needs more inspection. Due to the fact that the higher distance of ethylenic group from the end methyl group causes the FAME to be eluted first [5,6], therefore, the peak 4 could probably be related to $C_{18:1\omega11}$.

The resolved chromatograms and related mass spectrum of minor components of A and B clusters together with the corresponding standard spectra are shown in Figs. 5 and 6, respectively.

The quality of resolution techniques can be further ascertained with the application of WTTFA. This technique that is a combination of target factor analysis and FSMWEFA attempts to determine if response profile of a target analyte (the mass spectrum of the pure compound) lies within the response subspace of a time window containing unresolved chromatographic peaks [25]. The procedure has been performed simply by projection of target vector into PCA subspace (matrix of eigenvectors) and then obtaining the angle between the two vectors (target and projected vectors). Once this angle has been calculated for the target

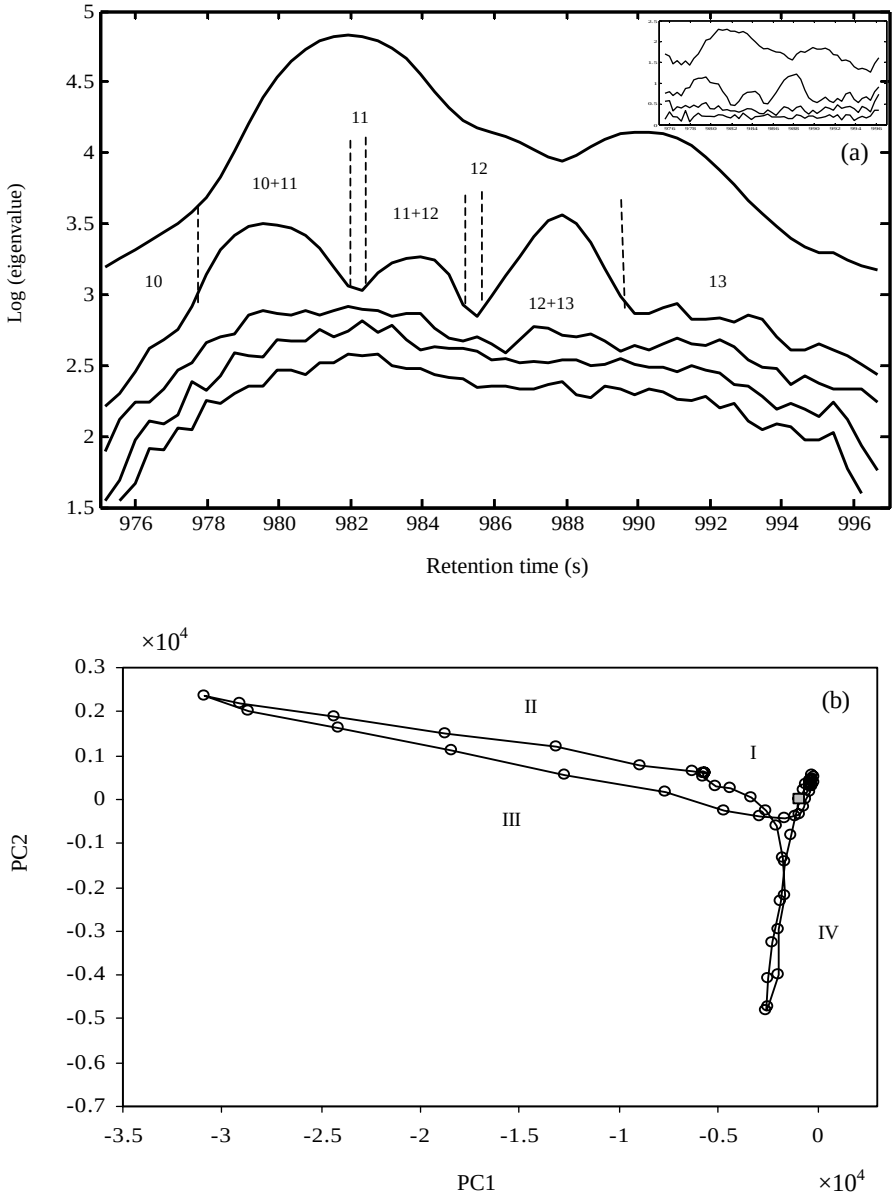

Fig. 9. FSWM plots of peak clusters D (a) after correcting heteroscedastic noise in Fig. 8. The horizontal lines represent the noise level. The regions 10, 10 + 11, 11, 11 + 12, 12, 12 + 13, 13 in (a) show the pure region of the component 10, overlapping region of the components 10 and 11, the pure region of the component 11, overlapping region of the components 11 and 12, the pure region of the component 12, overlapping region of the components 12 and 13, the pure region of the component 13, respectively. The same trend has been found for the peak cluster C and components 6, 7, 8, 9 (it is not shown here). The eigensubtraction plot that is inserted for the cluster D as a small plot in the right side of (a) clearly indicates three co-eluted regions. (b) ELPG for the cluster D. The regions I, II, III and IV show the selective and co-eluted regions for components 11–13. (Indicates the initial point of graph.)

spectrum, the window is moved to the next retention time and the calculation is repeated for the new window. This process is repeated until the end of the chromatogram and a plot of angles versus the position of the center of window is created. A minimum in this plot with a small angle (below 10°) will satisfy the presence of the target spectrum at the window. Because the window moves sequentially through the chromatogram, WTTFA allows a relative assessment of match quality. The angle versus the retention time plot for three FAMEs of $C_{16:1\omega9}$, $C_{18:4\omega3}$ and $C_{18:1\omega9}$ supports the presence of these compounds (curves a–c in Fig. 7).

Fig. 8a and b show the TIC of the peak cluster C (35 × 330 matrix), within 780–793 s (or between 1811 and 1846 scan points) and peak cluster D (55 × 330 matrix), within 975–996 s (or between 2302 and 2356 scan points) of Fig. 1. Likewise, Fig. 8a appears to be a mixture of two components, but by using the similarity searches many compounds were found in this cluster. For example 11-eicosenoic, 11,14-eicosadienoic, 15-tetracosenoic acid methyl esters and many other compounds, could be directly matched (with low quality index) in NIST MS database. Also in Fig. 8b the appearance of three constituents can be seen. On the other hand, the results of matching with NIST library confirm the presence of the compounds of 11 and 13-docosenoic acid, 10-nonadecenoic acid and 15-tetracosenoic methyl ester, etc. with low match index. However, it is not possible to discriminate between the two positional isomers of 13 or 11-docosenoic acids.

However, four constituents containing 11-eicosenoic acid methyl ester, 11,14-eicosadienoic acid methyl ester and two non-fatty acid components (methyl dihydromalvalate and a derivate of 1-pyrroline) are resolved for the cluster C. Also for the cluster D, four constituents containing two positional isomers of 9-docosenoic acid and 11-docosenoic acid methyl ester and two non-fatty acid constituents (9-octadecen-1-ol and a derivate of cyclobuta-dithiophene) are resolved by means of the HELP resolution method. Since, the fatty acid components are important in this study, only the quantitative analysis of FAMEs is investigated in this work.

For the C and D parts of the TIC chromatogram (Fig. 1) similar to the previous clusters, background shifting exists and the correction step seems to be essential before any resolution. After removing the background and correction of heteroscedastic noises, the FSWM plot for the peak clusters C and D are obtained. Fig. 9a shows FSMW plot for the peak cluster D (because of similarity in these two plots, the plot for the cluster C containing components of 7, 8, 9 and 10 is not shown). As can be seen from this plot, there are four components and the selective region for each component and the co-eluted parts of the chromatogram are clearly depicted. The eigensubtraction method [32], which is another correction method, is also applied on this FSWM plot for further confirming of the co-eluted regions of the cluster D and is inserted as a small plot on the right side of Fig. 9a. The ELPGs for the above clusters are also carried out and are shown in Fig. 9b for the cluster D. These plots clearly

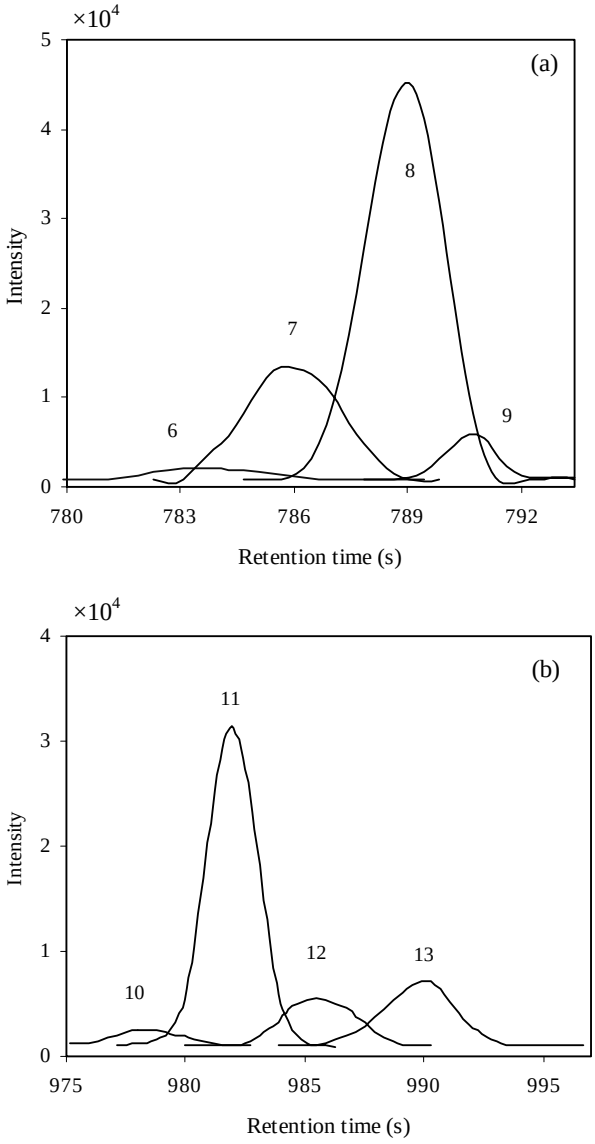

Fig. 10. Resolved chromatograms of peak clusters C (a) and D (b) in Fig. 8.

indicate that these systems are more complicated compared with the previous peaks of A and B. The ELPG in Fig. 9b shows a four-component system for the cluster D. The results obtained from FSMW and EPLGs are consistent with each other. After determination of the selective and zero concentration region of each component, the unique resolution of each component into chromatograms and mass spectra is performed on the two-dimensional data. Fig. 10 shows the resolved chromatograms of the clusters C and D. Fig. 11 shows the resolved mass spectra of the components and the related standard mass spectra. The high matching index between the resolved and standard mass spectra obtained from the library indicates (Fig. 11) the high quality of the resolution. The WTTFA is also carried out on the cluster C and D based on pure $C_{20:1\omega9}$ and $C_{22:1\omega9}$ as the targets, respectively. These results support the presence of the above

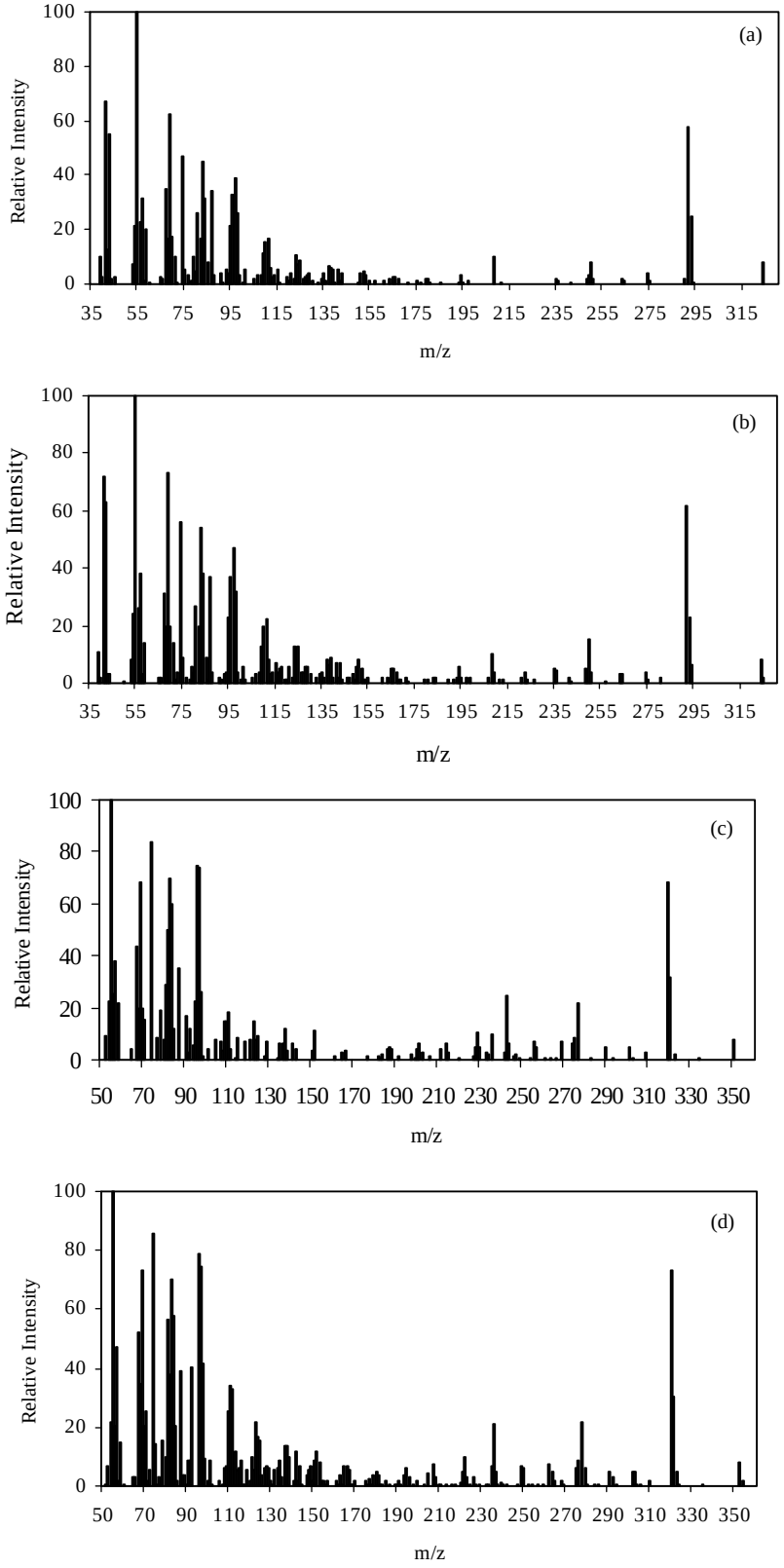

Fig. 11. Recovered and their standard mass spectra of some components of peak cluster C and D in Fig. 8. Resolved (a) and standard (b) mass spectra of $C_{20:1\omega9c}$. Resolved (c) and standard (d) mass spectra of $C_{22:1\omega9c}$.

Table 1
Identity and the proportions of all fatty acids in the commercial fish oil and PUFA No.1 (marine source); expressed as percentage of the total fatty acids of the sample oils

No.	Component	Retention time (s)	Abbreviated form	Content (% of total fatty acids, PUFA No. 1)	Content (% of total fatty acids, commercial oil)
1	Tetradecanoic acid	126.02	$C_{14:0}$	6.93	5.21
2	*cis*-9-Hexadecenoic acid	351.13	$C_{16:1\omega7c}$	12.84	9.65
3	6,9,12,15-Octadecatetraenoic acid	353.92	$C_{18:4\omega3}$	1.28	0.97
4	Hexadecanoic acid	375.79	$C_{16:0}$	2.20	19.85
5	*cis*,*cis*,9,12-Octadecadienoic acid	569.09	$C_{18:2\omega6c}$	3.31	2.50
6	7-Octadecenoic acid	575.46	$C_{18:1\omega11}$	–	3.50
7	*cis*-9-Octadecenoic acid	577.05	$C_{18:1\omega9c}$	24.68	18.54
8	11-Octadecenoic acid	582.61	$C_{18:1\omega7}$	5.67	4.26
9	Octadecanoic acid	605.68	$C_{18:0}$	0.08	3.02
10	5,8,11,14,17-Eicosapentaenoic acid	752.06	$C_{20:5\omega3}$	11.10	8.31
11	*cis*,*cis*-11,14-Eicosadienoic acid	783.47	$C_{20:2\omega6}$	–	0.20
12	*cis*-11-Eicosenoic acid	789.03	$C_{20:1\omega9}$	10.61	7.97
13	4,7,10,13,16,19-Docosahexaenoic acid	937.78	$C_{22:6\omega3}$	9.53	7.16
14	7,10,16,19-Docosapentaenoic acid	950.91	$C_{22:5\omega3}$	2.01	1.51
15	11-Docosenoic acid	981.93	$C_{22:1\omega11}$	7.77	5.85
16	*cis*-13-Docosenoic acid	985.51	$C_{22:1\omega9}$	1.54	1.16
17	15-Tetracosenoic acid	1196.96	$C_{24:1\omega9}$	0.45	0.34

components in the clusters, around 789 s and 981–986 s, respectively, which are consistent with the retention data.

Other peaks in the TIC, at different scan points are determined qualitatively in a similar way as described for the clusters A–D. For example, for the octadecaonic acid methyl ester ($C_{18:0}$), there was severe co-elution problem that was resolved. Seventeen fatty acid methyl esters are found and all of them were analyzed in this work. However, because the aim of the present study was resolution and determination of fatty acids, the non-fatty acid components were not determined in this work. For comparison of the relative peak areas, the HELP method is also applied on some parts of the PUFA No. 1 TIC.

3.2. Quantitative analysis of FAMEs in the fish oil

The quantitative analysis of GC–MS data that is usually performed using peak area integration and peak splitting, in the case of overlapping peaks based on the TIC, gives inaccurate results. Therefore, in the present work we have used the method of OVI for this purpose [22,23]. For the resolved chromatogram and mass spectrum of each component obtained from GC–MS two-way data, the peak area integration was calculated at every *m/z* for the total mass spectrum range. Its sum, which is called the overall volume, is directly proportional to the content of the corresponding component [22,23]. The reason for using this method in the present work was unavailability of some of the total standards of PUFAs and also their different isomers. The OVI method is certainly superior to the common method of analysis. Fatty acid compositions and quantitative results for the commercial fish oil and the standard sample of PUFA No. 1, based on the percentage of the total fatty acids, are summarized in Table 1. As can be seen from Table 1, the presence of fatty acids with more than four double bonds is a unique property of the fish oils [10] and there must be a clear dominance of the non-saturated fatty acids over the saturated ones. Comparison of the results for the PUFA No. 1 sample with the sample analyzed in this work shows that the presence of $C_{16:0}$ is relatively high in the later. However, it is shown that in the vegetable oils the presence of ω3 poly unsaturated fatty acids is negligible [10]. In contrast, the sample analyzed contains 8.31 and 7.16 % of total fatty acids of $C_{20:5\omega3}$ and $C_{22:6\omega3}$, respectively.

4. Conclusion

The combination of hyphenated chromatographic techniques with chemometric resolution methods can provide a complementary method, or in several cases as an alternative method for the quick and accurate analysis of real multicomponent systems such as mixtures of fatty acids (as their methyl esters). The high ability of these methods is more obvious especially in the case of the resolution of the positional isomers that have a similar mass spectral pattern. It is clear that, the different locations of double bonds in unsaturated fatty acids (i.e. erucic acid, $C_{22:1n9}$), cause different properties for them and therefore their resolving is very important. However, resolution and determination of these isomers require the hyphenated method of GC–MS coupled with the chemometric resolution techniques.

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