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SCIENCE DIRECT
Talanta 68 (2006) 888–894
Talanta
www.elsevier.com/locate/talanta

Statistical evaluation of an analytical GC/MS method for the determination of long chain fatty acids

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Received 26 April 2005; received in revised form 11 June 2005; accepted 11 June 2005
Available online 20 July 2005

Abstract

In-depth evaluation of an analytical method to detect and quantify long chain fatty acids (C_8–C_{16}) at trace level concentrations (25–1000 μg/l) is presented. The method requires derivatization of the acids with methanolic boron trifluoride, separation, and detection by gas chromatography–mass spectrometry. The calibration experiments passed all the tested performance criteria such as linearity, homoscedasticity, and ruggedness. The detection limits and related quantities were computed by applying the method detection limit, and the calibration line approximation. The values obtained by applying the latter approach were more reliable and consistent with the actual statistical theory of detection decisions and yielded the following concentrations: C_8, 87.6 μg/l; C_{10}, 45.2 μg/l; C_{11}, 39.9 μg/l; C_{12}, 37.7 μg/l; C_{14}, 41.4 μg/l and C_{16}, 40.6 μg/l. Two different gas–liquid chromatographic columns were tested and similar results achieved, which shows the ruggedness of the method.

Keywords: Long chain fatty acids; Calibration; Linearity; Detection limit

1. Introduction

Among the different kinds of lipidic biomolecules, the most abundant compounds are fats and oils. Fats are the main constituent of the storage fat cells in animals and plants and, chemically, are triacylglycerols, i.e., carboxylic esters derived from the single alcohol, glycerol. Each fat consists of glycerides derived from diverse carboxylic acids with C_8–C_{18}, being the most abundant long chain fatty acids. The diverse chemical composition of fats initiated their use as raw materials for many and distinct industrial products such as foods, detergents, surfactants, or drying oils [1]. Therefore, there are important economic activities surrounding these compounds, which require transportation, storage, treatment of wastes, etc. For this reason, in a 1997 Federal Register announcement [2], the U.S. Environmental Protection Agency (EPA) issued an opinion that non-petroleum oils (i.e., vegetable oils and animal fats) should not be exempted from regulations that govern the cleanup of oil spills. EPA's Office of Research and Development (ORD) is responsible for conducting research that addresses the issue of non-petroleum oils and to obtain scientifically sound information on the fate and effects of such oils in the environment, with special attention paid to the biodegradability and toxicity of vegetable oil before, during, and after exposure to degrading microbial populations in the aqueous phase.

The principal components of vegetable oils are triglycerides, and their main intermediates during the biological activity are long chain fatty acids [3,4]. Several methods have been proposed for analyzing these organics in seawater and sewage sources [5–9]. These procedures involve either liquid–liquid extraction (LLE) or solid phase extraction (SPE), derivatization of the fatty acids, and gas chromatographic separation and detection. Such investigations involve time-consuming procedures, lack a meticulous statistical study of the chemical measurement process (CMP)

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doi:10.1016/j.talanta.2005.06.031

[10], and do not report cumulative blank contributions of palmitic or stearic acid. Hence there is a need for a procedure to analyze fatty acids in water. When dealing with new methods, the first action consists of determining the physical and chemical properties of the target compound, its matrix, and the estimated concentration in which the former is present in the latter. Then the analytical procedure can be designed in order to establish a CMP. In the core of this process, two main issues are essential: precision and accuracy. For this reason, the quantitative potential of the method has to be corroborated by means of an entire evaluation of its efficiency and validation. This step requires the definition of a set of performance criteria. The primary criteria are precision, bias, accuracy, and the detection limit. The secondary criteria are linearity, range, quantification limit, selectivity, sensitivity, and ruggedness ([11], Chapter 13). Not all the performance characteristics are determined in preliminary studies. At this point, the indispensable criteria are precision, which is given by repeatability, linearity, and linear range, estimated from the regression analysis of the calibration curve, and the lowest limits of the method, obtained from blank measurements, low concentration samples or calibration curves [12].

The objective of this work is to carry out a detailed evaluation of a CMP for quantifying long chain fatty acids in water (C_8–C_{16}). Due to the low solubility of these compounds in aqueous matrices [13], a calibration procedure is designed in the parts-per-billion concentration range (μg/l). This protocol consists of derivatization of the fatty acids to obtain the corresponding fatty acid methyl esters (FAMEs), separation by gas chromatography, and detection by mass spectrometry (GC/MS). The derivatization step is required due to the presence of carboxylic groups, which lack of a suitable gas chromatography behavior. The procedure transforms the acids into methyl esters that possess both lower polarity and vapor pressure, improving their separation and quantification by GC [14,15]. The results of the quantification and detection limits will be very useful to predict and optimize an SPE procedure for the analysis of large volume environmental samples [16].

2. Experimental

2.1. Chemicals

The target compounds caprylic (C_8), capric (C_{10}), undecanoic acid (C_{11}), lauric (C_{12}), myristic (C_{14}), and palmitic (C_{16}) acids, and the internal standard, tridecanoic acid (C_{13}), were purchased from Sigma (St. Louis, MO, USA). All the fatty acids were analytical reagent grade with minimum 99% purity. The boron trifluoride (BF_3) methanol solution was acquired from Aldrich (Milwaukee, WI, USA). Methanol, methylene chloride, and hexane were obtained as Optima grade from Fisher Scientific (Pittsburg, PA, USA).

2.2. Standard solutions and derivatization

Two sets of stock solutions were prepared using methanol as solvent: (a) the target compounds (20 and 5 mg/l), and (b) the internal standard (IS, 100 mg/l). Six standard solutions (1000, 400, 200, 100, 50, and 25 μg/l) containing the target compounds were prepared to perform the calibration of the GC/MS. The IS was added at a concentration of 500 μg/l in all of the calibration standards. The solutions were prepared in 2 ml Pyrex® flasks with caps (Corning, NY, USA), by spiking the desired concentration of stock solution in 2 ml of methylene chloride. These samples were evaporated to dryness under a gentle stream of nitrogen. After the evaporation step, 0.5 ml of BF_3 methanolic solution was added and the flask was then heated at 55 °C for 30 min. After cooling, 1 ml of hexane was added and the reaction flask was then shaken for 30 s in a Vortex mixer. The organic extract was transferred to an autosampler vial and analyzed. Derivatization blanks were prepared in the same way but the internal standard was the only spiked compound.

2.3. Chromatographic equipment and experimental conditions

GC/MS analyses were performed with an Agilent (Palo Alto, CA, USA) 6890 Series GC system equipped with a 7683 Series injector and a 5973 Network Mass Selective Detector. One microliter of the extract was injected in pulsed on-column mode in a single taper direct connect liner, 4 mm i.d., obtained from Agilent (Palo Alto, CA, USA). The liner, following an in-house procedure, was deactivated and packed with 5 mg of glass wool before every run. The inlet temperature was 310 °C. The carrier gas was ultra high purity helium.

Two different chromatography columns were tested. The first column was a 30 m HP-5MS (J&W, Palo Alto, CA, USA) (cross-linked 5% Phenyl Methyl Siloxane; 0.250 mm i.d., 0.25 μm film thickness). The GC oven temperature was maintained at 50 °C for the first minute, then ramped to 300 °C at 10 °C/min and kept at 300 °C for 15 min. The flow rate of helium flow was 1 ml/min. The second column was a SP-2380 (Supleco, Bellefonte, PA, USA) (30 m, 0.25 mm i.d., 0.20 μm film thickness). For this column, the GC oven temperature was maintained at 50 °C for the first minute, then first ramped to 170 °C at 5 °C/min and then to 250 at 20 °C/min. It was kept at 250 °C for 15 min. The flow rate of helium flow was 0.9 ml/min.

Detection and data acquisition was performed in selected ion monitoring mode (SIM) under a dynamic mass calibration. Under this mode the mass spectrometer is able to determine a SIM ion value to within 0.1 amu. The target molecular ions for the compounds (C_8–C_{16}) were selected to be (m/z) M^+ $[M-43]^+$, 87, and 74. The mass spectrometer parameters were: interface temperature 300 °C, ion source 230 °C, and quadrupole 150 °C. The ionization energy was 70 eV. The software used for the control of the GC/MS and for data acquisition was Environmental ChemStation G1701CA

by Agilent Technologies (Palo Alto, CA, USA). The statistical treatment of the data was done with Microsoft® Excel 2002.

3. Results and discussion

3.1. Calibration procedure

Each fatty acid was quantified using a six-point calibration of mixed standard solutions covering a concentration range from 25 to 1000 μg/l as described above. The internal standard method was used in the quantitation. A linear regression model of the response ratio, RR (standard response/IS response) against concentration ratio, CR (standard concentration/IS concentration) with intercept was chosen. The data were fitted using an unweighted least square curve. Three assumptions were made, i.e., measurement errors followed a Gaussian distribution, variances were independent of the concentration (condition of homoscedasticity) and the detection of a multicomponent sample was approached by treating each compound as a pseudo one-component problem. Each calibration event was performed by injection, in a randomized arrangement, of duplicates for each concentration level. Two derivatization blanks were also analyzed. In this way, instrument drifts taking place during the calibration run would not affect the pure error variance [17]. When dealing with on-column or splitless injection modes ([18], Chapter D6), matrix effects may affect the quantitative results. For this reason, a matrix enhancement technique was used for calibration at these low levels. A real sample, an extract from sediments without fatty acids, was injected at the beginning of the sequence and in between each standard. The compounds in the extract passivate the liner, and the adsorption of the FAMEs on its surface is minimized. As an added benefit, carry-over contamination was avoided. A total ion chromatogram for a 400 μg/l standard is shown in Fig. 1.

Two calibration events were carried out with each of the capillary columns described previously. The parameters obtained are presented in Table 1. The data show that the assumption of linearity is valid in the concentration range studied, since the $F_{0.05;4,6}$ values for the lack-of-fit tests are below 4.757. The slopes for C_8 and C_{10} were lower than the ones obtained for the rest of the esters, which means that the sensitivity of the MS decreases for these compounds. This can be further explained by the mass discretion effect obtained in the chromatograms for the shorter FAMEs. This phenomenon occurred because losses through the septum purge are larger for the volatile components while the ones with high-boiling points are partly retained on surfaces in the liner and glass wool and diffuse less rapidly ([18], Chapter D2).

A Cochran's test ([11], Chapter 6) was performed to ensure the homoscedasticity of the results. Four replicates of the highest and the lowest calibration points were injected three times. The results are shown in Table 2. It follows that the data are considered to be homoscedastic since the Cochran criterion, C, was lower than the critical value 0.768 (C critical value (3,4) = 0.768 at 5% level of significance). The normality and homoscedasticity of data ratios for an internal standard least squares calibration can be ensured by means of the internal standard R.S.D. [19]. In the calibration events, the R.S.D. for the internal standard response was below 5% (data not shown). Such a low R.S.D. value guarantees the assumptions of normality and homoscedasticity.

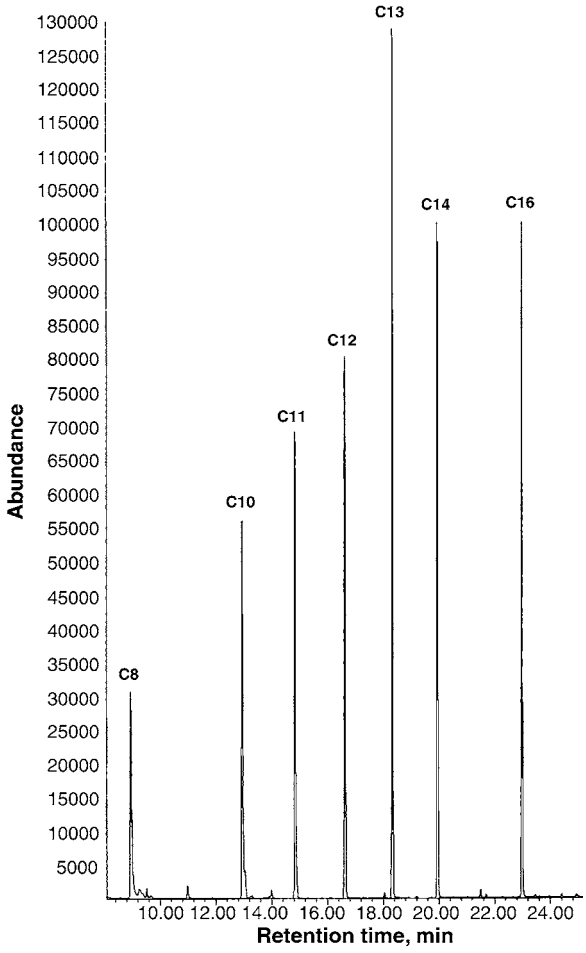

Fig. 1. Total ion chromatogram for 400 μg/l FAME standard. Internal standard (C_{13}) 500 μg/l.

The response ratios of the standard samples in the Cochran's test were used to check the repeatability of the method. The R.S.D. for the 25 and 1000 μg/l levels are included in Table 2, indicating an acceptable repeatability of the method.

Finally, the data from the four calibration events were fitted to the *zero intercept model* by testing the hypothesis that the intercept does not differ from zero [20]. All the calibration curves passed the test except C_{16} (see Table 3). This fact agrees with the values of the intercept (see Table 1) obtained for this analyte, which are always positive and larger than the results for the rest of the methyl esters. It is speculated that this behavior could be due to background contamination of palmitic acid. This will be explained latter.

Table 1
Statistical parameters of response ratio against concentration ratio

FAME	a[a]	s_a[b]	b[c]	s_b[d]	r[e]	s_{yx}[f]	F[g]
C_8	0.0074	0.0082	0.4200	0.0091	0.995	0.0214	0.61
	−0.0073	0.0055	0.4518	0.0061	0.998	0.0143	0.19
	−0.0068	0.0069	0.5537	0.0077	0.998	0.0181	1.56
	−0.0300	0.0115	0.4584	0.0128	0.992	0.0300	4.18
C_{10}	−0.0006	0.0021	0.3112	0.0023	0.999	0.0054	1.05
	−0.0064	0.0032	0.3234	0.0036	0.999	0.0084	0.38
	−0.0018	0.0022	0.3876	0.0024	1.000	0.0056	1.19
	0.0054	0.0036	0.3371	0.0040	0.999	0.0094	3.62
C_{11}	−0.0009	0.0042	0.7385	0.0047	1.000	0.0110	2.01
	−0.0113	0.0073	0.7541	0.0082	0.999	0.0192	0.62
	−0.0027	0.0028	0.8877	0.0031	1.000	0.0073	1.04
	−0.0007	0.0064	0.8201	0.0071	0.999	0.0167	0.06
C_{12}	0.0019	0.0029	0.7583	0.0033	1.000	0.0077	0.96
	−0.0047	0.0072	0.7644	0.0080	0.999	0.0187	0.66
	0.0024	0.0047	0.8947	0.0052	1.000	0.0121	0.93
	−0.0009	0.0074	0.8663	0.0082	0.999	0.0192	0.33
C_{14}	0.0029	0.0031	0.7702	0.0034	1.000	0.0080	0.72
	−0.0029	0.0066	0.7557	0.0073	0.999	0.0171	0.26
	0.0174	0.0052	0.9060	0.0058	1.000	0.0137	0.49
	0.0087	0.0106	0.9333	0.0118	0.998	0.0277	2.66
C_{16}	0.0239	0.0048	0.7348	0.0053	0.999	0.0124	0.47
	0.0146	0.0062	0.7023	0.0069	0.999	0.0162	0.24
	0.0360	0.0056	0.8662	0.0062	0.999	0.0145	1.45
	0.0209	0.0087	0.9160	0.0097	0.999	0.0228	4.29

For each FAME, the two first lines correspond to HP-5 column and the last two to SP column.
[a] a, intercept.
[b] s_a, intercept standard deviation.
[c] b, slope.
[d] s_b, slope standard deviation.
[e] r, correlation coefficient.
[f] s_{yx}, regression standard deviation.
[g] F-ratio for lack-of-fit test. Significant level $F_{0.05;4,6} = 4.757$ [11].

Table 3
Statistical parameters of response ratio against concentration ratio for the zero intercept model

FAME	b[a]	s_b[b]	r[c]	s_{yx}[d]	F[e]
C_8	0.4254	0.0068	0.995	0.0212	0.81
	0.4464	0.0047	0.998	0.0148	1.78
	0.5488	0.0058	0.998	0.0180	0.96
	0.4365	0.0119	0.997	0.0371	4.83
C_{10}	0.3108	0.0017	0.999	0.0052	0.08
	0.3139	0.0030	0.998	0.0095	3.94
	0.3862	0.0018	1.000	0.0056	0.00
	0.3411	0.0032	0.998	0.0099	2.23
C_{11}	0.7378	0.0034	1.000	0.0105	0.05
	0.7536	0.0053	0.999	0.0165	0.19
	0.8857	0.0023	1.000	0.0073	0.96
	0.8195	0.0051	0.999	0.0159	0.01
C_{12}	0.7597	0.0024	1.000	0.0075	0.40
	0.7609	0.0059	0.999	0.0182	0.43
	0.8965	0.0038	1.000	0.0117	0.27
	0.8657	0.0059	0.999	0.0183	0.01
C_{14}	0.7724	0.0026	1.000	0.0080	0.89
	0.7536	0.0053	0.999	0.0165	0.19
	0.9187	0.0061	0.999	0.0189	1.09
	0.9397	0.0088	0.998	0.0273	0.68
C_{16}	0.7523	0.0071	0.998	0.0212	25.28
	0.7130	0.0062	0.998	0.0193	5.51
	0.8926	0.0101	0.997	0.0315	41.83
	0.9313	0.0088	0.998	0.0273	5.75

For each FAME, the two first lines correspond to HP-5 column and the last two to SP column.
[a] b, slope.
[b] s_b, slope standard deviation.
[c] r, correlation coefficient.
[d] s_{yx}, regression standard deviation.
[e] F-ratio for zero intercept model. Significant level $F_{0.05;1,10} = 4.965$ [20].

3.2. Detection and quantification limits

The detection limit is defined as that concentration which gives an instrument signal significantly different from the blank signal ([21], Chapter 5). The ambiguity of the words "significantly different" has led to several interpretations and definitions. In 1995, IUPAC [10] gave recommendations regarding detection and quantification capabilities of analytical methods. That document included the definitions of critical value, L_C, detection limit, L_D, and quantification limit, L_Q (and their equivalents in the concentration domain: x_C, x_D, and x_Q, respectively) derived from the theory of hypothesis testing and the probability of false positives and false negatives. This requires a good estimate of the mean and the standard deviation of the blank. However, it is well known that for analytical methods, which involve the measurement of a peak on a noise base line (e.g. chromatography), the computation of uncertainties and detection limits applying the IUPAC criteria requires a complex and difficult treatment of the signal [22,23]. Three approaches may be applied to avoid such problems when computing detection limits. The first is the method detection limit (MDL) [24], the second is the determination from linear calibration curves [25], and the third is the integration of the background noise by using extrapolated values of the base–peak width at low concentrations [26]. Finally, Kaus [27] did not recommend the calculation of the detection limit from blank analysis results because these data differ from the statistical sample of the calibration standards and are often not normally distributed.

Table 2
Cochran's test results for 25 and 1000 μg/l and repeatability of the method at these levels

FAME	C 25 μg/l[a]	R.S.D. 25	C 1000 μg/l[a]	R.S.D. 1000
C_8	0.369	6.4	0.608	4.4
C_{10}	0.602	5.1	0.474	2.0
C_{11}	0.737	4.8	0.558	1.7
C_{12}	0.768	7.4	0.293	1.7
C_{14}	0.477	10.6	0.639	2.1
C_{16}	0.447	17.2	0.666	2.4

[a] Cochran's C; C critical value (3,4) = 0.768 at 5% level of significance [11].

To calculate the MDL, four standard samples, 25 μg/l in concentration, were prepared and then injected three times per sample. The MDL was obtained by applying Eq. (1) below:

$$\mathrm{MDL} = t_{0.01;n-1}s \tag{1}$$

where t is Student's t value at $n-1$ degrees of freedom ($n=4$) and $\alpha=0.01$ (one-sided), and s is the standard deviation of the four replicates. The calculation of the concentration limits from the calibration curves shown above was performed following the statistical approach developed by Vogelsand and Hädrich [28]. The critical limit (x_C) was calculated using Eqs. (2) and (3):

$$y_C = a + s_y t_{f;\alpha}\sqrt{1+\frac{1}{n}+\frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}} \tag{2}$$

$$x_C = \frac{(y_C-a)}{b} = \frac{s_y}{b}t_{f;\alpha}\sqrt{1+\frac{1}{n}+\frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}} \tag{3}$$

where y_C: upper confidence limit (one-tailed) when the analyte concentration is zero, a: intercept of the calibration curve, b: slope of the calibration curve, s_y: residual standard deviation, $t_{f;\alpha}$ = quantile of t distribution single-sided for $f=n-2$ degrees of freedom and probability of 95%, and $\bar{x}=$ mean value of the concentration ratios for all the calibration points.

To compute the detection limit (x_D), the value was assumed to be twice as high as x_C. This approximation yielded similar results to the one proposed by the AOAC ([11], Chapter 13). Finally, the lower edge of the Gaussian distribution around the quantification limit (x_Q) is x_C. The quantification limit was calculated considering the confidence limits at a particular concentration ratio for a linear calibration (Eqs. (4) and (5)):

$$y_Q = \bar{y} + b(x_D-\bar{x}) + s_y t_{f;\alpha}\sqrt{1+\frac{1}{n}+\frac{(x_D-\bar{x})^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}} \tag{4}$$

where y_Q: predicted response value for the detection limit. $\bar{y}=$ mean value of the response ratio of all calibration points.

$$x_Q = \frac{y_Q-a}{b} \tag{5}$$

The results from the four calibration events (two with each column) and the MDL from the repetitive injection of the 25 μg/l standards are given in Table 4. It has been found that, when comparing the results based on the regression approach (x_C, x_D, x_Q), the values are different. This result is expected because the limits are calculated depending upon the confidence interval of the concentration ratio (from 25 to 1000 μg/l) through inversion of the linear calibration. In other words, y_C and x_D are random variables and estimates only. That is why different results are obtained for each realization of the calibration curve [29]. The critical limit, x_C is close to the lowest calibration point (25 μg/l) for all the compounds except for C_8, which is higher. This could be due to a combination of a lower sensitivity and a higher residual standard deviation. No significant differences were found when comparing the performance of the two columns ($p>0.05$).

Table 4
Analytes detection limits from regression approach and method detection limit from 25 μg/l standards

FAME	x_C (μg/l)	x_D (μg/l)	x_Q (μg/l)	MDL (μg/l)
C_8	49.4	98.8	147.4	3.1
	30.7	61.4	91.8	
	31.7	63.3	94.6	
	63.5	126.9	189.2	
C_{10}	16.9	33.8	50.6	1.6
	25.2	50.4	75.4	
	21.0	42.1	63.0	
	27.2	54.3	81.2	
C_{11}	14.4	28.8	43.2	3.5
	24.7	49.3	73.8	
	21.0	41.9	62.7	
	19.8	39.5	59.1	
C_{12}	9.8	19.7	29.5	7.3
	23.8	47.5	71.1	
	20.3	40.6	60.8	
	21.5	43.0	64.4	
C_{14}	10.1	20.3	30.4	10.8
	23.8	47.5	69.0	
	20.0	40.1	60.0	
	28.8	57.6	86.2	
C_{16}	16.4	32.8	49.1	26.0
	22.4	44.9	67.2	
	18.2	36.4	54.5	
	24.0	48.1	64.1	

x_C: critical limit; x_D: detection limit; x_Q: quantification limit; MDL: method detection limit. For each FAME, the two first lines correspond to HP-5 column and the last two to SP column.

The MDL, by definition, is equivalent to the critical value established by IUPAC, i.e., the x_C obtained by the calibration approach in this paper. The results are based on the standard deviation of the mean values of the four standards for the lowest calibration point. In this case, because the analytical method achieved a high repeatability (see Table 2), the MDLs for all the esters are much lower than the statistical detection limits but for palmitic acid. Nevertheless, these values are less reliable than the ones obtained from the calibration curve (x_C, x_D, x_Q, see Table 4), because the MDL procedure does not determine whether the variance is a function of the analytes' concentration. Besides, it was found that several derivatization blanks had a higher concentration for the C_{16} methyl ester than the MDL (see Table 4). In addition, this value is the highest one among the analytes due to the random background contamination of this organic, which affects its standard deviation. Finally, Kirchmer [30] claims MDL is misleading because the value depends upon instrument sensitivity, the nature of the samples, and the skill of the analyst. However, AOAC [31], IUPAC [32], and the German

Normative Institute [33] have recommended the regression approach. In the present case, the regression approach yielded the following detection limits for the analytes: C_8, 87.6 μg/l; C_{10}, 45.2 μg/l; C_{11}, 39.9 μg/l; C_{12}, 37.7 μg/l; C_{14}, 41.4 μg/l and C_{16}, 40.6 μg/l.

Lower detection limit concentrations than the values presented here have been reported [34]; i.e., C_8, 10 μg/l; C_{10}, 12 μg/l; C_{12}, 10 μg/l; C_{14}, 14 μg/l and C_{16}, 8 μg/l. Nevertheless, those results require several observations. The authors used the third approach described above, which is based on the integration of the background noise. In this approach the detection limit was calculated by using an IUPAC definition from the 1970s, namely, three times the standard deviation of the blank responses divided by the sensitivity of the analytical method ([11], Chapter 13), which is not the recommended one for this organization in 1995 [10]. The standard deviation used to compute the detection limit was not obtained from the blank responses, as required, but estimated from the calibration curve at zero concentration, which can only be determined by an approximate formula ([21], Chapter 5) and strongly depends upon the number of calibration points and replicates analyzed and not on the blank signal itself. The calibration curves were run in a high concentration range from 2.5 to 10 mg/l and with a single injection for each calibration point. The homoscedasticity was not studied in spite of the high concentration levels. The chromatographic parameters were not appropriate for studies in the trace level—injection volume was 2 μl in splitless mode and the detection was in SIM, but no dynamic mass calibration of the MS was performed. Finally, the detection limit, as calculated from the old definition, provides protection against type I errors (false positive), yet it does not take in account type II (false negative). For these reasons, the detection limits presented in this paper are more reliable and consistent with the actual statistical theories about detection limit and the related quantities.

3.3. Derivatization blank

The analysis of fatty acids is complicated because they are omnipresent in nature and are constituents of commercial plastics, surfactants, and lubricants [35]. For this reason, several researchers [36–39] have reported cumulative blank contributions of palmitic acid that can exceed the quantity of these compounds in the analyzed samples. In the present case, this issue is critical due to the low concentration range studied. The effect of the C_{16} background contamination can be determined by computing the response factor (RF) during the calibration events (see Table 5). From Table 5, the RF values for the palmitic acid values increased with decreases in the concentrations of standards. This suggests a dramatic impact of contamination in the lowest concentration range. However, the concentration values for C_{16} in the derivatization blanks were not relevant. The described calibration procedure is reliable when plastic products are avoided and all the glassware used is cleaned.

Table 5
C_{16} FAME response factors for different calibration curves

Concentration (μg/l)	C_{16} FAME response factor			
1000	0.737	0.758	0.725	0.692
400	0.762	0.750	0.730	0.734
200	0.832	0.786	0.722	0.728
100	0.912	0.862	0.770	0.724
50	0.882	0.916	0.940	0.861
25	1.120	1.338	1.004	0.945

3.4. Ruggedness of the method

The comparison of two capillary columns provided an idea of how robust the method is. In this case, the stationary phases are different, although both are poly(siloxanes). The HP-5MS has poly(dimethyldiphenylsiloxane) (PMPS-5) as the liquid phase, while the SP-2380 is coated with poly(90% biscyanopropyl/10% cyanopropylsiloxane), a poly(cyanopropylsiloxane) (PCPS). The difference lies in the nature of the substituted radical in the polymer chain, which leads to different solvation properties of the stationary phase. In this case, the selectivity of both columns towards the FAMEs is distinct because the presence of diphenylsiloxanes increases the ability of the stationary phase to take part in dipole-type interactions. However, the introduction of cyanoalkyl groups augments the dipole-type interactions as well as its hydrogen-bond basicity ([40], Chapter 2). In other words, the HP-5MS column has a stationary phase with lower polarity than the SP-2380 one, and, therefore, the selectivity of the chromatographic system is different. As was noted in earlier sections, no relevant differences have been detected between both columns; consequently, the ruggedness of the method is demonstrated.

4. Conclusions

A calibration procedure is provided for the quantitation of long chain fatty acids in the trace concentration range based on their solubility values in water. The efficiency was evaluated by means of an in-depth study of precision, linearity, range, detection, and quantification limits. All of them were obtained from the calibration data, which passed all the required statistical tests such as lack of fit, homoscedasticity, and ruggedness. The critical value and the detection limit of the method were calculated using the MDL and the regression approach, the latter one being more reliable and rigorous from a statistical point of view. All these results were possible because a careful selection of chromatographic parameters, like on-column injection, matrix enhancement technique, and dynamic mass calibration, was performed. The detection limits for the analytes were C_8, 87.6 μg/l; C_{10}, 45.2 μg/l; C_{11}, 39.9 μg/l; C_{12}, 37.7 μg/l; C_{14}, 41.4 μg/l and C_{16}, 40.6 μg/l.

The ruggedness of the method was demonstrated by comparing the performance of two different stationary phases, which yielded similar results.

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