Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 194–199
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Evaluation of five methods for derivatization and GC determination of a mixture of very long chain fatty acids ($C_{24:0}$–$C_{36:0}$)

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Received 15 May 2007; received in revised form 31 August 2007; accepted 8 September 2007
Available online 19 September 2007

Abstract

D003 is a new active ingredient consisting of a mixture of very long chain saturated fatty acids ($C_{24:0}$–$C_{36:0}$) in a definite proportion, which shows antioxidant, antiosteoporotic, antiplatelet and cholesterol-lowering effects in experimental models. Five derivatization methods for determining these fatty acids by gas chromatography (GC), using diazomethane, sulphuric acid–methanol, hydrochloric acid–methanol, boron trifluoride–methanol and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide were evaluated. GC analysis was carried out using a BPX-5 wide-bore column and 1-nonadecanoic acid ($C_{19:0}$) as internal standard. Methods were similar on account of the fatty acid content determined (84.2–86.6%). However, whereas the hydrochloric acid–methanol method needed 90 min to complete the derivatization, the other methods only required 10 min. Considering costs, speed, safety and GC response, the method using sulphuric acid–methanol was found the most appropriate for determining these fatty acids. The validation of this method: linearity over a range 40–160%, accuracy assessed through a recovery study, precision within day and inter-day, and specificity, even for samples subject to stress conditions, proved it is suitable for quality control and stability studies of the very long chain fatty acids composing this active ingredient.

Keywords: D003; Very long chain fatty acids; Derivatization methods; Capillary gas chromatography; Validation

1. Introduction

Although some reports exist about the determination of long chain fatty acids (LFAs, from 12 to 24 carbon atoms) by high-pressure liquid chromatograpy, gas chromatography (GC) is the technique most widely used with this aim. In this sense, LFAs must be converted to convenient volatile derivatives previous to their analysis. There are many derivatization methods for GC, the majority of them will function quite well when care is taken to use properly [1]. Initially, two organisations that mark rules in the analytic methods: Association of Official Analytical Chemists and American Oil Chemists Society, recommended the use of sulphuric acid–methanol reagent [2,3] for preparing fatty acids methyl esters (FAMEs). However, both organisations accepted later the use of the boron trifluoride–methanol reagent [4,5]. Other methods for methylation of LFAs with good results involve the use of hydrochloric acid–methanol [6], and diazomethane [7], whereas, the methods that employ silylating agents are less used [8].

On the contrary, the GC analysis of very long chain fatty acids (VLFAs, higher than 24 carbons atoms) has had a little interest. This is probably because the VLFAs are less common in the human diet, nor have had a pharmacological interest. However, the development of D003 active ingredient, purified from sugar cane (*Saccharum officinarum* L.) wax has caused a turn on this topic. This natural product consists of a mixture of free saturated VLFAs, from 24 to 36 carbon atoms [9], in a definite proportion with cholesterol-lowering, antioxidant, antiplatelet [10,11], and antiosteoporotic effects [12,13].

As part of the chemical characterization and quality control of D003 at research and development stage, appropriate GC analytic methods were validated for the determination of its content of VLFAs [14–16]. To our knowledge, all these methods are the first in which VLFAs are derivatized to FAMEs using the hydrochloric acid–methanol reagent. However, because of the long time consumption of this acid-catalyzed reaction, with the subsequent delay to deliver the quantitative result, these methods were not considered practical for the routine of quality control.

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doi:10.1016/j.jpba.2007.09.015

In this sense, taking into account the experience provided by previous GC works with LFAs, other derivatization methods for the analysis of D003 were studied.

The GC determinations of these VLFAs, after derivatization using diazomethane, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), sulphuric acid–methanol, boron trifluoride–methanol, and hydrochloric acid–methanol reagents, including a kinetic evaluation of these reactions, are shown in this paper. The validation of the GC method using the derivatization process that was found as more suitable for this active ingredient is also presented.

2. Experimental

2.1. Materials

D003 (batch 990703) was provided by the National Center for Scientific Research (Havana, Cuba); all other chemicals were analytical reagent grade: hydrochloric acid (37%), sulphuric acid (98%), methanol, toluene, ether, boron trifluoride–methanol (14% solution in methanol), hydrochloric acid (0.1 M), hydrogen peroxide (30%), sodium hydroxide (0.1 M), *n*-hexane, chloroform and sodium hydroxide (99%, Merck, Darmstadt, Germany), and MSTFA (Sigma, St. Louis, USA). The diazomethane was generated from *N*-methyl-3-nitro-1-nitrosoguanidine (99%, Riedel-de-Haën, Seelze, Germany) in a mini-diazomethane generator.

Stock solution comprised of tetracosanoic ($C_{24:0}$), pentacosanoic ($C_{25:0}$), hexacosanoic ($C_{26:0}$), heptacosanoic ($C_{27:0}$), octacosanoic ($C_{28:0}$), nonacosanoic ($C_{29:0}$), triacontanoic ($C_{30:0}$) and hentriacontanoic ($C_{31:0}$) acids (Sigma, St. Louis, USA) was prepared as previously described [15].

The nonadecanoic acid ($C_{19:0}$), approximately 99% pure by GC (Sigma, St. Louis, USA), was used as internal standard (IS) at 1 mg ml^{-1} in two solutions, one in chloroform (IS solution A) and another in *n*-hexane (IS solution B). These solutions were found to be stable for at least 1 month when stored at +8 °C.

2.2. Chromatographic conditions

The GC system consisted of a GC-14B with a flame ionization detector (Shimadzu, Kyoto, Japan). A BPX-5 wide-bore fused silica capillary column (25 m, 0.53 mm i.d., 1.0 μm D_f; SGE, Texas, USA) was used, from 220 °C to 320 °C at 5 °C min^{-1} and isothermal for 10 min at 320 °C. Injector and detector were set at 320 °C. Carrier gas (H_2) flow was 11 ml min^{-1}. To form the flame, hydrogen gas flow, 40 ml min^{-1}, and air gas flow, 400 ml min^{-1}, were used.

The GC–Mass Spectrometry system (GC/MS) consisted of a GC 8000 coupled to a MD800 series (Fisons, Manchester, England) with a capillary column SPB-5 (30 m, 0.25 mm i.d. and 0.25 μm D_f; Supelco, Bellefonte, USA). Operating conditions: column programmed from 100 °C to 200 °C at 40 °C min^{-1}, from 200 °C to 320 °C at 10 °C min^{-1} and isothermal for 30 min at 320 °C. Helium carrier gas flow was 1 ml min^{-1}. Injector, ion source, and interface temperatures were 320 °C, 250 °C, and 250 °C, respectively. Ionization energy was 70 eV. The mass spectrum was continuously acquired from 40 to 600*m*/*z* with a scan speed of 1 s/decade in full scan mode.

2.3. Sample preparation

Hydrochloric acid–methanol: 1 ml of the IS solution A was added into a 4 ml vial containing previously 10 mg of D003, then the solvent was evaporated to dryness at 80 °C under a gentle air flow. One millilitre of the methylating reagent (5% aqueous hydrochloric acid–methanol, v/v) was added. The vial was heated at 80 °C with occasional shaking. Afterwards, the sample was evaporated to dryness at 80 °C under a gentle air flow. Then, 1 ml of toluene was added and the vial was again tightly closed and heated at 80 °C for 3 min.

Diazomethane: 1 ml of the IS solution A was added into a 4 ml vial containing previously 10 mg of D003, then the solvent was evaporated to dryness at 80 °C under a gentle air flow. One millilitre of the ethereal diazomethane reagent was added. The vial was left at room temperature. Afterwards, the sample was evaporated to dryness at 45 °C. Then, 1 ml of *n*-hexane was added and the vial was heated at 80 °C for 3 min.

Boron trifluoride–methanol: 1 ml of the IS solution A was added into a 4 ml vial containing previously 10 mg of D003, then the solvent was evaporated to dryness at 80 °C under a gentle air flow. One millilitre of the methylating reagent (methanol containing 14% (w/v) boron trifluoride) and 1 ml of *n*-hexane were added. The vial was heated at 60 °C with occasional shaking. Before the analysis of the *n*-hexane phase the sample was allowed to rest for 5 min.

Sulphuric acid–methanol: 1 ml of the IS solution B and 1 ml of the methylating reagent (2% sulphuric acid–methanol, v/v) were added into a 4 ml vial containing previously 10 mg of D003. The vial was heated at 80 °C with occasional shaking. Afterwards, 0.25 ml of the neutralising aqueous solution (sodium hydroxide at 1 M) was added and it was smoothly shaken. Before the analysis of the *n*-hexane phase the sample was allowed to rest for 5 min.

MSTFA: 1 ml of the IS solution A and 50 μl of MSTFA were added into a 4 ml vial containing 10 mg of D003 and it was heated at 60 °C.

In all cases five reaction times were evaluated and 1 μl portions were analysed by GC.

2.4. Identification and calibration

FAME identification criterion was the relative retention calculated from a D003 sample, which was previously analysed by GC/MS. Quantitative analysis was based on the IS method, previous determination of the relative mass response factor (f_i^m) from samples prepared using the stock and the IS solutions, according to the following equation:

$$f_i^m = \frac{A_{is} \times m_i}{A_i \times m_{is}}$$

where A_{is} is the peak area of the IS, m_i the mass of component i (mg), A_i the peak area of the component i and m_{is} is the mass of IS (mg).

The content (%) of each acid in this active ingredient was calculated through the following equation:

$$C_i\,(\%) = \frac{A_i f_i^{\mathrm{m}} m_{\mathrm{is}}}{A_{\mathrm{is}} m_{\mathrm{m}}} \times 100$$

where C_i is the content of component i (%) and m_{m} is the mass of D003 sample (mg).

Because of commercial standard of the acids from $C_{32:0}$ to $C_{36:0}$ acids were unavailable, the f_i^{m} of $C_{30:0}$ was used for the quantitative analysis of the even acids and the f_i^{m} of $C_{31:0}$ for the odd ones. The total content (%) of VLFA in D003 was determined by the summation of each acid percentage.

2.5. Kinetic evaluation of the methylating reactions

In order to determine the time that each method requires for completing the derivatization process, the next reaction times: 10, 30, 60, 90 and 120 min were evaluated ($n = 5$). To compare the results of the five evaluated times for each method, the Student's t-test was applied for dependent samples ($P = 0.05$).

2.6. Validation of test procedure

The method using the methylation process found as more practical was subject to validation following recommendations of the International Conference on Harmonisation (ICH) [17].

2.6.1. Specificity

To stimulate the formation of degradation products, D003 active ingredient was subject to thermolysis (105 °C, 2 weeks), base and acid hydrolysis (0.1 M sodium hydroxide and 0.1 M hydrochloric acid, at 1 g in 10 ml, at 105 °C, 1 day), oxidation (30% hydrogen peroxide, at 1 g in 10 ml, at 25 °C, 1 week), and photolysis (254 nm UV light, at 25 °C, 1 week). These tests were performed in neutral glass ampoules, which were flushed with nitrogen and sealed ($n = 3$). Chromatograms of the IS, D003, and D003 stressed under degradation conditions were compared to prove the specificity. Purity of each peak was checked by GC/MS.

2.6.2. Linearity of the method

The linearity was assessed at five concentration levels, from 40% to 160% of the nominal concentration ($n = 3$). For that reason, from a working standard solution of D003 (2.2 mg ml^{-1} in chloroform) the following volumes: 2, 3, 5, 7 and 8 ml were taken and transferred to test tubes. They were evaporated to dryness at 80 °C under a gentle air flow and the procedure continued as previously described.

The regression lines were obtained from total content of VLFAs calculated (y) versus the masses of D003 active ingredient analysed (x). Evaluation was made by linearity and proportionality tests for $P = 0.05$, taking into account the following acceptance criteria: correlation coefficient (r) ≥ 0.99; relative standard deviation of response factor (R.S.D.$_{\mathrm{f}}$) $\leq 5\%$, where response factor is defined as y/x; and relative standard deviation of slope (R.S.D.$_b$) $\leq 2\%$, with

$$\mathrm{R.S.D.}_b\,(\%) = \frac{\mathrm{S.D.}_b}{b} \times 100$$

where b is the slope and S.D.$_b$ is the standard deviation of the slope.

To prove no bias the zero value should be included in the confidence intervals (CI) of the intercept (a), and the CI was calculated as follows:

$$\mathrm{CI} = a \pm t \times \mathrm{S.D.}_a$$

where S.D.$_a$ is the standard deviation of the intercept and Student's t for (0.05; 13).

2.6.3. Accuracy

Accuracy was assessed by a recovery study over the range 97–104% of the nominal concentration. Volumes of 4.0 ml of the working standard solution were spiked with 0.3, 0.4, and 0.5 ml of the stock solution, and another blank group was not spiked ($n = 3$). All the samples were evaporated to dryness at 80 °C under a gentle air flow. Afterwards, the samples were analysed as previously described.

Through the calculated difference on account of VLFA content, between blank and spiked samples, the total content of fatty acids present in each volume of stock solution was determined. Mean recovery was checked to 100% with the Student's t-test for $P = 0.05$. The experimental t (t_{exp}) value was calculated as follows:

$$t_{\mathrm{exp}} = \frac{|100 - \mathrm{recovery}|\,\sqrt{n}}{\mathrm{R.S.D.}}.$$

2.6.4. Precision

An analyst, who performed eight replicates under the same conditions in a day, assessed the repeatability. The R.S.D. values were evaluated by comparison with the Horwitz's criterion [18]. On the other hand, two analysts assessed intermediate precision in 3 days ($n = 5$); significant differences between results were determined by Fisher's (F) and Student's (t) tests for $P = 0.05$.

3. Results and discussion

3.1. Kinetic evaluation of the derivatization processes

The total VLFA content determined in D003 active ingredient was similar by all evaluated methods, within the interval 84.2–86.6% (Table 1), with suitable precisions (R.S.D. < 2%). However, the methods using: diazomethane, boron trifluoride–methanol, MSTFA and sulphuric acid–methanol only required 10 min to complete the methylating reaction, whereas hydrochloric acid–methanol method needed at least 90 min.

The hydrochloric acid–methanol method was the first one used to analyse D003 samples, however, because of its long time-consuming as described above, it was ruled out. Diazomethane, MSTFA and boron trifluoride–methanol methods are fast, but

Table 1
Total VLFA content ± S.D. (%) determined from each time by means of the evaluated methods

Method	Time (min)				
	10	30	60	90	120
Hydrochloric acid–methanol	25.4 ± 0.9[a]	56.7 ± 0.9[b]	82.3 ± 0.96[b]	86.1 ± 0.4[b]	85.9 ± 0.6
MSTFA	84.5 ± 0.6[b]	84.9 ± 0.8[b]	84.2 ± 1.1[b]	85.5 ± 0.6[b]	84.6 ± 0.8
Boron trifluoride–methanol	85.9 ± 0.8[b]	85.9 ± 1.1[b]	85.6 ± 1.0[b]	86.4 ± 0.8[b]	86.4 ± 0.7
Diazomethane	85.2 ± 1.1[b]	85.5 ± 1.6[b]	85.8 ± 1.4[b]	85.1 ± 1.0[b]	85.6 ± 1.3
Sulphuric acid–methanol	86.6 ± 0.6[b]	86.4 ± 0.9[b]	85.5 ± 0.6[b]	85.9 ± 1.0[b]	85.9 ± 1.4

[a] Quantitative result with outstanding difference to the result of the subsequent time ($P < 0.05$).
[b] Quantitative result without outstanding difference to the result of the subsequent time ($P < 0.05$).

they have some inconvenient for routine analysis (e.g. quality control process).

While various papers indicate that the higher acids are not completely esterified by the diazomethane method [1,19], in the case of D003 VLFAs it was just observed the contrary. However, the truth is that diazomethane is an extremely toxic and explosive compound, even when it is diluted into an ethereal solution. It is known that this reagent should be used only if absolutely necessary.

The main drawback of the trimethylsilylation is the hydrolysis of the trimethylsilyl (TMS) derivatives that occurs in the presence of trace amounts of water in the samples [20]. In fact, a decrease in the VLFA peak areas obtained by the MSTFA-method was appreciated when these were compared with the peak areas obtained by other methods. This problem increased with the number of carbon atoms of the chain as observed in works with lower acids as TMS derivatives [21].

As previously mentioned, the boron trifluoride–methanol method allows a fast and effective methylation. However, although that reagent is very popular for FAME preparation, it is expensive, and does not have a long shelf-life, even when refrigerated.

Finally, taking into account that the sulphuric acid–methanol method is fast, it is neither expensive nor dangerous, and it has a good GC response, it was considered as the most appropriate for the routine of D003 quality control. Because of this approach, it was decided to validate this method for VLFA determination in D003 active ingredient using 15 min as derivatization time.

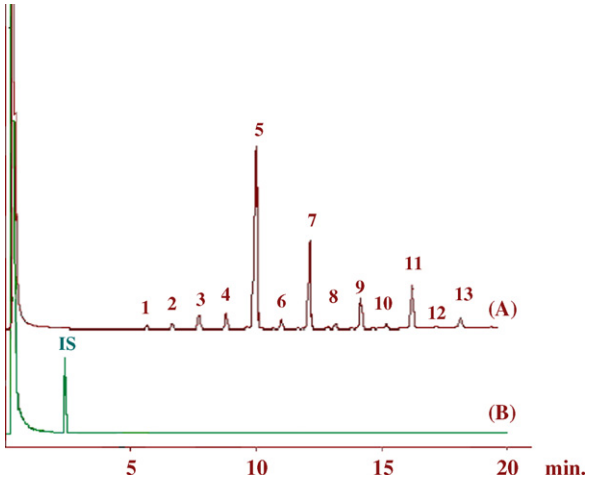

Fig. 1. GC profiles of the D003 sample (A) and IS sample (B) obtained by means of the sulphuric acid–methanol method. Peaks: (1) $C_{24:0}$, (2) $C_{25:0}$, (3) $C_{26:0}$, (4) $C_{27:0}$, (5) $C_{28:0}$, (6) $C_{29:0}$, (7) $C_{30:0}$, (8) $C_{31:0}$, (9) $C_{32:0}$, (10) $C_{33:0}$, (11) $C_{34:0}$, (12) $C_{35:0}$, and (13) $C_{36:0}$.

Table 2
Linearity of determination of VLFAs present in D003 by sulphuric acid–methanol method

VLFA	$y = (b \pm t \times \text{S.D.}_b)x \pm (a \pm t \times \text{S.D.}_a)$	r	R.S.D.$_f$ (%)	R.S.D.$_b$ (%)
$C_{24:0}$	$y = (0.014 \pm 0.001)x + (0.0003 \pm 0.001)$	0.9995	2.0	0.8
$C_{25:0}$	$y = (0.010 \pm 0.0001)x + (0.0001 \pm 0.001)$	0.9992	1.5	0.6
$C_{26:0}$	$y = (0.029 \pm 0.0007)x + (0.0058 \pm 0.007)$	0.9991	2.7	1.1
$C_{27:0}$	$y = (0.024 \pm 0.0007)x + (0.003 \pm 0.008)$	0.9972	2.1	1.3
$C_{28:0}$	$y = (0.323 \pm 0.0069)x + (0.039 \pm 0.076)$	0.9997	1.7	0.9
$C_{29:0}$	$y = (0.017 \pm 0.0007)x - (0.005 \pm 0.008)$	0.9984	3.9	1.5
$C_{30:0}$	$y = (0.178 \pm 0.0001)x + (0.0002 \pm 0.001)$	0.9990	3.9	1.2
$C_{31:0}$	$y = (0.011 \pm 0.0001)x - (0.0002 \pm 0.001)$	0.9997	2.0	0.4
$C_{32:0}$	$y = (0.090 \pm 0.0032)x + (0.030 \pm 0.035)$	0.9974	1.0	2.0
$C_{33:0}$	$y = (0.013 \pm 0.0023)x + (0.008 \pm 0.025)$	0.9991	4.6	0.4
$C_{34:0}$	$y = (0.108 \pm 0.0001)x + (0.002 \pm 0.010)$	0.9988	3.6	1.3
$C_{35:0}$	$y = (0.006 \pm 0.0001)x - (0.002 \pm 0.010)$	0.9987	4.7	1.4
$C_{36:0}$	$y = (0.037 \pm 0.0007)x - (0.003 \pm 0.007)$	0.9996	2.2	1.2
Total	$y = (0.860 \pm 0.020)x + (0.100 \pm 0.210)$	0.9994	1.4	1.1

a: intercept; b: slope; tabulated $t = 2.16$ (0.05; 13); r: correlation coefficient; R.S.D.$_f$: relative standard deviation of response factor; R.S.D.$_b$: relative standard deviation of slope.

Table 3
Accuracy of determination of VLFAs in D003 by sulphuric acid–methanol method

Amount added (mg)	Amount found (mg)			Mean recovery ± S.D. (%)	t_{exp} [a]
	1	2	3		
0.83	0.85	0.82	0.83	100.4 ± 1.8	0.387
1.11	1.12	1.13	1.14	101.8 ± 0.9	3.543
1.38	1.42	1.43	1.39	102.4 ± 1.5	2.847
Total				101.5 ± 1.5	1.755

[a] Experimental t; tabulated $t = 4.303$ (0.05;2); tabulated $t = 2.306$ (0.05; 8).

3.2. Validation of the sulphuric acid–methanol method

3.2.1. Specificity

There was no coincidence among the IS, FAME and other impurity peaks from D003 samples. In addition, new peaks were not observed in the chromatograms of samples subject to stress conditions (Fig. 1). All that was proved by GC/MS analysis, where characteristic fragments of the FAMEs were obtained (mostly, m/z 74, 87, 143 and M^+) and the data were also compared with that observed in mass spectral library. Taking into account these results, the method can also be used in stability studies.

3.2.2. Linearity

The regression line for determining the total VLFA content was $y = (0.86 \pm 0.02)x + (0.10 \pm 0.21)$. Table 2 shows the values obtained from calculating several statistical parameters, which allowed evaluating the linearity of the method for each VLFA and for the total of them. In all the cases the zero was included in the CI of the intercept ($P = 0.05$), thus all the lines passed through the origin. Moreover, r, R.S.D.$_f$ and R.S.D.$_b$ parameters fulfilled the acceptance criteria. Finally, the evaluated method can be considered as linear and proportional in the studied range.

3.2.3. Accuracy

The individual mean recoveries from spiked samples were between 100.4% and 102.4%, whereas the total mean recovery ($n = 9$) was 101.5%. In all cases the t_{exp} values were lower than tabulated t for $P = 0.05$ (Table 3), so the recoveries and 100% value were not significantly different, neither for each concentration nor for the total average recovery. Thus, the method can be considered accurate.

Table 4
Repeatability of determination of VLFAs in D003 by sulphuric acid–methanol method ($n = 8$)

VLFA	Mean ± S.D. (%)	R.S.D. (%)
$C_{24:0}$	1.4 ± 0.03	2.1
$C_{25:0}$	1.0 ± 0.01	1.0
$C_{26:0}$	2.9 ± 0.04	1.38
$C_{27:0}$	2.4 ± 0.04	1.67
$C_{28:0}$	32.3 ± 0.32	0.99
$C_{29:0}$	1.7 ± 0.03	1.76
$C_{30:0}$	17.8 ± 0.19	1.07
$C_{31:0}$	1.1 ± 0.02	1.82
$C_{32:0}$	9.0 ± 0.12	1.33
$C_{33:0}$	1.3 ± 0.02	1.54
$C_{34:0}$	10.8 ± 0.18	1.67
$C_{35:0}$	0.6 ± 0.01	1.66
$C_{36:0}$	3.7 ± 0.07	1.88
Total	86.2 ± 0.88	1.02

Table 5
Intermediate precision of determination of VLFAs in D003 by sulphuric acid–methanol method ($n = 30$)

Analyst	Replicate	Day		
		1 (%)	2 (%)	3 (%)
1	1	85.5	86.1	84.6
	2	86.1	86.4	84.8
	3	85.2	85.3	85.8
	4	85.4	85.2	85.4
	5	84.7	86.4	85.1
2	6	86.1	85.4	85.8
	7	86.5	85.8	84.8
	8	86.0	84.8	86.4
	9	85.2	86.3	84.8
	10	85.3	85.2	85.4
Mean ± $t \times$ S.D./$n^{1/2}$		85.5 ± 0.22		
R.S.D. (%)		0.68		

Tabulated $t = 2.045$ (0.05; 29).

3.2.4. Precision

Good results were obtained in the repeatability study (Table 4), within day R.S.D. values of quantification for each VLFA and the total content of them were lower than the Horwitz's criterion; these results prove that the method is repeatable. No significant differences were found in the intermediate precision (Table 5), obtained from two analysts. It was demonstrated through the experimental F and t values (1.035 and 0.468, respectively), which were lower than the tabulated values (2.460 and 2.045, respectively) for $P = 0.05$.

4. Conclusions

Diazomethane, hydrochloric acid–methanol, boron trifluoride–methanol, MSTFA and sulphuric acid–methanol can be used as methylating reagents for determining the VLFAs from C_{24} to C_{36} that compose D003 active ingredient. However, the hydrochloric acid–methanol needed more time than the others, and the sulphuric acid–methanol reagent integrally performed well in terms of costs, speed, safety and GC response, which allows to consider it as the most suitable. The GC method for D003 determination, using sulphuric acid–methanol for derivatization, was subject to a validation process, proving fulfils the parameters of specificity, linearity, precision and accuracy. For these reasons, it can be used in the quality control and stability studies of this mixture of VLFAs.

Acknowledgement

We thank Prof. Jesús Núñez for the critical revision of the manuscript.

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