Gas chromatographic method for determining very long chain fatty acids that compose D003 in 1 to 10 mg/ml suspensions used in pharmacological and toxicological studies

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Palabras Clave: D003, ácidos grasos, métodos de identificación y cuantificación, suspensiones, validación.
Key words: D003, fatty acids, identification and quantification method, suspensions, validation.

RESUMEN: D003 es una mezcla de ácidos grasos de alto peso molecular ($C_{24:0}$ to $C_{36:0}$), que muestra actividad hipolipemiante, antiagregante plaquetaria y antitrombótica en modelos experimentales. Ha sido desarrollado y validado un método donde se emplea la cromatografía gaseosa usando una columna Widebore DB-5 y ácido nonadecanoico como estándar interno para determinar la presencia y el contenido de D003 en las suspensiones acuosas, que presentan una concentración de 1 a 10 mg/mL, utilizadas en los estudios farmacológicos y toxicológicos. Los ácidos grasos fueron analizados como sus ésteres metílicos, preparados utilizando 5 % HCl-metanol. Este método fue específico para estas suspensiones de D003. La determinación del contenido total de ácidos grasos presentó un CV de 0,39 %. Los coeficientes de correlación (r) fueron mayores que los límites de aceptación (0,99) tanto para los ácidos individuales como para el total de estos, indicando que las correlaciones son positivas para una probabilidad mayor del 99,9 %. En el estudio de la repetibilidad los CV para el contenido total de D003 (1,55, 1,68 y 0,62 %, respectivamente) fueron menores al límite establecido, indicando que el método es repetible. En la evaluación de la precisión intermedia, para el contenido total de D003 se obtuvo un CV de 1,47 %, por lo que el método es reproducible. El recobrado promedio obtenido para el contenido total de D003 fue mayor del 99 % en todos los casos. Para el total de ácidos grasos $t_{exp} < t_{tab}$ para un 95 % de confiabilidad, no existen diferencias significativas entre el recobrado promedio y el 100 %, por lo que puede considerarse que el método es exacto. Este método permitió el control de la calidad y los estudios de calidad de estas suspensiones.

ABSTRACT: D003 is a mixture of fatty acids ($C_{24:0}$ to $C_{36:0}$), that shows antiplatelet, antithrombotic and cholesterol-lowering effects in experimental models. A gas chromatographic method using a DB-5 Widebore column and 1-nonadecanoic acid as internal standard was developed and validated in order to determine D003 in the aqueous suspensions, with concentrations from 1 to 10 mg/mL, used in pharmacological and toxicological studies. Fatty acids were analyzed as methyl esters derivatives, prepared using 5 % aqueous HCl-methanol. This method was specific for D003 determination in these suspensions. The determination of total content of fatty acids showed a CV of 0.39 %. The values of the correlation coefficients (r) were higher than that of the acceptation limit (0.99) for the individual fatty acids and for the total content of them, indicating that correlations were positives for a probability higher than 99.9 %. In the repeatability study the CV for total content of D003 (1.55, 1.68 and 0.62 %, respectively) were lower, indicating that the method has repeatability. The inter-mean precision for the total content of fatty acids (CV 1.47 %), demonstrated that the method is reproducible. The average recovery obtained for the total content of acids was greater than 99 % for all cases. Also, for all the fatty acids $t_{exp} < t_{tab}$ for a 95 % of releability, there were no significant differences between the average recovery and 100 %, then, it can be considered that this method is exact. The method was suitable for quality control and stability studies of these suspensions.

INTRODUCTION

D003 is a natural product consisting of a mixture of 13 very long-chain primary fatty acids ($C_{24:0}$-$C_{36:0}$). This product is isolated and purified from sugar cane (*Saccharum officinarum* L.) wax[1], the composition of D003 is highly reproducible from batch to batch.[2] D003 have demonstrated good cholesterol-lowering[3, 4] as well a

antiplatelet and antithrombotic[5] effects in experimental models. Also, was studied its acute and oral subchronic toxicity[6] where no drug-related toxicity has been observed after single or short term repeated administration of D003 to rats. It was also found that this product does not show evidences of cytotoxic or genotoxic activity on both somatic or germ cells in rodents.[7] In these studies were used aqueous suspensions of D003 formed using Acacia gum as vehicle. Suspensions were prepared because of the very low solubility of D003 in water and aqueous solutions.[8] To know the exact content of D003 in the suspensions used in these studies was necessary to develop a method that allows the pharmacologist to know the exact quantity of D003 administered to the animals.

Gas chromatography (GC) shows to be the best technique for determining fatty acids; and with this aim, they are usually converted to the simplest convenient volatile derivatives, often methyl esters (FAME) or other esters.[9, 10] However, to author's knowledge, published works are related to acids with lesser than 26 carbon atoms and contain limited information concerning the details of the quantitative determination of them.

In the present work was described the development and validation of a GC method for determining the fatty acids that compose D003 on the aqueous suspensions, from 1 to 10 mg/mL, used in the toxicological and pharmacological studies. The fatty acids were extracted from suspension and converted to methyl esters by an acid-catalyzed reaction.

This method is used for quality control of these suspensions, that were prepared using Acacia gum as vehicle.

MATERIALS AND METHODS

Apparatus

The gas chromatographic system (a) consisted of Shimadzu GC-14A with a flame ionization detector and Shimadzu C-R4A computerized data processor, (Shimadzu,Kyoto, Japan). The column used was a DB-5 Wide-bore fused-silica capillary column (30 m, 0.53 mm id, 1.5 μm D_f; J&W Scientific, Folsom, USA) set to the injection port intended for packed column (fitted with a 3.8 mm id sylanized quartz glass liner) by means of a wide-bore adapter. Operated at a program from 250 to 320 °C at 5 °C/min and isothermal for 10 min at 320 °C while injector and detector temperatures were 300 and 320 °C, respectively. Carrier gas (H_2) flow, 11.4 mL/min. To form the flame, hydrogen gas flow, 40 mL/min, and air gas flow, 400 mL/min, were used.

Chemicals

D003 (batches 990702 and 990703) was provided by National Center of Scientific Research (C.N.I.C.) (Havana, Cuba); all other chemicals were analytical reagent grade: hydrochloric acid (37 %), methanol, (Merck, Darmstadt, Germany), toluene and chloroform (Riedel-de-Haën, Seelze, Germany). Acacia gum (ucb, Leuven, Belgium).

1-Nonadecanoic acid (99 %, Sigma, St. Louis, MO, USA,), 1 mg/mL in chloroform, was used as internal standard.

The stock solution was prepared as follows: weighed 6.5 mg 1-tetracosanoic ($C_{24:0}$), 4.5 mg 1-pentacosanoic ($C_{25:0}$), 14 mg 1-hexacosanoic ($C_{26:0}$), 12 mg 1-heptacosanoic ($C_{27:0}$), 145.5 mg 1-octacosanoic ($C_{28:0}$), 8 mg 1-nonacosanoic ($C_{29:0}$), 80 mg1-triacontanoic ($C_{30:0}$) and 5 mg 1-hentriacontanoic ($C_{31:0}$) acids; all > 99 % GC, (Sigma, St. Louis, MO, USA) into a 100-mL volumetric flask complete volume with chloroform and mix in order to give final concentrations of 0.06, 0.04, 0.14, 0.12, 1.45, 0.08, 0.8, and 0.05 mg/mL, respectively. This solution was found to be stable for 1 month, when stored at +4 °C.

The methylation solution (MSoln) was prepared with hydrochloric acid: methanol (5:95, v/v). This solution should be weekly prepared and stored at + 4 °C.

Suspensions formulation

The test procedure was applied to D003 suspensions of different concentrations, which were prepared with the real concentration (Table 1). Suspensions were prepared as follows: the exact quantity of D003 was placed in a 150-mL Beaker and the vehicle was added drop by drop, stirring until the powder is moisturized. Then, the product was trasvased to a 250-mL volumetric flask with continuos washing of the Beaker with water and completing the volume.

Test procedure

In order to perform the analysis of each suspension was taken a quantity of suspension that corresponds to took 2 mg of D003 (4.0; 2.0 and 0.2 mL, respectively). From these new suspensions, 0.1 mL was transferred to a test tube with screw cap and 1 mL of the internal standard solution as well as 4 mL of chloroform were added, heating at 80 °C for 30 min, stirring occasionally. This suspension was transferred to a separator funnel and the organic phase was isolated from it. One milliliter of this organic phase was transferred to a test tube and evaporated to dryness with the help of a nitrogen flow. MSoln (0.1 mL) was added, heating to 80 °C for 30 min, evaporated to dryness with the help of a nitrogen flow. Later on, 0.04 mL of toluene were added and heated at 80 °C for 3 min.

The mass (mg) of each acid was obtained by the internal standard method[11] according to the following equation:

$$\textit{Mass of Compound } i = \frac{\textit{Area of compound } i \times \textit{Mass of internal standard} \times F_i^w}{\textit{Area of internal standard}}$$

where:

f_i^w relative mass response factor for compound i.

In order to determine f_i^w, 0.5 mL of the stock solution and 0.5 mL of the internal standard solution were transferred to a 1.8 mL crimp vial, the content was evaporated to dryness at 80 °C with a gently nitrogen stream. Afterwards, 1 mL of the methylation solution was added and the mixture was heated at 80 °C for 90 min. Content was evaporated to dryness, 250 µL of toluene were added and the mixture was heated at 80 °C for 3 min. This procedure was performed in triplicate, and f_i^w was calculated as follows:

$$f_i^w = \frac{\text{Area of internal standard} \cdot \text{Mass of compound i}}{\text{Area of compound i} \cdot \text{Mass of internal standard}}$$

Commercial standards of $C_{32:0}$, $C_{33:0}$, $C_{34:0}$, $C_{35:0}$ and $C_{36:0}$ acids are not available, then the f_i^w of $C_{30:0}$ acid was used for $C_{32:0}$, $C_{34:0}$ and $C_{36:0}$ acids, and for $C_{33:0}$ and $C_{35:0}$ acids was used that f_i^w of $C_{31:0}$ acid. The content of D003 in these suspensions corresponds to the sum of each one of the following fatty acids: 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 and 36 carbon atoms. The equations that were used for calculating the concentration of D003 in each suspension are shown in Table 2.

Validation of test procedure[12, 13]

Applicability of the system

Resolution: was determined using the following formula:

$$R_{i,j} = 2 \cdot \left(\frac{t_2 - t_1}{w_1 + w_2} \right)$$

where:

$t_2 - t_1$ Difference between the retention times of the analyzed compounds (min).
w Width of the peak at the base (min).
2 Mathematical constant.

Linearity of the method

Linearity test as well as the proportionality test was determined as described.[12]

Precision

Repeatability of the method and confidence limits

Confidence limits were determined using the following formula:

$$\bar{x} \pm t_{tab} \cdot S$$

where:

t_{tab} is the value of Student's t distribution for n-1 liberty degree for a probability P = 0.05.[13]

Intermean precision and confidence limits

In the gas chromatograph were changed the following conditions: A column DB-5 Wide-bore fused-silica capillary column (30 m, 0.53 mm id, 1.5 µm D_f; J&W Scientific, Folsom, USA) set to the injection port intended for packed column (fitted with a 3.8 mm id sylanized quartz glass liner) by means of a wide-bore adapter. a capillary column. Operated at a program from 250 to 320 °C at 5 °C min^{-1} while injector and detector temperatures were 320 and 340 °C, respectively. Carrier gas (H_2) flow, 11.2 ml . min^{-1}.

Accuracy

Accuracy was assessed by a recovery study. Recoveries were calculated according to the following equation:

$$Recover = \frac{Amount found}{Amount added} \cdot 100\%$$

Average recovery was checked to 100 % with the student t test. The experimental value of t was calculated as follows:

$$t_{exp} = \frac{|100 - Recovery|}{RSD} \sqrt{n}$$

The null hypothesis (the recovery is close to 100 % and the method is accurate) was accepted for a significance level greater than 5 %. To determine if the concentration factor affected the results, the Cochran test for p = 0.05 was used.

RESULTS AND DISCUSSION

Applicability of the system

The results of the chromatographic resolution between C_{28}-C_{30} acids, the total quantification of fatty acids (%) and the relative retention time of each fatty acid, when is used C_{19} acid as internal standard, are shown in Tables 3 and 4. As can be seen from these results, the resolution values were greater than the acceptance limits for a capillary column (R > 2), guaranteeing a correct separation of the chromatographic peaks, avoiding the interference of peaks that corresponds to other compounds present in the suspensions. The relative retention times were ranged in the acceptance limits, existing a concordance between the specific chromatographic conditions, for the method, and those analyzed in this research. When was determined the total content of fatty acids, in D003 suspensions, was

obtained a coefficient of variation of 0.39 %, lower than 0.5 %, the maximum accepted limit.

Linearity of the method

When there were studied the value of individual concentrations was possible to obtain the regression equations (Table 5). In this table is observed that the values of correlation coefficients (r) are higher than that of the acceptation limit (0.99) for the individual fatty acids, as well as the total of them, indicating that correlations were positives for a probability higher than 99.9 %. The cero value is included in the confidence limits of the intercepts for all the acids, except for C_{24}, C_{25} and C_{26} acids, because of its minor content in the mixture, and did not affect the confidence limit of the intercept for the total content of acids, while bias is not observed.

The coefficients of variation of the response factors, that were lower than 5 % for all fatty acids as well as for the total amount are shown in Table 6. The relative standard deviations of the slopes are lower than 2 %. As can be observed, in the statistical test of the slope, the value of t_{exp} for each one of the fatty acids was greater than t_{tab}, indicating that the null hypothesis is not fulfilled, existing a great probability that the slope should be different from cero. In the case of the statistical test of the intercepts, was observed that the values of t_{exp} were smaller than that of t_{tab} for all the fatty acids, as well as for the total amount, fulfilling the null hypothesis and existing a great probability than the intercept will be equal to cero.

Precision

Repeatability and confidence limits

It was determined the repeatability of 8 replicate analysis of each one of the suspensions used in order to establish the linearity of the method. As can be observed from Table 7 repeatability was given by coefficient of variation lower than the established acceptance limit for all the fatty acids and for total content of fatty acids (D003), indicating that the method have repeatability.

Inter-mean precision and confidence limits

The results of the analysis performed by two technicians in two different equipments with different columns and chromatographic conditions and with a sample of the suspension used in the study of the linearity of the method, corresponding to the mean concentration (1.0 $mg.mL^{-1}$) are shown in Table 8.

The coefficients of variation of each fatty acid in the inter-mean precision of the analyzed suspension were greater than that obtained in the repeatability test and, also lower than the acceptance limit, with the exception of that of fatty acids (C_{31}, C_{33} and C_{35}) of minor content, not affecting the inter-mean precision for the total content of acids whose coefficient of variation was smaller than that of the acceptance limit, then, it can be considered that the method is reproducible.

Accuracy

The results of the obtained concentration of each suspension as well as the recovery in each point of the linearity, corresponding to the total content of fatty acids are shown in Table 9. The average recovery obtained for the total content of acids was greater than 99 % for all the cases. Also, for all the fatty acids $t_{exp} < t_{tab}$ for a 95 % of confiability, there are not significant differences between the average recovery and 100 %. Then, it can be considered that this method is exact.

Specificity

The chromatograms of: a sample of 10 mg ml^{-1} suspension of D003, a sample of the D003 active principle, the internal standard and a blank sample are shown in Figure 1. As can be seen, any interference was produced between the internal standard, D003 and other components of the suspensions.

CONCLUSIONS

An analytical method was developed and validated to determine D003 in aqueous suspensions, concentrations from 1 to 10 mg/mL, that can be used in the quality control and stability studies of these suspensions.

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Table 1. Real concentration of the suspensions of D003.

Theoretical concentration of suspensions ($mg \cdot mL^{-1}$)	**Real concentrations of suspensions ($mg \cdot mL^{-1}$)**
10	10.00
1	1.01
0.5	0.49

Table 2. Equations used for calculus of D003 concentration.

Suspension	Calculus of D003 concentration
10 $mg \cdot mL^{-1}$	C ($mg \cdot mL^{-1}$) = m (RCOOH) . 5
1.0 $mg \cdot mL^{-1}$	C ($mg \cdot mL^{-1}$) = m (RCOOH) . 0.5

m (RCOOH) = mass of D003 obtained by the analysis of chromatograms.

Table 3. Results of the applicability of the system: resolution and quantification.

Reply	$R_{C28-C30}$	**M($RCOOH_T$)**
1	5.701	9.779
2	5.646	9.715
3	6.602	9.742
4	5.880	9.673
5	5.830	9.745
6	5.804	9.767
	$\bar{x}$	9.737
	DE	0.038
	CV(%)	0.393

Table 4. Results of the applicability of the system: Relative retention time.

Acid	$r_{i,p}$	**Acid**	$r_{i,p}$
C_{19}	-	C_{30}	4.45 - 4.52 - 4.59
C_{24}	2.42 - 2.44 - 2.47	C_{31}	4.74 - 4.81 - 4.89
C_{25}	2.75 - 2.78 - 2.81	C_{32}	5.07 - 5.15 - 5.23
C_{26}	3.10 - 3.13 – 317	C_{33}	5.35 - 5.44 - 5.53
C_{27}	3.44 - 3.48 - 3.52	C_{34}	5.67 - 5.76- 5.86
C_{28}	3.82 - 3.87 - 3.92	C_{35}	5.93 - 6.03- 6.14
C_{29}	4.10 - 4.16 - 4.22	C_{36}	6.22 - 6.33 - 6.43

n = 6

Table 5. Regression parameters and confidence limits of the linearity of the method.

Acid	Regression equation	R	r^2
C24	y = (0.0152± 0.0030)x + (0.0001±0.0018)	0.9994	0.9988
C25	y = (0.0134± 0.0002)x - (0.0002±0.0012)	0.9997	0.9993
C26	y = (0.0361± 0.0007)x - (0.0003±0.0038)	0.9995	0.9991
C27	y = (0.0310± 0.0006)x + (0.0001±0.0032)	0.9995	0.9991
C28	y = (0.3749± 0.0049)x - (0.0030±0.0286)	0.9998	0.9995
C29	y = (0.0214± 0.0002)x + (0.0001±0.0013)	0.9999	0.9997
C30	y = (0.2047± 0.0021)x + (0.0008±0.0121)	0.9999	0.9997
C31	y = (0.0104± 0.0001)x + (0.0002±0.0005)	0.9999	0.9998
C32	y = (0.1020± 0.0014)x + (0.0023±0.0079)	0.9998	0.9995
C33	y = (0.0119± 0.0002)x + (0.0002±0.0010)	0.9997	0.9994
C34	y = (0.1210± 0.0022)x + (0.0028±0.0127)	0.9995	0.9991
C35	y = (0.0051± 0.0001)x + (0.0001±0.0007)	0.9993	0.9985
C36	y = (0.0435± 0.0010)x + (0.0012±0.0059)	0.9992	0.9985
Total	y = (0.9907± 0.0121)x + (0.0044±0.0701)	0.9998	0.9996

Table 6. Linearity and proportionality of the linearity of the method.

	$t_{tab}(0.05;4) = 2.16$								
		Linearity test				Proportionality test			
	CV_i(%)	S_B^2	S_B	$S_{B(rel)}$	t_{exp}	S_a^2	S_a	$S_{a(rel)}$	t_{exp}
C_{24}	2.353	$2.1\cdot10^{-8}$	$1.4\cdot10^{-4}$	0.960	104.13	$7.2\cdot10^{-7}$	$8.0\cdot10^{-4}$	1575.6	0.063
C_{25}	2.595	$9.6\cdot10^{-9}$	$9.8\cdot10^{-5}$	0.732	136.63	$3.2\cdot10^{-7}$	$6.0\cdot10^{-4}$	354.32	0.282
C_{26}	1.982	$9.1\cdot10^{-8}$	$3.0\cdot10^{-4}$	0.837	119.53	$3.1\cdot10^{-6}$	$1.8\cdot10^{-3}$	698.96	0.143
C_{27}	2.124	$6.7\cdot10^{-8}$	$2.6\cdot10^{-4}$	0.835	119.82	$2.3\cdot10^{-6}$	$1.5\cdot10^{-3}$	1358.8	0.074
C_{28}	1.713	$5.2\cdot10^{-6}$	$2.2\cdot10^{-3}$	0.608	164.48	$1.7\cdot10^{-4}$	$1.3\cdot10^{-2}$	448.45	0.223
C_{29}	1.823	$1.0\cdot10^{-9}$	$1.0\cdot10^{-5}$	0.467	213.93	$3.4\cdot10^{-7}$	$6.0\cdot10^{-4}$	820.85	0.122
C_{30}	1.612	$9.2\cdot10^{-7}$	$9.6\cdot10^{-4}$	0.469	213.09	$3.1\cdot10^{-5}$	$5.6\cdot10^{-3}$	691.95	0.145
C_{31}	1.800	$1.9\cdot10^{-9}$	$4.3\cdot10^{-5}$	0.422	236.88	$6.0\cdot10^{-7}$	$3.0\cdot10^{-4}$	143.28	0.698
C_{32}	2.362	$3.9\cdot10^{-7}$	$6.3\cdot10^{-4}$	0.614	162.98	$1.3\cdot10^{-5}$	$3.6\cdot10^{-3}$	159.33	0.628
C_{33}	2.140	$6.1\cdot10^{-9}$	$7.8\cdot10^{-5}$	0.653	153.09	$2.0\cdot10^{-7}$	$5.0\cdot10^{-4}$	257.05	0.389
C_{34}	2.505	$1.0\cdot10^{-6}$	$1.0\cdot10^{-3}$	0.834	119.85	$3.4\cdot10^{-5}$	$5.9\cdot10^{-3}$	211.52	0.473
C_{35}	3.595	$2.9\cdot10^{-9}$	$5.4\cdot10^{-5}$	1.057	92.72	$1.0\cdot10^{-7}$	$3.0\cdot10^{-4}$	382.69	0.261
C_{36}	2.947	$2.2\cdot10^{-7}$	$4.7\cdot10^{-4}$	1.079	119.54	$7.5\cdot10^{-6}$	$2.7\cdot10^{-3}$	222.02	0.450
Total	1.723	$3.1\cdot10^{-5}$	$5.6\cdot10^{-3}$	0.564	177.32	$1.1\cdot10^{-3}$	$3.3\cdot10^{-2}$	728.02	0.136

Table 7. Results of the repeatability study.

Suspension	10 mg . mL^{-1}		1 mg . mL^{-1}		0.5 mg . ml^{-1}	
Acids	**Mean**	**CV (%)**	**Mean**	**CV (%)**	**Mean**	**CV (%)**
C24:0	0.1508± 0.0097	2.75	0.0155± 0.0004	1.20	0.0074± 0.0002	0.01
C25:0	0.1335± 0.0664	2.07	0.0135± 0.0004	1.49	0.0064± 0.0002	0.01
C26:0	0.3586± 0.0204	2.38	0.0361± 0.0012	1.37	0.0178± 0.0010	0.02
C27:0	0.3077± 0.0175	2.39	0.0316+ 0.0014	1.76	0.0153± 0.0007	0.02
C28:0	3.7281± 0.1638	1.85	0.3751± 0.0147	1.66	0.1849± 0.0085	0.23
C29:0	0.2127± 0.0088	1.76	0.0217± 0.0010	2.01	0.0106± 0.0005	0.01
C30:0	2.0433± 0.0609	1.26	0.2088± 0.0085	1.73	0.1020± 0.0052	0.13
C31:0	0.1036± 0.0028	1.15	0.0108± 0.0005	1.83	0.0053± 0.0002	0.01
C32:0	1.0227± 0.0377	1.56	0.1068± 0.0045	1.77	0.0518± 0.0028	0.07
C33:0	0.1189± 0.0055	1.90	0.0123± 0.0007	2.12	0.0060± 0.0005	0.01
C34:0	1.2177± 0.1404	2.05	0.1267± 0.0055	1.84	0.0615± 0.0036	0.08
C35:0	0.0512± 0.0036	2.92	0.0053± 0.0002	2.22	0.0026± 0.0002	0.003
C36:0	0.4385± 0.0265	2.57	0.0458± 0.0021	1.88	0.0224± 0.0014	0.03
Total	9.8873± 0.3631	1.55	1.0099± 0.0403	1.68	0.4942± 0.0244	0.62
			t_{tab} (0.05;7) = 2.37			
			n = 8			

Table 8. Results of inter-mean precision study.

Suspension	1.0 mg . ml^{-1}	
Acids	**Mean**	**CV (%)**
C24:0	0.0153± 0.0007	2.04
C25:0	0.013± 0.0019	5.99
C26:0	0.0352± 0.0026	3.19
C27:0	0.031± 0.0012	1.71
C28:0	0.3796± 0.0168	1.88
C29:0	0.0218± 0.0009	1.74
C30:0	0.2093± 0.0073	1.47
C31:0	0.0126± 0.0045	**15.29**
C32:0	0.1065± 0.0038	1.51
C33:0	0.0142± 0.0045	**13.51**
C34:0	0.1267± 0.0043	1.41
C35:0	0.0063± 0.0026	**16.74**
C36:0	0.0449± 0.0026	2.48
Total	1.0165± 0.0355	1.47
	t_{tab} (0.05;7) = 2.37	
	n = 16	

Table 9. Results of the accuracy study.

		t_{tab} (0.05;4) = 2.78			
Suspension (mg . mL^{-1})	**Real concentration**	**Statistic**	**Obtained concentration**	**Recovery (%)**	**t_{exp}**
10.00	10.00	$\bar{x}$	9.913	99.109	1.17
		SD	0.169	1.688	
		CV	1.70	1.70	
1.0	1.01	$\bar{x}$	1.005	99.934	0.88
		SD	0.020	1.957	
		CV	1.96	1.96	
0.5	0.49	$\bar{x}$	0.493	99.130	1.11
		SD	0.009	1.743	
		CV	1.76	1.76	

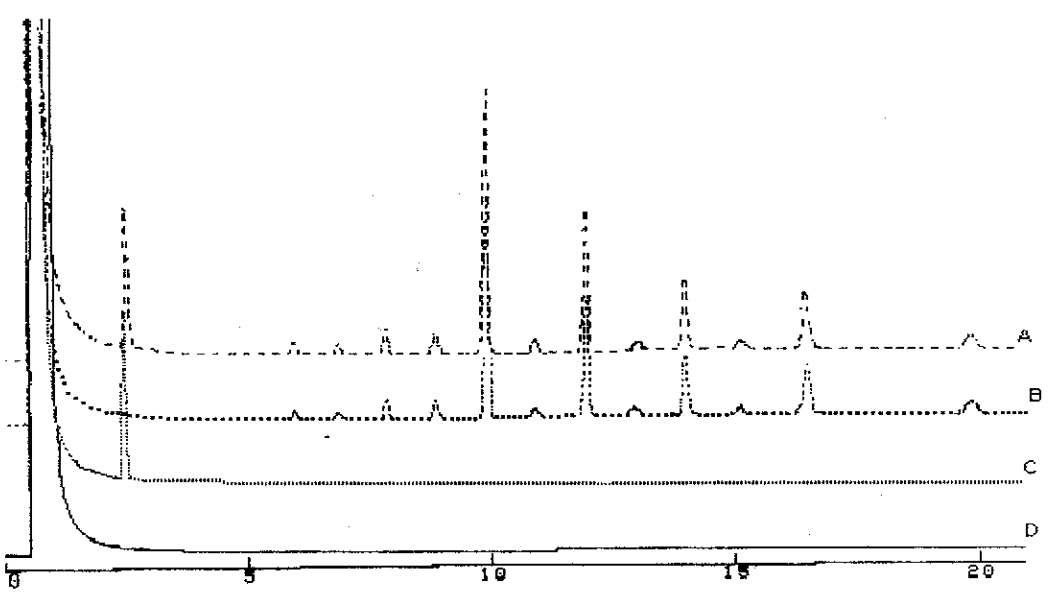

Fig. 1: A) chromatogram of a 10 mg/mL suspension of D003 with the internal standard, B) chromatogram of a sample of D003 (active principle), C) chromatogram of the internal standard and D) chromatogram of a blank sample of a suspension with Acacia gum.