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DEVELOPMENT AND VALIDATION OF A GAS CHROMATOGRAPHY METHOD FOR QUANTITATIVE ANALYSIS OF FATTY ACIDS IN VEGETABLE OILS

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ABSTRACT. The major fatty acids present in cosmetics are the unsaturated fatty acids from triglycerides, especially essential fatty acids: linoleic acid (omega-6) and α-linolenic acid (omega-3).The purpose of the study was to develop a simple and precise gas chromatography-flame ionization detection method, using an OPTIMA-WAX (macrogol 20000) capillary GC column (30m x 0.32mm x 0.25µm) with a run time of 17min, for the analysis of fatty acids composition from vegetable oils and macerated oils. The method was validated for quantifying four major fatty acids: palmitic, stearic, oleic and linoleic acids, as methyl esters. The quantification was performed by internal standardization, using the methyl ester of nonadecanoic acid as internal standard. The esterification reaction was carried out on a magnetic stirrer at a temperature of 80°C and with continuous stirring, in hermetically sealed vials.

Keywords: *fatty acid, vegetable oils, gas-chromatography, method validation*

INTRODUCTION

The benefits of natural products have been known since ancient times when our ancestors used various herbal mixtures to treat different skin diseases. Even if, at that time, they could not explain the beneficial effects, they trusted nature.

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Fatty acids play a key role in metabolism: energy substances, components necessary for all membranes and play a role in regulating the transmission of intracellular messages. They represent 30-35% of total energy consumption in several industrial countries, the most important source of fatty acids being vegetable oils, dairy products, meat products, cereals, fish fats or oils.

In the last decade, vegetable oils, such as argan oil, avocado oil, sunflower oil, broccoli oil, olive oil, have begun to be used for their emollient, moisturizing and nourishing effects. Nowadays, a very important role in the industry has been attributed to omega fatty acids, especially linoleic acid, an omega-6 acid, which is a very important element in ceramides, contributing to maintaining the structure and function of the epidermis [1-6]. They form a protective layer over the skin, reducing local inflammation. Moreover, they play a very important physiological role in the body, sustaining in the synthesis of eicosanoids or local hormones (prostaglandins, prostacyclines, thromboxanes). Therefore, deficiency of fatty acids and corresponding lipids significantly affects vascular fragility, reduces immune function and interferes with the coagulation process. Moreover, the oils are incorporated into the cell membrane and regenerate the lipid barrier [7-14]. Unsaturated fatty acids have pronounced healing effects on dermatoses, such as atopic skin inflammation and are used in creams, emulsions, ointments, hair conditioners, cosmetic masks, lipsticks, nail polishes and other personal care formulas [15-17].

In the last decade, numerous studies have been carried out regarding the fatty acid composition of vegetable oils through different chromatographic techniques: GC-MS, GC-FID, LC-MS [18, 19].

The GC-FID method is a common method for the analysis of fatty acids. Such methods are also described in the European Pharmacopoeia and United States Pharmacopoeia [20, 21].

Songul Kesen [23] describes a technique for sample preparation that involves the methylation of 0.1g of oil with 0.2mL of 2N methanolic potassium hydroxide solution and vigorously shaking. The upper phase was injected in GC.

Qiwen Young et al. [24] describe a technique for the extraction of the fatty acids from sunflower seed oil. The fatty acids were extracted with an acidification method and after the methyl esterification, the separation of the saturated fatty acid methyl ester was realized with an urea encapsulation method and the content of unsaturated fatty acid methyl ester was up to 99,67%.

The aim of this study was to develop and validate a simple method for the simultaneous determination of major fatty acids in vegetable oils.

The chromatographic method was developed on argan oil, broccoli oil, olive oil, sunflower oil, avocado oil and raspberry oil. The method was validated on sunflower oil because it is widely studied in dermatology for two main reasons: firstly, it is a cheap natural source of oil and secondly it contains lipids similar to those from the composition of the corneum layer. It contains predominantly linoleic acid, tocopherols, lecithin and carotenoids. Sunflower oil helps moisturize the skin and creates a barrier against infections [23-26].

RESULTS AND DISCUSSION

Validation results

Specificity

As can be seen in Figure 1 no interfering peaks were found at the retention times of the analytes of interest into the blank sample. The retention times of fatty acid methyl esters in the chromatogram of the test sample were confirmed by comparing with those in the standard chromatogram. All fatty acids were adequately resolved from each other.

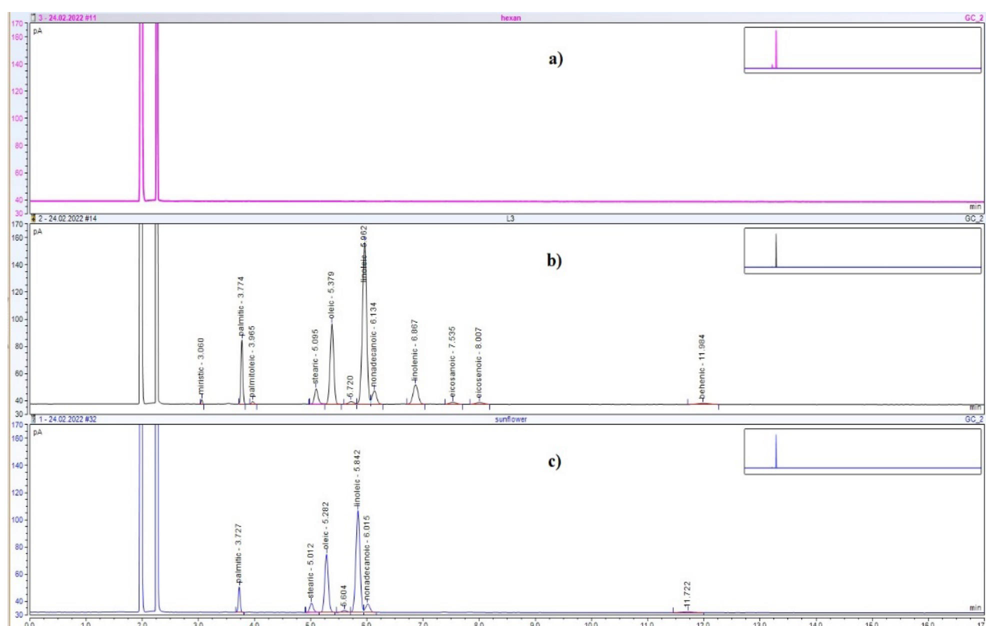

Figure 1. a) Blank (Hexane) Chromatogram, b) Standard Chromatogram and c) Sample Chromatogram

System suitability

The detailed results for the system suitability are represented in Table 1.

Table 1. System suitability

Component and chromatographic elution order	RSD %	Tailing factor	Resolution	Theoretical plates
methyl tetradecanoate (miristic, C14:0)	1.6	1.01	-	96321
methyl hexadecanoate (palmitic, C16:0)	0.7	1.00	13.96	57299
methyl palmitoleate (C16:1)	1.8	1.08	2.91	52830
methyl octadecanoate (stearic C18:0)	1.1	1.19	12.30	31289
methyl oleate (oleic C18:1)	0.8	0.97	2.42	32568
methyl linoleate (C18:2)	0.8	0.93	1.63	30046
methyl linolenate (C18:3)	0.9	1.02	4.92	26663
methyl eicosanoate (arahidic, C20:0)	1.3	0.98	3.58	21537
methyl 11 eicosenoate (C20:1)	1.4	1.01	2.20	20357
methyl docosanoate (behenic C22:0)	1.6	1.03	13.28	16248

Linearity

The linearity was assessed by plotting the ratio of the analyte peak area to the internal standard peak area versus the corresponding concentration.

ANOVA statistical analysis showed, in all cases, that there was a proportionality relationship between chromatographic response and concentration at 95% confidence level. The correlation coefficient of the regression line ranged from 0.9910 to 0.9987. The confidence interval of the intercept includes zero value. The statistical significance of the slope is checked by the Student test ($t_{calculated} > t_{critical}$), thus demonstrating the linearity of the curve. An additional element confirming the linearity of the method is the fulfilment of the condition that the experimental value of the Fisher test is greater than the critical value. Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the residual standard deviation of the calibration curve (SD) and the slope of the calibration curve (b), where LOD=3.3xSD/b and LOQ=10xSD/b [32].

The results of linearity are summarized in Table 2.

Table 2. Linearity verification by ANOVA test

Statistical characteristics of the regression	Acceptability criteria	Myristic acid	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Eicosanoic acid	Eicosenoic acid	Behenic acid
Regression line equation	-	y=0.4040x +0.0662	y=0.4465x + 2.0511	y=0.4111x +0.9425	y=0.521x -0.9657	y=0.4620x +3.2940	y=0.4562x +17.0250	y=0.4624x -1.1542	y=0.4651x -0.2394	y=0.4694x +0.1605	y=0.4582x +0.9139
Correlation coefficient	>0.9900	0.9987	0.9967	0.9907	0.9922	0.9947	0.9942	0.9932	0.9947	0.9945	0.9910
Determinations, n	-	5	5	5	5	5	5	5	5	5	5
Degrees of freedom, n-2	-	3	3	3	3	3	3	3	3	3	3
Standard error of regression	-	0.2097	7.2238	0.5835	5.1310	22.0799	53.7179	8.6435	0.9890	1.0200	1.2760
Standard error of slope	-	0.0083	0.0149	0.0230	0.0267	0.0195	0.0202	0.0222	0.0195	0.0201	0.0252
Student's test $t_{n-2}(1-\alpha/2)$	$t_{calculated} > t_{critical}$ = 3.1824	48.79	30.06	17.85	19.56	23.75	22.59	20.87	23.82	23.31	18.19
Confidence interval for slope (95% confidence)	does not include zero value	0.3776÷ 0.4303	0.3992 ÷ 0.4938	0.3378 ÷ 0.4844	0.4364 ÷ 0.6060	0.4001 ÷ 0.5240	0.3919 ÷ 0.5205	0.3918 ÷ 0.5329	0.4030 ÷ 0.5272	0.4053 ÷ 0.5335	0.3780 ÷ 0.5383
Standard error of intercept	-	0.2199	7.5763	0.6120	5.3814	23.1576	56.3398	9.0654	1.0373	1.070	1.3383
Confidence interval for intercept (95% confidence)	include zero value	-0.6338÷ 0.7663	-22.0601 ÷ 26.1624	-1.0052 ÷ 2.8903	-18.0918 ÷ 16.1604	-70.4039 ÷ 76.9918	-162.2731 ÷ 196.3237	-30.0043 ÷ 27.6958	-3.5405 ÷ 3.0618	-3.2440 ÷ 3.5650	-3.345 ÷ 5.1728
Fisher test (95% confidence) $F_{critical}=F_{1,n-2}(1-\alpha)$= 10.128	$F_{calculated} > F_{critical}$	2380.3	903.66	318.47	382.41	563.85	510.15	435.41	567.53	543.51	330.88
LOQ (µg/mL)		1.7129	53.3898	4.6839	32.4871	157.7140	388.5775	61.6859	7.0172	7.1709	9.1899
LOD (µg/mL)		5.1906	161.7870	14.1936	98.4459	477.9199	1177.5077	186.9269	21.2642	21.7299	27.8481

Accuracy

The average percentage recovery obtained in the range 90-110% are summarized in Table 3.

Table 3. Accuracy results

Component	The average percentage recovery %
methyl tetradecanoate (miristic, C14:0)	94.13
methyl hexadecanoate (palmitic, C16:0)	96.35
methyl palmitoleate (palmitoleic C16:1)	98.97
methyl octadecanoate (stearic C18:0)	95.62
methyl oleate (oleic C18:1)	96.98
methyl linoleate (linoleic C18:2)	96.39
methyl linolenate (linolenic C18:3)	96.51
methyl eicosanoate (arahidic, C20:0)	100.25
methyl 11 eicosenoate (C20:1)	97.11
methyl docosanoate (behenic C22:0)	95.20

These values demonstrate a good accuracy of determination of fatty acid methyl esters in sunflower oil by the proposed analytical procedure.

Precision

The relative standard deviation obtained for the results of 6 samples of sunflower oil analysis was less than 5% which is the value set for testing precision. The detailed results are represented in Table 4.

Table 4. Precision results

	Component			
	palmitic acid	**stearic acid**	**oleic acid**	**linoleic acid**
Concentration mg/100g	4.98	3.78	26.94	41.97
RSD, %	2.60	3.73	2.59	2.62
Standard error	0.0530	0.0575	0.2848	0.4496
Confidence level (95.0%)	0.14	0.15	0.73	1.16
Confidence interval	4.84-5.12	3.63-3.93	26.21-27.67	40.81-43.13

After validating the method, it can be seen that it meets all the criteria: the method is specific, linear and precise.

Table 5. Theoretical Concentration (%) of fatty acids in the studied oils from USP, EP and Technical data sheet (TDS)

Component	Theoretical Concentration (%)					
	Sunflower (USP29-NF24)	**Argan (TDS)**	**Avocado (TDS)**	**Olive (EP)**	**Broccoli (TDS)**	**Raspberry (TDS)**
methyl tetradecanoate (miristic, C14:0)	-	-	-	-	-	-
methyl hexadecanoate (palmitic, C16:0)	3-10	10-15	5-25	7.5-20	<5	2
methyl palmitoleate (C16:1)	-	-	1.0-12.0	0.3-3.5	-	0.2
methyl octadecanoate (stearic C18:0)	2-8	4.3-7.2	3	0.5-5	<5	1
methyl oleate (oleic C18:1)	14-24	43-50	45-75	56-85	10-20	13
methyl linoleate (C18:2)	40-74	29-37	5-20	3.5-20	10-20	59
methyl linolenate (C18:3)	-	-	3.0	1.0	5.0-10.0	24
methyl eicosanoate (arahidic, C20:0)	-	-	-	0.6	-	0.4
methyl 11 eicosenoate (C20:1)	-	-	-	0.5	5.0-10.0	-
methyl docosanoate (behenic C22:0)	-	-	-	0.2	-	-

In Table 5 we have the theoretical concentration of fatty acids in the oils examined described in USP, EP and in the technical data sheet from the oils manufacturer [20,22].

Comparing the results obtained (Table 6) with those from the literature (Table 5), it can be seen that this method can be used for the analysis of vegetable oils, being a faster and easier method.

Table 6. Concentrations of fatty acids methyl esters in the vegetable oils

Component	Concentration %					
	sunflower	argan	avocado	olive	broccoli	raspberry
methyl tetradecanoate (miristic, C14:0)	-	0.13	-	-	-	-
methyl hexadecanoate (palmitic, C16:0)	4.98	13.95	15.27	11.09	2.53	2.02
methyl palmitoleate (C16:1)	-	0.10	5.28	0.88	0.15	-
methyl octadecanoate (C18:0)	3.76	7.02	1.64	3.97	1.38	1.02
methyl oleate (C18:1)	26.94	50.05	54.88	78.32	15.07	11.79
methyl linoleate (C18:2)	41.97	31.47	7.85	3.29	12.13	48.82
methyl linolenate (C18:3)	-	0.08	0.56	0.41	5.57	21.57
methyl eicosanoate (arahidic, C20:0)	-	0.51	-	0.47	1.07	0.65
methyl 11 eicosenoate (C20:1)	-	0.50	0.13	0.27	11.92	0.15
methyl docosanoate (C22:0)	-	-	-	-	-	-

CONCLUSIONS

A gas chromatographic method with flame ionization detection for simultaneous analysis of the major fatty acids in vegetable oils and their quantification as methyl esters was developed and validated based on standards using an internal standard.

The method required a small amount of sample, a small volume of solvent and a short time for esterification.

This study revealed that in sunflower oil linoleic acid is in the highest concentration, followed by oleic, palmitic and stearic acids as seen in Table 6.

The highest concentration of linoleic acid is found in raspberry oil and the lowest concentration is in the olive oil.

The results of the validation tests proved that the analytical method presents a degree of linearity, accuracy, precision and specificity within the proposed limits and can be used for the determination of fatty acids in sunflower oil.

The experimental values of fatty acids from the analyzed oils are comparable to the theoretical values described in the literature.

EXPERIMENTAL SECTION

In the method development, several derivatization procedures were tested using 14% boron fluoride (BF_3) solution in methanol, 0.5M sodium hydroxide in methanol - 14% BF_3 in methanol mixture and 0.5M sodium hydroxide in methanol solution. The best results were obtained using 0.5M sodium hydroxide solution in methanol and a mixture of this and 14% BF_3 solution in methanol.

Methyl esters were identified by comparing the retention times in the test sample with those in the standard solution.

In this study, the quantification of methyl esters was carried out by the internal standard method which is more accurate and precise than the external standard method, the latter being more often used in case of a simple sample preparation. Nonadecanoic acid methyl ester, an acid that does not naturally occur in lipids, was used as internal standard; it was introduced in the derivatization step of the vegetable oil sample in the same concentration as in the standard solutions for the calibration curves.

Materials

Avocado oil, argan oil, broccoli oil, raspberry oil, olive oil obtained by cold pressing (Mayam), sunflower oil obtained by cold pressing from a local producer in the Valcea area; Fatty acid standard Mixture ME 275 (Larodan), Internal standard nonadecanoic acid methyl ester (Dr. Ehrenstorfer, purity 99.5%), Hexane (Carlo Erba, HPLC grade), Methanol (Riedel de Haen, HPLC grade), Sodium hydroxide (Lach Ner), Sodium sulphate anhydrous (Cristal R Chim), Polar stationary phase capillary column Optima-Wax 30m x 0,32mm x 0,25µm (Macherey-Nagel).

Analytical instruments

An Agilent 6890N Gas Chromatograph (Agilent Technologies) with flame ionization detection and split/splitless injector, Mettler Toledo electronic analytical balance, Parker hydrogen gas generator and Peak nitrogen gas generator were used for method development and validation.

The chromatographic conditions were established in the laboratory starting from European Pharmacopoeia Chapter 2.4.22 (Composition of fatty acids by gas chromatography) recommendations [20].

GC-FID system

The analysis was performed using an Agilent 6890N Gas Chromatograph (Agilent Technologies) with flame ionization detection and split mode for sample injection. For chromatographic separation a fused-silica capillary column (Macherey-Nagel, Optima-Wax 30m x 0,32mm x 0,25μm with macrogol 20000 as stationary phase) was used.

The gas carrier was nitrogen with a flow rate of 1.3 mL/min. The oven temperature was held at 200°C for 17minutes. The temperature of the injector was 250°C and the detector temperature was 260°C. The injection volume was 1μL, with split ratio of 50:1.

Preparation of solutions

Internal standard solution

A solution of nonadecanoic acid methyl ester with a concentration of 2000μg/mL in hexane was prepared.

Stock standard solution

A stock standard solution of fatty acid methyl esters in hexane was prepared with the concentrations shown in Table 7.

Table 7. Concentrations of fatty acids methyl esters in the standard Mixture ME 275

Component and chromatographic elution order	Composition %	Concentration mg/mL	Concentration range of linearity μg/mL
methyl tetradecanoate (miristic, C14:0)	0.50	0.08	8.01 ÷ 40.05
methyl hexadecanoate (palmitic, C16:0)	9.60	1.54	153.79 ÷ 768.96
methyl palmitoleate (C16:1)	0.50	0.08	8.01 ÷ 40.05
methyl octadecanoate (stearic C18:0)	3.80	0.61	60.88 ÷ 304.38
methyl oleate (oleic C18:1)	22.40	3.59	358.85 ÷ 1794.24
methyl linoleate (C18:2)	52.50	8.41	841.05 ÷ 4205.25
methyl linolenate (C18:3)	7.70	1.23	123.35 ÷ 616.77
methyl eicosanoate (arahidic, C20:0)	1.00	0.16	16.02 ÷ 80.10
methyl 11 eicosenoate (C20:1)	1.00	0.16	16.02 ÷ 80.10
methyl docosanoate (behenic C22:0)	1.00	0.16	16.02 ÷ 80.10

Standard calibration solutions

Five calibration standard solutions were prepared by dilution of the stock standard solution. To each solution, 500µL of internal standard was added.

Test solution

A test solution was prepared by weighing 25-30 mg oil sample into a 20mL glass vial over which 500µL of internal standard solution, 5mL hexane, 5mL methanol and 1mL 0.5M sodium hydroxide solution in methanol were added. The vial was closed and heated to 80°C with continuous shaking for 30 minutes on a magnetic stirrer. After it cooled down and the two phases were separated, 3mL were taken from the upper layer and filtered on anhydrous sodium sulfate. 1µL was injected from this solution.

Validation of the analytical method

The aim of the validation of the described method was to demonstrate that it is suitable for the quantitative analysis of fatty acids in sunflower oil. The validation characteristics used in the validation were specificity, linearity, accuracy, precision [29-32].

The linearity of the major fatty acids in sunflower oil: palmitic, oleic, stearic and linoleic was established. Linear regression analysis was carried out using the least squares method. The correlation coefficient of the calibration lines was determined; graphs of response (ratio of analyte area to internal standard area) versus each corresponding concentration of fatty acid were plotted.

The accuracy of the method was investigated by recovery tests using 3 standard solutions containing known amounts of analytes. These were analysed as unknown samples. The criterion adopted for evaluation was the percentage recovery in the range 90-110%.

The precision of the method was established by repeatability test: preparation and analysis of 6 samples. The relative standard deviation (RSD, %) of the results was determined. The criterion adopted for evaluation was RSD ≤ 5%.

Specificity refers to the ability of the analytical method to provide a different response for the analyte of interest in the presence of other compounds which may be expected in the sample matrix. It involves assigning identity to the analytes of interest under the experimental conditions of the method by obtaining positive results for the sample containing the analytes of interest when compared to the standard substances. A blank sample, a standard sample and a test sample were analyzed. In the blank sample there should be no signals interfering with those in the standard and test samples.

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