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Article

# High Performance Liquid Chromatography Determination of Fatty Acids in Drying Oils Following Lipase Action

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**This paper describes a quantitative analytical procedure to determine the fatty acid composition in drying oils like linseed, walnut and poppy seed. The procedure required the enzymatic hydrolysis of the oil triacylglycerol families by the action of *Candida rugosa* lipase. The fatty acids (FFAs) produced (linolenic, myristic, linoleic, palmitic, oleic and stearic) were extracted with *n*-heptane and derivatized with α-bromoacetophenone. Their separation and quantitative determination were performed by high-performance liquid chromatography employing a C18 column and an isocratic elution method coupled to ultraviolet detection. The analytical enzymatic procedure is sensitive for < 0.5 μg/mL of FFAs in a reduced sample of 0.1 mg of drying oil.**

## Introduction

Oils have been used for many years in many sectors as a base for perfumes and in medical and cosmetic unguents, whereas drying oils are especially used in paintings as a medium for pigments. The latter are composed of a mixture of triacylglycerols, esters formed of a molecule of glycerol and three primary molecules of unsaturated fatty acids of various lengths. The fatty acids generally found are monounsaturated, such as oleic acid (18:1), and polyunsaturated, such as linoleic acid (18:2) and linolenic acid (18:3), and also include saturated fatty acids such as palmitic acid and stearic acid (1). The drying power of an oil is in relation to its chemical composition. The saturated components of the original oils are not normally involved in the drying and ageing processes because they are chemically stable; polyunsaturated acids like linoleic and linolenic acids are extremely reactive and tend to form a solid film by means of a process of oxidative polymerization. The properties of these pictorial films (cohesion, flexibility, resistance), however, are often altered by the restoration interventions that paintings periodically undergo. These include cleaning operations during which the use of inappropriate substances such as solvents can lead to the leaching of the liquid glycerides caught inside the reticulated structure of the oil.

The use of drying oils such as *Linum usitatissimum* (linseed), *Junglans regia* (walnut) and *Papaver sonniferum* (poppy seed) dates back to thirteenth century North European and Flemish painting and to the beginning of the fifteenth century in Italy. Since the sixteenth century, linseed oil has been the most widely used due to its excellent film-forming characteristics of elasticity and resistance, although it tends to yellow more with age.

However, even if the type of oil used is characteristic of a particular historical period, the choice of the medium also depends on the school and painting technique. The identification of the binder in a painting is an important step in facilitating the choice of reagents to be used in the restoration and even occasionally for assigning a particular period to the painting (2). Therefore, it is obvious that scientific research is interested in developing an adequate methodology for their identification. In addition, quality control of the products usually employed in restoration is of fundamental importance.

In paintings and works of art, oil analysis is usually carried out by gas chromatography after the derivatization of fatty acids obtained by the thermal and chemical degradation of triacylglycerols and their transformation into methyl or trimethylsilyl esters (3–5).

Although this technique is reliable and efficient, it takes a long time to prepare the sample and to reach high temperatures.

At present, high-performance liquid chromatography (HPLC) represents a useful alternative to gas chromatography (GC), because it is a sensitive method that is easier to utilize after improvements related to the derivatization of fatty acids: for example, nitrophenylhydrazides (6–8) and 4--bromomethyl-7-methoxycoumarin derivatives (9). It also presents the advantage of separating fatty acids derivatives not only on the basis of their chain lengthbut also the unsaturation degree and the position of terminal double bond (10).

However, both techniques have the disadvantage of using extremely high temperatures for a prolonged length of time to hydrolyze the triacylglycerols, thus risking the degradation of the polyunsaturated fatty acids. In fact, it is well known that the stability of each single acid is significantly influenced by the temperature, which, when increased, can facilitate the saturation of the unsaturated bonds. A useful alternative to the acid hydrolysis is the use of a highly versatile enzyme like lipase, which has been exploited as the cheap and versatile catalyst to degrade lipids in more modern applications in the food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries (11). Lipase is suitable because it catalyzes the almost complete hydrolysis of fats to fatty acids and glycerols at temperatures below 40°C, because it denatures rapidly at higher temperatures.

The scope of this work is to carry out a relatively simple and reliable analytical method to study drying oils such as walnut, linseed and poppyseed oils by analysis of the fatty acids obtained enzymatically in milder conditions than those generally

employed. The fatty acids thus formed are then derivatized with bromoacetophenone and chromatographically separated using a reversed-phase HPLC system and ultraviolet (UV) detector for the quantitative determination. *Candida Rugosa* was chosen from among the lipases of microbial origin produced from the fermentation of different funguses and bacteria because it hydrolyzes all the ester bonds of triacylglycerols to fatty acids without any specificity, and consequently, it is particularly versatile.

## Materials and Methods

### *Reagents*

The fatty acids used to prepare the standard solution were: linolenic acid (18:3) (9,12,15−octadecatrienoic), myristic acid (14:0) (tetradecanoic acid), linoleic acid (18:2) (9,12−octadecadienoic), palmitic acid (16:0) (hexadecanoic ), oleic acid (18:1) (9-octadecenoic) and stearic acid (18:0) (octadecanoic) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile HPLC reagent was purchased from Baker (Amsterdam, the Netherlands) and α-bromophenacyl bromide > 99%, triethylamine 99%, *n*-heptane > 99%, from Fluka (St. Louis, MO). Acetic acid, $Na_2HPO_4$ and $NaH_2PO_4$ were obtained from Merck (Darmstadt, Germany). Ultra pure water was used from Milli-Q system, Millipore. Lipase enzyme, from *Candida rugosa* (700−1500 U/mg), was purchased from Sigma-Aldrich (St. Louis, MO). Linseed, walnut and poppy seed oils were obtained from Kremer Pigmente GmbH & Co. KG (Aichstetten, Germany).

## Experimental Procedure and Sample Preparation

### *Standards and solutions*

The individual standard solution of fatty acids was prepared by dissolving appropriate amounts of the selected fatty acids in *n*-heptane at concentration level 280 mg/L. The solution was stored at 4°C and is stable for 2 months. A standard solution mixture was obtained by diluting appropriate amounts of the individual solution with *n*-heptane to obtain a concentration of 28 mg/L and used for generating a calibration curve.

The lipase solution was prepared by dissolving 25 mg of lipase powder in 25 mL of phosphate buffer 10 mmol/L at pH 7. The solution was stored at 4°C.

### *Sample preparation*

Enzymatic hydrolysis of drying oils: linseed, walnut and poppy seed and extraction of fatty acids produced.

Small quantities (0.1−0.5 mg ± 0.01) (Analytical balance, Gibertini E50/S, Milan, Italy) of oil standards were placed in the vials and hydrolysed with lipase solution in 500 μL in phosphate buffer 10 mmol/L at pH 7. The solution was incubated at temperature 30°C for 30 min. After the reaction, the fatty acids produced in the resulting solution were extracted with 500 μL of *n*-heptane (shaken vigorously for 5 minutes). Then, an aliquot of the supernatant solution, organic extract 200 μL containing the fatty acids was placed in another vial and were then evaporated to dryness by nitrogen stream and followed by the derivatization process.

### *Extraction of the fatty acids*

The recoveries were calculated using standard fatty acids (FFAs) 0.1 mM diluted in the same solvent of hydrolysis reaction. Results of the extractions were calculated using the method of additions (by spiking). The operating conditions were as follows:

(1) A mixture of fatty acids 0.1 mM is added to 500 μL of phosphate buffer. The extraction is carried out using 500 μL of *n*-heptane. The results obtained by triplicate measurements vary between 80−90% ± 1.5% standard deviation (SD).
(2) Fifty μL of 0.1 mM myristic acid, internal standard, is added to 500 μL of oils, hydrolyzed by the action of the enzyme lipase. The results of the extraction, carried out in this case also using 500 μL of *n*-heptane, vary between 80−90% ± 1.8% SD ($n = 3$).

### *Derivatization of fatty acids*

Phenacyl bromide derivatives of the fatty acids were obtained in the presence of triethylamine in acetone solution according to the procedure described by Mehta (12).

After derivatization, the final solution was dried with a current of nitrogen and reconstituted in the mobile phase (200 μL). The solution was centrifuged and 20 μL were injected into the HPLC apparatus. The mobile phase was acetonitrile−water, 85−15 (v/v) and the column temperature was maintained at 45°C ± 1.

These derivatives are stable for three days at room temperature and for one month at 4°C. Detection was performed by monitoring the absorbance signals at 242 nm. External standard methods were used for quantification.

## Standard Preparation

### *Calibration curves*

After derivatization, phenacyl esters of mixed fatty acid standard were analyzed to obtain the calibration curve and the other calibration parameters.

### *Instrumentation and chromatographic conditions*

HPLC was carried out using an apparatus consisting of a pump (Model LC10AT and FCV-10al) and DGU-14A degasser (Schimadzu, Tokyo, Japan), equipped with a UV-vis detector model L-4250 (Merch Hitachi, LTD Tokyo, Japan). Data acquisition was performed by an interface Model Data Apex CSW32 (Prague, Czech Republic) with a PC. A reversed phase Supelcosil column LC18 (15 cm x 4.6mm, ID 3 μm particle size), and a Supelguardcartridge column (Supelco, Bellafonte, PA) were used for the chromatographic analysis. Absorbance of the eluted compounds was monitored at 242 nm. Mobile phase was acetonitrile−water 85−15 (v/v) at a flow rate of 1 mLmin.

**Table I**
Calibration Data for the Standard Solution

| Fatty acid (phenacyl bromide) | Linearity interval mg $L^{-1}$ | Determination coefficient $r^2$ |
|---|---|---|
| Linolenic acid | 95−380 | 0.9914 |
| Linoleic acid | 17−67 | 0.9962 |
| Myristic acid | 1.4−5.5 | 0.9981 |
| Oleic acid | 65−268 | 0.9714 |
| Palmitic acid | 1.1−4.4 | 0.9634 |
| Stearic acid | 1.2−4.8 | 0.9878 |

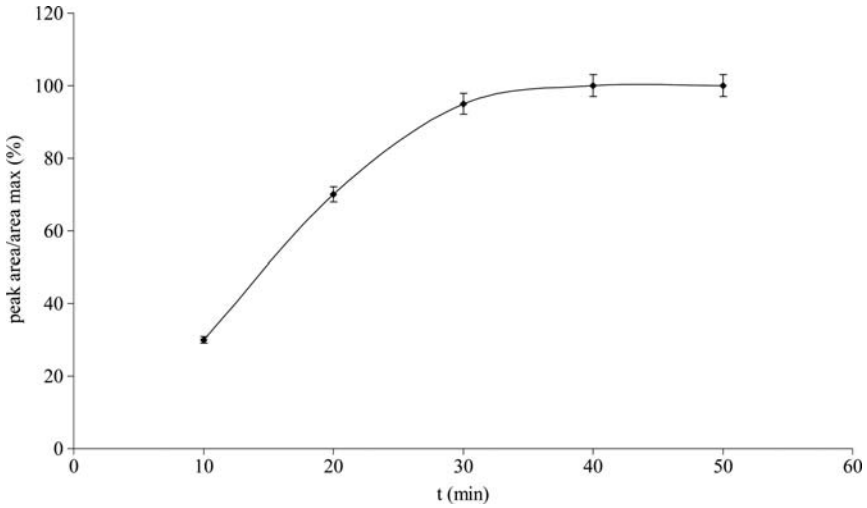


**Figure 1.** Hydrolysis of the walnut oil by Lipase enzyme action, formation of the linoleic acid, expressed as % value normalized with respect to its maximum value.

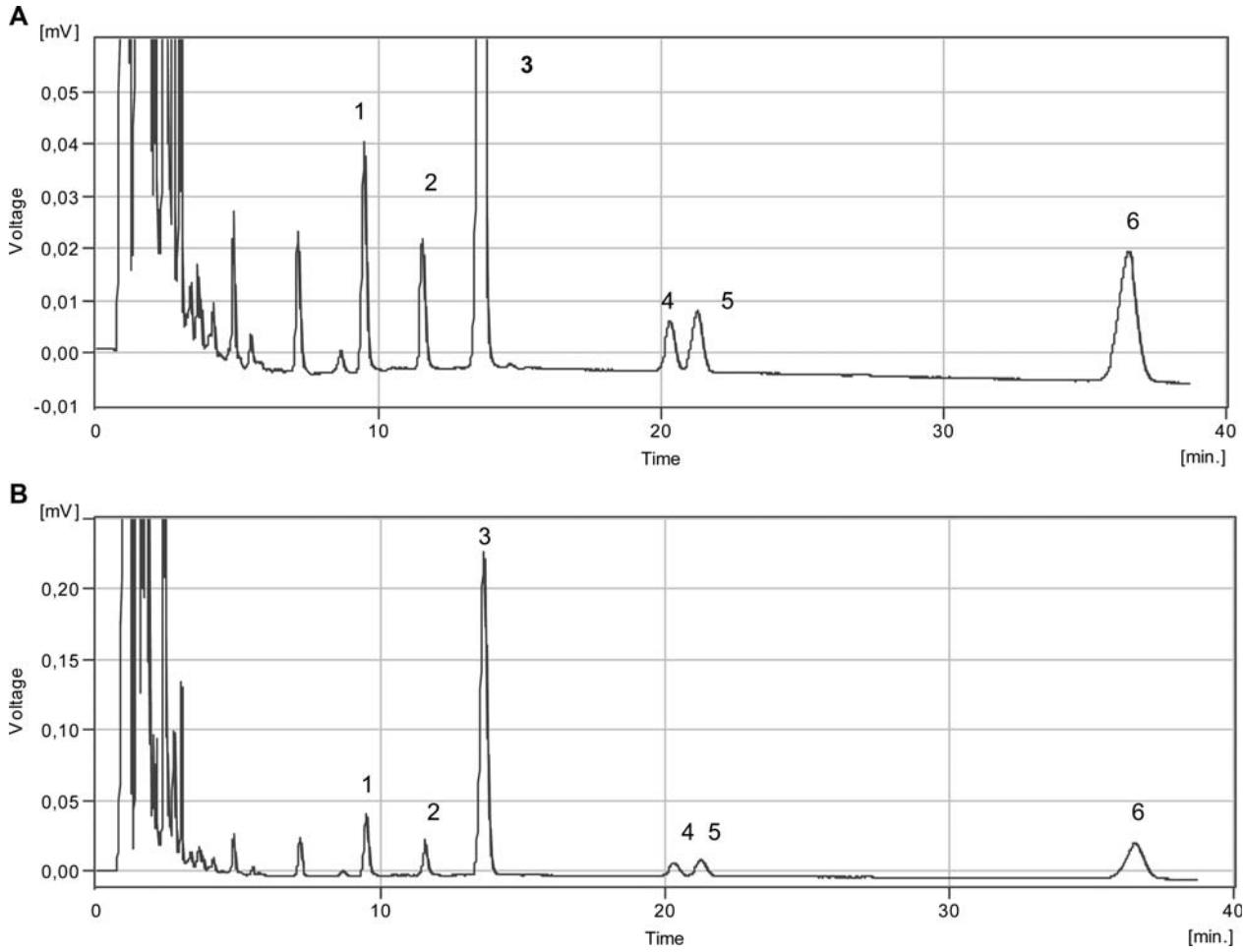


**Figure 2.** Representative chromatograms of fatty acids standard mixture derivatized at different attenuation (A) 0.00−0.06 mV- (B) 0.00-0.30 mV Peaks: 1-linolenic, 2-myristic, 3-linoleic, 4-palmitic, 5-oleic, 6-stearic. Isocratic elution with acetonitrile-water (85−15)(v-v), column temperature 45 °C, λ = 242 nm.

The peaks were identified by their retention times in comparison to the external standards. The concentrations of individual fatty acids were determined using the calibration curve of the mixture of free fatty acids.

## Results and Discussion

The procedure is based on the following steps: hydrolysis of the triacylglycerols through the catalytic action of lipase, extraction of the fatty acids and their derivatization with

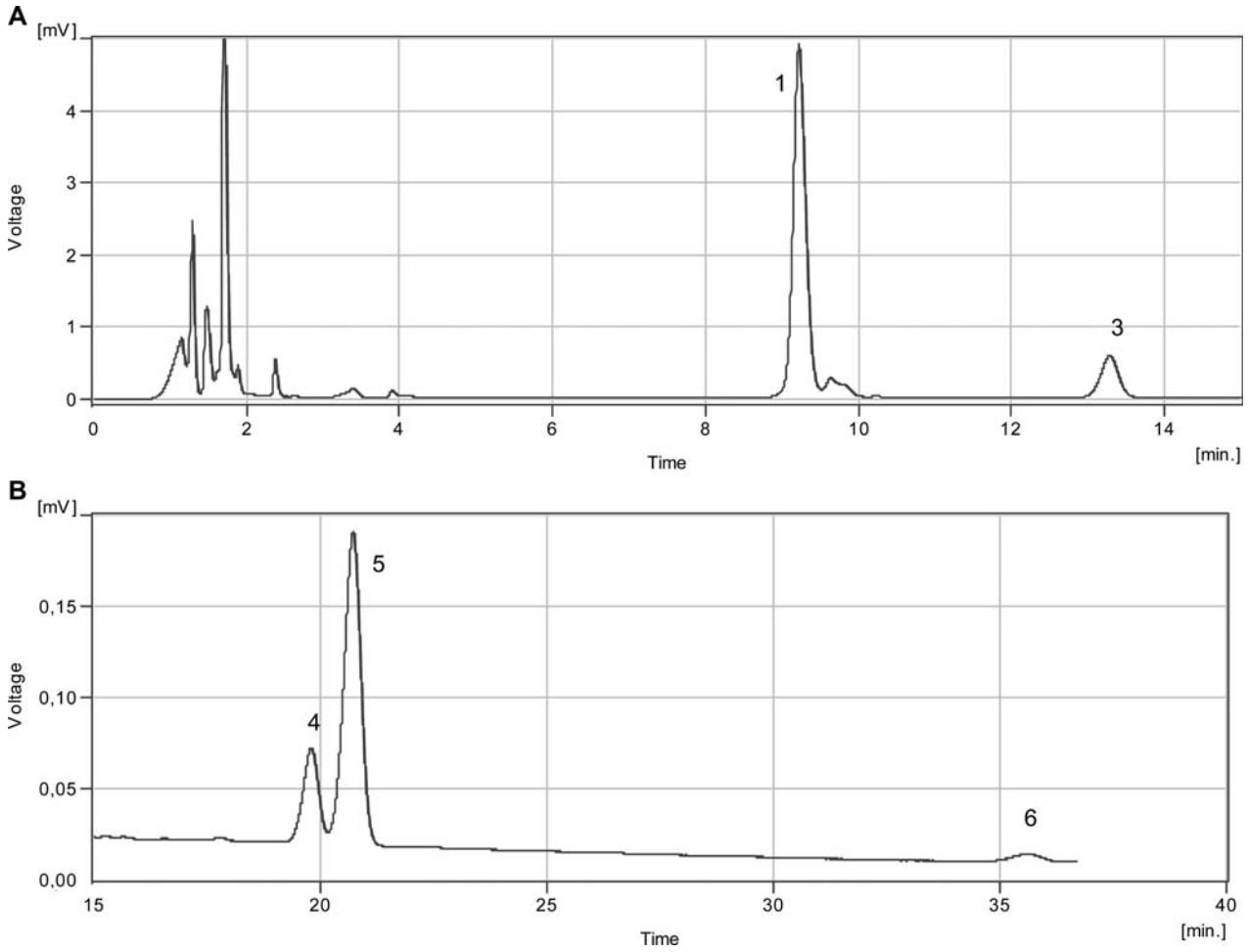


**Figure 3.** Representative chromatograms of fatty acids after enzymatic hydrolysis of linseed oil. (A) analysis time. 0–15 min, attenuation: 0–5 mV: peaks: 1-linolenic, 3-linoleic. (B) analysis time. 15–40 min, attenuation 0.00–0.20 mV, peaks: 4-palmitic, 5-oleic, 6-stearic. Isocratic elution with acetonitrile-water (85–15)(v–v), column temperature 45 °C, λ=242 nm.

phenacylbromide and analysis by HPLC. This method allows the determination of 6 fatty acids: linolenic, myristic, linoleic, palmitic, oleic and stearic acids. Chromatographic determination is carried out by using a method of external calibration.

The calibration curves show a linearity in the range of 1.2–380 mg/L with a determination coefficient $r^2$ 0.096–0.099 ($n = 3$) Table I. The limits of determination ranged from 0.24–0.36 μg/mL, corresponding to a signal-to-noise ratio of 10. The hydrolysis of the triacylglycerols present in the drying oils, walnut, linseed and poppy seed, is carried out using the action of the enzyme lipase of microbial origin, *Candida Rugosa*, which possesses the characteristic common to all lipases namely catalysing the reaction of hydrolysis in a biphasic system (oil/water). In fact, they undergo an activation defined as "interfacial activation," which make them active at the moment they bind to the interface of an organic phase and an aqueous phase (13–14) .

The experimental conditions of the hydrolysis reaction are pH 7 and 30°C corresponding to optimum conditions reported in literature for the catalytic activity of the lipase from *Candida Rugosa*. The reaction time was studied by carrying out the hydrolysis of the walnut oil by enzyme action at different times. Figure 1 shows an example of the evolution of the formation of the linoleic acid, expressed as a percent value normalized with respect to its maximum value, depending on the incubation time. The plateau is reached after 30 minutes, which is consequently the optimum incubation time.

### *Derivatization of fatty acids*

Saturated fatty acids do not contain chromophore groups and the contribution of double bonds from the unsaturated ones is limited for detection purposes under UV light. Therefore, it is necessary to derivatize them with appropriate UV-absorbing reagents to make them fully detectable. The derivatization procedure used in this study employed phenacylbromide in accordance with the procedure described by Mehta (12).

### *Quantitative determination*

The esters formed from linolenic, myristic, linoleic, palmitic, oleic and stearic acids are separated by reversed phase HPLC under isocratic conditions with a 45°C column temperature.

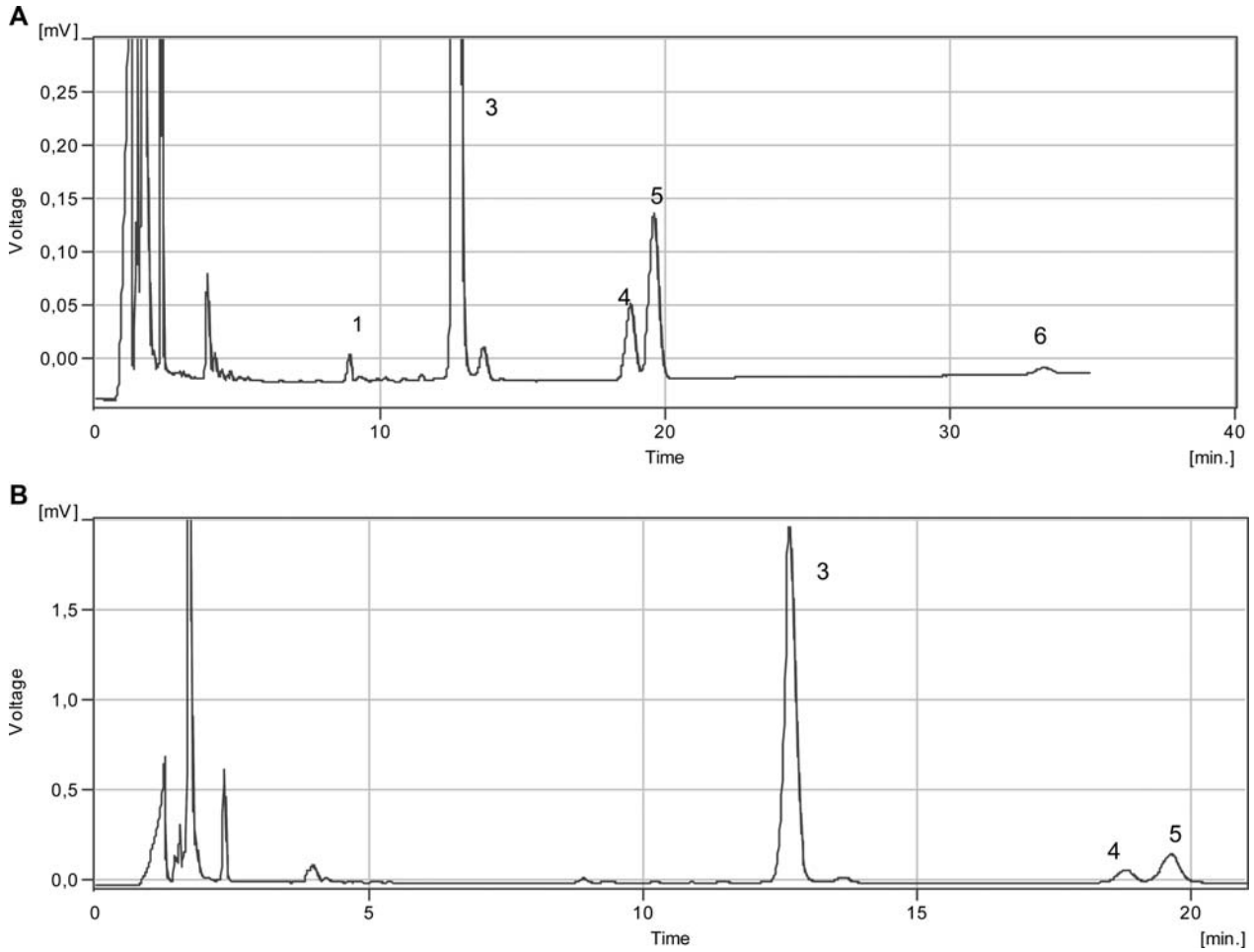


**Figure 4.** Representative chromatograms of fatty acids after enzymatic hydrolysis of walnut oil (A) analysis time. 0–40 min, attenuation: 0.00–0.30 mV, peaks: 1-linolenic, 3-linoleic, 4-palmitic, 5-oleic, 6-stearic. (B) analysis time. 0–21 min, attenuation: 0.0–2.0 mV, peaks: 3-linoleic, 4-palmitic, 5-oleic. Isocratic elution with acetonitrile-water (85–15)(v–v), column temperature 45 °C, λ=242 nm.

A representative chromatogram of the standard mixture of fatty acids is shown in Figure 2. The utilization of a C18 column, 15 cm in length and 3 μm, resulted in the advantage of a greater efficiency in the separation of the analytes, a reduction in the quantity of the mobile phase utilized with a consequent reduction in the cost of the solvents employed and the problems related to their disposal. Moreover, compared to GC methods (15), a higher resolution of fatty acids is obtained, particularly between oleic and stearic acids, which differ only in the unsaturation degree.

In general, the difficulty in identifying the nature of oils used as a pictorial medium is due to the small quantity of samples available and the complexity of the matrix. The procedure described in this study allows these problems to be overcome. Small quantities of samples from 0.1 to 0.5 mg can be used thanks to the selective action of the lipase, which acts on a specific substrate.

For this reason linseed, poppyseed and fresh walnut oils were examined. The chromatograms are shown in Figures 3–5. The results reported in Table II show the average mean of three determinations for each oil expressed as a percent of the total.

These results are in complete agreement with those shown in literature (16) highlighting the validity of the lipase-HPLC method used and confirming the diverse drying powers of the oils under examination. Poppyseed oil contains the least polyunsaturated acids (linolenic and linoleic) and therefore has less drying power than walnut oil and linseed oil. Results are shown in Table II. The identification of dried oils in the absence of polyunsaturated acids is based on the relationship between palmitic acid and stearic acid as shown in scientific literature. The proposed method was applied for the analysis of nine drying oils (such as walnut, linseed and poppyseed) utilized for restoration.

## Conclusions

This work describes the procedure for determining the fatty acid composition in drying oils such as linseed, walnut and poppyseed. The fatty acids produced by action of lipase enzyme at 30°C for 30 min are extracted by *n*-heptane, derivatized with α-bromoacetophenone and determined by HPLC on RP with UV detection at 242 nm at a 45°C column temperature. The primary advantages of this method are the specificity

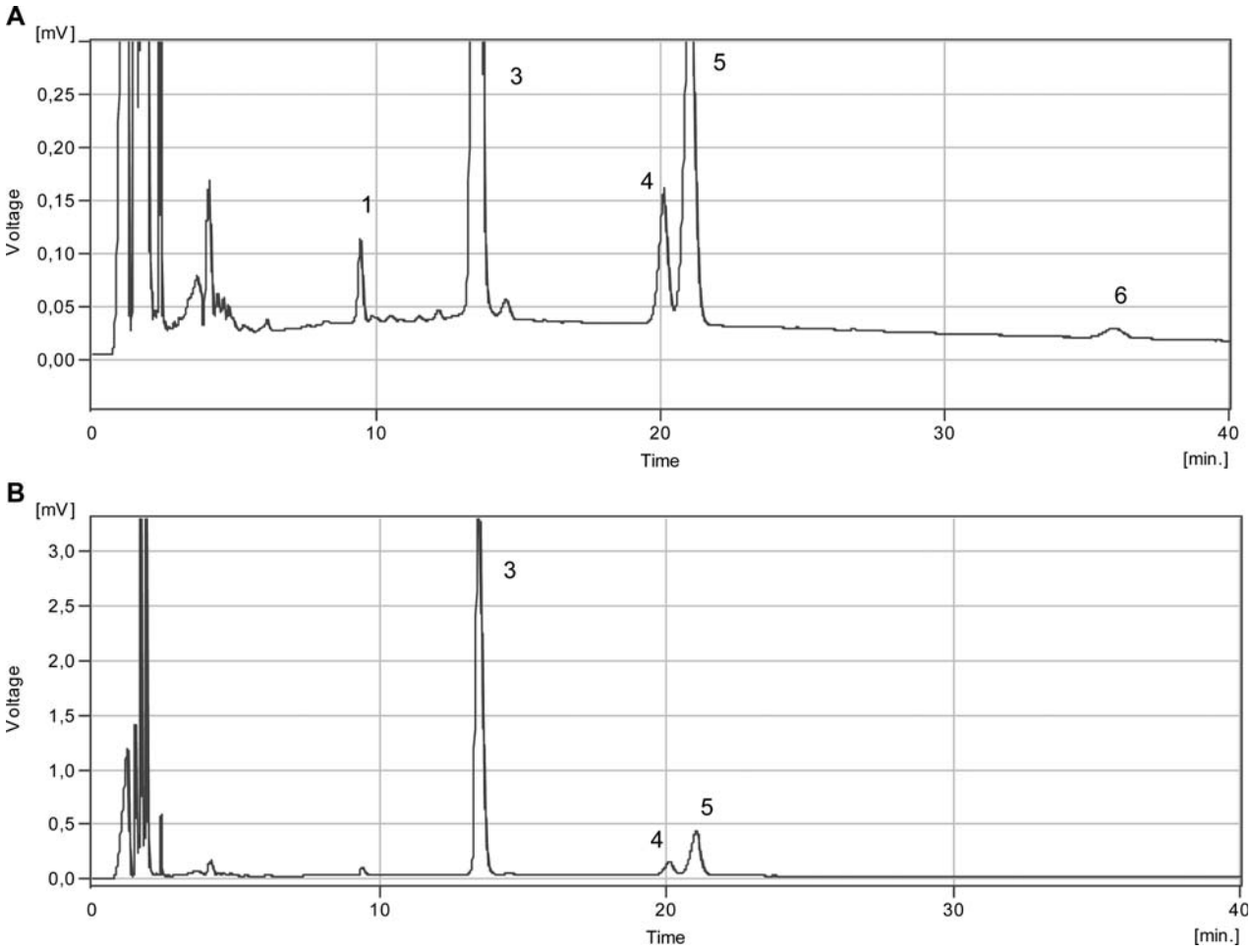


**Figure 5.** Representative chromatograms of fatty acids after enzymatic hydrolysis of poppy seed oil at different attenuation (A) 0.00–0.30, (B) 0.0–3.0. Peaks: 1-linolenic, 3-linoleic, 4-palmitic, 5-oleic, 6-stearic. Isocratic elution with acetonitrile-water (85–15)(v–v), column temperature 45 °C, λ=242 nm.

**Table II**
Content of Fatty Acid of the Drying Oil after Hydrolysis (Phenacyl Bromide Esters % w/w Total Fatty Acid)

| Drying oil | Linolenic acid C18:3 % ± SD | Linoleic acid C18:2 % ± SD | Myristic acid C14:0 % ± SD | Palmitic acid C16:0 % ± SD | Oleic acid % C18:1 % ± SD | Stearic acid C18:0 % ± SD |
|---|---|---|---|---|---|---|
| Linseed oil | 58 ± 1.2 | 16 ± 0.8 | – | 7 ± 1.1 | 16 ± 1.8 | 3 ± 0.1 |
| Walnut oil | 8 ± 0.3 | 72 ± 1.5 | – | 6 ± 0.6 | 12 ± 1.1 | 2 ± 0.2 |
| Poppy seed oil | 8 ± 0.7 | 70 ± 0.8 | – | 9 ± 0.5 | 10 ± 0.4 | 3 ± 0.2 |

Values are average mean n = 3 different hydrolysed drying oil.

of the hydrolytic action of lipase and that only small quantities of samples, 0.1 mg, must be employed. In the case of identifying the chemical nature of binding media, the use of small quantities of small samples is very important to avoid deterioration of the work.

Moreover, the utilization of a C18 column, 15 cm in length and 3 μm, resulted in the advantage of a greater efficiency in the separation of the analytes and a reduction in the quantity of the mobile phase utilized, with a consequent reduction of the solvents employed.

## References


1. Mills, J.; The gas-chromatographic examination of paint media; Part I. Fatty acid composition and identification of dried oil film; *Studies in Conservation*, (1966); 11: 92–106.
2. Johnson, M., Packard, E.; Methods used for the identification of binding media in Italian paintings of the fifteenth and sixteenth centuries; *Studies in Conservation*, (1971); 16: 145–169.
3. Tsakalof, A.K., Bairachtari, K.A., Chryssoulakis, I.D.; Pitfalls in drying oils identification in art objects by gas chromatography; *Journal of Separation Science*, (2006); 29(11): 1642–1646.
4. Cappitelli, F., Learner, T., Chiantonre, O.; An initial assessment of thermally assisted hydrolysis and methylation-gas chromatography/mass spectrometry for the identification of oils from dried paint films; *Journal of Analytical and Applied Pyrolysis*, (2002); 63: 339–348.
5. Marinach, C., Papillon, M.C., Pepe, C.; Identification of binding media in works of art by gas chromatography–mass spectrometry; *Journal of Cultural Heritage*, (2004); 5: 231–240
6. Chen, S.H., Chen, K.C., Lien, H.M.; Determination of fatty acids in vegetable oil by reversed-phase liquid chromatography with

fluorescence detection; *Journal of Chromatography A*, (1999); 849: 357–369.
7. Peris Vicente, J., Gimeno Adelantado, J.V., Doménech Carbo, M.T., Mateo Castro, R., Bosch Reig, F.; Identification of drying oils used in pictorial work of art by liquid chromatography of the 2-nitrophenylhydrazides derivatives of fatty acids; *Talanta*, (2004); 64: 326–333.
8. Miwa, H.; High-performance liquid chromatographic determination of free fatty acids and esterified fatty acids in biological materials as their 2-nitrophenylhydrazides; *Analytica Chimica Acta*, (2002); 465: 237–255.
9. Peris Vicente, J., Gimeno Adelantado, J.V., Doménech Carbo, M.T., Mateo Castro, R., Bosch Reig, F.; Identification of lipid binders in old oil paintings by separation of 4- parabromomathyl-7-methoxycoumarin derivatives of fatty acids by liquid chromatography with fluorescence detection; *Journal of Chromatography A*, (2005); 1076: 44–50.
10. Chen, S., Chuang, Y.; Analysis of fatty acids by column liquid chromatography; *Analytica Chimica Acta*, (2002); 465: 145–155.
11. Houde, A., Kademi, A., Leblanc, D.; Lipases and their industrial applications; *Applied Biochemistry and Biotechnology*, (2004); 118: 155–170.
12. Mehta, A., Oeser, A.M., Carlson, M.G.; Rapid quantitation of free acids in human plasma by high-performance liquid chromatography; *Journal of Chromatography B*, (1998); 719: 9–23.
13. Jeager, K.E., Eggert, T.; Lipases for biotechnology; *Current Opinions in Biotechnology*, (2002); 13: 390–397.
14. Linfield, W.M., O'Brien, D.J., Serota, S., Barauskas, R.A.; Lipid-lipase interactions. I. Fat splitting with lipase from *Candida Rugosa*; *Journal of the American Oil Chemists' Society*, (1984); 61(6): 1067–1071.
15. Gimeno-Aldebrando, J.V., Mateo-Castro, R., Domenech-Carbò, M.T., Bosh-Reing, F., Domenech-Carbò, A., Casa-Catala, M.J., *et al.*; Identification of lipid binders in painting by gas chromatography Influence of the pigments; *Journal of Chromatography*, (2001); 922: 385–390.
16. Mills, J.S., White, R.; The organic chemistry of museum objects. 2nd edition. Butterworth, Heinemann, London, (1994).