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# New Analytical Method using Coupled Enzymes for Determination of Polyunsaturated Fatty Acid Content in Olive Oil

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**Abstract** A new, simple, and original method is described for specific measurement of polyunsaturated fatty acid content in olive oil. This analytical system uses coupled enzymes, lipase and lipoxygenase. The system consists of lipase-catalyzed hydrolysis of triacylglycerol and subsequent lipoxygenation of liberated polyunsaturated fatty acids. The hydroperoxy-fatty acids formed were easily monitored by spectrophotometry at 234 nm. After being optimized, the method was validated in terms of linearity, precision sensitivity, and recovery. Linear calibration graph was obtained in the range 50–500 μg $mL^{-1}$, with a correlation coefficient higher than 0.921 and a detection limit (S/N=3) of 15 μg $mL^{-1}$. The precision of the method (relative standard deviation) for within and between days is better than 7% and 12%, respectively. The proposed method was successfully applied to the estimation of polyunsaturated fatty acids level in olive oil samples and results obtained were in excellent agreement with those obtained by the classical official method. The proposed method is accurate, simple, cheap, and can be satisfactorily used for routine analysis of edible oils.

**Keywords** Lipase · Lipoxygenase · Olive oil · Polyunsaturated fatty acids · Oxidative stability · Spectrophotometry

## Introduction

Virgin olive (*Olea europea* L.) oil is recognized as one of the higher quality plant oils given its nutritional benefits in the human diet [1, 2]. It is obtained from the fruit of several

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cultivars of olive trees. Each one of these cultivars exhibits specific physical and biochemical characteristics, providing oils with different compositions and performances [3]. The chemical composition of olive oil determines its intrinsic quality and could be influenced by other factors such as cultivars, environment, agronomic practices, processing, and oil storage conditions [4].

Olive oils are complex mixtures consisting of two main groups of substances: saponifiable substances which represent nearly 98% of the chemical composition, such as triglycerides, partial glycerides, esters of fatty acids, or free non-esterified fatty acids, and unsaponifiable substances, with many varied chemical structures and polarities, which represent only 2% of all olive oil composition [5].

One of the most severe quality problems of virgin olive oil is its oxidative rancidity due to oxidation of unsaturated fatty acids and subsequent formation of compounds that possess unpleasant taste and odor [5]. Oxidation of olive oil and other seed oils depends on their composition in polyunsaturated fatty acids; the oils with higher contents of unsaturated fatty acids, especially polyunsaturated fatty acids, are more susceptible to oxidation. Lipid oxidation occurs fairly slowly at room temperature; nevertheless, it is the main cause of olive oil quality deterioration and its reaction rate determines the shelf life of this product. It results in the losses of nutritional value of food products as well as changes in color, odor and flavor, texture, sensory, and other physiological properties [6, 7]. Since it has been shown that olive oil stability to oxidation was correlated to polyunsaturated fatty acid levels, products with high degree of unsaturation are more susceptible to lipid oxidation and hence off-flavor development. There is a growing need for a simple and accurate method for their detection and quantification in edible oil especially in olive oil samples.

To date, several analytical methods were suggested for fatty acid content determination in oil. The most known and widely used procedure, adopted as official standard in several laboratories, is based on gas chromatography analysis (GC). Although characterized by a good specificity, this method has several limitations, the most serious being the use of relatively large volumes of organic solvent. Another popular method is based on the determination of the iodine value (IV) to express the degree of unsaturation of fats and oils or their derivatives. The IV is defined as grams of iodine consumed per 100 g of fat. The standard IV determination by classical titration is time consuming and inaccurate [8]. A number of alternative methods and techniques for estimating polyunsaturated fatty acids have also been reported in the literature. Sreenivasan and Brown [9] proposed and improved a procedure to isomerize linoleic, linolenic, and arachidonic acids using potassium tertiary butoxide in *t*-butanol. Analysis of plant oil samples for component polyunsaturated acid was performed using UV spectrophotometric method to measure conjugated double bonds.

In this work, a new and original method is described for specific measurement of polyunsaturated fatty acid content in oil and especially in olive oil. This analytical system uses coupled enzymes, lipase and lipoxygenase, catalyzing two consecutive reactions in the lipoxygenase pathway [10]. In oil sample, after lipase catalyzed hydrolysis of triacylglycerols and fatty acid liberation, the lipoxygenase catalyzes the incorporation of $O_2$ into a (1Z, 4Z)-pentadiene system of polyunsaturated fatty acids, forming hydroperoxy-fatty acids [11]. Measurement of the conjugated double bond appearance was carried out at 234 nm [12]. The second reaction, being very fast and easily followed spectrophotometrically, allows an indirect determination of polyunsaturated fatty acid content. The reaction scheme of this analytical method is shown in Fig. 1. In oil sample, saturated or monounsaturated fatty acids as well as esterified fatty acids were not converted by the lipoxygenase and do not give any signal [12].

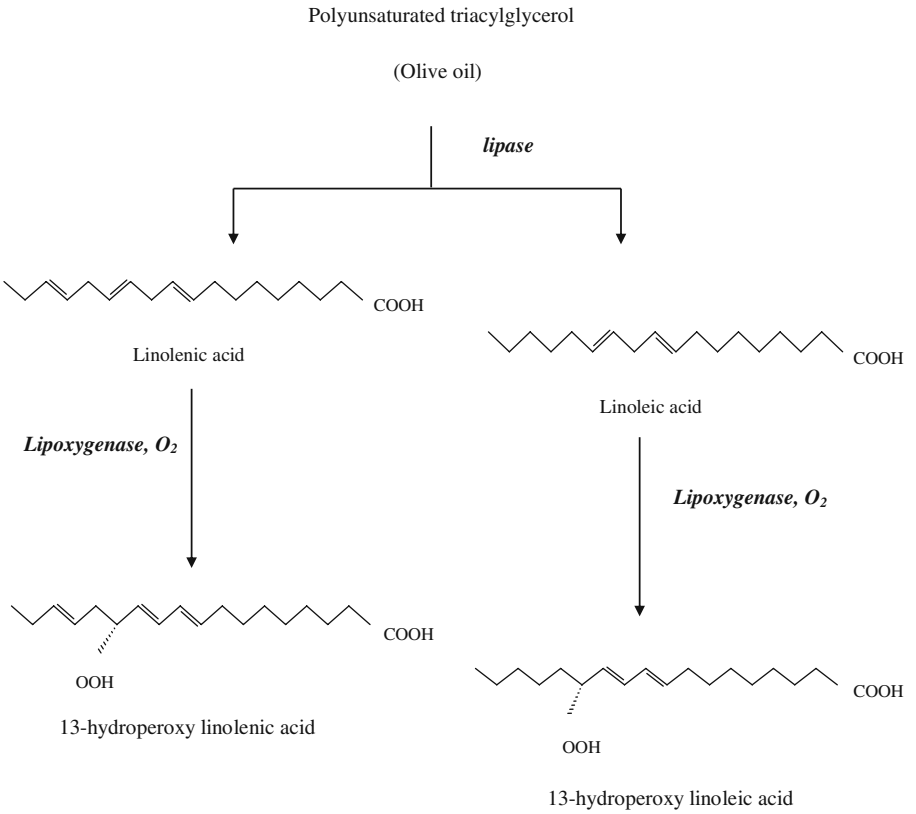


**Fig. 1** Schematic representation of the bi-enzymatic system (lipase-lipoxygenase) adopted for the determination of polyunsaturated fatty acid content in olive oil

The described method was optimized and the analytical features of this system proposed for polyunsaturated fatty acid determination were investigated. Optimization of pH, concentration of enzymes, and reaction time are illustrated and discussed.

## Experimental

### Sampling

Samples were obtained from homogenous olive fruits (*Olea europaea L.*). The studied cultivars were Chemlali, Chetoui-1, Chetoui-2, Zarrazi (Tunisian cultivars), and Arabequina (foreign variety). Oil samples were obtained by a cold extraction process using a laboratory mill. The samples were transferred into dark glass bottles and stored in the dark at 4 °C until analysis. Sunflower oil was purchased from the local market.

### Materials

All reagents used were of analytical reagent grade and were provided by Prolabo (Paris, France). HPLC-grade solvents were supplied by Sigma (France). Soybean lipoxygenase

type 1-B, linoleic acid (minimum 99%), trilinolein (grade >99%), trilinolenin (approx. 99%), triolein (grade >99%), tristearin (grade approx. 99%), and tripalmitin (approx. 99%) were purchased from Sigma Chemical (St. Louis, MO, USA). Lipases (triacylglycerol acyl hydrolases E.C. 3.1.1.3) from *Mucor javanicus* (lipase M), *Aspergillus niger* (lipase A), *Candida rugosa* (lipase AY), *Penicillum roqueforti* (lipase R), and *Penicillium camembertii* (lipase G) were kindly provided by Amano chemicals (Japan). Enzymes were used without further purification.

Determination of protein content was carried out according to the Bradford assay [13]. Protein content was estimated by mean of a calibration curve obtained using BSA (98%) as protein standard.

## Enzyme Activities

Lipase activity was measured by titrating fatty acids liberated from tributyrin with 0.05 mol $L^{-1}$ NaOH. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol fatty acid $min^{-1}$ at 30 °C and pH 7. The method was based on butyric acid titration after lipase catalyzed hydrolysis of tributyrin. The pH was maintained at 7.0 by titration with 0.05 M sodium hydroxide solution. Lipase-catalyzed hydrolysis reactions were carried out at 30 °C and pH 7 in 20 mL glycerol/water (5.4:4.6, v/v), containing gum arabic (6 g $L^{-1}$), NaCl (0.3 mol $L^{-1}$), $KH_2PO_4$ (3 mmol $L^{-1}$), and 0.25 mL tributyrin as substrate [14]. After preheating the mixture for 5 min, 2 mL lipase solution (0.2% w/v) was added into the reaction medium. Release of free butyric acid was monitored by continuous titration with 0.05 mol $L^{-1}$ NaOH.

Lipoxygenase activity was determined at 25 °C spectrophotometrically from the increase in absorbance at 234 nm. The reaction mixture (3 mL) contained 180 μL linoleic acid emulsion, 0.1 M glycine buffer pH 9, and 20 μL enzyme solution. The linoleic acid emulsion was prepared by mixing 70 mg linoleic acid and 70 mg Tween-20 in 25 mL water. One unit of lipoxygenase activity was defined as the amount of enzyme that increases the absorbance by 0.001 $min^{-1}$ at 234 nm and at 25 °C [15].

## Coupling of Hydrolysis and Lipoxygenation

By coupling both hydrolysis and lipoxygenation in the same reactor, we are able to perform two different reactions in the same system. This system allows the specific determination of polyunsaturated fatty acid content in oil samples.

Lipase-catalyzed triacylglycerol hydrolysis and the lipoxygenation of free polyunsaturated fatty acids were coupled in the same system. The second enzyme (lipoxygenase) catalyzed the conversion of the product of the first enzymatic reaction (lipolysis). The reaction mixture was magnetically stirred at 300 rpm and held at 30 °C. Samples (100 μL) were withdrawn periodically from the reaction mixture and the reaction was stopped with 1 mol $L^{-1}$ $H_2SO_4$ and finally diluted ten times with glycine buffer (0.1 mol $L^{-1}$ pH 9). Measurement of the appearance of hydroperoxy-fatty acid was carried out at 234 nm on a UV/visible Beckman spectrophotometer DU 530.

## Method Validation

Accuracy, precision, and linearity intra-day and inter-day assays were all determined on three different days. Recovery of analytes after sample preparation was determined at

different concentrations. The detection and quantification limits were determined as three and ten times the baseline noise (background levels), respectively.

### Fatty Acid Determination

Fatty acid composition of the oils was determined by GC as fatty acid methyl esters (FAMEs). FAMEs were prepared by saponification/methylation with $BF_3$ in methanol according to the method of Metcalfe et al. [16]. A chromatographic analysis was performed in a Hewlett-Packard gas chromatograph HP 6890 using an Innowax capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) equipped with a flame ionization detector. The carrier gas was nitrogen, with a flow rate of 1 mL $min^{-1}$. The column temperature was isothermal at 180 °C; the injector and detector temperatures were 230 and 250 °C, respectively. The injection volume was 1 μL. Fatty acids were identified by comparing retention times with standard compounds. Five fatty acids were considered in this study: palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. Each fatty acid was expressed as the percentage of total fatty acid peak areas.

### Quality Parameters

Free acidity, peroxide value, and UV spectrophotometric indices at 232 and 270 nm ($K_{232}$ and $K_{270}$) were determined following the analytical methods described in the regulation of the European Union Commission [17]. All parameters were determined in triplicate for each sample.

## Results and Discussion

### Lipase Activity Measurement

Different microbial lipases were used in the present work, namely, lipase AY, lipase M, lipase R, lipase A, and lipase G. Since they were commercial preparations with different purities, they were characterized regarding protein content and specific activity (Table 1). Protein content of the commercial preparations, determined by the Bradford assay, was in general quite low. The highest protein content (w/w) was shown with lipase M (5.70%) followed by lipase A (3.10%). The other commercial preparations, lipase AY, lipase R, and lipase G, showed protein content lower than 1% (Table 1). The lipase activity was determined by measuring the initial rate of tributyrin hydrolysis and was expressed in terms of units per gram (micromoles of butyric acid formed per minute and per gram of commercial lipase). Specific activity was then calculated as the activity (μmol $min^{-1}$) per milligram of proteins.

Since lipase M-10 from *M. javanicus* was known to be less selective and was already demonstrated to ensure a complete hydrolysis of olive oil samples [18], it has been chosen for further studies.

### Optimization of Analytical Method

The selectivity and the sensitivity of the proposed method depend on a number of experimental parameters such as lipase and lipoxygenase concentrations, pH of the working

**Table 1** Characterisation of commercial lipases.

| Lipase | Microbial source | Protein content (w/w %) | Activity (μmol $min^{-1}$ $g^{-1}$ commercial lipase) | Specific activity (μmol $min^{-1}$ $mg^{-1}$ protein) |
|---|---|---|---|---|
| M | *Mucor javanicus* | 5.70 | 1,028.00 | 18.03 |
| AY | *Candida rugosa* | 0.77 | 362.50 | 47.07 |
| A | *Aspergillus niger* | 3.10 | 97.01 | 3.13 |
| R | *Penicillium roqueforti* | 0.93 | 970.00 | 104.30 |
| G | *Penicillium camemberti* | 0.73 | 19.50 | 2.67 |

solution, and the reaction time. The optimization was carried out using a standard solution of trilinolein as substrate.

*Optimization of Lipase and Lipoxygenase Concentrations*

In order to optimize the analytical method for the determination of polyunsaturated fatty acid content in oil, the dependence of the bi-enzymatic system response on the amount of lipase M from *M. javanicus* in solution was studied. Figure 2 shows that the response increases with the increase of lipase concentration. Higher concentrations of lipase liberate free fatty acids in higher rates, subsequently leading to higher response due to hydroperoxy-fatty acid formation. It is necessary to use sufficient quantities of the active enzyme to achieve a complete hydrolysis of triacylglycerols in less than 20 min at 30 °C. For the complete hydrolysis of olive oil samples to produce free fatty acids especially polyunsaturated fatty acids, the enzyme should be as unspecific as possible.

In oil sample, after lipase catalyzed hydrolysis of triacylglycerols and fatty acid liberation, the lipoxygenase (EC 1.13.11.12, non-heme iron dioxygenase) catalyzes the incorporation of $O_2$ into a (1Z, 4Z)-pentadiene system of polyunsaturated fatty acids, forming hydroperoxy-fatty acids. The study of the effect of lipoxygenase concentration was investigated keeping the concentration of lipase constant (0.5 mg $mL^{-1}$). The dependence of the responses as a function of lipoxygenase amount is shown in Fig. 3. Experiments show that the lipoxygenase reaction is fast enough to immediately follow the lipase-

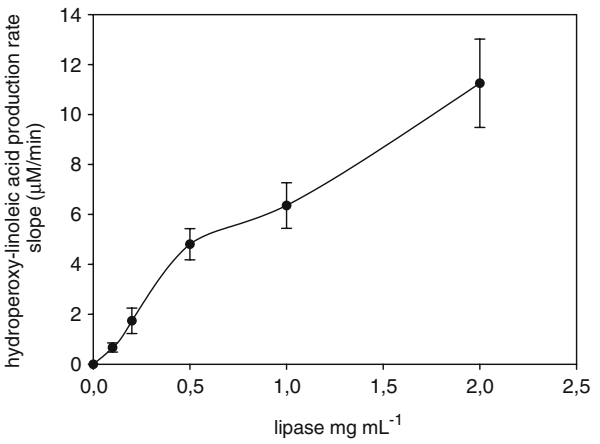


**Fig. 2** Effect of lipase M from *Mucor javanicus* concentration on the production of hydroperoxy-linoleic acid in the bi-enzymatic system from trilinolein. Experimental conditions: trilinolein concentration 0.5 mg $mL^{-1}$, [lipoxygenase]= 40 nM, 30 °C, glycine buffer 0.1 M pH 9, total volume=20 mL

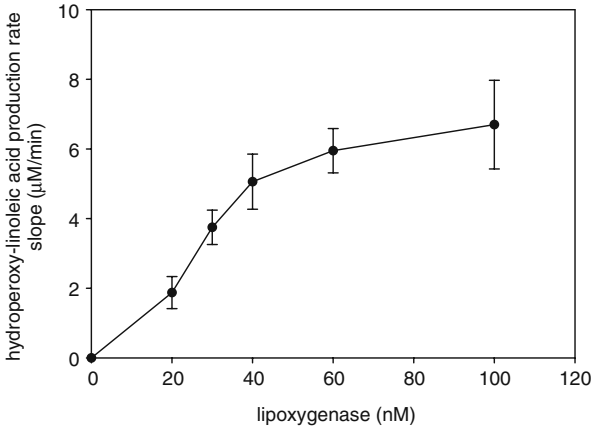


**Fig. 3** Effect of lipoxygenase concentration on the bi-enzymatic system response. Experimental conditions: trilinolein concentration=0.5 mg $mL^{-1}$, [lipase]=0.5 mg $mL^{-1}$, 30 °C, glycine buffer 0.1 M pH 9, total volume=20 mL

catalyzed hydrolysis reaction. Measurement of the conjugated double bond appearance was carried out at 234 nm. The second reaction, being very fast and easily followed spectrophotometrically, allows an indirect determination of polyunsaturated fatty acid content. This enzyme reacts specifically with free polyunsaturated fatty acids. A lipoxygenase concentration of 40 nM was chosen for further studies.

*pH Study*

It is well known that the enzyme activity is pH dependent and the optimum pH for an enzymatic assay must be determined empirically. In fact, the reaction medium pH can affect the overall enzymatic activity and stability since, like all proteins, enzymes have a native tertiary structure that is sensitive to pH, and denaturation of enzymes can occur at extreme pHs. Since the optimum pH values of the two enzymes used here are different, lipase M from *M. javanicus* optimum pH 8.0 [19] and soybean lipoxygenase optimum pH 9 [20], the pH effect on the bi-enzymatic system response was studied in the range 7.0–10 using phosphate (7.0 and 8.0), Tris (8.5), and glycine (9.0, 9.5, and 10.0) buffers. The reaction rate of the two-enzyme system was determined at 30 °C in stirred working buffer by following the release of hydroperoxy-fatty acid measured at 234 nm using the Beer–Lambert law according to the molar extinction coefficient of HPO ($\varepsilon$=25,000 $cm^{-1}$ $M^{-1}$). Figure 4 shows the pH profile of the hydrolysis-oxygenation reaction rate. The maximum activity was found at pH 9.0; this value was used for further studies.

In order to proceed with the final analytical system, the reaction time was studied under the optimum chemical variables determined previously. The spectrophotometric response was observed to increase with increasing reaction time (data not shown). An assay of 20 min was selected as optimal, making compromise between sensitivity and reaction time.

Based on these findings, all further measurements were performed under the above-mentioned optimal conditions.

## Fatty Acid Composition

Fatty acid composition of the oil may differ depending on the variety of the olive tree. Table 2 shows the variation of each fatty acid in the studied virgin olive oils. Oleic acid is

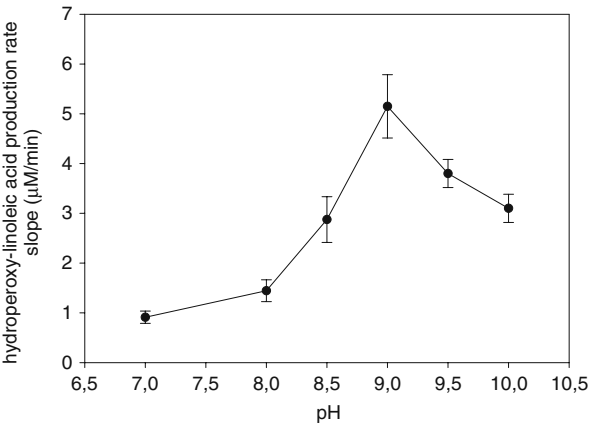


**Fig. 4** Effect of pH on the bi-enzymatic system lipase/lipoxygenase response. Experimental conditions: trilinolein concentration=0.5 mg $mL^{-1}$, [lipase]=0.5 mg $mL^{-1}$, [lipoxygenase]=40 nM, 30 °C, phosphate buffer 0.1 M for pH 7 and 8, Tris buffer 0.1 M for pH 8.5 glycine buffer 0.1 M for pH 9, 9.5, and 10, total volume=20 mL

the main monounsaturated fatty acid and is present in higher concentrations according to varieties (62.33–71%). The monounsaturated fatty acids have great importance because of their implication and effect on oxidative stability of oils. Palmitic acid, the major saturated fatty acid, showed a percentage that ranged between 11.66% and 20%. Concerning linoleic acid, the highest percentage was observed in Chetoui-1 oils (21.94%), whereas the lowest one was found in Zarrazi samples (14%). For the other fatty acids such as stearic ($C_{18:0}$) and linolenic ($C_{18:3}$) acids, their amounts were varied between 0.3% and 1.61% in the analyzed oil samples.

Oleic acid/linoleic acid ratio varied from 2.95 to 5.07 for all tested samples (Table 2). The ratio can be useful in olive cultivar characterization and stability interpretation [21].

Fatty acid composition has a relatively wide range due to the genetic and environmental factors. It has previously been used by a number of authors as a parameter for oil classification [22, 23]. It is an essential aspect of the qualitative assessment of olive oil.

**Table 2** Fatty acid composition and analytical characteristics of virgin olive oil samples.

| | Chetoui-1 | Chetoui-2 | Chemlali | Zarrazi | Arabequina | Sunflower oil |
|---|---|---|---|---|---|---|
| Palmitic acid ($C_{16:0}$) (%) | 11.66 | 15.32 | 18.2 | 14.65 | 20 | 9.41 |
| Stearic acid ($C_{18:0}$) (%) | 0.65 | 0.92 | 1.08 | 0.3 | 1.05 | 1.6 |
| Oleic acid ($C_{18:1}$) (%) | 64.69 | 67.67 | 62.33 | 71 | 63.23 | 33.42 |
| Linoleic acid ($C_{18:2}$) (%) | 21.94 | 15.52 | 17.5 | 14 | 14.11 | 53.59 |
| Linolenic acid ($C_{18:3}$) (%) | 1.06 | 0.6 | 0.9 | 0.46 | 1.61 | 2 |
| Polyunsaturated fatty acids (PUFAs %) | 23 | 16.12 | 18.4 | 14.46 | 15.72 | 55.53 |
| Oleic acid/linoleic acid ratio ($C_{18:1}/C_{18:2}$) | 2.95 | 4.36 | 3.56 | 5.07 | 4.48 | – |
| Free Acidity (%) | 0.45 | 0.72 | 0.36 | 0.3 | 0.55 | – |
| Peroxide Value (meq $O_2$ $kg^{-1}$) | 17.6 | 13.4 | 12.6 | 6.5 | 13.2 | – |
| $K_{232}$ | 1.24 | 1.35 | 1.68 | 1.12 | 0.98 | – |
| $K_{270}$ | 0.09 | 0.12 | 0.15 | 0.13 | 0.09 | – |

## Quality Indices

All the analyzed samples showed very low values for the regulated physicochemical parameters evaluated (acidity ≤0.8; peroxide value ≤20 meq $O_2$/kg; $K_{270}$≤0.22; $K_{232}$≤2.5) corresponding to the highest quality category: extra virgin olive oil (Table 2).

## Validation Assay

Method validation establishes that the method performance characteristics are suitable for the intended use. Various parameters of the method such as selectivity, accuracy, precision, linearity, sensitivity, detection limit, quantification limit, and recovery should be evaluated.

### *Selectivity*

The selectivity of the proposed method was tested using the coupled enzyme system by adding an amount of triacylglycerol corresponding to highest concentration found in olive oil. Amounts of each glyceryl trioleate, glyceryl tristearate, and glyceryl tripalmitate were added to 20 mL of reactional media. The results obtained showed no response, which indicates that these compounds does not interfere with trilinolein and trilinolenin. The selectivity of the method was further assessed by separate analysis of triacylglcerol samples, and no significant absorbance was observed at 234 nm. These results indicate that the proposed method is suitable for selective determination of polyunsaturated fatty acids in olive oil samples.

### *Linearity and Calibration Graph*

The linearity of the proposed method was investigated by replicate analysis ($n$=3) at five concentrations, i.e., 50, 100, 150, 250, and 500 μg $mL^{-1}$ trilinolein, over three different days. The absorbance obtained at each concentration was plotted against the concentration of hydroperoxy-fatty acids using molar extinction coefficient of HPO ($\varepsilon$=25,000 $cm^{-1}$ $M^{-1}$) and the linear regression equation was evaluated by statistical treatment of calibration data (absorbance at 234 nm versus the concentration of linoleic acid equivalent in triacylglycerol).

The equation of the curve $y=3.274\ 10^{-4}\ x+0.054$, where $y$ is the absorbance and $x$ is the linoleic acid concentration (μg $mL^{-1}$), was used to calculate the level of polyunsaturated fatty acids in the real olive oil samples. Least squares analysis showed a correlation coefficient values ($R^2$) higher than 0.921 in the considered concentration range.

### *Precision and Sensitivity*

The intra- and inter-day precision of the proposed method was evaluated as the relative standard deviation (RSD) of six repeated determinations of 250 μg $mL^{-1}$ trilinolein standard solution. The precision was found to be satisfactory with an average of intra- and inter-day RSD values of 7% and 12%, respectively.

The limit of detection, calculated as three times the noise at blank level, was determined by nine replicate measurements of a reference solution containing the same medium including lipoxygenase but without the addition of lipase. It was found to be 15 μg $mL^{-1}$. The absorbance detected at 234 nm was due to oxidation of compounds that were not produced by the lipase-catalyzed hydrolysis of trilinolein.

The limit of quantification was defined as the lowest amount detectable with a precision of less than 15% and an accuracy of ±15%. The limit of quantification was found to be 45 μg $mL^{-1}$.

*Application to the Analysis of Real Samples and Accuracy*

The proposed method has been applied for the determination of polyunsaturated fatty acids in six real oil samples. The obtained results were compared with those obtained with the official chromatographic method (Table 3).

The accuracy of the proposed assay was evaluated by adding known amounts of standard polyunsaturated fatty acids (100 and 150 μg $mL^{-1}$) to oil samples and determining the recovery. The calculated mean recoveries were varied between 86% and 109%.

The accuracy of the proposed procedure was further investigated by comparing the values obtained with those estimated by the chromatographic official method. The correlation of the analytical results between the proposed enzymatic and the official chromatographic methods varied from 77.74% to 117.43% with an average of 92.16%. A highly significant linear regression was obtained ($y$=1.055 $x$+0.576) with a regression coefficient of 0.978, indicating a good agreement between the two methods.

All these results clearly indicate the good accuracy of the developed method and its interesting potential as an alternative to the official chromatographic method. It offers interesting assets such as rapidity, simplicity, and reproducibility relative to the official method.

## Conclusion

The proposed enzymatic spectrophotometric method has been successfully applied for the determination of polyunsaturated fatty acids content in olive oil samples with a precision and accuracy similar to the official method. The method is useful for the quality control and routine analysis since there is no interference between the excipients and additives. The sensitivity of the analytical system could be sufficient for the application of this approach for determination of polyunsaturated fatty acid levels in biological sera and food preparations. Work is in progress in our laboratory in order to develop the adopted methods.

**Table 3** Determination of polyunsaturated fatty acid level (%) in oil samples using the proposed enzymatic spectrophotometric procedure compared statistically with the official chromatographic method.

| | Official chromatographic method (A) | Proposed spectrophotometric method (B) | (B/A)×100 |
|---|---|---|---|
| Chetoui-1 | 23 | 20.99±1.00 | 91.26 |
| Chetoui-2 | 16.12 | 18.93±0.66 | 117.43 |
| Chemlali | 18.4 | 15.72±0.67 | 85.44 |
| Zarrazi | 14.46 | 12.82±0.46 | 88.66 |
| Arabequina | 15.72 | 12.22±0.45 | 77.74 |
| Sunflower oil | 55.53 | 51.31±2.84 | 92.40 |

The data reported in the table represent the average of triplicate

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