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Olive Oil Quality Evaluation and Analysis of Phenols in Other Fats by Means of an Enzyme Sensor Directly Operating in n-Hexane Solutions

Procjena kakvoće maslinovog ulja i analiza fenola u drugim mastima s pomoću enzimskog senzora u otopinama n-heksana

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Summary

A method for the determination of phenols in oils and fats, based on a tyrosinase biosensor, where the enzyme is inmobilized on a κ-Carrageenan gel, is described. The sensor can operate directly in a non aqueous solvent and is particularly suitable for the determination of phenols in olive oil. An evaluation of the olive oil quality is proposed and the phenols content of other edible oils and fats is determined. Finally, a comparison with a classical spectrophotometric method, usually employed for the same purpose, is reported.

Sažetak

Opisan je postupak određivanja fenola u uljima i mastima, s pomoću biosenzora s tirozinazom, u kojem je enzim imobiliziran u κ-karageanskom gelu. Senzor može djelovati izravno u nevodenom otapalu, a osobito je prikladan za određivanje fenola u maslinovom ulju. Predloženo je procjenjivanje kakvoće maslinova ulja, a određen je i udjel fenola u drugim jestivim uljima i mastima. Na kraju je uspoređen predloženi postupak s klasičnim spektrofotometrijskim postupkom uobičajenim za određivanje fenola.

Introduction

Recently, a number of studies have been performed in our laboratory, using the tyrosinase enzyme, to develop a suitable probe sensitive to phenols, and capable of working in organic solvents *(1–3)*. At that time to achieve this goal, a Clark oxygen electrode as indicating sensor, the tyrosinase enzyme immobilised in a dialysis membrane and n-hexane as non aqueous solvent, have been used. A comparison of the sensor response in n-hexane, chloroform and water saturated chloroform *(2)* or other organic solvents *(3)*, has been also performed, since some other researchers *(4)* preferred to work in chloroform.

We carried out preliminary applications *(5)* to the analysis of different types of olive oils using a tyrosinase sensor, working in n-hexane. The results indicated that it is possible to check very easily the quality of olive oil by assaying its phenols content.

The present paper reports the results obtained using a new kind of a tyrosinase sensor, where the enzyme is well immobilized in κ-Carrageenan gel, recently developed by us *(3)*, to determine the phenols content in different types of olive oil, by operating directly in n-hexane. Also, phenols analyses in other type of edible oils and fats are considered; in addition, the results concerning the comparison between the method herein proposed and a classical spectrophotometric method, employed for the same purpose by other authors *(6)*, are reported.

Experimental

Materials

The κ-Carrageenan and phenols were purchased from Fluka AG, Buchs (Switzerland), while tyrosinase (EC 1.14.18.1) from mushroom (2400 U/mg), the Folin-Ciocalteau reagent (art. F9252) and the dialysis membrane (art. D-9777) were supplied by Sigma, St. Louis Mo (U.S.A.). The n-hexane (RPE) and chloroform (Chromosolv, stabilised with amylene), both containing only 0.01 % (mass fraction)

of water according to the Karl-Fischer method, were supplied, respectively, by Carlo Erba, Milan (Italy) and Riedel de Haen, Seelze, Hannover (Germany). Methyl alcohol (HPLC grade), used for sample treatment in the spectrophotometric analysis was from Carlo Erba, Milan (Italy), while the phenols standard solution ($\gamma = 0.5$ g/L) was supplied from Poli Diagnostici, Milan (Italy).

All other chemicals were of »analytical reagent grade« and were purchased from Farmitalia-Carlo Erba, Milan (Italy).

Samples

Olive oil from different samples was tested (»extra-virgin olive oil«, »virgin olive oil«, »olive oil«, »oil of olive oil residues«, »olive oil of olive paste«, »olive oil of tuna fish«, »olive oil of sardines«); samples were purchased as sealed glass packages or tins on the Italian market, or directly from a small producer in the Rome area. For the analysis by the enzyme sensor, the olive oil samples were not subjected to any preliminary treatment before the measurement, while the oil of olive paste and the oil from tuna fish and sardines were obtained by filtering the commercial products and by centrifuging the oil, which is then diluted, if necessary. The samples of extra-virgin and virgin olive oil were diluted 1:3 or 1:6 (volume ratios) with n-hexane prior to analysis, while the other samples of olive oil were generally diluted 1:1; otherwise, they were analysed without any preliminary dilution. Solid fat samples (margarine), purchased on the Italian market, were analysed by partitioning a weighted amount (about 3 g) of a commercial sample with three different portions of n-hexane (about 3 mL each). After centrifugation, the liquid fraction was separated and the total volume was adjusted to 10 mL. Refined walnut oil and almond oil were obtained from Italian producer (A. Cruciani, Rome, Italy). Peanuts oil, soy oil and sunflower oil were purchased as sealed tins on the Italian market: these samples were generally analysed directly without any preliminary dilution.

Apparatus

The oxygen electrode used was a Mod. 4000-1 purchased from Universal Sensors Inc., New Orleans (U.S.A.), and modified by substituting the original membrane with a teflon membrane and the O-ring with the one in teflon. The external body of the electrode was also replaced by a suitable home-made teflon body.

The detection apparatus consisted of a 200 Digital pH-meter (Bell Engineering, U.S.A.), connected to a 868 recorder (Amel, Italy). Lastly, a potentiostatic power supply, model 641 VA-Detector (Metrohm, Switzerland), which also provides the transformation of the »current« signal into a »tension« signal, was used.

The solution was stirred magnetically using a Velp Scientifica (Italy) microstirrer and temperature control was carried out by means of a Julabo (Germany), model VC 20B thermostat. The thermostat was connected to a 25 mL glass cell equipped with a thermostatic jacket.

A Lambda 15 Perkin Elmer (Norwalk, U.S.A.) spectrophotometer with 1 cm pathlength quartz cells were used for the spectrophotometric analysis.

Enzyme immobilisation

5.0 mg of tyrosinase enzyme were weighed and placed in an Happendorf tube; 200 μL of phosphate buffer ($c = (1/15)$ mol/L), pH = 7, were added to the enzyme and the tube stirred in a vortex until complete dissolution was obtained. A 2 % (mass fraction) solution of κ-Carrageenan was prepared by dissolving, while gently warming, a proper amount of the polysaccharide in distilled water under magnetic stirring. 100 μL of the above warm solution were placed dropwise in a Petri dish. Before the drop solidified completely and its temperature approached room temperature, 25 μL of the concentrated solution of tyrosinase were injected into the drop, by means of an automatic pipette. After the drop had become a layer, the Petri dish was left at 5 °C overnight. Once the layer was dried, it was rehydrated by dipping it into a phosphate buffer solution, pH = 7, for about 15 min before use.

Biosensor assembly

The biosensor was made of an amperometric gas diffusion oxygen electrode able to measure the oxygen concentration in non aqueous solution, *i.e.* equipped with a teflon cap suitable for use in organic solvents as above described, coupled with a κ-Carrageenan layer entrapping the tyrosinase enzyme prepared as illustrated above.

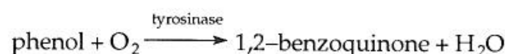
As shown in Fig. 1, the layer entrapping the enzyme was placed between the gas permeable membrane of the electrode and a dialysis membrane; the whole system was fixed to the teflon cap by means of a teflon O-ring.

Before use, the biosensor was immersed for about 15 min in phosphate buffer; after use, it was first washed with the used solvent, then with phosphate buffer before being stored at 5 °C in a humid atmosphere. If degradation of the dialysis membrane occurs, the O-ring must be removed and the teflon cap should be dipped into phosphate buffer for a short time so that the dialysis membrane can be easily removed without disturbing the layer containing the enzyme. Then a new dialysis membrane and the same enzyme-containing layer can be mounted on the cap using the teflon O-ring.

Methods

Enzyme sensor method

When the biosensor is immersed in an organic solvent, containing the phenolic substrate, the phenol is converted by the tyrosinase enzyme into orthoquinone, according to the following reaction:



$$\text{phenol} + O_2 \xrightarrow{\text{tyrosinase}} \text{1,2-benzoquinone} + H_2O$$

Thus, phenol was determined by measuring the oxygen consumption occurring during the enzymatic reaction, using the gaseous diffusion indicator electrode for oxygen. A calibration graph was constructed by means of successive additions of small volumes of standard non aqueous solution of substrate, to 25 mL of non aqueous solvent, contained in a glass cell thermostated at 25 °C

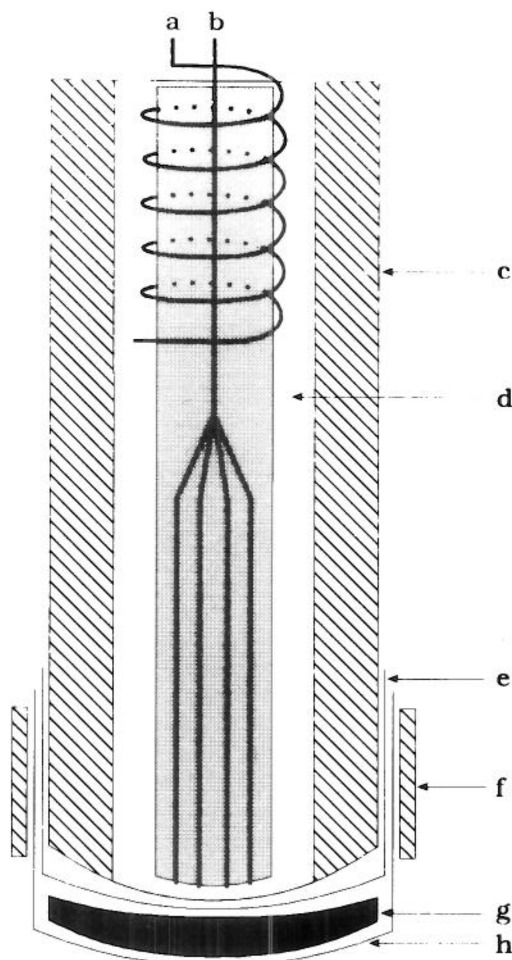


Fig. 1. Biosensor assembly: a: reference electrode Ag/AgCl; b: platinum electrode; c: teflon body; d: inner solution (KCl, c = 0.1 mol/L, phosphate buffer, c = 0.06 mol/L, pH = 6.6); e: gas permeable membrane; f: teflon O-ring; g: tyrosinase enzyme in κ-Carrageenan gel; h: dialysis membrane.

Slika 1. Biosenzorski uređaj: a) referentna elektroda Ag/AgCl; b) platinska elektroda; c) teflonska posuda; d) otopina unutar uređaja (KCl, c = 0,1 mol/L, fosfatni pufer, c = 0,06 mol/L, pH = = 6,6); e) membrana propusna za plinove; f) teflonski obruč; g) enzim tirozinaza u κ-karageanskom gelu; h) membrana za dijalizu.

and magnetically stirred and by recording the signal variation on the measurement apparatus after each addition. The small variations of volume thereby introduced were taken into account.

The same procedure was followed in all the tests carried out for the analysis of authentic samples of olive oil or other fat samples, but adding first of all the sample, appropriately diluted with n-hexane, to the non aqueous solvent contained in the glass cell.

Spectrophotometric method

The phenol solutions used to perform the calibration curve, were obtained by diluting the phenols standard solution from Poli with methanol-water, up to a final volume ratio of 60:40. A calibration graph was obtained as follows: 5 mL of Folin Ciocalteau reagent, followed by 15 mL of 20 % sodium carbonate solution were added to 10 mL of standard solution. The mixture was then diluted to 50 mL with water and held in a water bath at 35 °C for 20 min to develop the colour completely *(7)*. The absorbance was measured at λ = 760 nm.

For the spectrophotometric analysis of authentic samples, a proper amount (10–40 g) of sample was dissolved in n-hexane (1:5 mass/volume ratio), and partitioned three times with a methanol/water solution 60:40 (volume ratio) *(6)*. The hydroalcoholic solutions collected each time, were pooled and then analysed following the aforementioned procedure. The phenolic content was determined using the direct method, by means of the calibration graph.

Results

The working conditions, used in this research and described in Table 1, are the same as described and optimised in a previous paper *(3)*; these conditions were maintained constant throughout all the tests. Table 2 shows the results of the electroanalytical characterisation and the calibration graph data, carried out using n-hexane or chloroform as non aqueous solvent and phenol as substrate for the enzymatic reaction. The results point out that the sensor response is lower in chloroform than in n-hexane and that the sensor lifetime is shorter when

Table 1. Main working conditions of the enzyme electrode in n-hexane and chloroform

Tablica 1. Uvjeti rada enzimske elektrode u n-heksanu i kloroformu

Indicating electrode	Oxygen gas diffusion electrode
Immobilised enzyme	Tyrosinase, in phosphate buffer (c = (1/15) mol/L), pH = 6.5
Immobilisation method	Entrapment in κ-Carrageenan gel
Substrate	Phenol
Working temperature	25 °C
Working conditions	25 mL of non aqueous solvent, in a thermostated cell under magnetic stirring

Table 2. Characterisation data of the enzyme electrode in n-hexane and chloroform

Tablica 2. Karakteristični podaci enzimske elektrode u n-heksanu i kloroformu

	n-hexane	chloroform
Response time / min	1.5	2.0
Linearity range / (μmol/L)	1 ... 37	10 ... 350
Minimum detection limit / (μmol/L)	0.2	2.0
Relative precision (pooled RSD/%)	8.5	6.9
Relative inaccuracy/%	–1.7 ... + 2.2	–7.8 ... + 3.7
Calibration graph: y =ax + b		
a / (L/μmol)	19.5 ± 0.3	3.3 ± 0.1
b	15.5	35.0
Correlation coefficient	0.999	0.999

Table 3. Comparison of phenol content in different olive oil samples obtained by the tyrosinase enzyme sensor directly working in n-hexane and by the classical spectrophotometric method; found values are the mean of at least five determinations

Tablica 3. Usporedba utvrđenih udjela fenola u raznim uzorcima maslinova ulja dobivenih postupkom s tirozinaznim biosenzorom u n-heksanu i klasičnim spektrofotometrijskim postupkom. Dobivene vrijednosti prosjek su od najmanje pet određivanja.

Sample	Spectrophotometric method		Biosensor		
	Found value c^{sp} / (μmol/L)	RSD/%	Found value c^{bs} / (μmol/L)	RSD/%	$\left(\frac{c^{bs}-c^{sp}}{c^{sp}}\right)/\%$
Extra virgin olive oil †	791	4.0	796	1.6	+0.6
Virgin olive oil †	423	7.1	382	8.7	–9.7
Virgin olive oil †	343	4.3	349	6.2	+1.7
Olive oil*	268	2.4	232	5.1	–13.4
Oil of olive paste*	1008	3.2	945	7.9	–6.3
Olive oil from tuna fish*	152	10.0	170	8.0	+11.8

* : commercial product; † : non commercial product

measurements are carried out in chloroform (≈ 8 days instead of ≈ 3 months).

Moreover, phenols determination of olive oils and other edible oils and fats was performed in n-hexane rather than in chloroform because of the excellent solubility of olive oil in the first solvent, of the greater sensitivity displayed by the sensor in n-hexane and of the lowest limit of detection in this solvent, but, above all, because of the longer lifetime and reliability of the biosensor when n-hexane rather than chloroform is used. Nevertheless, determination in chloroform (or even better in water saturated chloroform *(2)*) can be carried out when it is required to assay samples with high phenol concentrations, in view of the larger linearity range displayed by the sensor in chloroform and in water saturated chloroform. In this case dilution prior to analysis is not necessary.

Table 4. Recovery of phenol, by standard addition method, in different oil samples. Data obtained using the tyrosinase biosensor and n-hexane as non aqueous solvent

Tablica 4. Utvrđivanje udjela fenola postupkom standardnog dodavanja fenola u raznim uzorcima ulja. Podaci su dobiveni primjenom tirozinaznog biosenzora u n-heksanu kao nevodenom otapalu.

Sample	Found value° c / (μmol / L) [RSD/%]	Standard phenol added c / (μmol/L)	Total found value c / (μmol / L) [RSD/%]	Recovery/%
Extra virgin olive oil †	7.81 [1.6]	1.97	9.81 [5.1]	100.3
		3.95	11.80 [5.9]	100.4
Extra virgin olive oil*	3.79 [4.3]	1.97	5.78 [3.4]	100.3
		3.93	7.71 [1.9]	99.9
Virgin olive oil †	4.85 [8.7]	1.97	6.92 [5.3]	101.5
		3.95	7.60 [5.0]	101.0
Olive oil*	2.88 [5.1]	1.97	4.73 [6.2]	97.5
		3.95	6.90 [3.6]	100.1
Oil of olive oil residues*	1.00 [4.3]	1.97	2.95 [9.5]	99.3
		3.93	5.07 [7.7]	102.8
Oil of olive paste*	9.38 [6.0]	1.97	11.32 [3.0]	99.7
		3.93	13.68 [2.5]	102.8
Olive oil from tuna fish*	2.83 [2.2]	1.93	4.81 [2.0]	101.0
		3.86	6.84 [0.4]	102.2
Olive oil from tuna fish*	1.76 [9.9]	1.97	3.69 [4.7]	98.9
		3.93	5.76 [3.3]	101.2
Margarine from olive oil*	0.63 [5.6]	1.97	2.49 [4.1]	95.8
		3.93	4.72 [0.9]	103.5

* : commercial product; † : non commercial product

° : samples were appropriately diluted before analysis; values represent the final concentration in the measurement cell

The main analytical properties, when the sensor is used for the determination of different phenol derivatives in n-hexane solution, have been studied in previous papers *(1,2)*

The biosensor has been applied in the determination of the phenol content in different commercial samples of olive oil, or other edible oils and fats.

For this aim, the various oil samples were analysed using the enzymatic sensor dipped in n-hexane and following the same procedure as described in the »Method« section. In practice, each sample was added directly to the non aqueous solvent in which the biosensor was immersed, either as such or after proper dilution with the same solvent. The results obtained in the analysis of different samples and the correlation with the spectrophotometric method, are summarised in Table 3. Accuracy data, which were evaluated as recoveries by the standard addition method, are shown in Table 4, while the results of phenols content (expressed as mg of phenol per kg of oil) found in all edible oils and fats analysed until now, are summarised in Table 5; in the same table, precision, as percent relative standard deviation, for all the measurements in authentic matrices, is reported.

Discussion

A tyrosinase biosensor has been described, with the enzyme immobilized in κ-Carrageenan gel, that shows better analytical properties than the one we presented previously, where the enzyme was immobilized in a dialysis membrane *(1,2)*.

Table 5. Results of phenols content determination and repeatability data, in different edible oils and fats samples, obtained by the tyrosinase biosensor using n-hexane as non aqueous solvent. Each value is the mean of at least five determinations.

Tablica 5. Rezultati određivanja količine fenola i ponovljivosti rezultata u raznim uzorcima jestivih ulja i masti, dobiveni tirozinaznim biosenzorom, koristeći n-heksan kao nevodeno otapalo. Svaka vrijednost prosjek je od najmanje pet određivanja.

Sample	Mean found value $10^6 \cdot w$ (phenol)	RSD/%
Extra virgin olive oil †	104.5	1.6
Extra virgin olive oil*	128.2	3.1
Virgin olive oil †	44.7	8.7
Virgin olive oil †	40.8	6.2
Olive oil*	27.0	5.1
Oil of olive oil residues*	11.9	8.5
Oil of olive paste*	102.0	7.9
Olive oil from tuna fish*	14.3	2.7
Olive oil from tuna fish*	17.4	8.0
Olive oil from sardines*	≤ 1 (□)	–
Margarine from olive oil*	9.5	5.8
Margarine*	≤ 1 (□)	–
Almond oil*	≈ 1.6 (□)	–
Walnut oil*	≈ 1.2 (□)	–
Peanuts oil*	≤ 1 (□)	–
Soy oil*	≤ 1 (□)	–
Sunflower oil*	≤ 1 (□)	–

*: commercial product; †: non commercial product
□: value of the same order of the minimum detection limit of the method

The application of the biosensor in the analysis of different types of olive oil has indicated a simple way of determining the phenol content of the oils, as already described in the literature *(6)* and also of evaluating their quality; the latter is done by correlating the oil quality with phenols content. In fact, the results of the tests carried out on different olive oil samples (Table 5), reveal the »treatments« and »blending« to which the lower quality oils have been subjected. Indeed, these treatments are known to produce a decrease in the amount of phenolic compounds present in the product obtained by simple mechanical pressing of the olives. The products denoted as »olive oil«, or »olive oil residues«, are found to contain less phenols than the samples respectively denoted as »extra-virgin« and »virgin«; this is indicative of the corrective treatments to which these samples have been subjected.

On the other hand, the results reported in Table 5 show that the sensor is able to detect the phenols content in different olive oils and in several edible oils and fats. It should be pointed out that, for oils different from olive oils, the values of phenol content should be considered only as approximate values since they are near to the minimum limit of detection of the method. Again, these low values can be explained with the extraction and refining processes which these oils undergo before sale.

The method described is reliable and yields data in good agreement with those obtained by the spectrophotometric method (see Table 3). The apparatus is simple, inexpensive and does not require specially-trained operators. Further, the use of the biosensor eliminates the disadvantages encountered when spectrophotometric methods are applied to turbid samples or when pigment formation occurs. Lastly, smaller amount of sample, simpler sample treatment and shorter analysis time are required with respect to the spectrophotometric method.

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