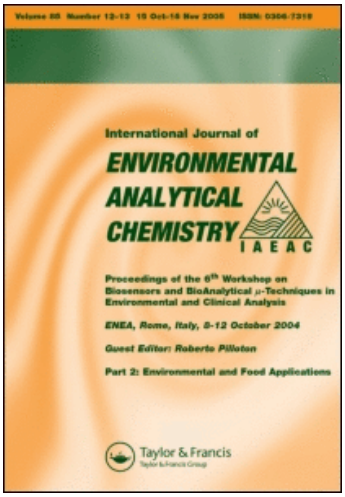

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Biosensors are very versatile devices that can be used to solve various kinds of problem that are increasingly found in the various branches of chemistry, particularly in the fields of foodstuffs and the environment. In recent years, there has been considerable development in a new biosensor sector, that of OPEEs (organic phase enzyme electrodes). These are biosensors able to function also in organic solvents or in mixtures of several different organic solvents. One of these enzymatic biosensors that has recently proved to be particularly versatile is the tyrosinase biosensor, of which several different versions have been developed. Our group in particular has in recent years developed both a version that operates in aqueous solutions and one suitable for organic solvents. These tyrosinase biosensors are essentially made up of an amperometric transducer for oxygen (Clark type), coupled with the tyrosinase enzyme, which is suitably immobilized according to the solvent in which it must operate. In this article the possibility was assessed of using them to determine the polyphenol 'pool' in olive oil mill wastewater. The method has been optimized as regards both the solvent and the type of enzymatic immobilization to be used. Results have been compared with those obtained using the Folin–Ciocalteau method, which is chosen as reference method.

Keywords: Tyrosinase biosensor; Polyphenols; Analysis; Olive oil mill wastewater

1. Introduction

There are essentially three different kinds of oil extraction systems: pressure (discontinuous cycle), centrifuging (continuous cycle), and percolation. Olive oil mill byproducts consist of husk and olive oil mill wastewater (MWW). The latter consists of effluent from the oil mills composed of the water contained in the olives plus the water added in the course of processing. Olive oil mill wastewaters are classified as a highly polluting effluent in the agro-food sector [1–3]. The toxicity of MWW is believed to be principally due to the polyphenol component [4,5].

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This water was [6] and often still is disposed of by discharging it on farmland, thus inhibiting many microorganisms, reducing seed germination and varying several soil characteristics, such as porosity and humic composition.

In the present work tyrosinase biosensors were used to determine the polyphenol component present in olive oil mill wastewater. In some preliminary research [7] the possibility was raised of using the tyrosinase biosensor operating in organic solvent (OPEE, organic phase enzyme electrode) [8, 9] to determine the polyphenol 'pool' in olive oil mill wastewater, although the validity of this approach to the problem is still very uncertain owing to the gaps in the research due to its preliminary nature. In the present work it was therefore attempted to experimentally verify the real behaviour of the tyrosinase biosensor operating in both aqueous solution and organic solvent (dichloromethane), as well as in water–acetonitrile mixtures of different ratio by volume, in order to determine polyphenols in olive oil mill wastewater. From the results of the work carried out by numerous researchers it has clearly emerged over the past few years that a large number of enzymatic sensors, when properly constructed, are able to operate also in suitable organic solvents [10–12], above all in non-polar solvents, and in some cases also in moderately polar solvents [13]. Systematic research was thus performed to evaluate the true validity of the biosensor method, for this purpose also constantly comparing the results obtained on real samples with those found using the well-known Folin–Ciocalteau spectrophotometric method [14].

2. Experimental

2.1 *Reagents and materials used*

Acetonitrile, chloroform, dichloromethane, potassium chloride, dibasic and monobasic potassium phosphate and anhydrous monobasic RPE were purchased from Carlo Erba Reagenti; Phenol and dialysis membrane (art.D-9777) from Sigma-Aldrich; Kappa–Carrageenan and Tyrosinase (EC 1.14.18.1) from mushrooms 3216 U/mg from Fluka.

2.2 *Apparatus used*

The transducer used in the biosensor-based determination of total polyphenols in MWW was an amperometric electrode for oxygen from Universal Sensor Inc., New Orleans (USA), Mod. 4000-1. Measurements were made using a Mod. 3001 ABD potentiostat supplied by the same firm. The signal was recorded using an Amel (Milan, Italy) mod. 868 analog recorder. The starting solution, to which the sample to be analysed had been added, was contained in a cell thermostated at 23°C and constantly stirred by a magnetic stirrer (Amel Instruments: mod. 291/lf).

For the spectrophotometric measures a Lambda 5 model UV-VIS Perkin Elmer Sp equipped with a Perkin Elmer Printer/Plotter was used together with a quartz cuvette with a 1.0 cm optical path.

2.3 *Sampling and sample conservation*

Sampling was carried out on 'fresh' olive oil mill wastewater, taken from the extraction process outlet. All the MWWs analysed in the present work came from

(continuous cycle) centrifugation processes and the samples were taken by us at several mills in central Italy. All the samples were conserved in dark plastic bottles and subjected to analysis without any pretreatment other than dilution.

3. Methods

3.1 *Determination of total polyphenols*

Total polyphenol determination was performed on the MWW 'as is' using a tyrosinase enzymatic biosensor. As well as being highly specific, the enzymatic processes have the advantage of taking place under mild and thus relatively non-polluting conditions, while being very effective. Tyrosinase is an oxidase that contains copper [15], which catalyses the reduction of molecular oxygen by numerous electron-donating molecules, such as phenol compounds [16].

The method is thus based on the oxidation of phenols to quinones as catalysed by the tyrosinase enzyme. Detection is achieved by measuring the decrease in dissolved oxygen concentration, which occurs as a result of the enzymatic reaction:

$$\text{Phenol} + O_2 \xrightarrow{\text{tyrosinase}} o\text{-quinone} + H_2O$$

The variation in oxygen is determined using a Clark type gaseous diffusion amperometric electrode.

3.2 *Tyrosinase biosensor assembly operating in aqueous solution or in water–acetonitrile mixtures*

Three different methods of enzymatic immobilization were tested for the purpose of constructing the tyrosinase biosensors used, and three biosensors having different characteristics and responses were obtained. In the first method, enzyme immobilization was performed using a dialysis membrane [17]. In the second method, immobilization was achieved using a cellulose triacetate membrane (TAC) [18] and in the third method Kappa–Carrageenan gel [8, 9, 13].

In the case of the first method, the biosensor (figure 1) was made by sandwiching 2 mg of tyrosinase (3216 U mg^{-1}), moistened with two drops of phosphate buffer, pH 7.0, between a dialysis membrane and a PTFE gas-permeable membrane placed in contact with the electrode. The whole assembly was fixed using an O-ring to a cap screwed onto the head of an amperometric electrode for oxygen. This transducer was composed of a platinum cathode (working electrode) and of an Ag/AgCl anode (reference electrode).

The cathode was polarized at a potential of −650 mV *versus* the anode. Any variation in current due to the reduction of the oxygen at the cathode was recorded according to the reaction:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

The reaction at the anode was instead:

$$Ag + Cl^- \rightarrow AgCl + e^-$$

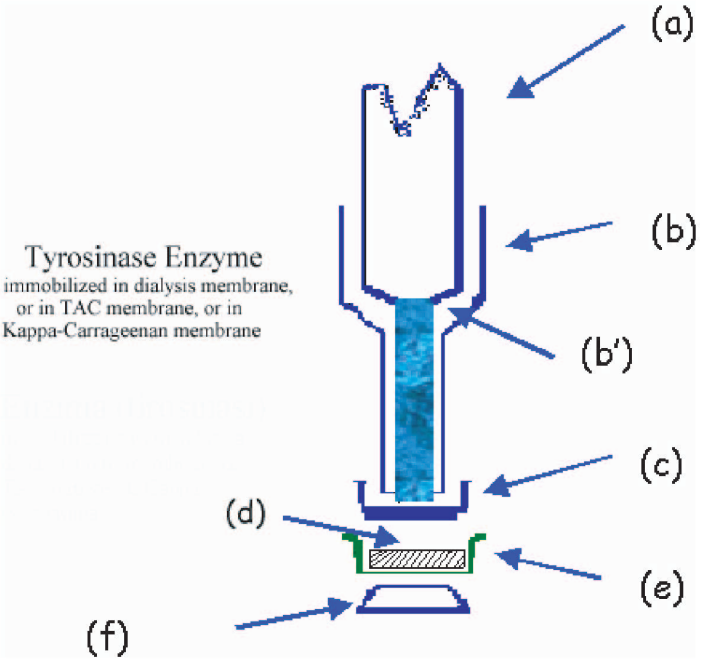

Figure 1. Representation of tyrosinase biosensor used: (a) body of O_2 electrode; (b) PTFE cap; (b′) inner phosphate buffer solution; (c) PTFE gas-permeable membrane; (d) enzyme immobilized in dialysis membrane, or in TAC membrane, or in a Kappa–Carrageenan gel like disk; (e) dialysis membrane, or nylon net; (f) PTFE O-ring.

Signal variation, recorded operating in conditions of limiting diffusion current, was proportional to the species being reduced at the cathode, i.e., the oxygen.

In the case of the second method, the biosensor was prepared (figure 1) by coupling the same gaseous diffusion indicator electrode for oxygen to the tyrosinase enzyme immobilized in a cellulose triacetate membrane. The TAC membrane, prepared as described in preceding papers [18, 19], was sandwiched between a dialysis membrane and a PTFE gas-permeable membrane. The whole assembly was fixed by means of an O-ring to a cap screwed on to the head of the amperometric transducer. For enzyme immobilization 5.0 mg of tyrosinase enzyme (3216 U mg^{-1}) were weighed out and placed in an Eppendorf test tube and dissolved in 200 μL of phosphate buffer 0.06 mol L^{-1} at pH = 7. A 50 μL aliquot of the enzyme solution was placed on top of one of the previously prepared cellulose triacetate disks and allowed to stand at 4°C until completely dry. In the third method the biosensor was obtained (figure 1) using the same type of transducer as in the preceding two methods, coupling it to the tyrosinase enzyme immobilized in Kappa–Carrageenan gel. The Kappa–Carrageenan disk containing the enzyme was sandwiched between a PTFE gas-permeable membrane and a dialysis membrane. The whole assembly was fixed by means of an O-ring to a cap screwed to the electrode. In practice, 3.75 mg of tyrosinase enzyme (3216 U mg^{-1}) were weighed out, placed in an Eppendorf test tube and dissolved in 100 μL of phosphate buffer 0.06 mol L^{-1} at pH = 7. In order to immobilize the enzyme one of the previously prepared Kappa–Carrageenan disks,

as described in previous papers [8, 9], was rehydrated by placing a 50 μL aliquot of enzyme solution on it and allowing it be absorbed for 48 h at 4°C in the polysaccharide gel.

3.3 *Construction of calibration straight lines in the different water–acetonitrile solvent mixtures with the tyrosinase biosensor*

Standard phenol solutions in water, or in acetonitrile, or in water–acetonitrile mixtures at different percentages by volume: 3+1 (v+v), 1+1 (v+v), 1+3 (v+v) were prepared, all have the same phenol concentration, namely 1.0×10^{-2} mol L^{-1}. The calibration curves, obtained using a biosensor with the enzyme immobilized in dialysis membrane, were constructed by successively adding small volumes (200 μL) of standard substrate solution, that is, of one of the phenol solutions described above, to 10 mL of a water, or acetonitrile, or mixed solvent solution having the same percentage by volume of water–acetonitrile as the added standard solution placed in a glass cell thermostated at 23°C and kept under constant magnetic stirring. After each addition, a variation in the order of several tens of nA was recorded using the tyrosinase biosensor with the enzyme immobilized in the dialysis membrane immersed in this mixture. The measurement time was ≈5 min, and the response time ≤1 min. The final concentration of the standard phenol solution introduced into the measuring cell was calculated taking into account the small variations in volume due to the successive additions of substrate solution.

Figure 2 shows the calibration straight lines obtained in the various water–acetonitrile solvent mixtures and in the two respective pure solvents.

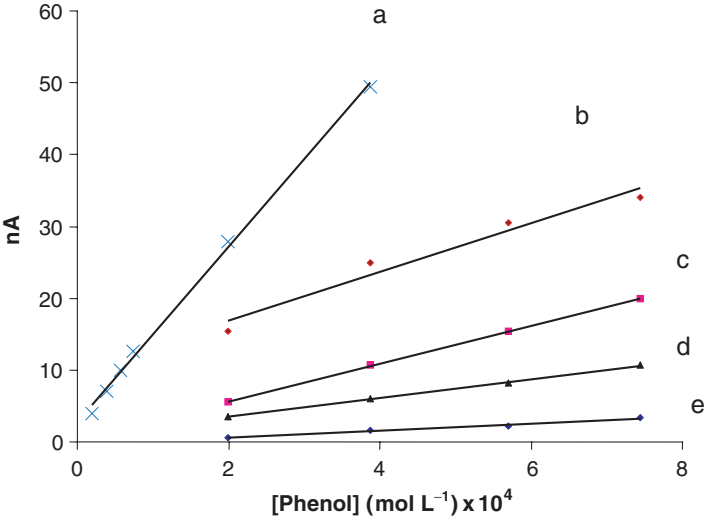

Figure 2. Calibration straight lines of tyrosinase biosensor *vs.* standard phenol solutions for the different water–acetonitrile solvents used. (a) Calibration straight line in water. (b) Calibration straight line in water–acetonitrile (3+1) (v+v). (c) Calibration straight line in water–acetonitrile (1+1) (v+v). (d) Calibration straight line in water–acetonitrile (1+3) (v+v). (e) Calibration straight line in pure acetonitrile.

3.4 *Polyphenol content determination in MWW samples using the tyrosinase biosensor*

After optimization of the solvent mixture and of the enzymatic immobilization method, polyphenol content determination in the MWW samples was carried out using the same procedure as described in the preceding section, adding 200 μL of sample to 10 mL of the water–acetonitrile solvent mixture 1+1 (v+v) and using a 1.0×10^{-2} mol L^{-1} phenol mixture in water–acetonitrile 1+1 (v+v) as standard.

3.5 *Optimization of the enzymatic immobilization method and tyrosinase OPEE assembly, operating in dichloromethane*

Also in this case, in order to construct the tyrosinase OPEE, the same three different immobilization methods as described above were tested.

The first method thus involved immobilization of the enzyme in a dialysis membrane, the second method in a cellulose triacetate membrane and the third in Kappa–Carrageenan gel.

In the case of the first method the biosensor (figure 1) was prepared by sandwiching 2 mg of tyrosinase (3216 U mg^{-1}) between a nylon net and a PTFE gas-permeable membrane placed in contact with the electrode. The whole assembly was fixed by means of a PTFE O-ring to a PTFE cap screwed on to an amperometric electrode for oxygen.

In the second case, the biosensor (figure 1) was prepared by coupling a gaseous-diffusion indicator electrode for oxygen determination to the tyrosinase enzyme, which had previously been immobilized in a cellulose triacetate membrane. The triacetate membrane was sandwiched between a nylon net and a PTFE gas-permeable membrane. The whole assembly was fixed by means of a PTFE O-ring to a PTFE cap screwed on to the head of the amperometric electrode.

In the third case, the biosensor (figure 1) was prepared by using the same type of transducer as for the two methods described above, which was then coupled to the tyrosinase enzyme, immobilized in a gel-like disk of Kappa–Carrageenan ($\Phi = 0.5$ cm). In order to immobilize it, 5.0 mg enzyme (3216 U mg^{-1}) were weighed out, placed in an Eppendorf test tube and dissolved by adding 200 μL of phosphate buffer 0.06 mol L^{-1} at pH = 7. One of the previously prepared Kappa–Carrageenan disks was taken and, using a procedure previously developed in our laboratory [8, 9], the disk was rehydrated by placing it on a 50 μL aliquot of the enzyme solution, and then allowing the enzyme to penetrate the polysaccharide gel for 48 h at 4°C. The Kappa–Carrageenan disk was then sandwiched between a PTFE gas-permeable membrane and a nylon net. The whole assembly was fixed by means of an O-ring to a PTFE cap screwed on to the electrode.

3.6 *Construction of a calibration straight line in dichloromethane with the tyrosinase OPEE*

After optimizing the immobilization method, the tyrosinase OPEE was definitively assembled in order to perform all the measures in dichloromethane, using the enzyme adsorbed on to the Kappa–Carrageenan membrane. Before use, the biosensor was placed in a cell thermostated at 23°C containing 10 mL of dichloromethane and

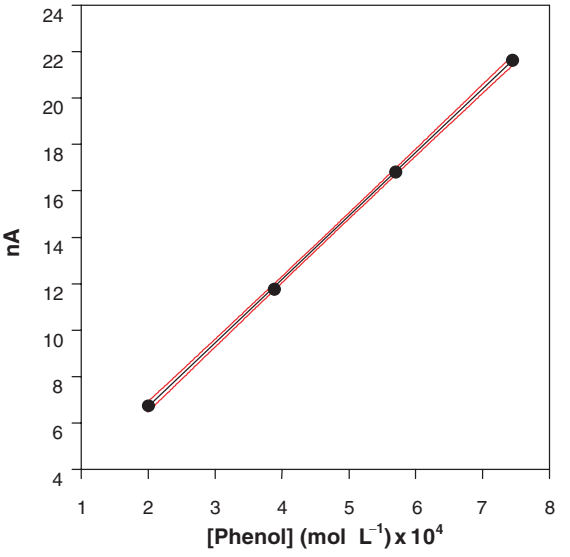

Figure 3. Calibration straight line *vs.* phenol in dichloromethane, obtained using a tyrosinase OPEE.

Table 1. Mean calibration straight line equation ($n = 4$) and correlation coefficient r^2.

$y = (2.736 \pm 0.052)x + (1.24 \pm 0.27)$
$r^2 = 0.9999 \quad (1 - \alpha) = 0.90 \quad t = 2.92$
$x = \text{mol L}^{-1} \quad y = \text{nA}$
Linear range from 2.0×10^{-4} mol L^{-1} to 7.4×10^{-4} mol L^{-1}
Low detection limit 0.5×10^{-4} mol L^{-1}
'Pooled standard deviation' 10%

allowed to stabilize under constant magnetic stirring. Successive additions of 200 µL of a standard solution of phenol, 1.0×10^{-2} mol L^{-1}, in dichloromethane were made. After each addition a current variation of the order of several nA was recorded with the biosensor immersed in this organic solvent. The measurement time was ≈6 min, and the response time ≤1.5 min. The final concentration of the standard phenol solution introduced into the measuring cell was calculated taking into account the small variations in volume caused by the successive additions of substrate solution.

Figure 3 shows the calibration straight line obtained using a standard phenol solution in dichloromethane while table 1 shows the principal analytical data and relative equation of the calibration straight line.

3.7 *Polyphenol determination in MWW after sample extraction using dichloromethane*

Extraction was performed in dichloromethane in the ratio of MWW/organic solvent 3 : 1 (v/v), in accordance with the procedure described in previous works carried out in our laboratory [20]. During extraction an emulsion formed was removed by centrifuging after adding ≈1 mg of sodium chloride. The extraction operation of the aqueous phase was repeated three times following the same procedure. Each time the two

phases were separated. Then the three dichloromethane extracts were combined and the subsequent biosensor analysis to determine polyphenol content in each of the phases (aqueous and organic) was performed.

Determination of the polyphenols contained in the aqueous phase was performed, as described previously, using a tyrosinase biosensor with the enzyme immobilized in a dialysis membrane.

Determination of the polyphenols contained in the organic phase (dichloromethane) was performed using the biosensor with the enzyme immobilized in Kappa–Carrageenan and operating in dichloromethane. In practice, to 10 mL of CH_2Cl_2 placed in a cell thermostated at 23°C, alternating additions were made, each time waiting for the signal to stabilize, 200 μL of standard phenol solution in dichloromethane and 200 μL of the extract in the organic phase as previously described, recording the respective variations in current intensity after each addition. Polyphenol concentration was then computed by comparing the signals obtained after the successive additions of equal volumes (200 μL) of the standard and of the extracting organic phase. The same operating procedure was used for all the samples analysed by us.

3.8 *Polyphenol determination using the Folin–Ciocalteau spectrophotometric method*

The polyphenol content of the various MWW samples was also determined by using the tyrosinase biosensor, again by the spectrophotometric method involving the use of Folin–Ciocalteau reagent [21]. Folin–Ciocalteau reagent (phosphotungstic-phosphomolybdic acid) oxidizes the –OH phenol group. This reaction produces a compound having a characteristic blue colour, the absorbance of which is read off at 760 nm. The total quantity of polyphenols in the MWW is determined from a comparison of the sample absorbance (at 760 nm), with that of a standard phenol solution, the absorbance of which is read off at the same wavelength.

3.9 *Execution of measures using the Folin–Ciocalteau method*

To 0.5 mL of each suitably diluted sample (the dilution performed was 1+20 (v+v) with deionized water) were then added 4.2 mL of deionized water and 0.5 mL Folin–Ciocalteau reagent. After stirring the solution for about 1 min, 1 mL of a sodium carbonate solution 20% (p/v) and 4.2 mL of deionized water were added. The mixture was allowed to react for 2 h in darkness in a cell thermostated at 25°C. The absorbance of the solution was then read off at 760 nm *versus* a blank solution composed of distilled water. The same procedure was repeated using standard phenol solutions in order to obtain a calibration straight line. In particular, 0.5 mL was taken respectively from each of the following, specifically prepared, standard phenol solutions (7.0×10^{-3} mol L^{-1}; 1.0×10^{-2} mol L^{-1}; 3.0×10^{-2} mol L^{-1}; 5.0×10^{-2} mol L^{-1}). Then, in order to perform each measure, the same procedure as described above was followed.

The calibration straight line shown in figure 4 was obtained by plotting the absorbance read off as a function of the concentration of the standard phenol solutions used. The measures were performed using a UV-VIS dual-beam dual-grid spectrophotometer. Scattering radiation was less than 0.0001% at 220 nm. The resolution was 0.07 nm; the cuvettes were made of quartz, 1.0 cm. The relevant analytical data are summarized in table 2.

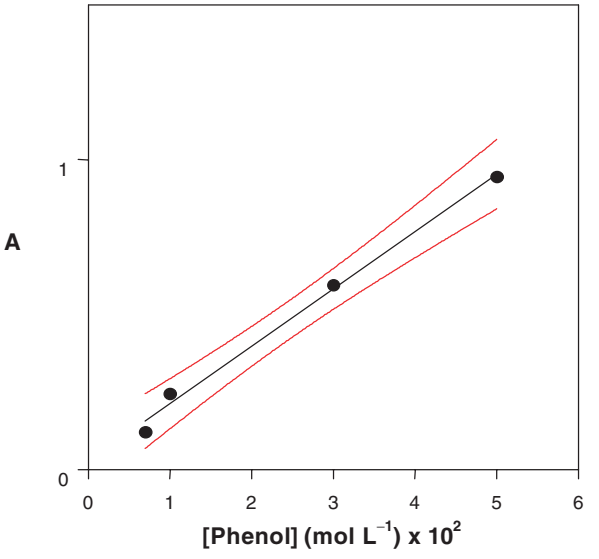

Figure 4. Spectrophotometric calibration straight line *vs.* phenol, obtained using the Folin–Ciocalteau method.

Table 2. Mean of calibration straight line equation ($n = 4$) and correlation coefficient r^2.

$y = (0.185 \pm 0.030)x + (0.027 \pm 0.009)$
$r^2 = 0.9938 \quad (1 - \alpha) = 0.90 \quad t = 2.92$
$x = \text{mol L}^{-1} \quad y = \text{A}$
Linear range from 7.0×10^{-3} mol L^{-1} to 5.0×10^{-2} mol L^{-1}
Low detection limit 0.5×10^{-3} mol L^{-1}
'Pooled standard deviation' 5%

3.10 *Recoveries using the standard addition method*

Both in the case of biosensor measures and spectrophotometric measures carried out using the Folin–Ciocalteau method on real matrixes, an assessment was also made of the possible effect of the matrix using the standard addition method. To this end to the suitably diluted sample a known volume of phenol sample was added such that the added final concentration was of the same order of magnitude as the polyphenol concentration initially contained in the test sample. The measures were then performed using exactly the same procedure as described above, both before and after the addition.

4. Results and discussion

4.1 *Optimization of the best solvent mixture (water–acetonitrile) for total polyphenol determination in MWW using the tyrosinase biosensor*

The problem that arose immediately was how to determine experimentally which was the best solvent in which to carry out polyphenol concentration measures in the

MWW sample. It was indeed necessary to take account of the peculiar nature of the MWW sample, which is essentially an aqueous sample, but which contains not negligible traces of oil and of different kinds of different organic compounds, above all polyphenols, several of which are more soluble in water, others in organic solvents. In some cases, they are also contained in the lipid droplets present in the sample or even contained in micelles. It is therefore quite clear that it was above all necessary to establish experimentally whether the best biosensor response was obtained in water, in organic solvent or in a mixture of these two solvents. Clearly, the solvent in which the response is greatest is the one best able to dissolve part or all of the polyphenol compounds present, whether those most soluble in water or those contained in oil drops or micelles, and that, once dissolved, can then be determined using the tyrosinase biosensor. To this end, the acetonitrile appeared to be the most suitable solvent and, even though it is not one of the best possible organic solvents for use in OPEEs [8, 13], it can nevertheless dissolve many liposoluble compounds. Above all, however, it can be used mixed with water at any ratio by volume without 'immiscibility gaps' being formed. The most appropriate experiment to perform thus seemed to be to determine in which solvent or which water–acetonitrile mixture the biosensor response was greatest. However, from recently published papers [8–12] on OPEEs it clearly emerged that the level of OPEE response itself varies according to the solvent or solvent mixture used. The problem therefore, as we saw it, had to be approached in two stages. In the first instance it was necessary to study biosensor response to a typical phenol compound that was completely soluble both in water and in acetonitrile. It was then necessary to address the case of the real MWW sample which, in addition to what was said above, displayed the same implications as mentioned earlier with reference to the different solubility in the aqueous and organic phase of the phenol compounds contained in it. In the first place, therefore, a study was made of simple biosensor response to the phenol both in water and in acetonitrile as well as in different mixtures of the two solvents. For this purpose, figure 2 contains the calibration straight lines for the tyrosinase biosensor *versus* phenol obtained, using for the addition a phenol standard 1.0×10^{-2} mol L^{-1} in distilled water, or else a phenol standard 1.0×10^{-2} mol L^{-1} in different water–acetonitrile mixtures, or finally a phenol standard 1.0×10^{-2} mol L^{-1} in acetonitrile. The straight lines were obtained by means of successive 200 μL additions of each of these standard phenol solutions to 10 mL of the same solvent (or solvent mixture) contained in a cell thermostated at 23°C and under magnetic stirring. After each addition the current variation in nA was read off after about 60 s (biosensor response time). All the curves obtained in this way, that is, by operating in the two solvents and solvent mixtures considered, are shown in figure 2, while the relative equations and corresponding analytical data are summarized in table 3.

As may easily be seen, from the trend in the calibration curves thus obtained, the biosensor's greatest sensitivity towards phenol is obtained when operating in water. The sensitivity decreases on going from water to acetonitrile. However, it should be noted that these straight lines were obtained when the added substrate (phenol) was contained in the same solvent (or solvent mixture) as was used to perform the measure (i.e., contained in the thermostated cell) in such a way that, after the addition, the composition of the solvent (or solvent mixture) did not vary. However, it was necessary to take into account the fact that, on going on to the real case, i.e., after adding the MWW sample to the solvent contained in the measuring cell, in practice one was

Table 3. Analytical data referring to the calibration straight lines of the tyrosinase biosensor, obtained when operating in the different solvent mixtures: (a) distilled water; (b) water–acetonitrile 3+1 (v+v); (c) water–acetonitrile 1+1 (v+v); (d) water–acetonitrile 1+3 (v+v); (e) acetonitrile 100%.

(a)	$y = (12.2 \pm 0.62)x + (2.7 \pm 1.1)$ $r^2 = 0.9977$ $(1-\alpha) = 0.90$ $t = 2.132$ $x = \text{mol L}^{-1}$ $y = \text{nA}$ Linear range from 0.74×10^{-3} mol L^{-1} to 2.0×10^{-2} mol L^{-1}
(b)	$y = (3.4 \pm 1.4)x + (10.1 \pm 7.2)$ $r^2 = 0.9612$ $(1-\alpha) = 0.90$ $t = 2.920$ $x = \text{mol L}^{-1}$ $y = \text{nA}$ Linear range from 0.74×10^{-3} mol L^{-1} to 2.0×10^{-2} mol L^{-1}
(c)	$y = (2.64 \pm 0.47)x + (0.38 \pm 0.25)$ $r^2 = 0.9999$ $(1-\alpha) = 0.90$ $t = 2.920$ $x = \text{mol L}^{-1}$ $y = \text{nA}$ Linear range from 0.74×10^{-3} mol L^{-1} to 2.0×10^{-2} mol L^{-1}
(d)	$y = (1.31 \pm 0.08)x + (0.87 \pm 0.43)$ $r^2 = 0.9991$ $(1-\alpha) = 0.90$ $t = 2.920$ $x = \text{mol L}^{-1}$ $y = \text{nA}$ Linear range from 0.74×10^{-3} mol L^{-1} to 2.0×10^{-2} mol L^{-1}
(e)	$y = (0.503 \pm 0.083)x + (0.47 \pm 0.43)$ $r^2 = 0.9936$ $(1-\alpha) = 0.90$ $t = 2.920$ $x = \text{mol L}^{-1}$ $y = \text{nA}$ Linear range from 0.74×10^{-3} mol L^{-1} to 2.0×10^{-2} mol L^{-1}

adding an essentially aqueous sample to the selected solvent (the measures performed actually showed that the traces of oily substances in the MWW sample did not exceed 1–2% by volume on average). Therefore the addition of the MWW sample to the chosen solvent would lead to a signal variation due in part to the actual concentration of polyphenols present in the added sample, which represents the substrate of the enzymatic reaction, and in part to the variation in enzymatic response to the phenol, which is affected by the variation in the composition of the solvent mixture caused by adding the water contained in the aqueous sample; lastly, it is partly due also to the variation in dissolved oxygen concentration in the solvent (or solvent mixture) as its percentage water content varies. In order to verify this, several trials were performed to measure both the entire biosensor response obtained by adding a phenol standard in aqueous solution to the solvent comprising acetonitrile alone, and the biosensor response due to the variation in enzymatic response to the phenol caused by the variation in the water content of the solvent (acetonitrile) contained in the measuring cell. To this end, above all, 200 μL of phenol standard in acetonitrile alone were added and the biosensor response recorded. A further 200 μL of distilled water alone were then added (that is, practically the same volume of water contained in the MWW sample as is added when measuring real samples), again recording the new signal variation produced, which is precisely the one that in this case we are interested in measuring. Lastly, the response due to the variation in dissolved oxygen concentration with variation in the percent water in the solvent or solvent mixture used was recorded. To this end the oxygen sensor response was recorded, complete with the membranes used to construct the biosensor, but of course without the enzyme, and immersed in the acetonitrile solution when 200 μL of phenol standard in aqueous solution (or more simply 200 μL of simple distilled water) were added to the acetonitrile. These same experiments were also repeated following the same procedures but using

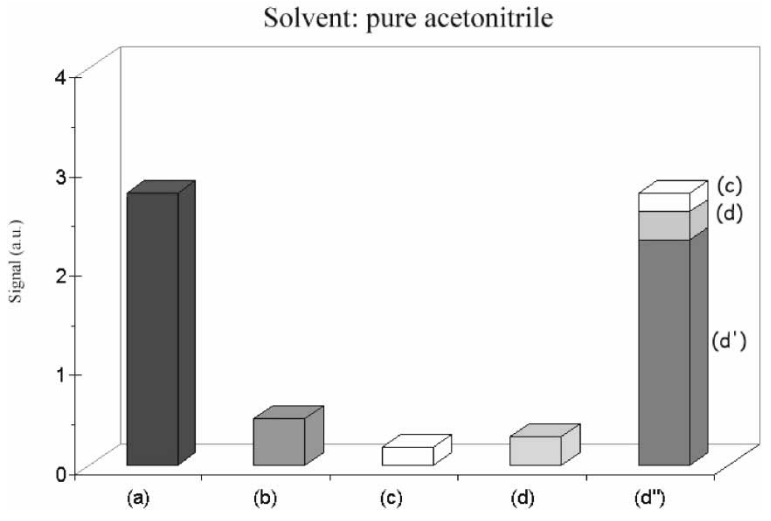

Figure 5. Different contributions to the tyrosinase biosensor response when operating in pure acetonitrile, with reference to a final phenol concentration of 10^{-4} mol L^{-1}. (a) Total signal (100% of total response). (b) (Sum of (c) + (d)): increase in signal due to the variation of the enzymatic response *vs.* phenol caused by the addition to the acetonitrile solution, of the water contained in the aqueous sample and of the variation in the concentration of dissolved oxygen in the solvent as the percent water contained in it varies (27% of total response). (c) Signal due exclusively to the variation in dissolved oxygen concentration in the solvent as the percent water contained in it varies after aqueous sample addition (7% of total response). (d) Increase in signal due exclusively to the variation of the enzymatic response *vs.* phenol caused by the addition of water contained in the aqueous sample (20% of total response). (d′) Signal due to true enzymatic response *vs.* phenol when operating in acetonitrile (73% of total response). (d″) Sum of different contributions to total signal [(73+20+7)% of total response].

as solvent contained in the thermostated cell a water–acetonitrile mixture 1+3 (v+v) instead of acetonitrile alone. Clearly, in this case, in order to measure the variation in the enzymatic response to phenol caused by the variation in the water content of the solvent, 200 μL of phenol standard in water–acetonitrile 1+3 (v+v) and then 200 μL of distilled water were added in the first instance. The results are shown in figures 5 and 6, and confirm what has already been stated: when operating in solvents such as pure acetonitrile or in water–acetonitrile mixtures, at least up to 75% (by volume) in acetonitrile, the contributions to biosensor response consist of the three already predicted: that due to the concentration of polyphenols present, which represents the substrate of the enzymatic reaction; that due to the addition of aqueous sample, which varies with the percentage content of water in the reaction solvent, thus causing the enzymatic reaction; response to vary; and that due to the variation in dissolved oxygen concentration. From the histograms obtained using acetonitrile alone it may be inferred that the first of the three contributions accounts for 75% of total biosensor response, the second for 20% of total response and the third for 7% of total response. On the other hand, in the case of the 1+3 (v+v) water–acetonitrile solution, these percentages were found to be 86, 13 and 2% of total biosensor response, respectively.

Carefully taking into account this aspect of the response to standard phenol solutions in different solvents or solvent mixtures, we moved onto the second part of the proposed experiment, namely to determine which solvent or solvent mixture was

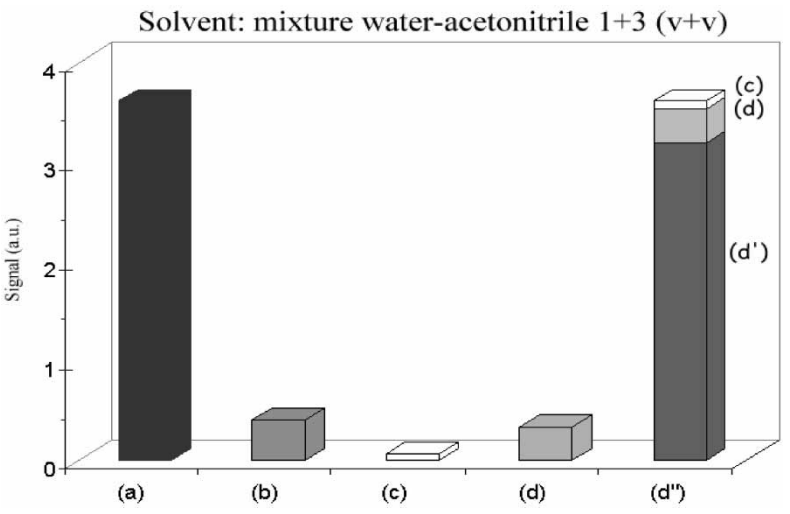

Figure 6. Different contributions to tyrosinase biosensor response when operating in acetonitrile–water (3+1) (v+v), with reference to a final phenol concentration of 10^{-4} mol L^{-1}. (a) Total signal (100% of total response). (b) (Sum of (c) + (d)), increase in signal due to the variation of the enzymatic response *vs.* phenol caused by the addition in the acetonitrile–water (3+1) (v+v) solution, of the water contained in the aqueous sample and of the variation in the concentration of dissolved oxygen in the solvent as the percent water contained in it varies (15% of total response). (c) Signal due exclusively to the variation in dissolved oxygen concentration in the solvent as the percent water contained in it varies after aqueous sample addition (2% of total response). (d) Increase in signal due exclusively to the variation of the enzymatic response *vs.* phenol caused by the addition of water contained in the sample (13% of total response). (d′) Signal due to true enzymatic response *vs.* phenol when operating in acetonitrile–water (3+1) (v+v) (85% of total response). (d″) Sum of different contributions to total signal [(85+13+2)% of total response].

optimal and effectively gave the highest biosensor response, that is, due either to the three contributions described above but also to the different solubility of the real MWW sample in the solvent or the solvent mixture considered. For this purpose the biosensor responses were recorded when 200 µL of the MWW sample were added to 10 mL of different solvents or solvent mixtures, and specifically to 100% solutions in water, or 3+1 (v+v), or 1+1 (v+v), or 1+3 (v+v) water–acetonitrile, and lastly in 100% acetonitrile. The trend obtained for the responses is shown in the histogram in figure 7.

Taking into account the results obtained from the curves in figure 2, from the histograms in figures 5 and 6 and from the analysis of the results shown in the histogram in figure 7, it may be concluded above all that, in the 100% acetonitrile solution, and in the water–acetonitrile solution 1+3 (v+v), the recorded signal is thus overestimated; since in these two mixtures the recorded signal is due not only to the concentration of polyphenols contained in the sample, the signal recorded in this solvent actually consists of the signal due to the polyphenols contained in the sample plus that due to the effect of the water contained in the sample added, plus that due to the variation in dissolved oxygen concentration. Moreover, figure 7 shows that the best biosensor response to the real MWW samples is that obtained when operating in the 1+1 (v+v) water–acetonitrile mixture; furthermore, in this solvent mixture, the response obtained may be entirely attributed to the enzymatic reaction involving the polyphenols present (indeed in this case, both the contribution due to the addition of water contained in the sample and that due to the variation in dissolved oxygen

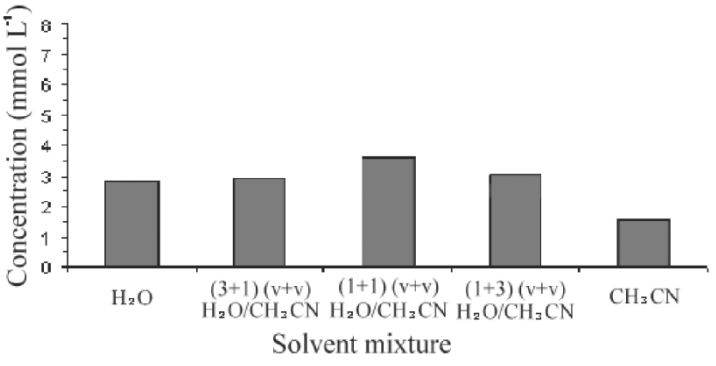

Figure 7. Histogram referring to the choice of optimal solvent or solvent mixture for the determination of polyphenols in the MWW sample. Tyrosinase biosensor response to a MWW sample, operating in different water–acetonitrile solvent mixtures.

concentration may be neglected) as we verified experimentally by means of experiments similar to those described in figures 5 and 6 but carried out in water–acetonitrile solutions 1+1 (v+v). In the first instance, therefore, it may be inferred that the 1+1 mixture (v+v) of water–acetonitrile seems also to be the best 'compromise' as far as the possibility of simultaneously dissolving both the polyphenols more soluble in water and those more soluble in acetonitrile is concerned.

4.2 *Selection of immobilization method for the biosensor operating in aqueous solvent or in water–acetonitrile mixtures*

Three different methods were also tested for the immobilization of the tyrosinase enzyme. They had previously been used in our laboratory [8, 9, 13, 17–19] to construct a large number of biosensors also of another type [22, 23]. The histograms in figures 8(a), (b) and (c) show the tyrosinase biosensor responses to a 1.0×10^{-2} mol L^{-1} solution of phenol, recorded when operating in a water–acetonitrile solution 1+1 (v+v) obtained using the biosensor assembled using three different methods of enzyme immobilization. In the first method the enzyme was immobilized in Kappa–Carrageenan, in the second in a dialysis membrane and in the third the enzyme was immobilized in a cellulose triacetate membrane. The tests were carried out over a period of four days.

Observing the results shown in the histograms in figures 8(a), (b) and (c), the following may be noted: the immobilization methods producing the best results under these operating conditions are those in which immobilization was performed in the dialysis membrane, or in Kappa–Carrageenan. When the enzyme was immobilized in a cellulose triacetate membrane the response was always much lower than when immobilization was carried out using one of the other two methods considered. In particular, immobilization in a dialysis membrane gives a better biosensor response during the first two days of operation starting from the biosensor assembly itself. However, immobilization in Kappa–Carrageenan enables the biosensor to provide good responses also from day two until day four. During this period it gives almost constant responses. Nevertheless, during the first two days, the responses of the biosensor using the latter immobilization method are slightly weaker than those produced by the biosensor with the enzyme

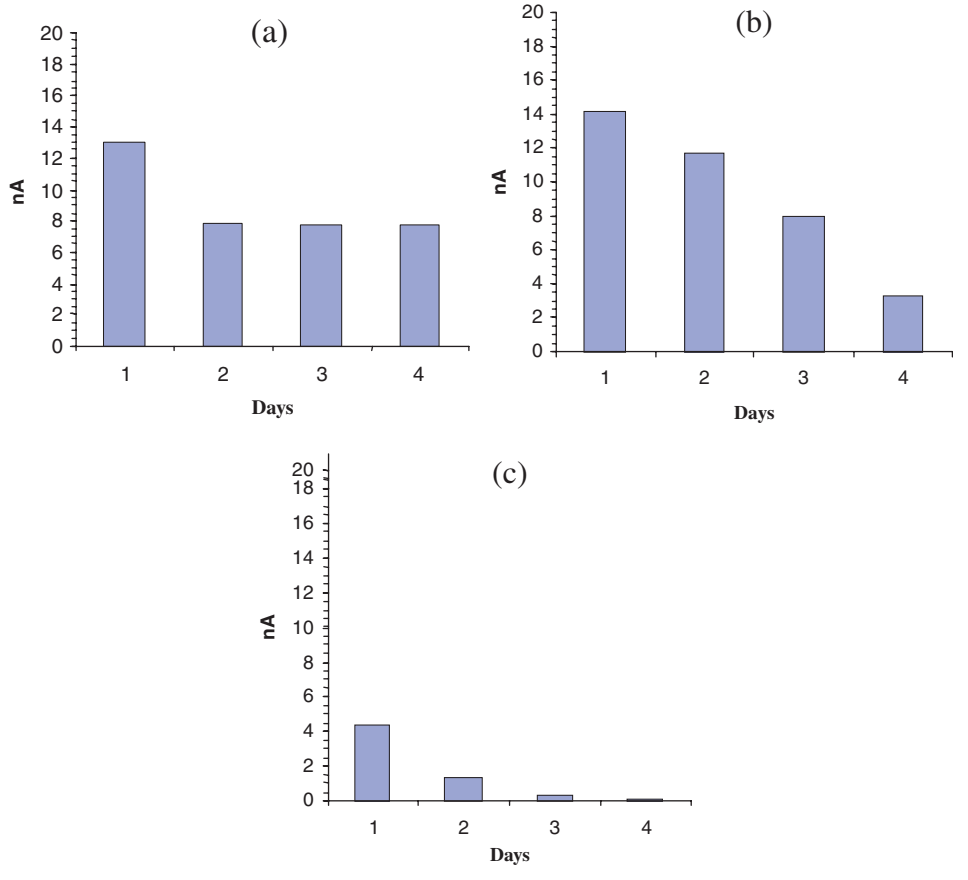

Figure 8. Signal variation (nA) of tyrosinase OPEE caused by the addition of 200 μL of a 1.0×10^{-2} mol L^{-1} phenol solution over a period of four days after biosensor preparation; solvent used (also for the phenol standard added), water–acetonitrile 1+1 (v+v): (a) immobilization of tyrosinase enzyme in Kappa–Carrageenan gel; (b) immobilization of tyrosinase enzyme in dialysis membrane; (c) immobilization tyrosinase enzyme in cellulose triacetate membrane.

immobilized in a dialysis membrane. Therefore, during the first two days' operation after assembly and operating in a 1+1 (v+v) water–acetonitrile mixture, the biosensor displays its greatest sensitivity when the enzyme is immobilized in the dialysis membrane. The same conclusion may be drawn also operating in water or in different water–acetonitrile mixtures with different composition ratios.

4.3 *Polyphenol 'pool' determination in six different MWW samples*

At this stage the optimal operating conditions for making biosensor measures on a MWW sample appeared clearly. For these measures a tyrosinase biosensor was therefore used with the enzyme immobilized in a dialysis membrane and operating in a 1+1 (v+v) water–acetonitrile mixture. In these experimental conditions six different MWW samples were analyzed. The results obtained for the polyphenol concentration contained in them are shown in table 4.

Table 4. Concentration of total polyphenols in six different MWW samples tested when operating with tyrosinase biosensor in acetonitrile–water (1+1) (v+v) ($n = 6$).

MWW samples	Polyphenol conc.[a] $(\text{mol L}^{-1}) \times 10^3$
Sample 1	2.29 ± 0.23
Sample 2	3.60 ± 0.14
Sample 3	3.46 ± 0.38
Sample 4	2.55 ± 0.26
Sample 5	5.90 ± 0.66
Sample 6	2.19 ± 0.18

[a]Expressed as phenol concentration.

Table 5. Evaluation of matrix effect in tyrosinase biosensor measures using the standard addition method.

MWW samples	Found[a] (in mg of phenol)	Phenol added (mg)	Total nominal value	Total experimental value	Recovery (%)
Sample 1	0.043	0.094	0.137	0.143	106
Sample 2	0.069	0.094	0.163	0.167	105
Sample 3	0.065	0.094	0.159	0.163	104
Sample 4	0.048	0.094	0.142	0.146	104
Sample 5	0.111	0.094	0.205	0.207	103
Sample 6	0.041	0.094	0.135	0.139	104

[a]Volume of sample MWW taken (=200 μL).

It can be seen that the percent standard deviations never exceed 12%. In order to determine the presence of possible interference due to complex matrices like olive oil mill wastewaters recovery tests using the 'standard addition method' were also carried out. In practice a fixed quantity of phenol, of the same order of magnitude as the average amount found in the polyphenol 'pool' sample, was added. The customary biosensor measure was then performed before and after the addition. The results are shown in table 5.

As can be seen, the recoveries obtained in five cases out of six do not differ by more than about 4% from the nominal value after the addition. In only one case was a difference in nominal value greater than 5% found. Therefore, in view of the good results obtained in terms of both precision and recovery tests, the problem of evaluating the method's accuracy was addressed. As no certified reference sample of olive mill wastewater was available on which to perform rigorous tests of method accuracy, we decided that the best thing to do was to analyse the test samples themselves using another method that is often cited in the literature and that is of course different from the biosensor method we used. The choice fell on the Folin–Ciocalteau spectrophotometric method. Although having many drawbacks this is one of the best known and most widely used methods, as is shown by the large number of citations in the literature and in handbooks dedicated to this type of analytical problem. The spectrophotometric measures performed as described in the 'Methods' section, for the six different MWW samples tested produced the results shown in table 6. The standard deviation is found to be very good, never exceeding 1% (as RSD%).

In this case recovery tests were also carried out using the standard addition method (table 7).

Table 6. Concentration of total polyphenols in MWW samples determined using the Folin–Ciocalteau method ($n = 4$).

MWW samples	Polifenol conc.[a] $(\text{mol L}^{-1}) \times 10^2$
Sample 1	1.56 ± 0.01
Sample 2	2.37 ± 0.02
Sample 3	2.72 ± 0.01
Sample 4	3.36 ± 0.01
Sample 5	4.35 ± 0.02
Sample 6	3.05 ± 0.01

[a]Expressed as phenol concentration.

Table 7. Evaluation of matrix effect in measures using the Folin–Ciocalteau method, with standard addition method.

MWW samples	Found[a] (in mg of phenol)	Phenol added (mg)	Total nominal value	Total experimental value	Recovery (%)
Sample 1	0.146	0.141	0.287	0.296	106
Sample 2	0.223	0.235	0.458	0.481	110
Sample 3	0.256	0.282	0.538	0.551	96
Sample 4	0.316	0.329	0.645	0.656	104
Sample 5	0.409	0.423	0.832	0.784	92
Sample 6	0.401	0.423	0.824	0.856	108

[a]Volume of diluted 1+20 (v+v) MWW sample taken (=500 μL).

Although not brilliant, the results were acceptable, differing from the nominal value after addition by a factor of about −8% and +10%. On the other hand, a considerable difference was found in polyphenol concentration in each of the samples analysed compared with that found using the biosensor method. The concentration was always found to be one order of magnitude higher than that found using the biosensor method.

4.4 *Comparison of results obtained using the two different methods*

Analysis of the data contained in tables 4–7, i.e. the good (or fair) precision offered by both methods (biosensor and spectrophotometric) and lastly the good, or at least acceptable, results obtained in both cases also in the 'recovery' tests carried out using the standard addition method and, on the other hand, the difference found in polyphenol concentration found using the two respective methods, which differ by a mean difference of one order of magnitude for the six MWW tested, led us to believe that this difference is due to factors inherent in the complexity of the matrix, that is, in the way in which the polyphenols are contained in the MWW matrix, which seems to have a stronger influence on the results obtained using the biosensor method rather than the spectrophotometric one. Indeed, in addition to the effects due to the different solubility of the individual polyphenols in water or in a lipophilic matrix, a significant role is probably played also by the presence of micellar aggregates or in any case by polyphenol aggregates held together by various types of weak bonds.

It was therefore attempted to account for this discrepancy in the results obtained using the two different methods considered.

Determinations carried out on several MWW samples subjected to pretreatment, such as pH variation of the sample (in both acid and basic fields) before measurement caused no significant changes in polyphenol concentration determined by the biosensor. It was thus decided to pretreat the aqueous sample of MWW with an organic solvent so as to extract the more liposoluble polyphenols into the organic phase while the more hydrosoluble ones remained in the aqueous phase. It was thus assumed that this drastic pretreatment would largely disaggregate the polyphenol aggregates present in the original phase. In this way, the polyphenols now released could be determined separately but more accurately, again using the tyrosinase biosensor, although now operating directly in the two different phases – the water phase and the organic phase. The pretreatment of extraction and separation was carried out on each of the six MWW samples tested using dichloromethane as organic extractant, i.e., the solvent that, as reported in the literature [24, 25], was used also in chromatographic research carried out on vegetal matrices and found to be more efficient.

4.5 *Choice of immobilization method for OPEE operating in dichloromethane*

In order to perform biosensor measures directly in dichloromethane, it was obviously necessary to again optimize the enzyme immobilization method in view of the quite different characteristics of the new solvent compared with the aqueous solvent or the water–acetonitrile mixtures previously considered. The histograms in figures 9(a), (b) and (c) show the responses to a $1.0 \times 10^{-2}\,\mathrm{mol\,L^{-1}}$ solution of phenol in dichloromethane obtained using a tyrosinase OPEE (operating in a dichloromethane solution) constructed using three different enzyme immobilization methods. In the first method, the enzyme was immobilized in Kappa–Carrageenan, in the second in a dialysis membrane and in the third, the enzyme was immobilized in a cellulose triacetate membrane. The tests were carried out over a period of four days. It was noted that, in these operating conditions, the best biosensor responses over the four days were certainly those obtained, operating with the biosensor using the enzyme immobilized in Kappa–Carrageenan. This confirms the findings made in recent years in investigations carried out in our laboratory concerning the development of different kinds of OPEE [8, 13, 22, 23]. It is also interesting to observe however that slightly less satisfactory but nevertheless appreciable results were obtained also using immobilization in cellulose triacetate. On the other hand, with immobilization in a simple dialysis membrane, an OPEE was obtained which had too short a working life (less than one full day).

4.6 *Measure of extracts in dichloromethane with the tyrosinase OPEE and final analytical comparison between the biosensor and spectrophotometric methods*

The measures of the dichloromethane extracts of the six samples tested were thus carried out in the same solvent (dichloromethane) with the tyrosinase OPEE in which the enzyme was immobilized in Kappa–Carrageenan. The results regarding polyphenol concentration in the six MWW samples tested obtained in both the aqueous phase, as a residue of the extraction process, and the extracting organic phase, as well as the sum of these two concentrations, are shown in table 8.

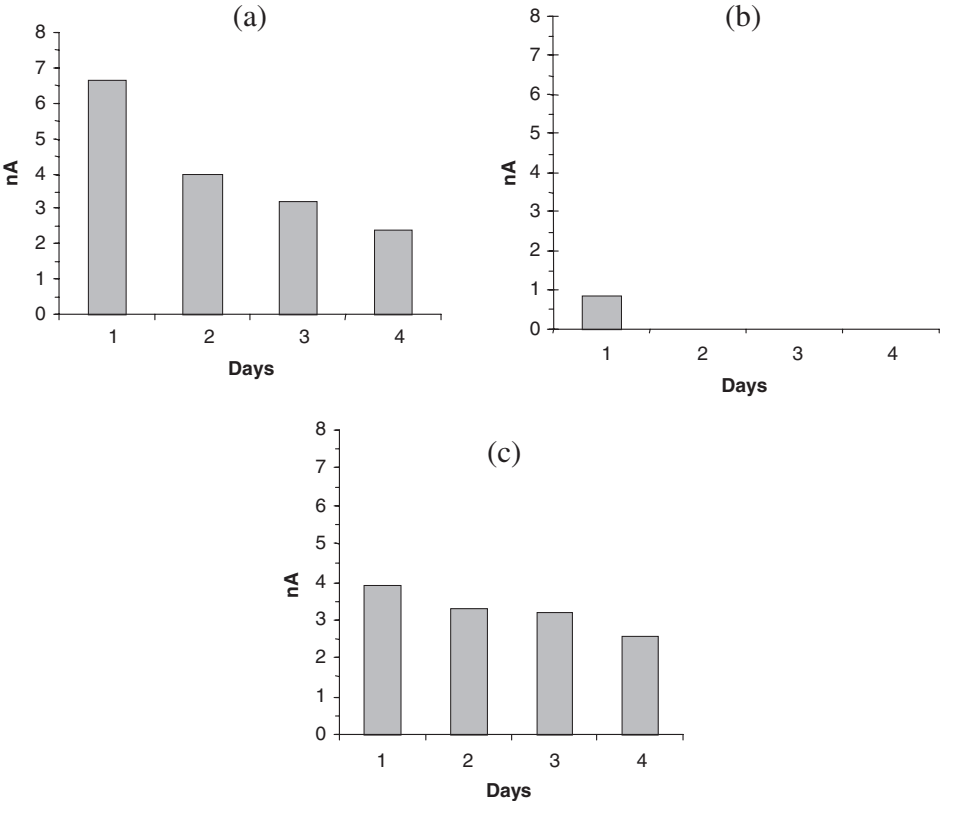

Figure 9. Signal variation (nA) of tyrosinase OPEE after addition of 200 μL of a 1.0×10^{-2} mol L^{-1} phenol solution over a period of four days after OPEE preparation; solvent used (also for phenol standard added) dichloromethane: (a) immobilization of tyrosinase enzyme in Kappa–Carrageenan gel (b) immobilization in dialysis membrane (c) immobilization in cellulose triacetate membrane.

Table 8. Results of total polyphenol determinations spread over aqueous and organic phase (CH_2Cl_2), obtained respectively using a tyrosinase biosensor and a tyrosinase OPEE ($n = 5$).

MWW sample	Experimental polyphenol[a] conc. in aqueous phase (mol L^{-1})$\times 10^2$	Experimental polyphenol[a] conc. in CH_2Cl_2 phase (mol L^{-1})$\times 10^2$	Experimental total[a] polyphenol conc. (mol L^{-1})$\times 10^2$
Sample 1	1.14 ± 0.07	0.39 ± 0.03	1.53 ± 0.10
Sample 2	1.39 ± 0.13	0.67 ± 0.06	2.06 ± 0.19
Sample 3	1.65 ± 0.16	1.54 ± 0.13	3.19 ± 0.29
Sample 4	2.53 ± 0.18	1.01 ± 0.08	3.54 ± 0.26
Sample 5	3.41 ± 0.33	0.41 ± 0.03	3.82 ± 0.36
Sample 6	2.05 ± 0.18	0.43 ± 0.03	2.48 ± 0.21

[a]Expressed as phenol concentration.

It is immediately apparent that, by summing the polyphenol concentrations found in the two phases for the various samples analysed, total concentrations are obtained that, in this case, are of exactly the same order of magnitude as those obtained using the Folin–Ciocalteau spectrophotometric method. Also in this case table 9 shows the results of recovery tests carried out using the standard addition method, operating in dichloromethane with the tyrosinase OPEE.

Table 9. Evaluation of matrix effect with tyrosinase OPEE operating in CH_2Cl_2; recoveries obtained using the standard addition method.

MWW samples	Found[a] (in mg of phenol)	Phenol added (mg)	Total nominal value	Total experimental value	Recovery (%)
Sample 1	0.073	0.094	0.167	0.166	99
Sample 2	0.126	0.094	0.220	0.221	101
Sample 3	0.290	0.300	0.590	0.585	98
Sample 4	0.190	0.188	0.378	0.370	96
Sample 5	0.077	0.094	0.171	0.181	111
Sample 6	0.081	0.094	0.175	0.177	102

[a]MWW sample volume taken (=200 μL).

Table 10. Comparison of values referring to total concentration of polyphenols present in the MWW analysed using the various different methods.

MWW samples	[a] Spectrophotom. method conc. (mol L^{-1}) $\times 10^2$ *	RSD %	[b] Biosensor method by extraction[a] conc. (mol L^{-1}) $\times 10^2$ *	RSD %	(a−b)/a %	[c] Biosensor method without extraction[b] conc. (mol L^{-1}) $\times 10^3$ *	RSD %
Sample 1	1.56	0.6	1.5	6.5	1.9	2.3	10.2
Sample 2	2.37	0.8	2.1	9.2	13.1	3.6	9.2
Sample 3	2.72	0.4	3.2	9.1	−17.3	3.5	11.0
Sample 4	3.36	0.3	3.5	7.3	−5.4	2.6	10.4
Sample 5	4.35	0.5	3.8	9.4	12.2	5.9	11.2
Sample 6	3.05	0.2	2.5	8.8	17.1	2.2	8.2

*Expressed as phenol concentration.
[a] Sum of experimental concentration of polyphenols in aqueous phase and in organic phase.
[b] Experimental concentration of polyphenols in water–acetonitrile mixture (1+1) (v+v).

Lastly table 10 shows a comparison between total polyphenol concentrations obtained using the Folin–Ciocalteau method and those obtained using the biosensor method. However, in the latter case both the values obtained using a double measurement in the two different phases (organic and aqueous) after extraction in CH_2Cl_2, and those performed using a single measurement in the 1+1 (v+v) water–acetonitrile mixture are shown.

There is clearly relatively good agreement between the spectrophotometric method and the biosensor method when, using the latter method, the double measurement is performed, that is, after the extractive treatment has been carried out. In this case the difference in value found between the two methods actually does not exceed 12–13% in four out of the six samples tested and about 17% in the other two cases. This result may be considered as relatively satisfactory in view of the complexity of the real matrix and the considerable differences in the two analytical methods used. Above all, these results seem to confirm that the differences in polyphenol concentration found using the two different methods in the MWW samples, when a single measure was made using the tyrosinase biosensor operating in (1+1) (v+v) water–acetonitrile mixture, are mostly dedicated in fact to problems of solubility and the formation of micelles, rather than to reduced, or even zero, sensitivity to certain polyphenols by the spectrophotometric method [26], but above all by the biosensor method [27, 28], even though the likelihood of this happening, even in part, cannot however be ruled out completely.

5. Conclusions

In conclusion, it may be pointed out that the tyrosinase biosensor, albeit assembled in two different ways, i.e., as biosensor operating in aqueous solvent (or solvent mixture) and in the organic phase (OPEE), allowed total polyphenol 'pool' determinations to be made in extremely complex real matrices of specific environmental interest. That is, it was possible to develop and optimize a method for determining the polyphenol content of olive oil mill wastewater with reasonable precision (RSD $\leq$ 10%), relatively fast and quite cheaply, and how it was possible to validate the results obtained by comparing them with those found by analyzing the same samples using the Folin–Ciocalteau spectrophotometric method.

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