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ARTICLE

Determination of an Antioxidant Capacity Index by Immobilized Tyrosinase Bioreactor

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Immobilized tyrosinase on aminopropyl-controlled pore glass support (AMP-CPG) was used to determine the antioxidant capacity index and the total phenol content in juices, integrators, infusions, jams, and drugs containing bilberry fruits. The method is based on the chromatographic determination of the decrease of total ingredients content, absorbing at 280 nm, by mushroom tyrosinase oxidation. In comparison with the widely used Folin−Ciocalteu method (FC), this enzymatic method appears to be more specific and rapid and as whole is not affected by interfering compounds such as citric acid and sugar. The results were also compared with those obtained by 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)/persulfate antioxidant assay, expressed as Trolox equivalents (TEAC) . Using all 17 samples, a good linear correlation ($r^2 > 0.94$) was observed between the enzyme assay and both the FC and TEAC assays. Poor correlation ($r^2 < 0.4$) with the other tested methods (pH-differential, vanillin index, and butanol−HCl assays) was evidenced. This shows that the enzymatic assay cannot be applied for a specific phenol class determination, but it is sensitive to the total phenolic content.

KEYWORDS: Bioreactor; antioxidant capacity; immobilized tyrosinase

INTRODUCTION

Different studies have shown that the free radicals present in humans cause oxidative damage to different molecules such as lipids, proteins, and nucleic acid (*1*, *2*) and are thus involved in the initiation phase of some degenerative illnesses (*3*−*8*). As a consequence, the antioxidant compounds that are capable of inhibiting the initiation or propagation of this oxidative chain reaction by the free radical neutralization may play an important role in the prevention of certain diseases (*9*). In addition, taking into account that in the United States and for most European Union countries, fruit juice and concentrates can be used without restriction, they became an important ingredient in the manufacture of many foods and beverages.

Several studies have shown that bilberry (*Vaccinium myrtillus*) and derivatives exhibit high antioxidant potentials, which are tightly connected with the total phenolic content (*10*, *11*). Bilberries are rich in anthocyans, which are a large and important group of the flavonoid compounds, and also contain phenolic acids and procyanidins (*12*−*15*). Therefore, analytical methodology able to determine their source is needed for quality control.

It is possible to determine the individual components by separative techniques, but these methods are time-consuming and expensive and require considerable effort to obtain standards, because many of them must be purified from natural sources. In addition, because there seems to be synergism between different antioxidants during oxidative stress, examining one in isolation from the rest may not accurately reflect their combined action. Interest has therefore focused on the measurements of antioxidant activity and of total phenolic contents. Generally, the determination of antioxidant capacity assays may be obtained by electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays and also by the cupric ion reducing antioxidant capacity (CUPRAC) method (*16*). Of the numerous electron transfer methods, which measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced, the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay has been highly employed because it is rapid and can be used over a wide range of pH values in both aqueous and organic solvent systems (*17*, *18*). The assay has adapted to use the preformed blue-green ABTS radical ($ABTS^{\bullet}$) obtained by oxidation with a strong oxidizing agent (potassium persulfate). The degree of decolorization, induced by the antioxidant compound that reflects the amount of $ABTS^{\bullet}$ scavenged after a fixed period of time, is related to that of Trolox.

Spectrophotometric methods are also available for the estimation of the total phenolic content and of particular fractions. Among these, the most common are the vanillin index for condensed flavanols with C6 and C8 free atoms, the pH-differential method for anthocyanin content, the butanol−HCl assay for condensed tannins, and the Folin−Ciocalteu (FC) assay for total phenolics. The latter is the most used, although it is known that the strong inorganic oxidants (phosphotungstic and phosphomolybdic acids) will react also with nonphenolic compounds. Consequently, the removal of interfering reductants must be done prior to the analysis.

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Various enzymatic assays and biosensors have been reported for phenolic compounds (*19*), but to our knowledge no practical application of these methods has gained acceptance to date.

In the present study we have investigated the use of a copper enzyme widely distributed in nature (*20*, *21*), such as mushroom tyrosinase (EC 1.14.18.1), to measure the total phenolic content in 17 samples containing bilberry (juices, integrators, infusions, jams, and drugs). The enzyme catalyzes the hydroxylation of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones, in both cases by molecular oxygen. The tyrosinase has been immobilized on aminopropyl-controlled pore glass support (AMP-CPG), as previously reported (*22*), to obtain a stable and reusable bioreactor that may be used in a HPLC system. The results have been compared with the values obtained by FC and TEAC methods, after a screening of the principal phenolic and acidic constituents and the identification of anthocyanidins, obtained by acid hydrolysis of the bilberry samples.

MATERIALS AND METHODS

Chemicals and Reagents. Mushroom tyrosinase, Folin−Ciocalteu reagent, 3,4-dihydroxyphenylalanine (D,L-DOPA), vanillin index, 2,2′-azinobis(3-ethylebenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), gallic, ferulic, caffeic, *p*-coumaric, and chlorogenic acids, (+)-catechin (C), epicatechin (EC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), and potassium persulfate were purchased from Sigma (Milan, Italy), whereas glutaraldehyde, methanol, acetonitrile, hydrochloric and phosphoric acids, *n*-butanol, and the salts $NaCNBH_3$, Na_2CO_3, Na_2HPO_4, NaH_2PO_4, KCl, CH_3COONa, and $FeNH_4(SO_4)_2 \cdot 12H_2O$ were from Carlo Erba (Milan, Italy). Malvidin, cyanidin, delphinidin, and (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Fluka (Milan, Italy), whereas aminopropyl-controlled pore glass support (AMP-CPG) was purchased from Millipore (Lincoln Park, NJ). All of the solutions were prepared using Milli-Q deionized water (Millipore Inc., Bedford, MA).

Apparatus. Chromatographic analysis was performed with a Kontron system (Milan, Italy), consisting of a model 422 pump and a UV−visible 432 detector, complete with a Rheodyne 7125 injector with a 20 μL sample loop (Rheodyne, Berkeley, CA). The absorbance values were registered by an integrator system constituting a PC equipped with an Intel processor, Pentium III 800 MHz CPU, and Agilent ChemStation software for LC version A.08.03(847) running under MS Windows NT 4.00.31 OS.

Spectra of peaks were obtained with a Thermo Quest Spectra Series HPLC (Thermoquest Inc., San Jose, CA) equipped with a Spectra Series UV 6000 photodiode array detector (Thermoquest Inc.) using Chrom Quest Software.

Spectrophotometric determinations were performed on a Kontron Uvikon 920 UV−vis spectrophotometer with cuvettes of 1 cm path length.

Free Tyrosinase Assay. A solution constituted by 20 μL of tyrosinase (0.3 mg/mL in phosphate buffer solution 10 mM, pH 7.2), 150 μL of 1 mM D,L-DOPA, and 0.1 M phosphate buffer, pH 6.5, to a final volume of 1 mL was spectrophotometrically monitored at 475 nm for determining the dopachrome formation. The activity is determined by the expression

$$\mathrm{U\ (nmol\ L^{-1}\ min^{-1})} = \frac{\Delta A/\mathrm{min}}{3716} \times 50 \times 10^6$$

were 3716 is the molar extinction coefficient and 50 is the dilution factor.

Immobilization Procedure. Tyrosinase has been coupled to the aminopropyl groups of CPG support, previously activated by reaction with glutaraldehyde by in situ immobilization technique. In brief, the method has been as follows (*23*): the support (70 mg of the AMP-CPG) was placed in a stainless steel column (size 50 × 2.1 mm), and glutaraldehyde solution (2.5% v\v in 50 mM phosphate buffer, pH 7) was recycled through it at 0.2 mL/min flow rate for 2 h. The column was washed with deionized water for 2 h at 0.2 mL/min flow rate to eliminate the surplus of glutaraldehyde. In these steps the temperature were maintained at 15 °C through a Thomson TLC Cryothermostat. Then, the enzyme (3 mg of tyrosinase (3.320 units/mg of solid) in 10 mL of 0.01 M phosphate buffer, pH 7.2, was recycled through the column for 4 h in the cold (4 °C). To eliminate nonbound protein, the bioreactors was washed with 10 mM phosphate buffer (1.5 h, 0.2 mL/min flow rate). Finally, the Schiff base was reduced by washing the column with sodium cyanoborohydride (1% w/v 0.1 M phosphate buffer, pH 6.5) for 1 h.

Catalytic activity and stability of the chromatographic reactor were evaluated using D,L-DOPA as substrate, and the immobilization's parameters have been determined as reported by Girelli (*22*) and Villanova (*23*).

Three distinct immobilized enzyme bioreactors (IMER) were prepared with the above-mentioned procedure. IMER 1 and IMER 2 were obtained by tyrosinase solutions with almost the same enzymatic activity, whereas IMER 3 was obtained by a solution with a higher activity.

Immobilized Tyrosinase Assay. The reactor (IMER), placed between the HPLC pump and the detector, is initially equilibrated with 50 mM phosphate buffer, pH 6.5, for 15 min. Then 20 μL of a 10 mM D,L-DOPA solution in 0.1 M phosphate buffer, pH 6.5, are injected into the HPLC system with a flow rate of 0.5 mL/min. The amount of dopachrome formed, detected at 475 nm, is obtained by integration of peak areas and by interpolation with a calibration curve obtained as follows. Standard solutions of dopachrome, generated by chemical oxidation of D,L-DOPA with a 2-fold excess of $NaIO_4$ at pH 6.5, are injected in the HPLC system at 475 nm (*14*). The calibration curve, obtained in the range of 30−250 μM o f dopachrome concentration, presents the equation $y = 1209.4x$ ($r^2 = 0.9979$).

The tyrosinase immobilized activity is determined as

$$\mathrm{activity\ (nmol\ min^{-1})} = \frac{\mathrm{dopachrome\ peak\ area}}{1209.4 \times t_r}$$

where 1209.4 is the coefficient of dopachrome calibration curve and t_r the retention time.

Determination of Protein Percent Immobilized. The amount of enzyme, as protein, immobilized on the support was calculated from the difference in the amount of protein contents in the solution before and after immobilization. The protein concentrations were determined by spectrophotometric measurements at 215 and 280 nm (*15*) and mathematically expressed as

$$Y_{\mathrm{IMER}}^{\mathrm{protein}}\ (\%) = \frac{P_i - P_f}{P_i} \times 100$$

where P_i and P_f are the initial and final UV absorbance values, respectively, of enzyme solution.

Removed Activity Ratio. The activity percent removed from the free tyrosinase as a consequence of the enzyme immobilization as well as its inactivation was determined spectrophotometrically by

$$R_{\mathrm{rem}}^{\mathrm{activity}}\ (\%) = \frac{U_i - U_f}{U_i} \times 100$$

where U_i and U_f are the total units of tyrosinase activity in the solution before and after the immobilization process, respectively.

Coupling Efficiency. The coupling efficiency is simply defined as the percent ratio of the total units of immobilized tyrosinase activity to the activity removed from the solution during the immobilization process:

$$\mathrm{coupling\ efficiency\ (\%)} = \frac{U_0^{\mathrm{IMER}}}{U_i - U_f} \times 100$$

Sample Preparation and Extraction Procedure. The samples purchased from a local grocery were juices, integrators, drugs, infusions, and jams.

The liquid bilberry samples were Zuegg (minimum bilberry amount 40%), Joga (32%), Brio (65%), Bio-Beutelsbacher (100%), and Pfanner juices. All of them have been only centrifuged at 3800 rpm for 10 min with an AIC 4212 centrifuge.

The syrup (S. Bernardo) and the solid samples such as integrators (BODY-SPRING, Arkocapsula and Equilibria), infusions (Lester House, Twining, Teekann), drugs (Tegens, Mirtilene Sife), and one integrator containing also tea (Vital) have been treated with 30 mL of boiling water for 10 min and then centrifuged at 3800 rpm for 10 min, taking the surnatant and discarding the solid.

The two jam samples (Conad and Fiorentini) (2.5 g) were, instead, treated by stirring with 25 mL of a methanol/acetic acid/water mixture (25:1:24 v/v/v) for 20 min at room temperature (*24*) and taking the surnatant.

After extraction, different aliquots of all the samples were immediately frozen at −15 °C in the dark for further use.

HPLC Separation. A modification of the HPLC assay reported by Escarpa (*25*) was employed. Briefly, the separation was obtained employing a column, C18 Kromasil (4.6 × 250 mm, particle size 5 μm) (Supelco, Italy) with a C18 Alltima guard column. The phenols were eluted with a gradient of 0.01 M phosphoric acid (solution A) and acetonitrile (solution B). In the first 5 min, acetonitrile was 2%, from 5 to 10 min B increased from 2 to15%, and from 10 to 35 min B increased to 25%. Then from 35 to 40 min acetonitrile decreased from 25 to 2% B. The total elution time was 40 min. Experimental conditions were flow rate = 1.3 mL/min, λ = 280 nm, room temperature, and 20 μL sample injection volume. Identification of the compounds was made by comparing their t_r values and UV spectra with those of standards.

HPLC Anthocyanidin Determination. To establish the anthocyanidin composition in a sample, the most reliable method is to release the aglycone portion of an anthocyanin molecule using acid hydrolysis. Optimal anthocyanin hydrolysis is obtained by incubating 2 mL of the samples in 2 mL of methanol and 1 mL of 12 M HCl (final concentration of 2−3 M HCl) in a boiling water bath for 50 min. The cooled samples are filtered and put into 10 mL flasks and brought up to the final volume with methanol (*26*). HPLC separation of anthocyanidins has been performed using a C18 Kromasil (4.6 × 250 mm, particle size 5 μm) column (Supelco, Italy) and a C18 Alltima guard column (Alltech, Italy). An isocratic elution with a mixture of acetic acid/acetonitrile/water (10.8:12:77.2 v/v/v) was performed. Other chromatographic conditions were as follows: flow rate, 1.2 mL/min; column temperature, room temperature; detection, 540 nm; 20 μL sample injection volume. Identification of anthocyanidins was based on retention times of reference compounds.

Vanillin Index Determination. The vanillin index has been determined according to the procedure reported in the literature (*27*). An aliquot of 0.5 mL of the samples was made to react in a tube, maintained in a cold water bath, with 3 mL of 4% (w/v) vanillin solution in methanol, to which 1.5 mL of concentrated hydrochloric acid was added after 3 min. Then after 15 min, the absorbance was measured at 500 nm against a blank prepared by substituting vanillin with methanol. The results have been expressed as (+)-catechin.

$$(+)\text{-catechin (mg/mL)} = \frac{\Delta A}{2.36} \times b$$

Here 2.36 is the absorbivity coefficient expressed as cm^{-1} mg^{-1} mL and b = 1 cm.

pH-Differential Assay. A modification of the pH-differential spectrophotometry INA method 116.000 (*28*) has been used to calculate the anthocyanin content in the samples. Because anthocyanic pigments exhibit their maximum color intensity at about pH 1 and are colorless at pH ∼4.5, an aliquot of each sample was diluted 1:50 with a pH 1 buffer solution (1.49 g of KCl into 100 mL of deionized water). Twenty-five milliliters of this solution was mixed with 67 mL of 0.2 M HCl solution, adjusted at pH 1.0 if necessary, and another aliquot with a sodium acetate solution adjusted with hydrochloric acid at pH 4.5. The difference in absorbance at 510 nm was proportional to the anthocyanin contents. The results were expressed as cyanidin equivalents

$$\text{anthocyanincontent (mg/mL)} = \frac{\Delta A \times \text{Df}}{33.54 \times b}$$

where $\Delta A = [(A_{510nm})_{pH1} - (A_{510nm})_{pH4.5}]$, Df = 50, b = 1 cm, and 33.54 is the absorptivity coefficient (cm^{-1} mg^{-1} mL).

Butanol−HCl Assay. This assay, specific for proanthocyanidins or condensed tannins (*29*), was based on acid-catalyzed depolymerization of condensed tannins into anthocyanidins. To 1 mL of samples, diluted with deionized water until the absorbance of samples was within the 1 unit value, were added 6 mL of HCl/*n*/butanol (5:95 v/v) reagent and 0.2 mL of the iron reagent (0.5 g of ferric ammonium sulfate in 25 mL of 2 N HCl). After mixing, the tubes were placed in a boiling water bath. After 50 min, absorbance at 550 nm was measured against a blank prepared by mixing the solvent sample (1 mL) with butanol−HCl (6 mL) and ferric reagent (0.2 mL) but without heating. Condensed tannin as cyanidin equivalent was calculated as

$$\text{condensed tannin content (mg/mL)} = \frac{A_{550}}{7.51} \times b \times \text{Df}$$

where 7.51 is the absorptivity coefficient expressed as cm^{-1} mg^{-1} L, b = 1 cm, and Df is the dilution factor.

Folin−Ciocalteu Assay. Total phenolic content was determined according to the FC method (*30*). In a tube, maintained at 20 °C, to an aliquot of 20 μL of samples were added in succession 150 μL of FC reagent, 1200 μL of 7.5% (w/v) Na_2CO_3 solution, and distilled water to a 3000 μL final volume. After 2 h, the absorbance increase was measured at 784 nm against the blank containing 20 μL of water instead of sample. The concentration of the total polyphenol content was expressed as delphinidin, (+)-catechin, and ferulic acid equivalents.

TEAC Assay. To determine the antioxidant capacity, the TEAC method, which is one of the most used methods (*31*), has been employed. It is based on a neutralization of radical cation formed by a single-electron oxidation of a synthetic ABTS chromophore to a strongly absorbing ABTS$^{\bullet}$ radical (700−550 nm). The radical was prepared through the reaction between 7 mM ABTS in pH 7.4 phosphate buffer (1.48 g of Na_2HPO_4, 0.43 g of NaH_2PO_4, and 7 g of NaCl in 1 L of distilled water) and potassium persulfate (2.45 mM). This solution was stored at room temperature in the dark for 12−14 h before use. The formed ABTS$^{\bullet}$ solution was then diluted with pH 7.4 phosphate buffer to obtain an absorbance of 0.70 ± 0.02 at 734 nm. The calibration curve has been built using Trolox as standard, and the linearity corresponded to an inhibition potential of about 40−80%. Ten microliters of the antioxidant Trolox (0−2.5 mM) was added to 990 μL of ABTS$^{\bullet}$ solution, and the absorbance at 734 nm was measured after 6 min. This was compared with a blank where 10 μL of the phosphate buffer, pH 7.4, was added to 990 μL of ABTS$^{\bullet}$ [equation: $y = (3.24 \times 10^{-1})x + 4.25 \times 10^{-2}$, $r^2 = 0.9900$].

The same procedure was utilized for the analysis of samples, each of them diluted in the same way.

The results were expressed by

$$\text{TEAC (mg/L)} = \left(\left(\frac{A_0 - A_s}{A_0}\right) - 4.25 \times 10^{-2}\right) \frac{\text{Df} \times \text{PM}}{3.24 \times 10^{-1}}$$

where A_0 and A_s are the absorbance of the blank and of the samples at 6 min of the reaction, Df is the dilution factor, and PM is the Trolox molecular weight.

Enzymatic Assay. The total phenolic content has been enzymatically determined by two steps: initially, an aliquot of sample, diluted (1:3) in 0.1 M phosphate solution, pH 5, was injected on a C18 precolumn in a chromatographic system and, then, another aliquot was injected on the bioreactor connected to the C18 precolumn. In this way the differences of peak areas measured (Δarea) were related to the phenols enzymatically oxidized. The chromatographic conditions were as follows: mobile phase, 0.1 M phosphate solution, pH 5; flow rate, 0.5 mL/min; injection volume, 10 μL; λ, 280 nm; temperature, 20 °C. For the quantification, the external standard method has been used employing three different reference standards: delphinidin, (+)-catechin, and ferulic acid.

RESULTS AND DISCUSSION

Bioreactor Performance. The characterization of the bioreactor was performed by determining its activity and stability. Taking into account that the enzyme can be inactivated by interaction

with the support and the coupling conditions, the protein percent immobilized, the activity percent removed, the immobilized enzyme activity, and the coupling efficiency have been determined. **Table** 1 presents the data obtained for three different immobilized tyrosinase bioreactors, prepared at the same experimental conditions: IMER 1 and IMER 2 from almost the same tyrosinase free activity to verify the repeatability of the immobilization procedure and time stability of bioreactors; IMER 3 from a solution with higher tyrosinase activity. It appeared that data of IMER 1 and 2 are in good agreement, whereas when free tyrosinase activity increased (U_i^{spec}), the bioreactor activity, as in IMER 3 (U_f^{IMER}) increased, but not to the same extent. This can be ascribed probably to the binding site saturation of the support. However, for all of the bioreactors, <7% of the initial enzyme activity remained in the solution ($R_{rem}^{activity} > 93\%$), whereas 83−87% of the protein was removed after coupling of the enzyme to AMP-CPG. The values of the coupling efficiency evidence that almost 40% of the initial enzymatic activity was immobilized. However, IMER 3 was employed for its highest immobilized activity value (117 nmoL/min) for further studies on bilberry samples.

The other critical performance indicator, like the stability of the bioreactor with respect to time, was evaluated by maintaining, when not in use, the immobilized and free tyrosinase under fixed operating conditions (4 °C in 10 mM phosphate buffer, pH 7.2) and monitoring their activity after fixed periods of time. As evidenced by data reported in **Figure 1** the storage stability of the bioreactor IMER 3, employed for the samples analysis, was 40% lower than that of IMER 1, never employed for the sample analysis, but in both cases the storage stability was higher than that shown by the soluble tyrosinase, which drastically decreased after 60 days of storage.

Table 1. Diphenolase Activity of Tyrosinase Solution, Immobilized Protein Percent, Removed Diphenolase Activity Percent, IMER Diphenolase Actitivity, and Coupling Efficiency Percent for Three Bioreactors Building

tyrosinase	IMER 1	IMER 2	IMER 3
diphenolase activity of free tyrosinase (U_i^{spec}) (nmol/min)	170	180	450
immobilized protein % ($Y_{IMER}^{protein}$%)	84	87	83
removed diphenolase activity % ($R_{rem}^{activity}$%)	93	93	94
IMER diphenolase activity (U_f^{IMER}) (nmol/min)	67	76	117
coupling efficiency %	40	44	39

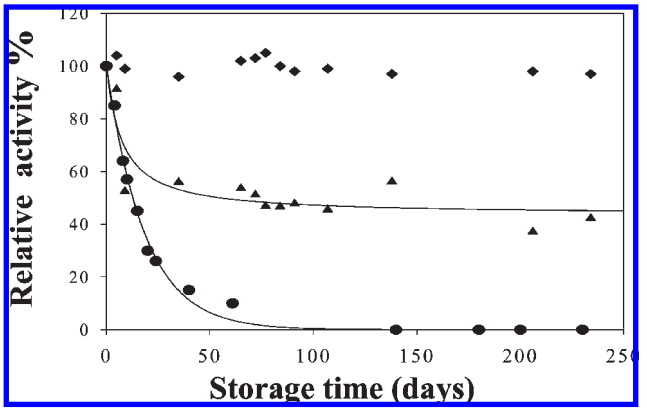

Figure 1. Storage stability of tyrosinase: (●) soluble; (▲) immobilized IMER 3 (utilized for the analysis of each samples); (◆) immobilized IMER 1 (utilized as reference).

Optimization of Analytical Method. The enzymatic method is based, at an opportune λ, on the chromatographic determination of the peak areas obtained using two different stationary phases: first, a C18 phase to monitor all of the absorbent species at the prefixed λ; second, the same C18 phase coupled with the tyrosinase bioreactor to monitor the species not reacted with the enzyme (**Figure 2**). The difference between the two values corresponds to the phenols, present in the samples, which have reacted with the immobilized tyrosinase. Therefore, λ operative and pH were the main factors to be regulated.

The choice of λ was made by evaluating the spectra obtained by injecting standard solutions of anthocyanidins and bilberry juice on the IMER coupled with a diode array. As known, the spectral behavior of all the flavonoids shows two UV absorption bands: Band II, with a maximum in the 240−285 nm range, arises from the A ring, whereas band Ib with a shoulder in the 300−550 nm range, presumably arises from the B ring (*32*). In **Figure 3**, for example, the action of tyrosinase bioreactor on cyanidin is reported: an absorbance decrease at 280 nm and an absorbance increase at 320 nm are shown. This might be ascribed to the quinones enzymatically formed because they have typical UV−visible absorption bands in the range of 300−500 nm.

In the case of bilberry fruit (**Figure 4**) a similar behavior is evidenced even if the spectra are more complicated for the presence of a high absorption band at 220−250 nm presumably due to the presence of non-flavonoid ingredients. In conclusion, the value of 280 nm was preferred; being the principal maxima of all flavonoids absorption (band II), it responds sensitively to the tyrosinase action as evidenced in **Figure 3**.

Because the pH influences not only the enzyme activity but also the stability of phenols, a preliminary study was made, employing free tyrosinase and bilberry juice as substrate, at different pH values. The data, expressed as normalized values with respect to the maximal value, are reported as a function of pH in **Figure 5** (curve a), and a bell-shaped profile with maximal activity at pH 5 is shown. Because, when the enzyme is immobilized, the pH−activity profile may modify, depending on the nature of the support material, a similar study was undertaken with the tyrosinase bioreactor (**Figure 5**, curve b). Therefore, it appeared that the immobilized enzyme activity plateaus at a pH level higher than 5, indicating that above this value the pH surface becomes independent of the bulk pH.

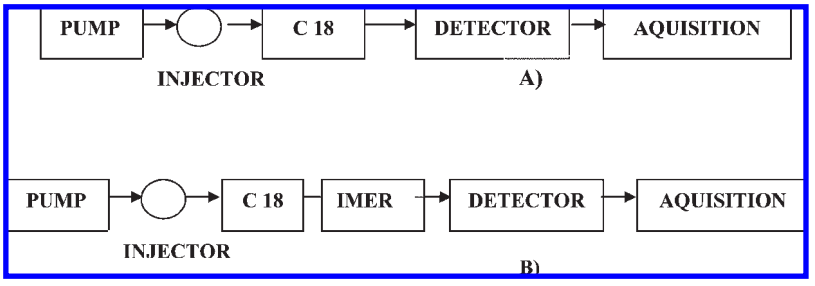

Figure 2. Scheme of the chromatographic system for the sample analysis employing the two assemblies without (**A**) and with (**B**) IMER.

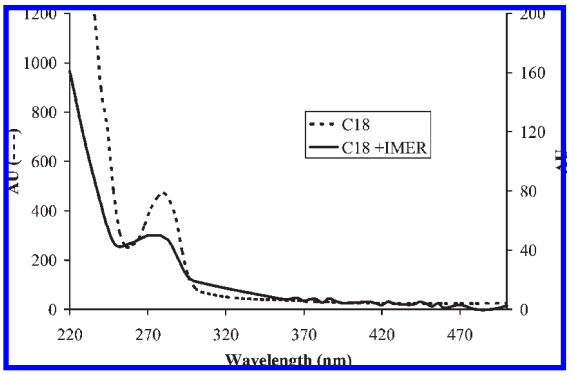

Figure 3. Spectra obtained by HPLC-DAD injecting cyanidin on the precolumn C18 and on the precolumn coupled with IMER. Experimental conditions: mobile phase, 0.1 M phosphate solution, pH 5; flow, 0.5 mL/min.

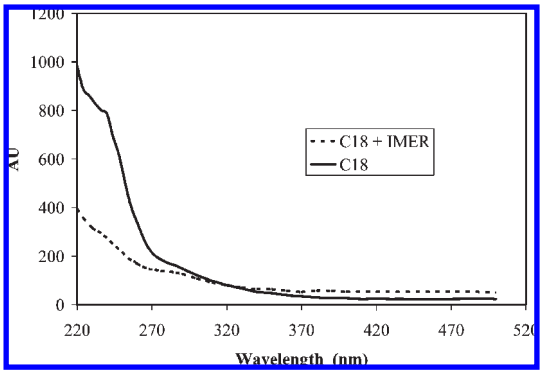

Figure 4. Spectra obtained by HPLC-DAD injecting a bilberry juice on the precolumn C18 and on the precolumn coupled with IMER. Experimental conditions: mobile phase, 0.1 M phosphate solution, pH 5; flow, 0.5 mL/min.

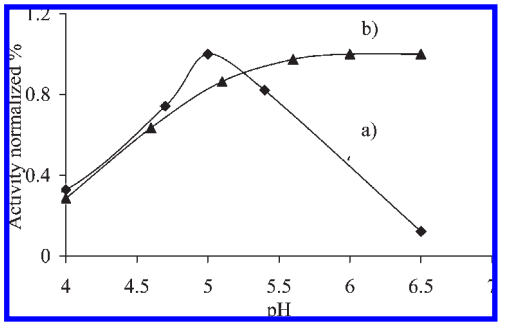

Figure 5. pH−activity profiles for free (curve a) and immobilized (curve b) tyrosinase.

Taking into account also the high flavonoid stability at acidic pH, the choice of pH 5, as operative condition, appeared to be more convenient even if it does not correspond to the maximum value.

Extraction of Solid Samples. To optimize the procedure of extraction, a study was undertaken employing the integrator BODY-SPRING and four different solvents: 0.2 M HCl (final pH 1), methanol, water at room temperature, and boiling water. Total phenol, flavanol, and anthocyanin amounts were determined in every extract. To homogenize all of the results, the data are expressed as normalized values with respect to their maximal values (**Figure 6**). The solvent most suitable for the extraction of total phenols, including catechins, condensed tannins, and anthocyanins, appeared to be boiling water. The acidic solvent was

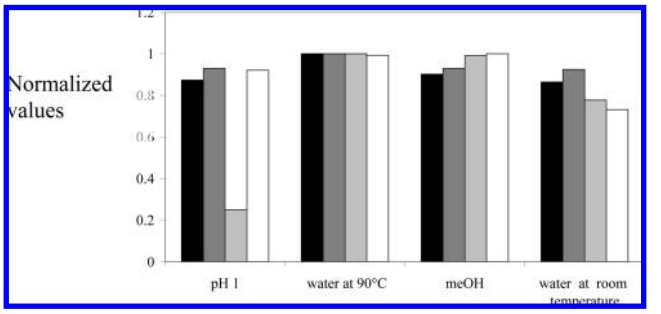

Figure 6. Effect of solvent on the total phenol content, determined by both Folin−Ciocalteu (black bars) and IMER (dark gray bars) assays and flavanol (light gray bars) and anthocyan (white bars) extractions.

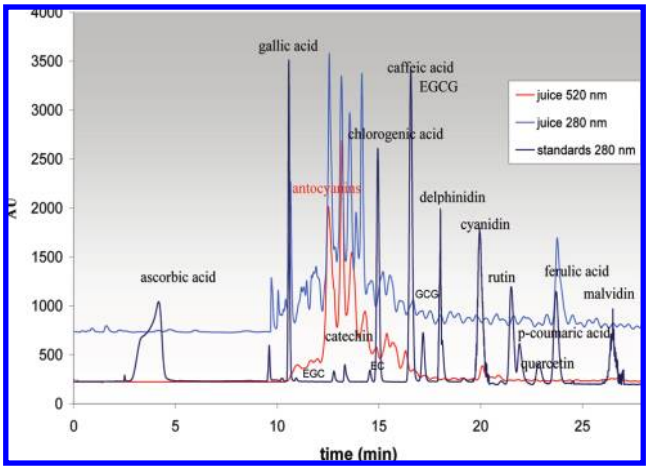

Figure 7. Chromatographic profile of (black) standards at 280 nm, (blue) juice at 520 nm, and (red) juice at 280 nm. Solvent system: solvent A, 0.01 M phosphoric acid; solvent B, acetonitrile. Elution gradient: gradient started with 2% B to reach 15% at 10 min and 25% at 35 min. Solvent flow rate was 1.3 mL/min, and the injected volume was 20 μL.

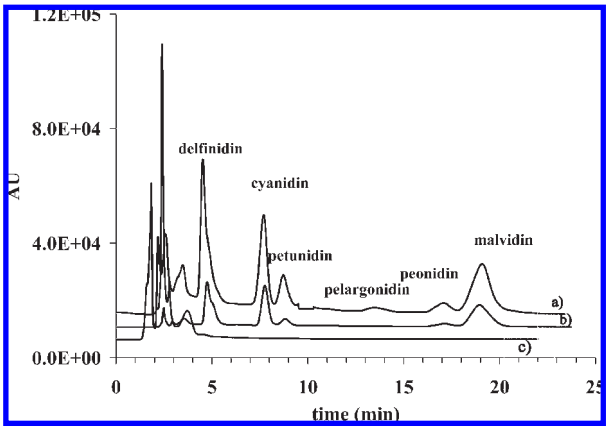

Figure 8. Chromatographic profile of (**a**) anthocyanidin standards, (**b**) hydrolyzed Bio-Beutelsbacher juice, and (**c**) unhydrolyzed Bio-Beutelsbacher juice. Mobile phase, acetic acid/acetonitrile/water = 10.8:12:77.2 (% v/v); flow rate, 1.2 mL /min; injected volume, 20 μL; λ, 540 nm.

not particularly suitable for the condensed tannins extraction because it favored their hydrolysis to species such as anthocyanidins, which do not react in the vanillin index assay.

In addition, the good agreement of enzymatic data with FC results can also be noted.

HPLC Analysis. To have a profile of antioxidant substances in the samples, a chromatographic separation of the main phenolic

classes, phenolic acids, catechins, anthocyanin, and anthocyanidins on a reversed phase column was realized. Identification of the substances was made by comparison of UV−vis spectra and t_r with those of standards. As can be seen from **Figure 7a**, all of the utilized standards, except of caffeic acid and EGCG, were separated within 30 min, employing a gradient elution at room temperature. The presence of the anthocyanins has been underlined at 520 nm, where they have a typical absorption band even if they are not fully separated (**Figure 7b**). The chromathographic analysis of all examined samples, at 280 nm, has shown a similar profile: (i) the predominance of chlorogenic and ferulic acids, as hydroxycynnamic acids; (ii) the almost absence of ascorbic acid, probably due to its thermal instability (*35*); and (iii) the absence of anthocyanidins, but only of their glycoside form (anthocyanins) (**Figure 7a**,**c**). To establish the nature of the aglycons, the anthocyanins were treated with acid hydrolysis. The obtained anthocyanidins were separated by HPLC in a reasonable time (<25 min), by an isocratic elution at room temperature. In this way the anthocyanidins identification was easily achieved (**Figure 8a**). In the same **Figure 8**, for example, the chromatograms of the Bio-Beutelsbacher juice hydrolyzed (**Figure 8b**) and not (**Figure 8c**) are reported.

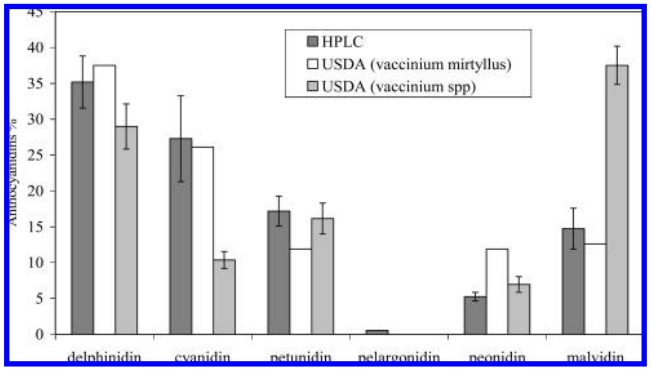

Figure 9. Comparison of HPLC results with those reported by the USDA for two species of bilberry: *Vaccinium myrtillus* and *Vaccinium* ssp.

In the histogram (**Figure 9**), the percent average values of the anthocyanidin peak areas, determined for all of the samples, with the data reported (*33*) by the U.S. Department of Agriculture (USDA) for two types of bilberry (*Vaccinium myrtillus* and *Myrtillus* ssp.) are shown. From the results it appears that the obtained distribution, delphinidin > cyanidin > petunidin > malvidin > peonidin > pelargonidin, is very similar to the *V. myrtillus* profile.

Total Content of Phenols. Experimental results of the enzymatic method for each sample, expressed as Δarea, were compared with the equivalent absorbance values obtained by (i) Folin−Ciocalteu method; (ii) pH-differential method; (iii) acid−butanolic assay; and (iv) vanillin index. The IMER method was well correlated with the FC method (r^2 = 0.9445) (**Figure 10a**) with the exception of three samples (Tegens, Tekann, and Lester house infusions). The poor correlation with the other methods ($r^2 \cong 0.4$) (**Figure 10b−d**) shows that the enzymatic assay can be applied to determine an index of the total phenolic content, but not for a specific phenol class determination.

The determined contents of phenolic compounds are shown in panels **a** and **b** of **Figure 11** for the liquid samples, such as juices, and for the solid samples, such as integrator, drugs, infuses, and jams, respectively, together with the data obtained by the spectrophotometric methods. Because it was impossible to use the same standard for all of the individual methods, the content of flavanols was expressed as (+)-catechin, that of anthocyanins and proanthocyanins as cyanidin, and the total phenolic content by both FC and enzymatic methods standardized using delphinidin. The choice of this standard was made because both

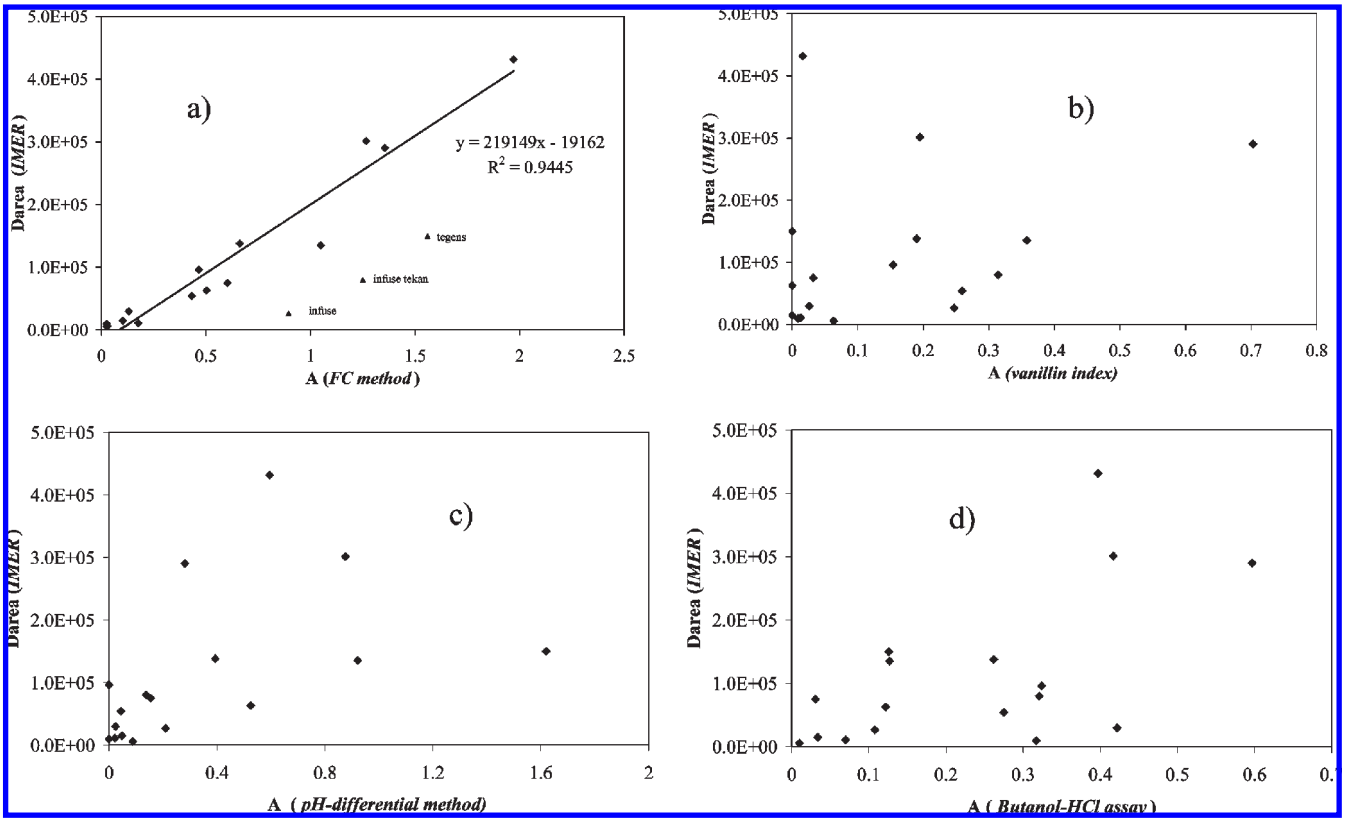

Figure 10. Correlation between the experimental IMER response and those obtained with (**a**) the Folin−Ciocalteu method, (**b**) the vanillin index, (**c**) the pH-differential method, and (**d**) the butanol−HCl assay.

methods presented the higher reactivity of phenolics with this reagent with respect to the others tested. In **Table 2** the coefficients of the linear calibration curves obtained for FC and enzymatic methods, using ferulic acid, (+)-catechin, and delphinidin, as standards, are reported. It appeared that catechin and delphinidin reacted 3-fold with FC reagent with respect to ferulic acid. This was in agreement with the literature (*34*, *35*), in which it is reported that the final absorbance values are proportional to the number of phenolic hydroxyl reacting groups. For IMER, instead, less variability of calibration coefficients is observed, evidencing a lesser influence of standard chemical structure.

By comparison of the results obtained by FC and enzymatic methods it appeared that for the samples (such as juices) with 5−25% anthocyanin content, a great difference of total phenolic amount is obtained, whereas the content of samples such as drugs and integrators, except the Arko capsule, with 60−100% anthocyanin content, obtained by FC assay, is correlated significantly with the enzymatic method developed by us. This different behavior might be due to the choice of an anthocyanidin, such as delphinidin, as standard, which, having a high number of hydroxylic reactive groups, presents a high reactivity toward FC reagent; however, if high amounts of compounds with a low reactivity are present, the real value could be underestimated. This hypothesis is supported also by the good correlation, shown previously, between the signals obtained by both methods.

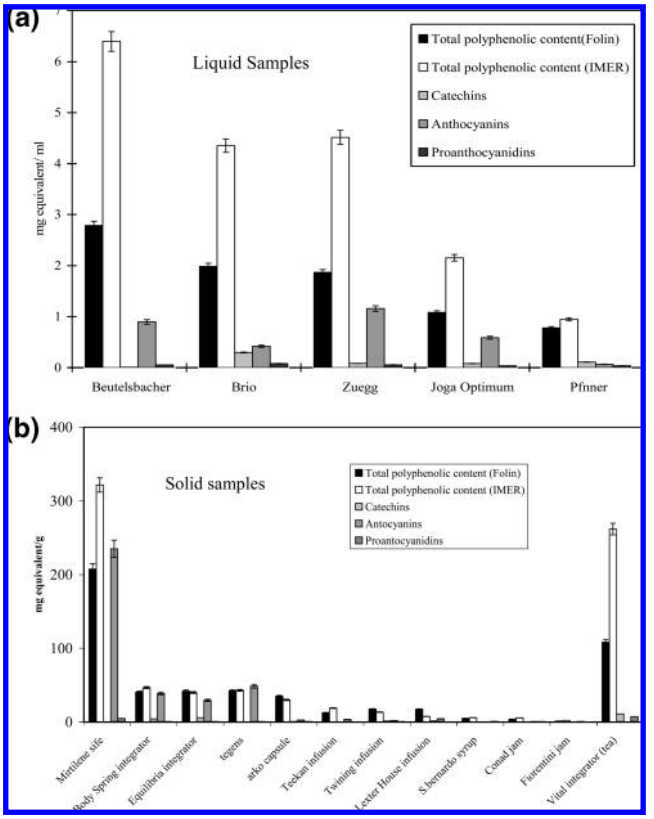

Figure 11. Content of total phenols, flavanols, anthocyanins, and proanthocyanidins, respectively determined by the Folin−Ciocalteu method, IMER, vanillin index, pH-differential method, and acid butanolic assay, for (**a**) liquid samples and (**b**) solid samples.

Infuses and jams presented very low total content of phenolic compounds.

Antioxidant Capacity. Antioxidant capacity was determined by TEAC method, which is the most reactive one in the reaction with phenolic compounds. It is based on a neutralization of radical cation formed by a single-electron oxidation of a synthetic ABTS compound to a strongly absorbing ABTS$^{\bullet}$ radical. The radical reacts quickly with electron/ hydrogen donors to form a colorless ABTS.

As shown in **Figure 12**, the total phenolic content, obtained by enzymatic method, was well correlated with the antioxidant capacity (r^2 = 0.9403) with exception of only one sample, Tegens. This drug contained the ammonium glycyrhizinate, an inhibitor of tyrosinase (*36*).

This result confirms that IMER is able to give antioxidant capacity of samples. Indeed, the polyphenol oxidase (PPO), as tyrosinase, that preferably acts on ortho hydroxyl groups can act via an indirect way also on nonideal substrates (monophenols, *o*-diphenol, and glycoside polyphenol) in the presence of *o*-diphenol. Many authors (*37*, *38*) have proposed a scheme of sequential reactions to explain the effect of mediating diphenol. The mechanism involved the oxidation of *o*-diphenol by PPO to the corresponding *o*-benzoquinone, which can oxidize successively other phenols. Therefore, the diphenolase activity of the bioreactor is influenced by the totality of phenolic substances in the samples. With this mechanism, the enzymatic oxidation of anthocyanins, poor substrates of PPO, in the presence of chlorogenic acid (*39*) and catechin (*40*) has been explained.

Evaluation of Interferences. The nonphenolic compounds, such as citric acid, ascorbic acid, and glucose, are important interfering substances in the FC method (*35*). They contribute to increase the absorbance value, reacting with FC reagent, and, if not correctly subtracted, can give an overestimated value of polyphenol content. For this reason, some measurements on two samples (yoga juice and black bilberry integrator) without and with the addition of citric acid or glucose were made, employing both FC and enzymatic methods. Ascorbic acid is not examined because it appeared from the HPLC analysis that it is not present in a significant amount.

According to FC measurement at 20 °C, 1 mg/mL citric acid increases by 25% the absorbance of the samples, whereas glucose does not interfere because sugar does not react appreciably at room temperature (*35*).

From the experimental data it appears that the enzymatic method is not affected by the presence either of glucose, up to 200 mg/mL, or of citric acid, up to 5 mg/mL (value fixed by Italian Rule DPR.18.5.1982 no. 489 for juices).

Repeatability. The repeatability of 10 antioxidant capacity measurements, employing Zuegg juice, by both enzymatic and FC methods, is reported in **Table 3**. It appears by their comparison that similar percent coefficient values (2−3%) are obtained.

Table 2. Calibration Curve Equations of Ferulic Acid, (+)-Catechin, and Delphinidin Obtained by Folin−Ciocalteu and IMER Methods

standard	Folin−Ciocalteu	IMER
ferulic acid (mM)	$Y = 0.0703x + 0.1251$ ($r^2 = 0.9735$)	$Y = 17181x - 14000$ ($r^2 = 0.9458$)
(+)-catechin (mM)	$Y = 0.2272x + 0.0611$ ($r^2 = 0.9810$)	$Y = 14474x + 3747$ ($r^2 = 0.9872$)
delphinidin (mM)	$Y = 0.2595x - 0.1658$ ($r^2 = 0.9728$)	$Y = 19048x - 7746$ ($r^2 = 0.9625$)

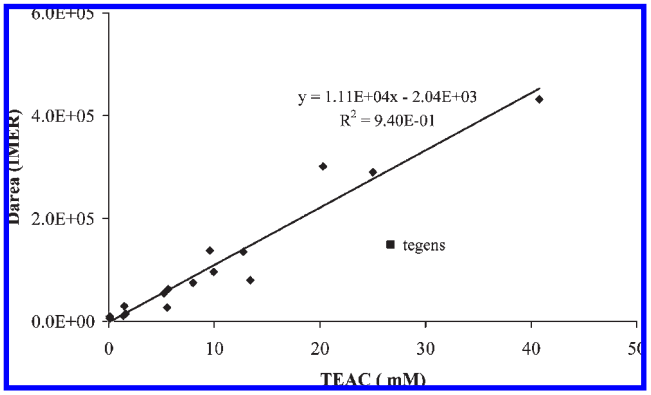

Figure 12. Correlation between IMER and TEAC values for all samples.

Table 3. Repeatability of 10 Measurements of Zuegg Juice by IMER and Folin−Ciocalteu Assays, As Reported in Experimental Conditions

	IMER Δarea	Folin−Ciocalteu *A*
1	81751	1.44
2	87101	1.52
3	89149	1.54
4	91085	1.54
5	85228	1.54
6	88212	1.55
7	94351	1.55
8	77555	1.48
9	83169	1.48
10	76648	1.51
$\bar{X}$	**85425**	**1.51**
σ	**2033**	**0.04**
CV%	**2.38**	**2.72**

Conclusion. The application of a tyrosinase-immobilized bioreactor has allowed the determination of total phenolic content of 17 samples, containing bilberry. The validity of this method has been tested by agreement of the results obtained, with those of FC assay, that gives the total polyphenolic content, and TEAC methods, for the antioxidant capacity. Poor correlation with the other methods (pH-differential, vanillin index, and butanol−HCl assays) shows that the enzymatic assay can be applied to determine an index of the total phenolic content but not for a specific phenol class determination. Moreover, the immobilization of tyrosinase on AMP-CPG is advantageous because the lifetime of the enzyme is increased. In this way the bioreactors can be utilized again in an automatic system, such as HPLC. Therefore, the rapidity and simplicity of the HPLC method, together with the specificity and stability of the bioreactor, render it suitable for a fast screening of the antioxidant capacity of a real matrix.

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