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Biosensor Measurements of Polar Phenolics for the Assessment of the Bitterness and Pungency of Virgin Olive Oil

JOHANNEKE L. H. C. BUSCH,*,[#] KAREL HRNCIRIK,[#] EMILY BULUKIN,[§] CLAIRE BOUCON,[#] AND MARCO MASCINI[§]

Unilever R&D Vlaardingen, Olivier van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands, and Dipartimento di Chimica, Università di Firenze, Polo Scientifico, Via della Lastruccia 3, 50019 Sesto Fiorentino, Firenze, Italy

Bitterness and pungency, sensory quality attributes of virgin olive oil, are related to the presence of phenolic compounds. Fast and reliable alternatives for the evaluation of sensory attributes and phenolic content are desirable, as sensory and traditional analytical methods are time-consuming and expensive. In this study, two amperometric enzyme-based biosensors (employing tyrosinase or peroxidase) for rapid measurement of polar phenolics of olive oil were tested. The biosensor was constructed using disposable screen-printed carbon electrodes with the enzyme as biorecognition element. The sensor was coupled with a simple extraction procedure and optimized for use in flow injection analysis. The performance of the biosensor was assessed by measuring a set of virgin olive oils and comparing the results with data obtained by the reference HPLC method and sensory scores. The correlations between the tyrosinase- and peroxidase-based biosensors and phenolic content in the samples were high ($r = 0.82$ and 0.87, respectively), which, together with a good repeatability (rsd = 6%), suggests that these biosensors may represent a promising tool in the analysis of the total content of phenolics in virgin olive oils. The correlation with sensory quality attributes of virgin olive oil was lower, which illustrates the complexity of sensory perception. The two biosensors possessed different specificities toward different groups of phenolics, affecting bitterness and pungency prediction. The peroxidase-based biosensor showed a significant correlation ($r = 0.66$) with pungency.

KEYWORDS: **Olive oil; phenols; sensory; bitter; pungent; taste; tyrosinase; peroxidase; biosensor; analysis**

INTRODUCTION

Virgin olive oil (VOO) is obtained from olives by employing mechanical processing only, and without further refinement steps these oils become available for consumption. VOO is associated with health benefits, such as protection against coronary heart diseases and cancer (*1*, *2*), and is recognized by its characteristic sensory properties: aroma, bitterness, astringency, and pungency (*3*).

Extensive sensory and analytical analyses have to be followed (*4*, *5*) to comply with the specific quality criteria of the European Union (EU) directives and International Olive Oil Council (IOOC) (*4*−*6*). In larger olive oil mills, sensory assessment during the harvesting season (November−March) requires many samples (>100) per day to be tasted by highly trained tasters. Replacement by instrumental analysis, to facilitate categorization or identification of low-quality samples, could accelerate this process, whereby typical drawbacks of the sensory evaluation, such as subjectivity, carry-over effects, fatigue of panel members, and high costs of training and maintenance of sensory panels, could be partially overcome.

The polar phenolic fraction of VOO consists of a mixture of compounds, which differ in chemical properties and impact on quality of VOO (*7*−*9*) (for structure of the major olive phenols, see **Figure 1**). Analytical procedures for the characterization and quantification of the complete profile of phenolic components of olive oils usually entail extraction (liquid−liquid or solid-phase) of phenolics from the oil, followed by RP-HPLC (*10*−*13*). The HPLC analysis, however, is quite laborious and time-consuming.). An alternative to chromatographic methods are nonspecific methods for the determination of total phenolics, of which the colorimetric assay employing the Folin−Ciocalteu reagent has been widely used (*14*, *15*).

Various secoiridoid derivatives of hydroxytyrosol and tyrosol have been shown to contribute to the bitterness of olive oil (*16*−*19*). In a recent study by Mateos et al. (*20*) correlations between bitterness (only oils with mild bitterness were included) and concentrations of secoiridoid derivatives (up to 500 μmol/kg) were determined, with a linear relationship ($r = 0.96$) found

* Author to whom correspondence should be addressed (e-mail johanneke.busch@unilever.com; telephone +31 10 4606213; fax +31 10 4605236).
[#] Unilever R&D Vlaardingen.
[§] Università di Firenze.

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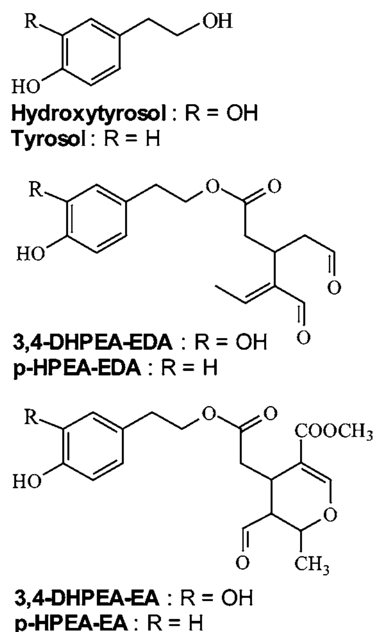
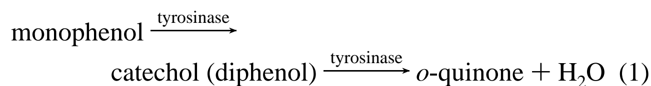


Figure 1. Structures of major polar phenolics occurring in olive oil: 3,4-DHPEA-EDA, dialdehydic form of decarboxymethyl oleuropein aglycone; *p*-HPEA-EDA, dialdehydic form of decarboxymethyl ligstroside aglycone; 3,4-DHPEA-EA, aldehydic form of oleuropein aglycone; *p*-HPEA-EA, aldehydic form of ligstroside aglycone.

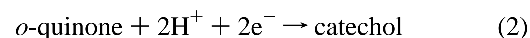
between bitterness and the aldehydic form of oleuropein aglycone (3,4-DHPEA-EA) (see **Figure 1**).

It was demonstrated in our laboratory that the pungency of VOO oil is caused by deacetoxy ligstroside aglycone (*p*-HPEA-EDA) (18), a finding which has recently been confirmed in an independent study of Beauchamp and co-workers, involving the de novo synthesis of the former aglycone (*21*, *22*). They attributed ibuprofen-like activity to *p*-HPEA-EDA in this study and speculated on the potential specific health effects of this aglycone (*21*).

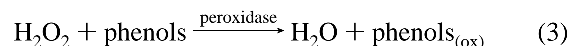
Sensors are a promising tool to supplement various analytical methods. With multisensor arrays of the electronic nose (EN) the volatiles of a sample (headspace) are assessed. ENs for olive oils typically employ metal oxide semiconductor or surface acoustic wave sensors, which can discriminate between different olive oil qualities and varieties (*23*, *24*). Multisensor systems for liquid analysis (electronic tongue) are an emerging application and can be considered as the taste equivalent of ENs (for recent reviews, see refs *25* and *26*). Biosensors form one class of (liquid) sensors. Their favorable characteristics, such as low cost, short analysis time per sample, small size, and selectivity, make them attractive for use in the rapid screening of a large number of samples. For the assessment of phenolics several biosensors based on the enzyme tyrosinase have been proposed (*27*−*30*). Tyrosinase, also called phenolic oxidase, is a bifunctional enzyme that converts monophenols to *o*-diphenols (hydroxylase activity) and *o*-diphenols to *o*-quinones (oxidase activity) (eq 1). The produced *o*-quinone is electrochemically active and can be reduced back to the catechol form at low applied potentials (eq 2). The phenolic substrate is enzymatically oxidized and subsequently regenerated through the electrochemical reduction of *o*-quinone, hence forming a bioelectrocatalytic amplification cycle (*31*). The resultant cathodic current can be related to the concentration of phenolic compound present in the solution. In addition, enzymes with lower specificity for phenolic compounds, such as peroxidases and NAD(P)H-independent dehydrogenases, have also been reported (*32*−*34*). In the case of peroxidase the phenolic compounds act as electron donors to the enzyme through mediated electron transfer (eq 3). The formed phenolic radicals may be reduced and the reduction current related to the phenolic compound present in solution (*35*).



$$\text{monophenol} \xrightarrow{\text{tyrosinase}} \text{catechol (diphenol)} \xrightarrow{\text{tyrosinase}} o\text{-quinone} + H_2O \quad (1)$$



$$o\text{-quinone} + 2H^+ + 2e^- \rightarrow \text{catechol} \quad (2)$$



$$H_2O_2 + \text{phenols} \xrightarrow{\text{peroxidase}} H_2O + \text{phenols}_{(ox)} \quad (3)$$

Recently, several types of enzyme immobilizations and additives have been suggested to influence the stability and activity of tyrosinase-based biosensors (*36*−*38*). In the case of the analysis of olive oil samples, several additional factors such as electrode fouling may influence the sensor performance. Immobilization of tyrosinase through physical entrapment in a protective biocompatible polymer matrix onto screen-printed electrodes is expected to overcome this problem. The use of screen-printing technology allows the production of low-cost, disposable electrodes avoiding traditional electrode surface renewal steps.

The main purpose of this work was to investigate the applicability of enzyme-based biosensors for the rapid prediction of sensory properties (bitterness and pungency) of VOO. The performance of the sensors, obtained by immobilization of the enzyme in a polymer matrix onto screen-printed electrodes, was tested by comparing their response with sensory scores and phenolic content of olive oil samples. The response of the tyrosinase-based biosensor to olive oil samples was compared with the response obtained with a peroxidase-based biosensor. As the suitability of these biosensors for this application is based not only on the accuracy of the biosensor to measure phenolic content but also on the underlying correlation of the measured phenolics with bitterness and pungency, this correlation was addressed here and related to biosensor specificity.

MATERIALS AND METHODS

Samples. Forty-eight samples of VOO from different regions of Greece, Italy, and Spain were subjected to measurement with the tyrosinase-based biosensor and HPLC (phenolic profiles) and evaluation of sensory scores. Of these olive oils a representative set of 16 olive oils was used for additional measurements on the tyrosinase- and peroxidase-based biosensors. All oils were harvested in the 2002 season and were stored at −20 °C until the start of the experiments.

Materials and Chemicals. All solvents and reagents were of analytical grade. All solutions and buffers were prepared with water, obtained from a Milli-Q system (Millipore, Milford, MA). Mushroom tyrosinase (2590 units/mg of solid) and horseradish peroxidase (1100 units/mg of solid) were purchased from Sigma (Milan, Italy). Nafion perfluorinated ion-exchange resin, 5% (w/v) in a solution of aliphatic alcohol/water mixture (90:10, v/v), was purchased from Aldrich (Milan, Italy). Glutaraldehyde (aqueous solution 25%, v/v) grade I was obtained from Sigma (Milan, Italy). Catechol standard solutions were daily prepared from catechol (99%) purchased from Aldrich (Zwijndrecht, The Netherlands). Cresol standard solutions were prepared daily from *p*-cresol (99% purity) purchased from Aldrich (Milan, Italy). Stock standard solution was prepared in a water/methanol mixture (1:4, v/v). Tyrosol [2-(*p*-hydroxyphenyl)ethanol] was obtained from Acros (Geel, Belgium). PBS buffer (0.1 M phosphate, 0.1 M NaCl, pH 7.4) was prepared with disodium hydrogen phosphate dihydrate (Merck, Darmstadt, Germany), sodium dihydrogen phosphate monohydrate (Sigma, Zwijndrecht, The Netherlands), and sodium chloride (Sigma, Zwijn-

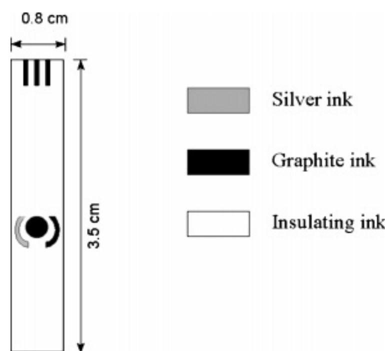


Figure 2. Design of a screen-printed electrode used as electrochemical transducer for the biosensor construction. See text for details.

drecht, The Netherlands). Methanol and hydrogen peroxide were obtained from Merck (Darmstadt, Germany).

Biosensor Preparation. *Screen-Printed Electrode Transducers.* The biosensors were assembled using a planar three-electrode strip, based on a carbon working electrode, a carbon counter electrode, and a silver pseudo-reference electrode (**Figure 2**). The electrode strips were prepared using a DEK 248 screen printer (DEK, Weymouth, U.K.). Graphite-based (Electrodag 423 SS) and silver-based (Electrodag PF-410) polymeric inks were purchased from Acheson Italiana (Milan, Italy), and insulating ink (Vinylfast 36-100) was purchased from Argon (Lodi, Italy). A polyester flexible film (Autostat CT5) obtained from Autotype (Milan, Italy) was used as the printing substrate. The silver ink was used for the conductive tracks and for the silver pseudo-reference electrode, whereas the carbon ink was used to obtain the working and the counter electrodes. After each printing step, the inks were cured at 120 °C for 10 min. The insulating ink was used to define the surface of the working electrode (3 mm i.d.). After printing of the insulating layer, the electrodes were cured for 20 min at 70 °C.

Tyrosinase Immobilization. Tyrosinase was stored in small aliquots in PBS buffer at −20 °C. Enzyme aliquots were allowed to reach room temperature before appropriate dilution with PBS buffer in order to obtain the desired number of enzyme units (nominal concentration 100 units) on the surface of the working electrode. The enzyme solution (5 μL) was subsequently directly immobilized onto the surface of the carbon-based working electrode. The enzyme-modified electrodes were allowed to air-dry (1 h). Thereafter, Nafion (3 μL) was deposited on the surface of the enzyme-modified electrodes. When dry (15 min), the electrodes were stored at 4 °C.

Peroxidase Immobilization. Horseradish peroxidase was stored in aliquots in PBS buffer at −20 °C. Glutaraldehyde (25%, v/v) was diluted in PBS (pH 7.0). The enzyme aliquots were allowed to reach room temperature and were subsequently diluted using the glutaraldehyde solution to give a final cross-linker concentration of 0.2%. Thereafter, the enzyme solution (5 μL) containing glutaraldehyde was deposited on the working electrode, giving a final enzyme amount of 13 units per electrode (nominal concentration). Modified electrodes were stored at 4 °C.

Analytical Methods. *Amperometric Measurements.* Amperometric measurements in FIA were carried out by fitting the enzyme-modified screen-printed electrode in a polyacrylate cell, which was connected to a Rheodyne four-way sample injector with PTFE (Teflon) tubes. The system was driven with a peristaltic pump [Gilson (Den Haag, The Netherlands), Minipulse 3] with Elkay Accu-Rated tubing. The substrate and carrier (PBS) were introduced into the cell with a flow rate of 0.5 mL/min. Samples and standards were added using a 100 μL sample loop (Rheodyne, Bensheim, Germany), and the subsequent current was recorded. Amperometric experiments were performed with a μAutolab with GPES software (Autolab, Utrecht, The Netherlands). The applied potential for calibrations was −0.2 V in the case of the tyrosinase-based biosensor and −0.05 V in the case of the peroxidase-based biosensor.

Biosensor Measurement of Olive Oils. VOO extracts were obtained by weighing 0.35 g of olive oil sample in Eppendorf vials (2 mL). To each vial was added 0.8 mL of PBS buffer, and the oil/PBS mixtures were shaken during 10 min using a Mini Shaker [IKA (Staufen, Germany), model MS1, 2000 min^{-1}], with an insert for multiple samples. After extraction, the aqueous phase was analyzed amperometrically using FIA, as described above. Each olive oil sample was measured in duplicate. The use of freshly prepared biosensors was restricted to a maximum of 30 injections and/or a duration of 3 h. Sensor-to-sensor repeatability of the tyrosinase-based biosensor was determined for six different sensors by additions from standard catechol solutions.

Tyrosinase-Based Biosensor Measurement. After injection of an olive oil extract into the flow cell, an appropriate standard catechol solution was selected, with a current closest to that of the olive oil extract, and injected twice into the flow cell. The signal (peak height) was determined by means of the peak search analysis module of the GPES software. Catechol equivalent values of olive oil extracts were calculated on the basis of electrical current values obtained for the standard catechol solutions and correcting for the exact weight of olive oil samples. Values were expressed as micromolar catechol equivalents.

Peroxidase-Based Biosensor Measurements. Experiments were performed in batch mode with a PalmSens (Palm Instrument BV, Houten, The Netherlands). During experiments the test solution was stirred with a magnetic stirrer. The sensor was immersed in PBS (2 mL) with direct additions of standard substrate solutions using a micropipet. The amperometric signal was recorded when the current response had reached a steady state. In all experiments hydrogen peroxide was added to a final concentration of 100 μM. The current response from the olive oil extracts were compared to the response to *p*-cresol and hence expressed as micromolar *p*-cresol equivalents.

Other Methods. Bitterness scores were determined by a trained sensory panel according to EU regulations (*4*). Phenolics were extracted from samples by aqueous methanol and separated by HPLC (*15*); individual phenolics (see **Figure 1** for their structures) were quantified using published response factors (*10*).

Statistical Analysis. Measurements were carried out in duplicate. Statistical analysis (mean, standard deviation) was performed using the Excel standard software package (Microsoft Corp., Redmond, WA). Pearson's correlations between sensor signal, phenolic concentration, and sensory scores were calculated using SAS v. 9.1 (SAS, Cary, NC). Correlation coefficients were considered to be statistically significant at $p \leq 0.05$ or $p \leq 0.10$, as indicated in the text and tables.

RESULTS AND DISCUSSION

Correlation of Tyrosinase-Based Biosensor Results with Bitterness and Phenolic Content. Several experiments were carried out to determine whether the tyrosinase-based biosensor is a useful instrument to predict bitterness of olive oils. It is the underlying relationship between bitterness and phenolics, combined with the specificity of the enzyme tyrosinase toward oxidation of olive phenolics, that determines the suitability of the biosensor as a bitterness sensor. Therefore, the biosensor response, phenolic composition determined by HPLC, and bitterness scores of 48 olive oils were compared.

First, the repeatability of the olive oil extraction and tyrosinase-based biosensor measurement was determined by measuring one olive oil sample 10 times on the same day, using one biosensor. The within-day repeatability of the tyrosinase-based biosensor was good (std = 6%). Sensor-to-sensor repeatability (std = 10%) was in line with values expected for such sensors.

The repeatability of the biosensor measurements (based on measurement of the 48 olive oils in duplicate) was also good (std = 6%). Comparison of biosensor signal, bitterness, and total phenolics showed that the correlation between bitter score and biosensor response (catechol equivalent) was significant, but low (r = 0.56, **Table 1**), as displayed in **Figure 3**. The correlation of the biosensor response with total phenolics, however, was higher (r = 0.82) and suggests that the tyrosinase-based biosensor can provide a reliable indication of the total phenolics content. The correlation of bitter score and total phenolics was somewhat lower (r = 0.71).

Table 1. Correlations of Phenolic Content, Tyrosinase-Based Biosensor Measurements, and Sensory Scores of 48 Olive Oils[a]

	tyrosinase-based biosensor	bitter	pungent
hydroxytyrosol		0.45**	0.23
tyrosol		0.35**	0.25*
3,4-DHPEA-EDA		0.58**	0.73**
p-HPEA-EDA		0.52**	0.73**
3,4-DHPEA-EA		0.47**	0.15
p-HPEA-EA		0.47**	0.26*
monophenols	0.68**	0.61**	0.68**
o-diphenols	0.78**	0.64**	0.40**
aglycones	0.76**	0.69**	0.61**
total phenolics	0.82**	0.71**	0.62**
bitter	0.56**		0.70**
pungent	0.38**		

[a] **, significant at 0.05 significance level; *, significant at 0.10 significance level. Relevant correlation coefficients of phenolics with the biosensor output are discussed in the text. Monophenols: sum of tyrosol, *p*-HPEA-EDA, and *p*-HPEA-EA. *o*-Diphenols: sum of hydroxytyrosol, 3,4-DHPEA-EDA, and 3,4-DHPEA-EA. Aglycones: sum of 3,4-DHPEA-EDA, *p*-HPEA-EDA, 3,4-DHPEA-EA, and *p*-HPEA-EA.

The highest correlations between bitterness and phenolic concentrations were obtained for total phenolic concentration ($r = 0.71$), *o*-diphenols ($r = 0.64$), and aglycones ($r = 0.69$). These correlations are given in **Table 1** and illustrated in **Figure 4**.

3,4-DHPEA-EA has been identified by Mateos et al. (*20*) as a significant contributor to bitterness, but only when considering moderately bitter olive oils. In our study, however, this phenol did not show a very high correlation with bitterness ($r = 0.56$) and was comparable with the correlation coefficients obtained for other aglycones (see also **Figure 4**). The correlation did not change significantly when only moderately bitter oils (bitter score < 3.5) or oils with up to 500 μmol/kg of 3,4-DHPEA-EA were taken into account ($r = 0.55$ and 0.50, respectively). On the basis of the correlation between bitterness and phenolic compounds of the set of 48 oils analyzed, it seems that the bitterness can hardly be attributed to one or two single species (**Table 1**). This is in agreeement with our earlier work (*18*), in which we could not identify large differences in bitterness when solutions of individual isolated phenolics were sensorially evaluated. The reasons for the different bitter relationships found in our work and that of Mateos et al. (*20*) are unclear, and further evidence is needed to confirm the role of 3,4-DHPEA-EA in the bitterness of olive oils.

As the high correlation between the biosensor response and total phenolics indicates contributions of all phenolic compounds to the biosensor signal, we primarily considered the correlations of the biosensor response with the total phenolics and sums of mono- and diphenols, representing structurally similar phenolics (**Table 1**). Only the correlation of hydroxytyrosol with the biosensor response is high ($r = 0.71$). The larger correlation with summed diphenols versus summed monophenols ($r = 0.78$ versus 0.66) may indicate that the one-step oxidation (of the diphenols) is more efficient on the time scale of the experiment than the two-step oxidation necessary for the monophenols (see eqs 1 and 2). Dose−response curves of phenolics, obtained via fractionation or synthesis, measured with the biosensor could provide further insight into the biosensor response of the individual phenolics.

In addition to the major phenols included in the correlation analysis, three minor phenolic compounds were determined by HPLC: hydroxytyrosolacetate, pinoresinol, and 1-acetoxypinoresinol. The concentrations of these phenolics were low, and the correlations with the biosensor response were poor ($r <$ 0.20). With the wide range of phenolic concentrations present in the tested olive oils (including low concentrations) it is unlikely that major sources of biosensor response, detectable by HPLC, have been excluded is this study.

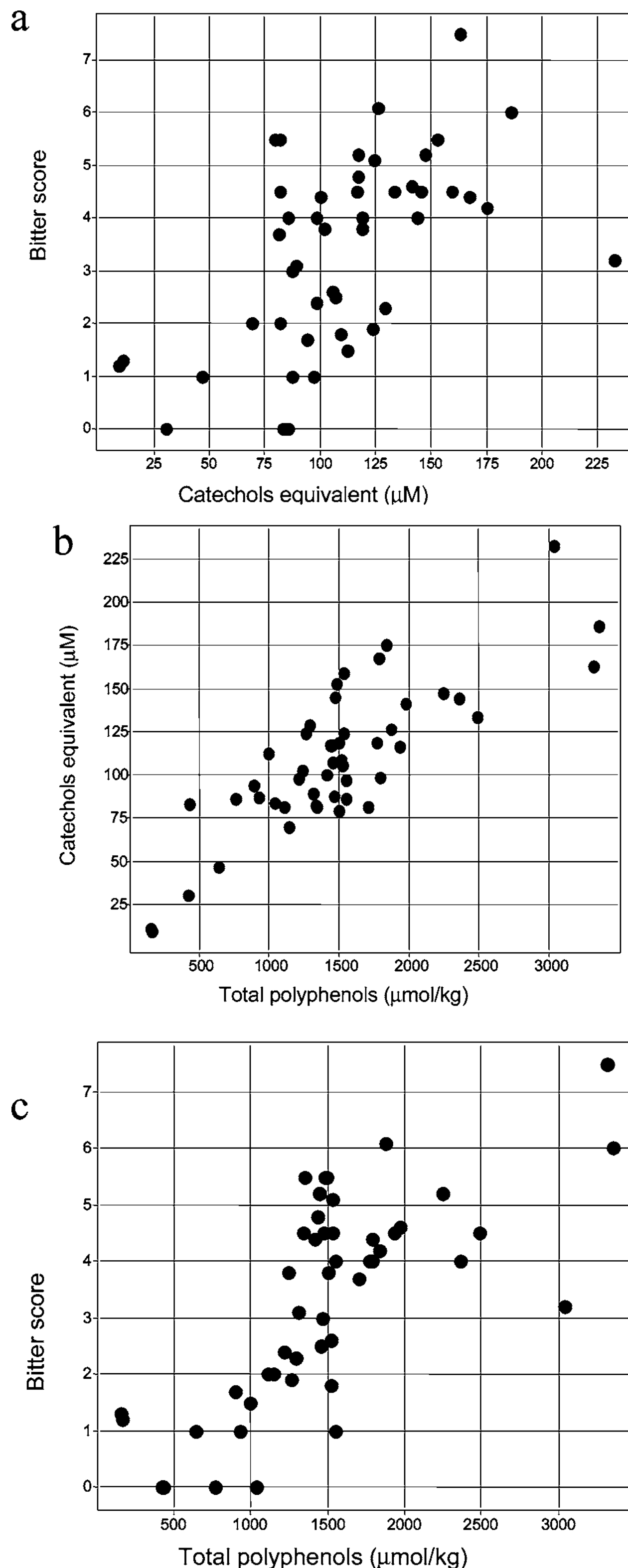


Figure 3. Correlation plots of tyrosinase-based biosensor measurements, phenolic content, and bitter scores of 48 olive oils: (**a**) biosensor response versus bitter scores ($r = 0.56$); (**b**) total phenolics versus biosensor response ($r = 0.82$); (**c**) total phenolics versus bitter scores ($r = 0.71$).

In general, the biosensor response appears to be a good measure for the total phenolics content ($r = 0.82$). However,

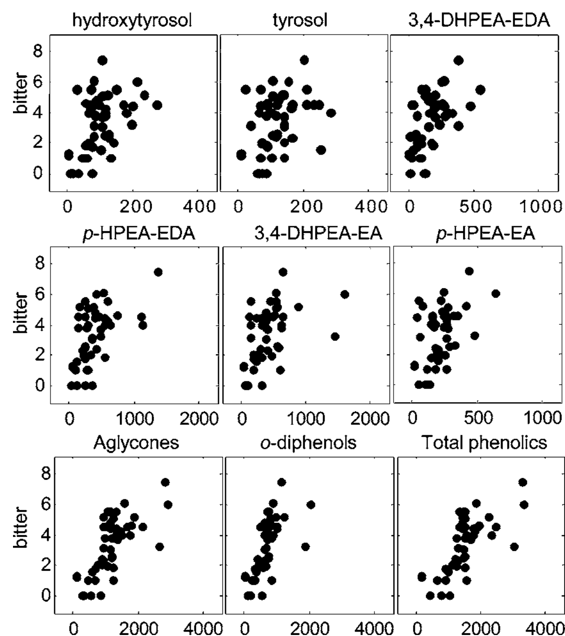


Figure 4. Scatter plots of bitter score for major (individual) phenolic concentrations (expressed in micromoles per kilogram), as determined by HPLC, based on the set of 48 olive oils.

the tyrosinase-based biosensor response of olive oils is not generated according to the same principles as phenolic bitterness in the mouth. The latter involves diffusion of phenolics from the oil into saliva; the phenolics are subsequently transported and then bind to one or more bitter receptors on the tongue (*39*, *40*). The biosensor output, on the other hand, is the result of the current produced upon reduction of the *o*-quinones generated in the samples by oxidation of *o*-diphenols (eqs 1 and 2), which comprise both native hydroxytyrosol-derived phenolics and oxidized tyrosol-derived phenols (eq 1). The different mechanisms behind the sensory and biosensor data are reflected in the different correlations for different types of phenolics obtained with these two methods, as detailed above (**Table 1**).

Comparison of Different Biosensors. In addition to the tyrosinase-based biosensor measurements, a small set of 16 olive oils was used for additional experiments also involving a peroxidase-based biosensor. Peroxidase is less specific for phenolic compounds, but as it displays different catalytic properties, we tested whether the combination of these two different biosensors could improve the correlation with phenolics and bitterness. The oils were subjected to measurements with both biosensors in parallel. Bitterness and pungency scores and the phenolic composition of these olive oils were used for comparison with the two sets of biosensor results.

As expected, the peroxidase-based biosensor showed a "correlation profile" with phenols different from that of the tyrosinase-based biosensor (**Table 2**). The peroxidase-based biosensor output was highly correlated with monophenols (0.93), total phenolics (0.87), and *o*-diphenols (0.84). The correlation of the tyrosinase-based biosensor was primarily in accordance with the findings of the larger data set; due to the lower number of oils included in this comparison the results slightly deviated from those determined with only the tyrosinase-based biosensor (**Table 1**).

The different correlation with various groups of phenolics for the two biosensors illustrates the different selectivities of these two biosensors. Particularly, the high correlation of the peroxidase-based biosensor with monophenols (r = 0.93) is remarkable, which appears to be linked to the significant correlation of this biosensor with pungency (r = 0.66). This correlation is clearly much higher than the correlation of the tyrosinase-based biosensor response with pungency (r = 0.38, **Table 1**; and r = 0.26, **Table 2**, respectively). The correlation of *p*-HPEA-EDA with pungency was high (r = 0.73, **Table 1**), which is in agreement with our previous study (*18*) demonstrating that the pungency of olive oils can be primarily attributed to this aglycone. The tyrosinase-based biosensor displayed a low correlation coefficient with *p*-HPEA-EDA (r = 0.50, set of 48 oils), which is in line with the low correlation of this biosensor with the pungency attribute. In contrast, the peroxidase-based biosensor displays a good correlation with *p*-HPEA-EDA (r = 0.82). The high correlation of the peroxidase-based biosensor with monophenols suggests an explanation for the significant correlation of the biosensor with pungency, attributed to the monophenol *p*-HPEA-EDA. On the basis of this data set we suggest that the peroxidase-based biosensor is suitable for predicting the pungency of olive oils, but additional experiments are needed to confirm this. The higher selectivity of the horseradish peroxidase enzyme toward olive monophenols appears to be a useful property for biosensor applications. We are not aware of publications on the selectivity of (horseradish) peroxidase toward (olive) phenols, which could confirm the different selectivity for mono- and diphenols.

Table 2. Correlations of Phenolic Content and Tyrosinase- and Peroxidase-Based Biosensor Measurements of 16 Olive Oils[a]

	tyrosinase-based biosensor	peroxidase-based biosensor
monophenols	0.64**	0.93**
o-diphenols	0.86**	0.65**
aglycones	0.81**	0.84**
total phenolics	0.83**	0.87**
bitter	0.48*	0.63**
pungent	0.26	0.66**

[a] **, significant at 0.05 significance level; *, significant at 0.10 significance level. Relevant correlation coefficients of phenolics with the biosensor output are discussed in the text. Monophenols: sum of tyrosol, *p*-HPEA-EDA, and *p*-HPEA-EA. *o*-Diphenols: sum of hydroxytyrosol, 3,4-DHPEA-EDA, and 3,4-DHPEA-EA. Aglycones: sum of 3,4-DHPEA-EDA, *p*-HPEA-EDA, 3,4-DHPEA-EA, and *p*-HPEA-EA.

On the basis of this set of olive oils the peroxidase-based biosensor correlates better with bitterness and pungency (r = 0.63 and 0.66, respectively) than the tyrosinase-based biosensor (r = 0.48 and 0.26, respectively). However, the two sensors are strongly correlated with each other, and combining the data could not improve bitterness prediction significantly.

The good correlation of the tyrosinase-based biosensor output with phenolic content (r = 0.82) and good repeatability indicate that this biosensor is suitable for measuring the total phenolic content and therefore might be used as an alternative to the traditional colorimetric assay (*14*). In an earlier experiment phenolic content in 14 olive oils was measured by HPLC, colorimetric assay, and tyrosinase-based biosensor. The correlation between data obtained by colorimetric assay and data obtained by biosensor was r = 0.93, and the correlation between colorimetric assay results and those obtained by HPLC was r = 0.91. This is comparable with values reported for the latter relation (*15*).

Indeed, particularly an instrument that can reliably predict the bitterness of VOO would have additional value, as it enables rapid screening of large numbers of samples on bitterness, for which human tasters are normally needed. Bitterness assessment,

more than other tastes, is highly influenced by different sensitivities of panel members for different bitter compounds (*41*, *42*), lingering in the mouth of bitterness (carry-over effects) (*43*, *44*), and, in the case of olive oil, mouthcoating by the oil matrix itself that may potentially reduce bitterness (*45*). The better repeatability of the tested biosensors, when compared with sensory assessment in general, is a justification for using an instrumental assessment for bitterness screening purposes once the biosensor performance is further optimized.

The modifications to the biosensors, that is, immobilization of the tyrosinase-based biosensor with Nafion and use of screen-printed electrodes for both biosensors, produced stable biosensors with good repeatability of the measurements, as compared to previous sensor designs (*36*−*38*). Coupling the off-line extraction of olive oil to flow injection analysis of the biosensor led to a simplification of the method and considerable reduction of the analysis time (3−5 min per sample), so that the method is comparable with the colorimetric assay. Moreover, the method is relatively simple and therefore could be implemented in a commercial electronic tongue system.

To improve both bitterness and pungency prediction by the biosensors studied, further optimization of the reported method, including optimization of the extraction procedure, is recommended. Such work may include testing of genuine VOO phenolics, either in a mixture or as single compounds instead of catechol. To verify whether significant sources of biosensor response that were not detectable by HPLC were overlooked in our approach, it is recommended that oils with low bitterness but displaying a biosensor response be included in such studies.

In conclusion, the correlation between the tyrosinase- and peroxidase-based biosensor and phenolic content in VOO was high, suggesting that these biosensors may represent a promising tool in the analysis of total phenolics of VOO. The two biosensors showed different specificities toward different groups of phenolics. The correlation with sensory attributes was lower; however, the peroxidase-based biosensor showed a significant correlation with pungency (likely associated with *p*-HPEA-EDA content), which is an interesting sensory attribute related to the quality of virgin olive oil.

ABBREVIATIONS USED

FIA, flow injection analysis; EN, electronic nose; PBS, phosphate-buffered saline; VOO, virgin olive oil; 3,4-DHPEA-EDA, dialdehydic form of decarboxymethylelenolic acid linked to (3,4-dihydroxyphenyl)ethanol; *p*-HPEA-EDA, dialdehydic form of decarboxymethylelenolic acid linked to (*p*-hydroxyphenyl)ethanol; 3,4-DHPEA-EA, aldehydic form of oleuropein aglycone; *p*-HPEA-EA, aldehydic form of ligstroside aglycone.

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