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Rapid Evaluation of Oxidized Fatty Acid Concentration in Virgin Olive Oils Using Metal Oxide Semiconductor Sensors and Multiple Linear Regression

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This works aims to set up a rapid and nondestructive method to evaluate the advanced oxidation of virgin olive oils (VOOs). An electronic nose based on an array of six metal oxide semiconductor sensors was used, jointly with multiple linear regression (MLR), to predict the oxidized fatty acid (OFA) concentration in VOO samples characterized by different oxidative status. An MLR model constructed using five predictors was able to predict OFA concentration with an average validation error of 9%.

KEYWORDS: Electronic nose; multiple linear regression; oxidative status; oxidized fatty acids; virgin olive oil

INTRODUCTION

Differently from other foods, oils and fats do not suffer microbiological problems during storage but suffer principally the oxidation process. The oxidation process is normally divided into three phases: primary phase (slow increase of oxidation); secondary phase (rapid propagation of oxidation), and termination phase. Each oxidation phase is characterized by the production of specific oxidized products (*1*, *2*), such as volatile compounds, oxidized polymers, and molecules that have a similar parent structure with respect to the starting molecules [i.e., oxidized fatty acids (OFA)] (*3*).

Virgin olive oils (VOOs) are characterized by a high oxidative stability with respect to other edible oils in terms of their fatty acid composition (a high oleic acid concentration) and antioxidant content (*4*). The formation of oxidation products during oxidation reactions depends on the fatty acid composition of oils and on antioxidant (phenolic compounds, tocopherols, carotenoids) and pro-oxidant factors (i.e., the presence of oxygen and metals, temperature, and light) (*5*, *6*).

The evaluation of secondary oxidation products represents a critical point to evaluate the storage status of fatty substances (*5*). The difficulty in determining these compounds is due to the fact that each fatty acid produces first hydroperoxides, followed by the production of different classes of compounds such as alcohols, ketones, and epoxides in various isomeric forms.

Several approaches have been attempted to find a reliable oxidation index that, combined with evaluation of primary oxidation products, would provide a realistic idea about the oxidation status of the fatty matrix (*7*). The secondary oxidation indices more widely applied to fat and vegetable oils are the *p*-anisidine value and thiobarbituric acid reactive substances as well as the content of hexanal or nonanal or their ratio (*8*, *9*). Among the chemical methods, the measurements of total polar compounds and polymerized triglycerides (in particular in oils subjected to heating) are the most common methods used (*10*) for the assessment of oil quality. In fact, the oxidized triglyceride and their polymers are good indicators of the oxidative level of oils and fats due to their high stability and low volatility. Nowadays, the official method used to measure the oxidation index entails gravimetric analysis of the polar products according to ISO 8420 (*11*).

An alternative method to analyze secondary oxidation products has been proposed by Rovellini, Cortesi, and Fedeli (*12*). This method evaluates the oxidation status of VOOs using RP-HPLC analysis of OFAs and permits reliable quantification thanks to the use of two reference standards. The method has been used to evaluate the oxidative status of VOOs in various areas of applied research (*2*, *13*, *14*).

This HPLC method (*12*, *14*) allows the identification and quantification of the main OFAs (hydroxy, keto, epoxy, and epidioxy) after a simple derivatization step with sodium benzyl oxide of the triglycerides. As these latter compounds are more stable than peroxides, OFAs seem to be a good index of oxidative changes in lipids. The disadvantages of this method include the long sample preparation time and the long HPLC analysis time (approximately 80 min). For this reason, other techniques that minimize these disadvantages and offer potentially rapid methods that can screen large numbers of samples are needed.

Metal oxide semiconductor (MOS) sensors have been shown to be valid instruments that are applicable in many fields of food control; these sensors have a low cost and can work online without sample pretreatment (*15*, *16*). Electronic noses have been used to detect a variety of sensory defects in VOOs (*17*−*21*) and

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to authenticate them according to the varietal or geographical origin of olives (*22*). In this regard, the oxidation level of VOO has been recently studied (*21*, *23*, *24*). As far as we are aware, no research has been carried out using MOS sensors coupled with multiple linear regression (MLR) to predict OFA concentration in VOOs.

In this work, an electronic nose based on an array of six MOS was used jointly with the application of MLR models to predict OFA concentration in VOOs characterized by different oxidative status. For this purpose, sensor signals were used as predictors.

MATERIALS AND METHODS

Reagents and Samples. The following reagents and standards were used: tricaproin, triheptadecanoin, sodium benzyl oxide in benzyl alcohol, *n*-hexane, 2-propanol, acetone (Sigma-Aldrich, St. Louis, MO), acetonitrile (ACN), anhydrous sodium sulfate (Merck, Darmstadt, Germany), and acetic acid (Fluka, Buchs, Switzerland).

A series of 72 VOOs were sampled from different Italian regions (Abruzzo, Emilia-Romagna, Puglia, Sicilia, and Toscana) during the harvest seasons 2006−2007, 2007−2008, and 2008−2009. All samples were analyzed between November 2008 and January 2009. The oils differed in terms of olive cultivar, degree of ripening, area of growth, production system (type, productive capacity, and manufacturer), and storage time.

Instrumentation and Working Conditions. An electronic olfactory system (EOS 507, Sacmi Imola S.C., Imola, Bologna, Italy) composed of a measuring chamber with six metal oxide sensors and a personal computer was used for the acquisition and analysis of the data generated by the EOS 507. The sensors used were as follows: sensor 1 (SnO_2); sensor 2 (SnO_2 + SiO_2); sensors 3, 4, and 5 (catalyzed SnO_2 with three different metals); and sensor 6 (WO_3). During the analysis, sensors were maintained at a temperature range of 350−450 °C. The EOS 507 was controlled by an integrated personal digital assistant equipped with proprietary software and was connected to an automatic sampling apparatus (model HT500H), which had a carousel of 10 sites for loading samples. Samples were kept at controlled temperature (37 °C) and placed in a chamber provided by a system that removes humidity from the surrounding environment.

OFA determination was performed using an 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA) provided with a binary pump delivery system, a degasser, an autosampler, and a diode array UV−Vis detector (DAD). The liquid chromatograph was also coupled (in series with the DAD) to an atmospheric pressure chemical ionization source from an HP 1100 series quadrupole mass analyzer (MS) (Agilent). OFA separation was carried out with a Luna C18 column (5 μm, 250 × 4.6 mm i.d., Phenomenex, Torrance, CA). Mobile phases were prepared by mixing ACN (A) and water (B) in gradient mode. The gradient elution was performed as follows: from 0 to 50 min, the A percentage was increased from 60 to 100%; an isocratic elution at 100% A was carried out from 50 to 70 min; an additional minute was used to decrease the A percentage from 100 to 60%; then, 60% A was maintained an additional 14 min to equilibrate the column. UV−Vis detection was performed at 255 ± 10 nm (reference 500 ± 50 nm). In all cases, 20 μL was injected at a flow rate of 1 mL min^{-1}. These conditions were adapted from the NGD C-88 official method published by Norme Grassi e Derivati (*25*). The MS working conditions were as follows: nebulizer gas pressure, 50 psi; drying gas flow, 9 L min^{-1} at 350 °C; vaporizer temperature, 300 °C; capillary voltage, 3 kV; corona current, 4 μA; and fragmentor voltage, 60 V. The mass spectrometer was scanned within the m/z 300−500 range in the positive-ion mode.

MOS Sensor Array Procedure. For each sample, 15 g was placed in a 100 mL Pyrex vial equipped with a pierceable silicon/Teflon cap. **Figure 1** represents the response of the six sensors for one of the samples employed in this study. For each sensor, the signal is divided in four parts: (A) conditioning phase (25 min period employed to obtain a constant baseline), (B) before injection phase (in which samples were incubated at 37 °C for 7 min before injection), (C) measurement cycle (in which the oil headspace, sampled with an automatic syringe, was then pumped over the sensor surfaces for 2 min during which the sensor signals were recorded; in this phase sensors were exposed to filtered air at a constant flow rate of 50 sccm (standard cubic cm per min) to obtain the baseline), and (D) recovery phase (another 7 min period applied to restore the original MOS conditions). Ambient air filtered with activated silica and charcoal was used as a reference gas during the recovery phase of the measurement cycle. The previous conditions ensured that the baseline reading had indeed been recovered before the next analysis was performed.

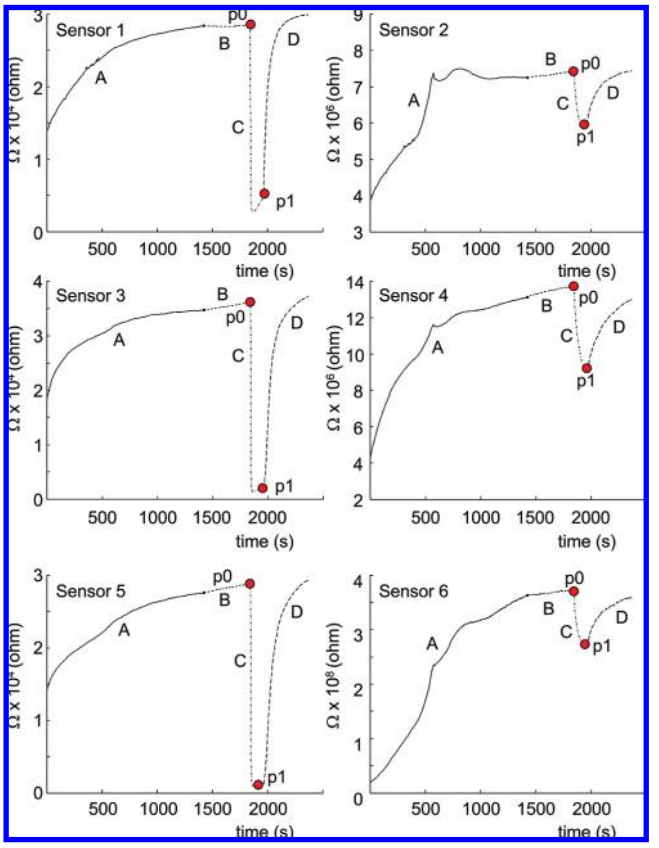

Figure 1. Plots representing the electrical resistance (Ω) of each MOS sensor during VOO evaluation: (A) conditioning phase; (B) before injection phase; (C) measurement cycle; (D) recovery phase.

The experimental conditions adapted from Camurati et al. (*18*) were used, and each sample was evaluated in duplicate.

Determination of OFAs. OFAs were prepared according to the literature (*14*, *25*), and analyzed by HPLC-DAD (*25*) and HPLC-MS after transesterification with 1.0 M sodium benzyl oxide in benzyl alcohol. Tricaproin and triheptadecanoin were used as internal standards (results are reported in percentages as g of total OFA expressed as benzyl heptadecanoate per 100 g of oil, whereas benzyl caproate was used as a control for the derivatization reaction).

Data Treatment and Construction of MLR Matrices. The data from the electronic nose were extracted and analyzed with the statistical package "Nose Pattern Editor" (Sacmi Imola S.C.). A feature extraction algorithm called "classical feature" was applied to the data before other statistical treatments. The response extracted by each sensor was defined by

$$X = p_1/p_0$$

where p_0 was the initial resistance of the sensor balanced in the air (see **Figure 1**), p_1 was the resistance (see **Figure 1**) of a sensor in the presence of the volatile compounds emitted from the VOO headspace (which decreased respect to p_0), and X was the response of each sensor recorded.

For MLR studies, calibration and external validation sets were constructed. The calibration matrix contained 60 objects (which were randomly selected), which corresponded to the average of the duplicates for each sample. The signal of the 6 sensors, which were used as predictors, was also added to this matrix. The external validation matrix was constructed with the remaining 12 objects also corresponding to the average of the duplicates of the samples. Also in this case, the signal of the 6 sensors was added to this matrix. A response column, containing the OFA concentration (obtained by HPLC), was then added to these matrices. Statistical analyses were performed using SPSS (v. 11.5, Statistical Package for the Social Sciences, Chicago, IL).

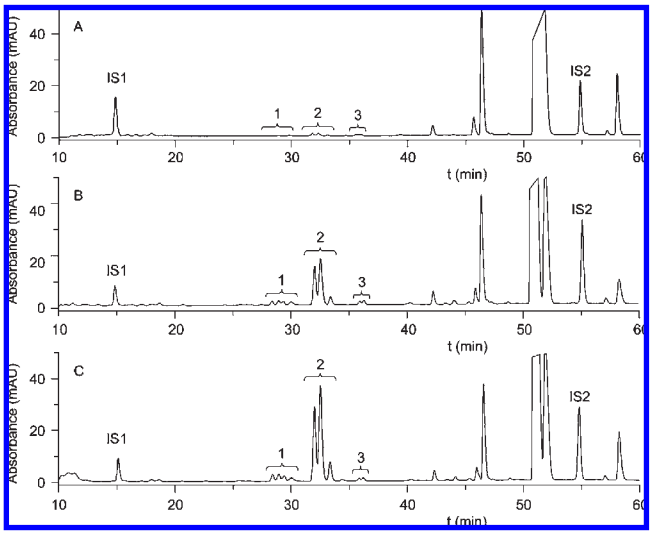

Figure 2. OFA HPLC traces of VOOs at (**A**) 2 weeks, (**B**) 16 months, and (**C**) 34 months after oil production. Detection was performed at 255 nm. Peak identification (as benzyl ester derivatives): 1, group of isomeric forms of ketolinolenic acid; 2, group of isomeric forms of ketolinoleic acid; 3, group of isomeric forms of keto-oleic acid. IS1 and IS2 are benzyl caproate and benzyl heptadecanoate, respectively.

RESULTS AND DISCUSSION

OFA Content in VOO Samples. The HPLC chromatograms in **Figure 2** show that the differences in OFA content (low, medium, and high) of three VOO samples are related to storage time (2 weeks, 16 months, and 34 months after oil production, which correspond to parts **A**, **B**, and **C**, respectively). On the basis of the study of MS spectra, three groups of OFAs were identified (see **Figure 2**): 1, for isomeric forms of ketolinolenic acid (m/z 383); 2, for isomeric forms of ketolinoleic acid (m/z 385); and 3, for isomeric forms of keto-oleic acid (m/z 387). All m/z values corresponded to the $[M + H]^+$ ions.

The OFA content was evaluated for the 72 VOO samples and was found to have a wide range, which varied from 0.3 to 6.5%. This can be attributed to the fact that the oil samples came from different harvest seasons and were analyzed at times ranging from 1 week to 36 months after production. Rovellini et al. (*14*) analyzed several VOOs and found that OFA percentages from 2 to 4% are typical for extra virgin olive oils stored from 2 to 18 months at room temperature, whereas oil samples characterized by a total OFA of >4% must be considered as "expired".

Taking into account the differences observed in OFA values, samples were grouped in four groups (**Figure 3**) on the basis of the OFA values (OFA < 1.0% for **Figure 3A**; 1.0% ≤ OFA < 2.5% for **Figure 3B**; 2.5% ≤ OFA < 4% for **Figure 3C**; and OFA ≥ 4% for **Figure 3D**). The 72 VOOs were subdivided as follows: a first group (G1, n = 23) with a mean of 0.6%; a second group (G2, n = 15) with a mean of 1.8%; a third group (G3, n = 23) with a mean of 3.0%; and a fourth group (G4, n = 11) with a mean of 5.3%. All of the samples produced within 1 month before analysis belonged to G1 with a very narrow range of OFA values (from 0.3 to 0.8%). In contrast, group G4 showed higher percentages and a wider range of variability (from 4.2 to 6.5%). These data confirm that it is possible to evaluate the freshness of VOOs with a simple OFA assay, thereby reducing the number of analyses (i.e., peroxide values or k_{232} for primary oxidation products and *p*-anisidine value or volatile content for secondary oxidation products).

Construction of MLR Models. The SPSS stepwise algorithm of the SPSS was used to select the variables to be included in the

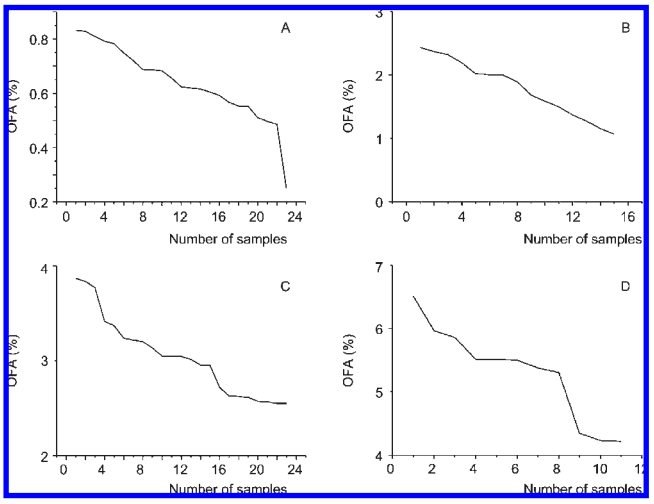

Figure 3. Plots representing the OFA values of the 72 VOOs employed in this study: (**A**) OFA < 1.0%; (**B**) 1.0% ≤ OFA < 2.5%; (**C**) 2.5% ≤ OFA < 4%; (**D**) OFA ≥ 4%. The first group (n = 23) shows a mean of 0.6%; the second group (n = 15), a mean of 1.8%; the third group (n = 23), a mean of 3.0%, and the fourth group (n = 11), a mean of 5.3%.

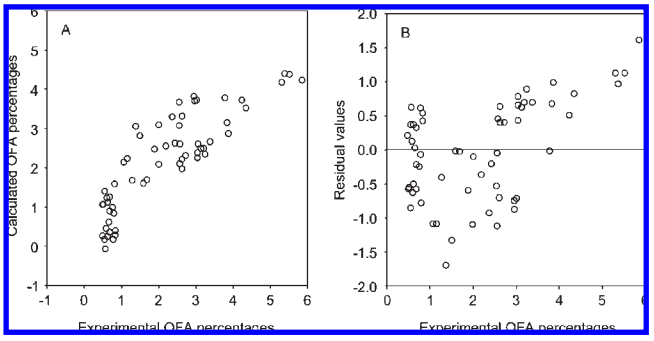

Figure 4. (**A**) Correlation plot of the calculated versus the experimental OFA percentages. (**B**) Plot of the residual values versus the experimental OFA percentages.

MLR models. For this purpose, the default probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted. Using the calibration matrix, two MLR models were constructed, both with and without the inclusion of an independent term (constant). The model including the constant gave lower linearity than the model without the constant (regression coefficient, r, of 0.961). For this reason, further studies were performed without the inclusion of the constant. The correlation plot of the calculated versus the experimental OFA percentages is shown in **Figure 4A**. When leave-one-out validation was applied, the average prediction error (calculated as the sum of the absolute differences between expected and calculated OFA concentrations divided by the number of predictions) was 30%. To obtain information regarding the fit of the model, residual values and/or the relative errors were examined. For this purpose, a plot representing the residual values against the experimental OFA percentages (**Figure 4B**) was obtained. A dependence of the residuals on the experimental values was observed and, therefore, heteroscedasticity (nonconstant variance) of the data. For this reason, the following variable transformations (*26*) were applied to the experimental OFA percentages: natural logarithm and square and cube roots. Homoscedasticity in the data distribution was obtained when the cube root transformation was used (see **Figure 5B**). Comparison of this plot with that in **Figure 4B** shows that homoscedasticity of the data

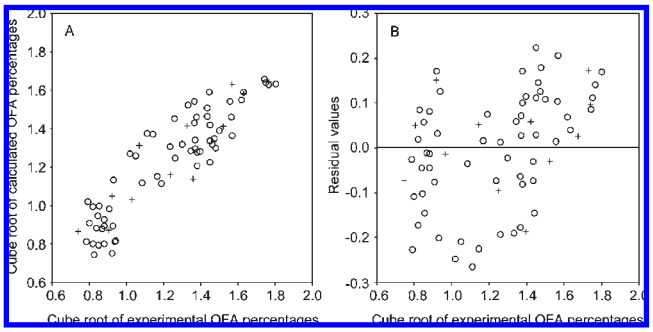

Figure 5. (**A**) Correlation plot of the calculated versus the experimental OFA percentages obtained after cube root transformation. (**B**) Plot of the residual values versus the experimental OFA percentages obtained after cube root transformation. For both **A** and **B**, samples are marked as calibration (○) and validation (+).

Table 1. Predictors Selected and Their Corresponding Nonstandardized Coefficients and Confidence Limits for the MLR Model Constructed with the Cube Root Transformation

predictor	coeff	confidence limits[a]
sensor 2	3.18	2.42, 3.94
sensor 3	10.12	7.67, 12.57
sensor 4	−8.61	−10.63, −6.58
sensor 5	−7.98	−10.12, −5.84
sensor 6	6.16	4.63, 7.69

[a] For a 95% confidence interval.

was observed. Using cube root transformation, an r of 0.995 was obtained with a squared correlation coefficient of 0.989. The correlation plot of the calculated versus the experimental OFA percentages obtained using the cube root transformation is shown in **Figure 5A**. The predictors selected for this model and their corresponding nonstandardized coefficients and confidence limits are detailed in **Table 1**. According to this table, the sensors selected corresponded to sensors 2−6, which corresponded to $SnO_2 + SiO_2$, SnO_2 catalyzed with three different metals, and WO_3. When leave-one-out validation was applied, the average prediction error was 8%. When the model was applied to the validation set, a good prediction capability was observed (see **Figure 4A**), the average validation error being 9%.

Thus, the possibility of estimating the oxidative status of VOO using OFA concentration as reference by means of electronic nose data has been demonstrated. After a cube root transformation of the experimental OFA percentages, an MLR model constructed using five predictors was able to predict OFA concentration with an average error of 9%. This method is useful, particularly considering the accordance with HPLC, the rapidity of analysis, and the lack of solvent consumption. This latter point should be taken into account considering the real problem of ACN shortage, which is the most widely solvent used in HPLC analysis.

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