JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Mathematical Model To Predict the Formation of Pyropheophytin *a* in Virgin Olive Oil during Storage

Ramón Aparicio-Ruiz,* María Roca, and Beatriz Gandul-Rojas*

Chemistry and Biochemistry Pigment Group, Department of Food Biotechnology, Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain

ABSTRACT: A mathematical model has been developed that describes the changes of pyropheophytin *a* (pyphy*a*) in virgin olive oil (VOO). The model has been created using multivariate statistical procedures and is used in the prediction of the stability and loss of freshness of VOO. An earlier thermokinetic study (Aparicio-Ruiz, R.; Mínguez-Mosquera, M. I.; Gandul-Rojas, B. Thermal degradation kinetics of chlorophyll pigments in virgin olive oils. 1. Compounds of series *a*. *J. Agric. Food Chem.* **2010**, *58*, 6200−6208) that looked at the characterization of the degradation of pheophytin *a* (phy*a*), the main chlorophyll compound in VOO and a precursor of pyphy*a*, allowed the authors to obtain the kinetic parameters necessary for mathematically expressing the percentage of pyphy*a*, according to the time and temperature of storage using the Arrhenius model. Data regarding the percentage of pyphy*a* obtained during the actual degradation of VOO in darkness, at room temperature and with a limited supply of oxygen, has allowed the mathematical prediction model to be validated. Using average monthly temperatures in the calculation of kinetic constants, theoretical data are obtained that are generally found to be within 95% confidence levels of experimental data.

KEYWORDS: *chlorophyll degradation products, degradation parameters, kinetic model, kinetic variations, mild deodorization, prediction model, pyropheophytin a, shelf life tests, stability chemical indices, storage, temperature dependence, virgin olive oil*

INTRODUCTION

The large demand for and financial value of extra virgin olive oil on an international level are justified by its excellent sensory and nutritional properties. These characteristics come from the quality of the fruit and from a careful process of extraction, which is carried out exclusively by mechanical processes[2] and under gentle thermal conditions, resulting in this oil being considered the natural juice of the olive.

For a consumer, one of the most important characteristics in a product is for it to be fresh or recently made. Generally, the freshness of a product is associated with quality and therefore safety. Virgin olive oil (VOO) is not affected by expiry dates as it can be consumed for years after its production with no associated health risks. However, its prized sensory and nutritional properties are significantly affected by oxidation, which is the main cause for depreciation in its quality. Spanish legislation (Spain is the main producer of extra virgin olive oil) makes it obligatory for the category of the oil and a "best-before date" to be indicated on the label. This date is set 12−18 months after the date of production, depending on the stability of the oil.[2,3]

All of the hard work carried out in the olive groves and mill to produce a high sensory quality olive oil can later be wasted if storage conditions are inadequate. Therefore, from a financial/commercial point of view, methods that can predict shelf life and stability are necessary.

Degradation of VOO can be monitored by measuring the kinetics parameters that are sensitive to small degrees of oil degradation. The values of these parameters and their kinetic variations will depend both on the operative conditions and on the compositional characteristics of the oil matrix. There is a wealth of data available that assesses the degradation of VOO. In addition, it has been reported that some empirical models are able to predict the time required to reach the upper limits (TRUL) of quality regulations, and always under specific operational conditions.[4−7] However, kinetic models are becoming more popular for studying the changes in the chemical composition of food. These models are capable of predicting shelf life in keeping with the different variables that can affect the degradation of the food item. The operational factors that significantly affect VOO depreciation are the presence of oxygen, exposure to light, and the combination of storage length/temperature.

Until relatively recently no studies had described the kinetic performance of any degradation parameters of VOO.[8,9] The first study of its kind[10] suggests apparent pseudo-zero-order kinetics for the changes in oxidation parameters as peroxide value (PV) and absorbance at 232 nm (K_{232}) and pseudo-first-order kinetics for the evolution of absorbance at 270 nm (K_{270}), using a Arrhenius model to describe the temperature dependence in all cases. A zero-order kinetic model to describe the evolution of PV has also been developed by Rahmouni et al.[9] and validated for the prediction of VOO shelf life when stored in high-oxygen atmospheres.

In an attempt to also look other factors relating to the composition of the oil matrix, in addition to operational variables, Zanoni et al.[11] has put forward a simple conceptual model to predict the stability of VOO based on the combination of three stability indicators: acidity, oleic acid

Received: March 14, 2012
Revised: June 14, 2012
Accepted: June 18, 2012
Published: June 18, 2012

dx.doi.org/10.1021/jf3010965 | *J. Agric. Food Chem.* 2012, 60, 7040−7049

content and bitter taste. This combination of indices was chosen using multivariate statistical analysis as the most favorable combination for predicting the stability of VOO. The model has been preliminarily validated by performing shelf life tests on VOO lots having similar and different value combination of stability indices.[7]

As well as the degradation markers that the olive oil companies frequently and easily monitor such as PV, K_{232}, K_{270}, minor polar components, and oxidized fatty acids, [11] other chemical parameters (volatile, phenolic, or pigment compounds) are applied to trace the deterioration of the quality of VOO under different storage conditions.[7,12−15]

Pigment content and type have been suggested as quality and authenticity indices for VOO.[16] The characterization and assessment of the transformation of pigments associated with the mechanical extraction of VOO have established a general qualitative profile of pigments that are intrinsic to a virgin olive oil, irrespective of the varietal source.[16−19] In addition, certain quantitative relationships between them are proposed as pigment indices, as for example in Spanish VOO: total chlorophylls/total carotenoids around 1.1 (between 0.5 and 1.4), minority carotenoids/lutein around 0.5 (between 0.2 and 1.2), and lutein/β-carotene > 1. These parameters are used as chemical authenticity indices[20] and as trace markers for a suitable processing and storage system for virgin olive oil.[13,17]

The pigment profile is also sensitive to small degrees of oil degradation that inevitably are produced during storage, including when under ideal darkness and controlled temperature conditions.[13] While VOO is stored, the pigment degradation reactions that started during the process of extraction advance [pheophytinization of the chlorophylls (chls) and a certain degree of allomerization], and a new reaction occurs, the formation of pyropheophytin (pyphy). It is formed from pheophytin (phy) due to the loss of the carbomethoxy group in C_{13} (Figure 1), and although it does

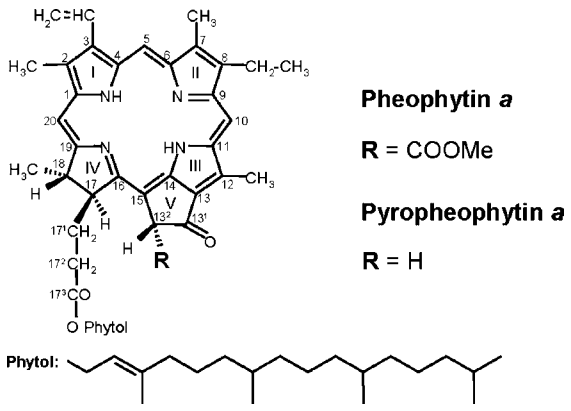

Figure 1. Structures of pheophytin *a* and pyropheophytin *a*.

not imply changes in its electronic absorption properties, its polarity decreases slightly and rapid separation is possible by using chromatographic techniques.[20−23] Thus, as it is a compound that is not produced during the oil extraction process, its formation over time was revealed as a useful parameter for monitoring the degradation of VOO[13,14] and has awakened an interest in studying its kinetic behavior with the aim of establishing a prediction model.

In this regard, our most recent research has focused on characterizing the kinetics of the degradation of phy*a* in VOO under conditions of darkness and lack of oxygen.[1] Of all the competitive degradation reactions, the formation of pyphy*a* was the one that showed significantly higher kinetic constants. An isokinetic study of these reactions did not show significant differences between VOO matrices with different pigment contents (high, medium, and low), which demonstrated that the mechanism of the formation/degradation of pyphy*a* was not affected at any stage by the type of oil matrix, which would allow these results to be extrapolated to any type of VOO matrix.

On the basis of that study, the aim of the present study was to design, using multivariate statistical procedures, a mathematical model to predict the stability of VOO. First, the research is conducted into how an increase and oscillation of the temperature influence the speed of modification of the chlorophyll pigments in VOO during a year of storage under the conditions generally used by the olive oil industry: room temperature, darkness, and limited oxygen. Second, this actual degradation test will allow the validation of a mathematical prediction model that is based on the description of the changes in the percentage of pyphy*a* as a function of temperature and time of storage.

MATERIALS AND METHODS

Chemicals and Standards. Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, The Netherlands). HPLC reagent grade solvents were purchased from Teknokroma (Barcelona, Spain), and analytical grade solvents were supplied by Panreac (Barcelona, Spain). For the preparation, isolation, and purification of chlorophyll pigments, analytical grade reagents were used (Panreac). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Bedford, MA). Standard of chlorophyll *a* (chl*a*) was supplied by Sigma-Aldrich Co. Standards of pheophytin *a* (phy*a*) and pyropheophytin *a* (pyphy*a*) were provided by Wako Chemicals GmbH (Neuss, Germany). The C-13 epimer of phy*a* was prepared by treatment with chloroform according to the method of Watanabe et al.[24] 13^2-OH-phy*a* was obtained by selenium dioxide oxidation of phy*a* at reflux heating for 4 h in pyridine solution under argon.[25] 15^1-OH-lactone-phy*a* was obtained from phy*a* by alkaline oxidation in aqueous media according to the method of Mínguez-Mosquera and Gandul-Rojas.[26]

Raw Materials. The study was carried out using six monovariety virgin olive oils (that is, each oil was extracted from fruits of a single variety) of four different olive cultivars from the main producing areas of Spain. The Blanqueta variety is cultivated in eastern Spain (in the provinces of Valencia and Alicante), and the Arbequina variety is cultivated in northeastern Spain (in the provinces of Lerida and Tarragona). The Cornicabra variety is characteristic of central Spain (mainly in the provinces of Toledo and Ciudad Real), and the Picual variety, approximately 20% of worldwide oil production, is native to southern Spain, mainly the provinces of Jaen and Cordoba.

Oils were requested from industry and extracted from fruits picked at the beginning (I), middle (II), and end (III) of the harvest to obtain the greatest possible variability in oil color (or pigment content). The samples provided were as follows: cv. Arbequina (A-III); cv. Blanqueta (B-II and B-III); cv. Cornicabra (C-I and C-III); and cv. Picual (P-II).

The ranges for quality characteristics of olive oil samples were within limits established for European Union for the extra virgin category: acidity (% oleic acid), 0.10−0.35; peroxide value (mequiv O_2 kg^{-1}), 6.4−14.60; K_{232} (absorbance at 232 nm), 1.54−2.38; and K_{270} (absorbance at 270 nm), 0.096−0.157.

Actual Degradation Test in VOO at Room Temperature. An immediate analysis (initial or time zero) was performed on the extracted oils. Next, the oils were distributed into amber glass jars of 65 mL capacity, with 3% (v/v) headspace. The jars were closed so as to be airtight and stored at room temperature in darkness. The study started in February, once the oil extraction season was completed, and

Table 1. Monthly Average of Temperature in the Storage Facilities (Seville, Spain) during the Period of Actual Degradation Test in VOO at Room Temperature

temp[a]	February[b]	March	April	May	June	July	August	September	October	November	December	January
x	11.4	15.6	19.2	22.3	26.2	28.2	27.8	24.1	20.4	13.7	12.2	10.3
SE	0.2	0.5	0.5	1.1	0.5	0.3	0.6	0.6	0.3	0.6	0.5	0.3

[a]*x*, mean value; SE, standard error. [b]The study started in February, once the oil extraction season was completed, and ended in January of the following year.

Table 2. Evolution of Chlorophyll Pigments in VOO Storage at Room Temperature during a Year for the Study of Picual (II) Variety[a,b]

	0	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
series *a*	12.26	12.25	12.20	12.17	12.00	11.85	11.79	11.70	11.73	11.67	11.70	11.66	11.47
pheo		0.01	0.01	0.01	0.01	0.01	0.03	0.03	0.03	0.03	0.06	0.07	0.06
lac-chl	0.11	0.11	0.01										
OH-chl													
chl	0.42	0.08	0.01										
lac-phy	0.18	0.23	0.30	0.35	0.44	0.49	0.64	0.54	0.54	0.65	0.71	0.69	0.76
OH-phy	0.66	0.92	1.03	1.14	1.40	1.43	1.53	1.58	1.50	1.55	1.67	1.63	1.72
phy	10.89	10.85	10.75	10.55	9.95	9.60	9.20	9.00	8.80	8.49	8.39	8.28	7.91
pyphy		0.05	0.09	0.12	0.20	0.32	0.39	0.55	0.86	0.95	0.87	0.99	1.02
allom	0.95	1.26	1.34	1.49	1.84	1.92	2.17	2.12	2.04	2.20	2.38	2.32	2.48
% pyphy[c]		0.46	0.83	1.12	1.97	3.23	4.07	5.76	8.90	10.06	9.40	10.68	11.42
series *b*	0.83	0.77	0.62	0.56	0.52	0.37	0.26	0.20	0.19	0.18	0.17	0.17	0.15
pheo													
lac-chl	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.04
OH-chl													
chl	0.68	0.57	0.40	0.33	0.28	0.19	0.13	0.10	0.10	0.08	0.07	0.07	0.07
lac-phy													
OH-phy													
phy	0.13	0.18	0.19	0.20	0.21	0.15	0.10	0.07	0.06	0.06	0.06	0.06	0.04
pyphy													
allom	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.04

[a]Data, expressed as μmol/kg, represent mean values for three determinations (CV < 14 in 90% of the cases). Epimers at C-13[2] are quantized together. [b]0, initial value; M, month; pheo, pheophorbide; lac, lactone; chl, chlorophyll; phy, pheophytin; pyphy, pyropheophytin; allom, allomerized. [c]Percentage of pyphy*a* with respect to the sum pyphy*a* + phy*a*.

ended in January of the following year. Table 1 shows the average monthly temperatures registered in the storage facilities (Seville, Spain), with a monthly minimum temperature of 10.4 °C and a maximum of 28.6 °C, and the average annual value standing at 19.3 ± 1.9 °C. The samples were analyzed monthly for one year.

Extraction and Analysis of Chlorophyll Pigments. All procedures were performed under green lighting to avoid any photooxidation of chlorophyll compounds. Pigment extraction was performed by liquid-phase distribution. This method was developed for olive oil by Mínguez-Mosquera et al.[27] The technique is based on the selective separation of components between *N*,*N*-dimethylformamide (DMF) and hexane and yielded a concentrated pigment solution that was oil free and could be adequately analyzed by chromatographic techniques.

HPLC analysis of chlorophyll pigments was performed according to the method described by Mínguez-Mosquera et al.,[27] using a reverse phased column (20 cm × 0.46 cm) packed with 5 μm C18 Spherisorb ODS2 (Teknokroma) and an elution gradient with the solvents (A) water/ion-pair reagent/methanol (1:1:8, v/v/v) and (B) acetone/methanol (1:1 v/v), at a flow rate of 1.25 mL/min. The ion-pair reagent was 0.05 M tetrabutylammonium acetate and 1 M ammonium acetate in water. The pigments were identified by cochromatography with the corresponding standard and from their spectral characteristics described in detail in previous papers.[22,27] The online UV−vis spectra were recorded from 350 to 800 nm with the photodiode array detector. Pigments were detected at the wavelength of maximum absorption (410 nm for phy*a*, 13[2]-OH-phy*a*, and pyphy*a* and 400 nm for 15[1]-OH lactone-phy*a*) and were quantified from the corresponding calibrate curves (amount versus integrated peak area). The calibration equations were obtained by least-squares linear regression analysis over a concentration range according to the levels of these pigments in VOO. Injections in duplicate were made for five different volumes at each standard solution.

Calculations and Statistical Data Analysis. Data were expressed as mean values from a triplicate and are accompanied by the standard error (SE) or the maximum coefficient of variation (CV). The data were analyzed for differences between means using one-way analysis of variance (ANOVA). A Brown and Forsythe[28] test was used as a post hoc comparison of statistical significance (p values < 0.05). Principal component analysis (PCA), least-squares, and nonlinear regression analysis were performed using Statistica 6.0 and Statgraphics Centurion XV for Windows (StatSoft, Inc., 2001).

RESULTS AND DISCUSSION

Changes of Chlorophyll Compounds in Virgin Olive Oils during Storage at Room Temperature. A randomized quantitative study was carried out into all of the transformations of the chlorophyll compounds in VOO during an actual degradation test at room temperature. Monthly analysis was carried out for one year on six VOOs of four different varieties (Tables 2 and 3). As an example, Table 2 includes the total of all the data referring to the Picual variety of oil, whereas those corresponding to the rest of varieties studied are

Table 3. Evolution of Chlorophyll Pigments in VOO Storage at Room Temperature during a Year for the Different Varieties Studied[a,b]

	Cornicabra I				Cornicabra III				Arbequina III				Blanqueta II				Blanqueta III			
	0	M4	M8	M12	0	M4	M8	M12	0	M4	M8	M12	0	M4	M8	M12	0	M4	M8	M12
series *a*	11.24	10.80	10.46	10.10	4.47	3.98	3.82	3.71	1.61	1.42	1.42	1.39	6.71	6.35	5.58	5.29	3.95	3.54	3.32	2.86
pheo									0.01	0.01	0.01	0.04	0.02	0.08	0.15	0.16	0.01	0.05		
lac-chl	0.08	0.24											0.02				0.04			
OH-chl	0.02	0.04											0.08	0.03			0.12			
chl	0.17	0.09							0.05				0.04				0.14			
lac-phy	0.20	0.21	0.27	0.41	0.20	0.28	0.28	0.27	0.03	0.04	0.05	0.04	0.07	0.10	0.11	0.12				
OH-phy	0.47	1.31	1.80	1.71	0.30	0.37	0.67	0.85	0.20	0.21	0.22	0.22	0.06	0.16	0.12	0.17	0.07	0.06	0.07	0.07
phy	10.30	8.73	7.64	7.02	3.97	3.22	2.62	2.33	1.32	1.14	1.01	0.96	6.42	5.84	4.69	4.18	3.57	3.37	3.01	2.51
pyphy		0.18	0.75	0.96		0.06	0.25	0.26		0.03	0.11	0.13		0.14	0.51	0.66		0.06	0.24	0.28
allom	0.67	1.52	2.07	2.12	0.50	0.65	0.95	1.12	0.23	0.24	0.26	0.26	0.11	0.26	0.23	0.29	0.07	0.06	0.07	0.07
% pyphy[c]	0.00	2.02	8.94	12.03	0.00	1.83	8.71	10.04	0.00	2.56	9.82	11.93	0.00	2.34	9.81	13.64	0.00	1.75	7.38	10.04
series *b*	0.81	0.47	0.24	0.24	0.27	0.14	0.07	0.06	0.04	0.03	0.01		0.58	0.29	0.10	0.10	0.37	0.29	0.16	0.14
pheo																				
lac-chl	0.02	0.02	0.02	0.02									0.03	0.02			0.02			
OH-chl	0.03												0.01							
chl	0.39	0.02			0.10								0.46	0.15			0.31	0.06		
lac-phy																				
OH-phy																				
phy	0.37	0.43	0.22	0.15	0.17	0.14	0.07	0.06	0.04	0.03	0.01		0.08	0.12	0.10	0.07	0.04	0.23	0.16	0.14
pyphy				0.07												0.03				
allom	0.05	0.02	0.02	0.02									0.04	0.02			0.02			

[a]Data, expressed as μmol/kg, represent mean values for three determinations (CV < 15 in 90% of the cases). Epimers at C-13[2] are quantized together. [b]0, initial; M, month; pheo, pheophorbide; lac, lactone; chl, chlorophyll; phy, pheophytin; pyphy, pyropheophytin; allom, allomerized. [c]Percentage of pyphy*a* with respect to the sum pyphy*a* + phy*a*.

dx.doi.org/10.1021/jf3010965 |J. Agric. Food Chem. 2012, 60, 7040−7049

summarized for simplicity in Table 3, which shows only results at 4-month intervals.

A qualitative profile of chlorophyll pigments and carotenoids is described that is common to virgin olive oils in general, irrespective of their geographic origin.[16,19,29−32] Thus, the initial samples of VOO (Tables 2 and 3) showed only differences in the total content of pigments or the individual proportion of each of them. With regard to the pigment content, it is described that the differences are in relation to the degree of ripeness (C-I vs C-III and B-II vs B-III) and variety of the fruit used in the virgin olive oil extraction.[16,33] Therefore, the oil obtained from a variety of olive with high pigmentation such as the Picual variety (Table 2) has pigment content quantitatively different from those that from varieties with low levels of pigmentation such as the Arbequina and Blanqueta varieties (Table 3). VOO also usually has a higher proportion of chlorophyll compounds in series *a* due to a higher destruction of series *b* derivatives during the extraction process,[34] which in this case is >95% of all the cases (Table 2). The VOO shows differences with the chl*a*/phy*a* or chl*b*/phy*b* relationships, which can first be associated with the degree of acidity that is released during oil extraction and which favors the pheophytinization reaction. However, this factor does not completely explain the differences, and it is thought that other substances with Mg-dechelating activities, which are functionally similar to an enzyme but different in nature,[35] could be present in the olive fruits and be involved in the pheophytinization reaction during the oil extraction process.[13]

Figure 2 shows the typical chromatograms in an extract of VOO pigments before and after the actual degradation test at

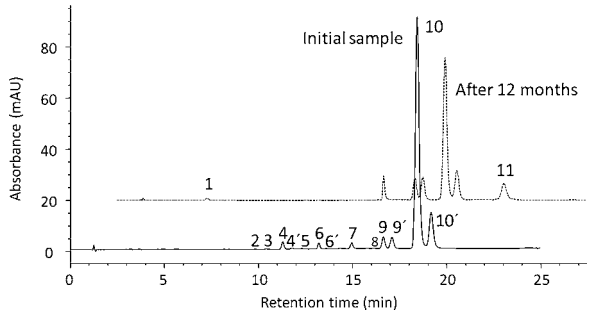

Figure 2. HPLC chromatograms of chlorophyll pigments from Picual virgin olive oil sample, at initial time and after 12 months of storage at room temperature. Detection was by absorption at 666 nm. Peaks: 1, pheo*a*; 2, lac-chl*b*′; 3, OH-chl*b*; 4, chl*b*; 4′, chl*b*′; 5, lac-chl*a*; 6, chl*a*; 6′, chl*a*′; 7, lac-phy*a*; 8, phy*b*; 9, OH-phy*a*; 9′, OH-phy*a*′; 10, phy*a*; 10′, phy*a*′; 11, pyphy*a*.

room temperature, with an average annual value of 19.3 ± 1.9 °C in the storage facilities (Seville). For simplicity, the HPLC shown were performed at 666 nm, the wavelength at which the absorption is due to chlorophyll pigments exclusively, and as an example the Picual variety of oil was chosen.

In the initial sample, the phy*a* and phy*a*′ peaks (peaks 10 and 10′), OH-phy*a* and OH-phy*a*′ (peaks 9 and 9′), lac-phy*a* (peak 7), chl*a* (peaks 6 and 6′), phy*b* (peak 8), and chl*b* (peaks 4 and 4′) stand out. At the end of the storage period, disappearance of the chls can be seen in general (*a* and *b*) as well as a decrease in phy*a* (peaks 10 and 10′). The increase in OH-phy*a* (peaks 9 and 9′) and pheophorbide *a* (pheo*a*) (peak 1) and above all the appearance of pyphy*a* (peak 11) stand out. There are similar transformations in the compounds of series *b*, although they develop to a lower level. The appearance of pyphy*b* was also detected, although this was not a general discovery for all the samples studied.

With regard to the structural modifications that maintain the structure of the chromophore, the most widespread transformation during the storage period was the conversion of chls to phys. As expected, this reaction affected both series *a* and *b*, although it was significantly quicker in the first series. The complete degradation of chl*a* took place in all of the samples during practically the first 4 months (Tables 2 and 3), irrespective of the initial content in the oils, whereas chl*b* was stable for longer: up to 4−6 months for the majority of the oils, and in some cases it was still detected at 12 months (Picual variety, Table 2). In general, the Mg-dechelating reaction shows kinetic constants of 2−10 times less for the chlorophyll compounds in series *b*, both in model systems and in food matrices.[36−39] Comparison of these results with a previous study on the storage of VOO carried out at a controlled temperature of 15 °C[13] revealed no significant differences in the speed of transformation of chl*a* to phy*a*, as this also occurred mainly during the first 3 months of storage. This is due to the fact that, although in general temperature does influence the speed of reactions, the average monthly temperatures when the pheophytinization reaction occurs—between the months of February and May—were 17.1 ± 2.3 °C (Table 1), not very different from the 15 °C used in the controlled-temperature study. From May a significant increase in the monthly average temperature was noted, with maximum temperatures being reached from July to September, which corresponds to the sixth to eighth months of storage. This increase in temperature did not affect the pheophytinization reaction of chl*a*, which had already occurred, but it did affect the rest of the modifications, which are discussed below.

The most important reaction was the formation of pyphy*a*. As can be seen from a previous study,[13] during prolonged storage of virgin olive oil at a controlled temperature of 15 °C, decarbomethoxylation occurs slowly in the C-13 of phy*a*, which generates pyphy*a* (Figure 1). This study was carried out on VOOs taken from different varieties of olives at different stages of ripeness and reported an annual formation of pyphy*a* not above 3.0% of the total chlorophyll compounds (or 3.5% with respect to the sum pyphy*a* + phy*a*), irrespective of the varieties and states of ripeness. In the research gathered during degradation at room temperature, the results showed a considerable increase in the percentage of formation of pyphy*a*, which in some cases tripled (10−14%) (Table 3). Another difference with regard to the storage of oil at a controlled temperature of 15 °C was the detection of the formation of pyphy*b* at trace levels in the Arbequina and Picual varieties and at approximately 0.6% of the total of chlorophyll compounds in the Blanqueta II and Cornicabra I VOOs.

With regard to the allomerization reactions, including the formation of 13^2-OH-chls, 13^2-OH-phys, 15^1-OH-lac-chls, and 15^1-OH-lac-phys, a significant percentage increase was also found, affecting further the Cornicabra variety oils, but it did not differ significantly from that found in the degradation trial carried out at 15 °C. As for the total content, a loss in colored chlorophyll compounds was found, which oscillated between 10.1 and 32.9% (average = 15%) depending on the oil variety,a loss that was not detected in the research carried out at 15 °C.[13]

The mathematical procedure of principal component analysis that allows for the number of initial variables to be reduced to a lesser number of factors was applied to the information

dx.doi.org/10.1021/jf3010965 | J. Agric. Food Chem. 2012, 60, 7040−7049

regarding the individual content of chlorophyll pigments to find out its overall development over the year of storage. Figure 3

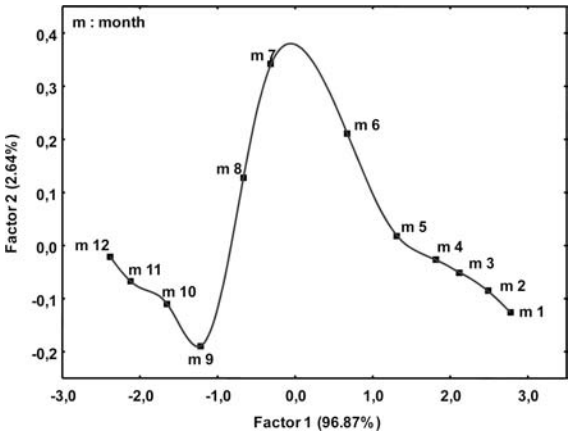

Figure 3. PCA of chlorophyll pigments in six monovarieties of Spanish virgin olive oil stored during a year at room temperature: score plots for the first two principal components (factor 1 vs factor 2). M, month; mathematical adjustment, spline.

shows how the quantitative development of the pigment profile is strictly related with the sequence of storage months. The statistical procedure allowed for all of the information to be reduced to two factors that explain the performance of pigment profile in the varieties studied. Thus, 96.87% of the variance is explained by the variable time (t), whereas only 2.64% of the variance is related to the variation in the quantitative profile of the pigments. This multivariate information indicates that the pigments develop over time and that this development is more pronounced during the summer months (July, August, and September), which are the sixth, seventh, and eighth months of storage. It was also observed that the variation is cumulative and, except for the months of summer, the variation in pigment profile is similar to that in the months before and after this period. This statistical analysis suggested that temperature had a significant effect on the speed of the transformation reactions of the chlorophyll compounds during VOO storage.

With regard to the stability of the chlorophyll molecule, it is described that prolonged heating produces decarbomethoxylation of carbon C-13[2], causing the formation of pyroderivatives.[25] Therefore, its presence in food products has always been associated with thermal treatments, above all in tinned and fermented products.[26,37] Indeed, in olive oils with organoleptic defects that cannot be sold as virgin oils and which are thermally treated via deodorization to eliminate the defects, it has been determined that this compound converts into the main chlorophyll derivative with a percentage level of >60%.[40] As for the formation of allomerized chlorophyll derivatives, this occurs when the chlorophyll compounds are oxidized by triple-molecular oxygen, involving a reaction mechanism via free radicals.[25] In this case, although an increase in temperature can have an impact, the speed of the reactions is more associated with the availability of oxygen present in the headspace of the oil containers.[12−14] This is the reason that the studies at 15 °C and at room temperature have similar values for both fractions of allomerized chlorophyll derivatives.

The formation of pyphy*a* is therefore a reaction that is sensitive to small degrees of oil degradation which occur even with limited availability of oxygen, and therefore it is revealed as an ideal parameter for tracing the deterioration of the quality of VOO under different storage conditions. In 2001 Serani and Piacenti[21] established an empirical correlation between the phy*a* and pyphy*a* content in VOOs from different sources and production dates, and they put forward an index (cold index) derived from the correlation that attempted to differentiate genuine VOO from olive oil that had been deodorized under mild conditions at low temperatures ("deodorato" oil). Aside from the error made in using chl*a* instead of pyphy*a* in the calibration of the method, later it was shown that the content in pyphy*a* is a very variable parameter that depends not only on the operational variables (time and temperature) but also on the initial content of phy*a* in the VOO.[13] Anniva et al.[14] found that the occurrence of phy*a* and/or pyphy*a* at appreciable levels is indicative of extended storage of the oil in the dark and/or exposure to elevated temperatures. These results led to a new traceability parameter being introduced: the relative content of pyphy*a* in relation to phy*a* (% pyphy*a* = 100[pyphy*a*/(phy*a* + pyphy*a*)],[13] which is independent of absolute quantities and can be calculated directly from the relationship between the corresponding areas of the peaks in the HPLC chromatogram, thereby making the calibration process unnecessary.

Figure 4 shows the development of this parameter in the degradation study carried out at room temperature for the

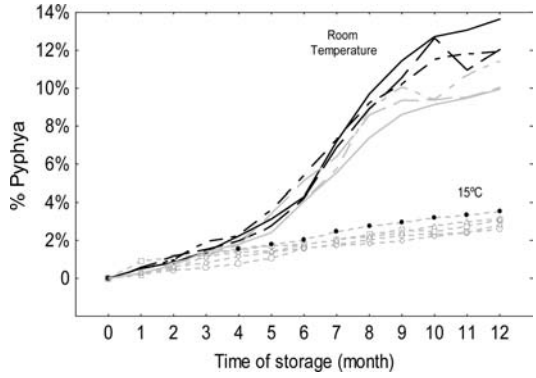

Figure 4. Percent of pyropheophytin *a* (pyphy*a*) in six monovarieties of virgin olive oil stored during a year at a controlled temperature of 15 °C and at room temperature (RT). % pyphy*a* = 100[pyphy *a*/(phy*a* + pyphy*a*)]. RT: Arbequina I (– · – ·), Blanqueta I (——), Blanqueta II (——), Cornicabra I (—— ——), Cornicabra II (—— ——), and Picual I (– • – •). Data at 15 °C were calculated from Gallardo-Guerrero et al.[13] and represent mean values (med) for two or three olive oil samples; each data sample is obtained in triplicate: Arbequina med (○), Blanqueta med (◇), Cornicabra med (□), Hojiblanca med (△), and Picual med (●).

different varieties of oils studied, and it compares it against the same study carried out by Gallardo-Guerrero et al.[13] at a controlled temperature of 15 °C. The most significant overall difference that can be observed is the increase in the percentage of pyphy*a* over the study period. Another finding is the slope change in the increase of the percentage of pyphy*a* during the summer months (6−8 storage months)—it was more pronounced—an effect that obviously did not occur during the controlled-temperature research. All of this supports the well-known effect that temperature has on the generation of pyphy*a* and that there is a need to research this dependence with the aim of developing a kinetic model that describes the changes in the pyphy*a* percentage parameter.

The mathematical equation necessary for predicting these changes in the composition of pyphy*a* depends on the chemical

reactions involved. Thus, experimental data are therefore needed to estimate the relevant parameters of this equation. With this aim, a corresponding thermokinetic study was carried out to characterize the degradation of phy*a*, the main chlorophyll compound in VOO and a precursor of pyphy*a*.[1] The degradation mechanism is not a simple one. It consists of various stages of competitive reactions, both with regard to the formation of oxidation products and degradation to colorless products. This study allowed us to obtain the necessary kinetic parameters to design a mathematical prediction model for the formation of pyphy*a* in VOO in general, which is based only on the storage temperature and time.

Design of the Pyropheophytin *a* Prediction Model. According to the mechanism proposed for the degradation of phy*a* (Figure 3 in ref 1) and in accordance with the kinetic study previously carried out,[1] the total degradation of phy*a* and the formation of pyphy*a* can be formulated following a first-order model, expressed as

$$[\text{phy}a] = [\text{phy}a]_0\, e^{-k_{ta}t} \tag{1}$$

$$[\text{pyphy}a] = \frac{k_1[\text{phy}a]_0}{k_2 - k_{t_a}}[e^{-k_{ta}t} - e^{-k_2 t}] + [\text{pyphy}a]_0\, e^{-k_2 t} \tag{2}$$

where $[\text{pyphy}a]$, $[\text{phy}a]$, $[\text{pyphy}a]_0$, and $[\text{phy}a]_0$ are the concentrations of pyphy*a* and phy*a* over time and initial pyphy*a* and phy*a* concentrations, respectively; k_{ta}, k_1, and k_2 are the constants of phy*a* total degradation, pyphy*a* formation, and pyphy*a* degradation, respectively.

Therefore, the percentage of pyphy*a* over time can be expressed as follows:

$$\%\text{pyphy}a(t) = \left[\frac{[\text{pyphy}a](t)}{[\text{pyphy}a](t) + [\text{phy}a](t)}\right] \times 100 \tag{3}$$

Substituting eqs 1 and 2 in eq 3 results in the following expression:

$$\%\ \text{pyphy}a(t) = \left[\frac{\frac{k_1[\text{phy}a]_0}{k_2 - k_{t_a}}[e^{-k_{ta}t} - e^{-k_2 t}] + [\text{pyphy}a]_0\, e^{-k_2 t}}{\frac{k_1[\text{phy}a]_0}{k_2 - k_{ta}}[e^{-k_{t_a}t} - e^{-k_2 t}] + [\text{pyphy}a]_0 e^{-k_2 t} + [\text{phy}a]_0\, e^{-k_{t_a}t}}\right] \times 100 \tag{4}$$

The equation obtained predicts the percentage of pyphy*a* in VOO in accordance with time variable and corresponding kinetic constants of phy*a* total degradation (k_{ta}), pyphy*a* formation (k_1), and pyphy*a* degradation (k_2) to uncolored products.

Each kinetic constant is a temperature function (T) that is adjusted to the Arrhenius model. Data used for its calculation included the values of the kinetic constants obtained by Aparicio-Ruiz et al.[1] for temperatures of 60, 80, 100, and 120 °C and the values corresponding to 15 °C, calculated using nonlinear regressions adjusted to the concentration−time development data obtained by Gallardo-Guerrero et al.[13] for the Hojiblanca variety. In this way, a wide variety of kinetic constants for the formation of pyphy*a* have been worked with, ranging from optimum storage conditions (15 °C) to temperatures that are too high (such as 120 °C), which are used in the mild deodorization processes.

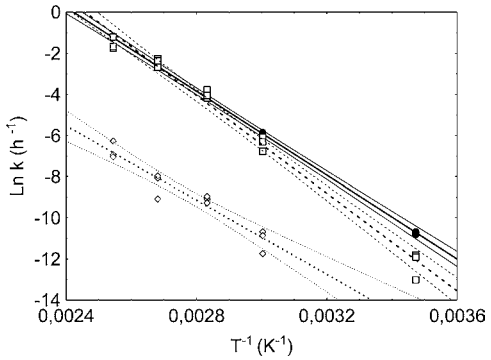

Figure 5. Arrhenius plot for pheophytin *a* degradation (D.Phy*a*) (●), pyropheophytin *a* formation (F.Pyphy*a*) (□), and pyphy*a* degradation to colorless products (D.Pyphy*a*) (◇) for VOO samples with low, medium, and high pigmentation.

The graphical representation of the values in the Arrhenius model (Figure 5) allows for the adjustment lines ($y = \alpha + \beta x$) or the Arrhenius lines to be determined, and the values of α and the slope (β), together with their standard error and the determination coefficient (R^2) have been protected by industrial license (Trade Secret License or Secret know-how).

The proposed mathematical functions that describe the dependence of the kinetic constants k_{ta}, k_1, and k_2 with temperature could be, for example

$$k_i(T) = e^{(\alpha_i - \beta_i/T)} \tag{5}$$

and are expressed as

$$k_{ta}(T) = e^{(\alpha_{ta} - \beta_{ta}/T)} \tag{6}$$

$$k_1(T) = e^{(\alpha_1 - \beta_1/T)} \tag{7}$$

$$k_2(T) = e^{(\alpha_2 - \beta_2/T)} \tag{8}$$

α_{ta}, α_1, α_2, β_{ta}, β_1, and β_2 are the values protected by industrial license.

By substituting these mathematical functions in the eqs 1 and 2, expressions are obtained that allow for the concentrations of phy*a* and pyphy*a* to be calculated in accordance with time.

$$[\text{phy}a](t, T) = [\text{phy}a]_0\, e^{-(e^{(\alpha_{ta} - \beta_{ta}/T)})t} \tag{9}$$

$$[\text{pyphy}a](t, T) = \frac{e^{(\alpha_1 - \beta_1/T)}[\text{phy}a]_0}{e^{(\alpha_2 - \beta_2/T)} - e^{(\alpha_{ta} - \beta_{ta}/T)}}\left[e^{-(e^{(\alpha_{ta} - \beta_{ta}/T)})t} - e^{-(e^{(\alpha_2 - \beta_2/T)})t}\right] + [\text{pyphy}a]_0\, e^{-(e^{(\alpha_2 - \beta_2/T)})t} \tag{10}$$

where the variables are T, the temperature in Kelvin (K); t, the time in hours (h); and $[\text{phy}a]_0$ and $[\text{pyphy}a]_0$, the initial concentrations of phy*a* and pyphy*a*, respectively.

By substituting eqs 9 and 10 in eq 4, an expression is obtained for calculating the pyphy*a* percentage that is formed during a given storage time (expressed in hours). This expression can be abbreviated further as fresh oil, obtained under suitable thermal conditions, should not present pyphy*a* ($[\text{pyphy}a]_0 = 0$).[13]

Therefore, the final equation can be expressed as

dx.doi.org/10.1021/jf3010965 | J. Agric. Food Chem. 2012, 60, 7040−7049

$$\%[\text{pyphy}a](t) = \frac{\frac{e^{(\alpha_1-\beta_1/T)}[\text{phy}a]_0}{e^{(\alpha_2-\beta_2/T)}-e^{(\alpha_{ta}-\beta_{ta}/T)}}\left[e^{-(e^{(\alpha_{ta}-\beta_{ta}/T)}t} - e^{-(e^{(\alpha_2-\beta_2/T)}t}\right]}{[\text{phy}a]_0\, e^{-(e^{(\alpha_{ta}-\beta_{ta}/T)}t} + \frac{e^{(\alpha_1-\beta_1/T)}[\text{phy}a]_0}{e^{(\alpha_2-\beta_2/T)}-e^{(\alpha_{ta}-\beta_{ta}/T)}}\left[e^{-(e^{(\alpha_{ta}-\beta_{ta}/T)}t} - e^{-(e^{(\alpha_2-\beta_2/T)}t}\right]} \quad (11)$$

Validation of the Mathematical Model. With the application of the model we can predict, from eq 3 and substituting the terms of eqs 9 and 10, the percentage of pyphy*a* that will form in a VOO during a year of storage (8760 h) at 15 °C (288 K) to validate the model with a real experience that was carried out in those conditions.[13] By applying eqs 6−8 the corresponding speed constants at said temperature are obtained, and by substitution in eq 4 the monthly prediction data at 15 °C are obtained. Figure 6

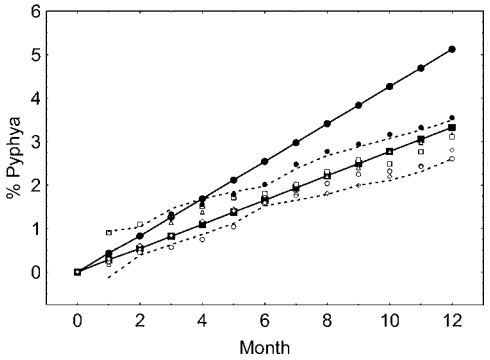

Figure 6. Evolution of pyropheophytin *a* percent in VOO during a year of storage at controlled temperature: comparison of the experimental data at 15 °C, with their confidence limits at −95 and 95% (- - -), and the prediction models for 12 °C (■) and 15 °C (●). Experimental data and their symbols are as in Figure 4.

compares these data with the corresponding empirical data, calculated using the experimental data obtained by Gallardo-Guerrero et al.[13] The kinetic model proposed predicts an annual formation at 15 °C of 5.37% of pyphy*a*, whereas the experimental data oscillated between 2.6 and 3.5 for the different oil varieties studied. The empirical increase in the percentage of pyphy*a* over the course of the year is approximately linear; however, a slight deviation can be seen with regard to the result of the prediction. The values that were predicted by the model are higher than those in the experiment, with a deviation of 3 °C. The values of the model for a temperature of 12 °C are within the margins of confidence (95%) for the experimental values at 15 °C.

With regard to storage at room temperature, in an initial overall approximation, the kinetic constants can be calculated for an average annual temperature in Seville of 19.3 °C, and by substitution of those values in eq 4, an annual prediction value at room temperature is obtained of 10.05% of pyphy*a*, whereas the experimental values obtained with the different stored VOO samples oscillated between 10.04 and 13.64% (Table 3).

This validation can be refined further. We could estimate an annual increase by adding the corresponding monthly estimates by using the monthly average temperatures instead of average annual temperatures in the application of the designed model. Applying eqs 6−8, the corresponding kinetic constants can be obtained for each of these monthly average temperatures, and by successive substitutions in eq 4 and additions of the monthly estimations, the corresponding prediction curve can be obtained (Figure 7, shown in black), which reproduces the empirical values obtained from six different varieties of oil

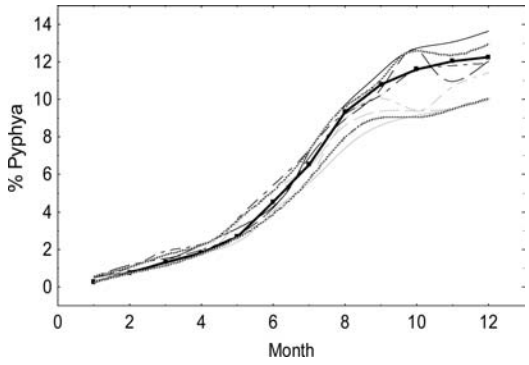

Figure 7. Evolution of pyropheophytin *a* percent in VOO during a year of storage at room temperature: comparison of the experimental data from six monovarieties of Spanish VOOs, with their confidence limits at −95 and 95% (- - -), and the values from the prediction (■) model using average monthly temperatures in the calculation of kinetic constants, adjustment by spline algorithm. Experimental data and layout of their trend lines are as in Figure 4.

(Figure 3). At room temperature the percentage of pyphy*a* does not increase linearly due to the variation in the room temperature over the course of the year. The evolution of the pyphy*a* percentage parameter in the oils used in the experiment is similar to a sigmoid, and the values predicted by the model are thereby adjusted, considering the average monthly temperatures. This performance is due to the higher temperature in the summer months, which translates into an increase in the slope, whereas at the beginning and the end of the year the temperatures are lower and tend to be quite similar. In general, the model data are found to be within 95% of the margins of confidence of the experimental data. Only the result in the first month is slightly outside the margin of confidence levels (±95%) for the experimental data.

Once validated, the model was used to develop a prediction graph between 15 and 35 °C (Figure 8). The prediction lines

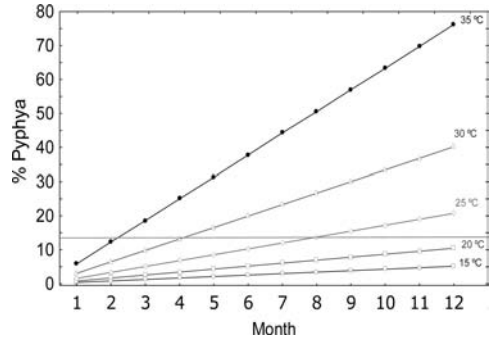

Figure 8. Calculating prediction of the pyropheophytin *a* percent that is expected in a VOO during a year of storage at different controlled temperatures (from 15 to 35 °C).

dx.doi.org/10.1021/jf3010965 | J. Agric. Food Chem. 2012, 60, 7040−7049

allow for the expected percentage of pyphy*a* in VOO to be calculated for a given storage temperature and length. Conversely, the maximum storage temperature theoretically cannot be exceeded to ensure that the pyphy*a* percentage parameter does not go above a reasonable limit. This limit could be set at 14%, which is the maximum value reached after a year of storage under real conditions at room temperature. In accordance with the prediction graph (Figure 8), the oils should be stored at a controlled temperature (or yearly average) of <22 °C to stop the percentage of pyphy*a* going above said limit.

The kinetic prediction model put forward is useful for the VOO producer and wholesaler so they can have, a priori, an estimation of the maximum storage time for VOO under controlled temperature, using annual, monthly, or daily predictions in keeping with the pyphy*a* percentage parameter.

This model provides the producer and/or wholesaler with a tool to determine the speed of the pyropheophytinization reaction as a function of temperature and the storage conditions that can slow it, facilitating the distinction between aged oil and oil that has been thermally treated. Furthermore, it is useful as a tracing tool: knowing the production date of a VOO, that is, the time for which it has been in storage, and taking into consideration the average monthly temperatures, the model allows for the expected percentage of pyphy*a* to be calculated (for suitable storage conditions, e.g., room temperature in the dark) and to trace whether the value that is obtained from the chemical analysis is correct or whether it is much higher than the expected value, which would indicate that it had undergone some unwanted process (e.g., mild deodorization, high storage temperature, or exposure to light).

AUTHOR INFORMATION

Corresponding Author

*Phone: 34-954-691054. Fax: 34-954-691262. E-mail: gandul@cica.es; aparicioruiz@cica.es.

Funding

This work was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT-EU, Spanish and European governments) and FEDER (CE) under Project AGL-2007-66139-21 C02-01 and by the Junta de Andalucía in the Project AGR 2010-6271.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Sergio Alcañiz-García for his technical assistance. We also thank Cooperativa La Paz (Estepa, Seville), Cooperatives Oleicoles Valencianes (Muro, Alicante), Aceites Toledo S.A. (Los Yébenes, Toledo), and Finca Venta del Llano (Menjibar, Jaen) for sample donations.

REFERENCES

(1) Aparicio-Ruiz, R.; Minguez-Mosquera, M. I.; Gandul-Rojas, B. Thermal degradation kinetics of chlorophyll pigments in virgin olive oils. 1. Compounds of series a. *J. Agric. Food Chem.* **2010**, *58*, 6200–6208.

(2) 1994. Regulation No. 2003/1989/CE. *Off. J. Commission Eur. Communities* **2003** (Nov 13), *L295*.

(3) 2000. Regulation No. 2000/13/CE. *Off. J. Commission Eur. Communities* **2000** (March 20), *L109*.

(4) Tsimidou, M.; Papadopoulos, G.; Boskou, D. Determination of phenolic compounds in virgin olive oil by reversed-phase HPLC with emphasis on UV detection. *Food Chem.* **1992**, *44*, 53–60.

(5) Gordon, M. H.; Mursi, E. A comparison of oil stability based on the Metrohm Rancimat with storage at 20 °C. *J. Am. Oil Chem. Soc.* **1994**, *71*, 649–651.

(6) Pagliarini, E.; Zanoni, B.; Giovanelli, G. Predictive study on tuscan extra virgin olive oil stability under several commercial conditions. *J. Agric. Food Chem.* **2000**, *48*, 1345–1351.

(7) Gómez-Alonso, S.; Mancebo-Campos, V.; Salvador, M. D.; Fregapane, G. Evolution of major and minor components and oxidation indices of virgin olive oil during 21 months storage at room temperature. *Food Chem.* **2007**, *100*, 36–42.

(8) Siliani, S.; Zanoni, B.; Fia, G.; Bertuccioli, M.; Mattei, A.; Lorenzini, O. Towards a conceptual model to predict extra virgin olive oil stability. *Riv. Ital. Sostaze Grasse* **2009**, *86*, 31–38.

(9) Rahmouni, K.; Bouhafa, H.; Hamdi, S. Development and validation of a mathematical model for olive oil oxidation. In *Proceedings of the 2nd Mediterranean Conference on Intelligent Systems and Automation*; Beji, L., Otmane, S., Abichou, A., Eds.; Zarzis, Tunisia, 2009; pp 49–53, ISBN: 978-0-7354-0642-1 .

(10) Mancebo-Campos, V.; Fregapane, G.; Salvador, M. D. Kinetic study for the development of an accelerated oxidative stability test to estimate virgin olive oil potential shelf life. *Eur. J. Lipid Sci. Technol.* **2008**, *110*, 969–976.

(11) Zanoni, B.; Bertuccioli, M.; Rovellini, P.; Marotta, F.; Mattei, A. A preliminary approach to predictive modelling of extra virgin olive oil stability. *J. Sci. Food Agric.* **2005**, *85*, 1492–1498.

(12) Psomiadou, E.; Tsimidou, M. Stability of virgin olive oil. 1. Autoxidation studies. *J. Agric. Food. Chem.* **2002**, *50*, 716–721.

(13) Gallardo Guerrero, L.; Roca, M.; Gandul-Rojas, B.; Mínguez Mosquera, M. I. Effect of storage on the original pigment profile of Spanish virgin olive oil. *J. Am Oil Chem. Soc.* **2005**, *82*, 33–39.

(14) Anniva, C.; Grigoriadou, D.; Psomiadou, E.; Tsimidou, M. Z. Pheophytin α degradation products as useful indices in the quality control of virgin olive oil. *J. Am. Oil Chem. Soc.* **2006**, *83*, 371–375.

(15) Kalua, C. M.; Bedgood, D. R., Jr.; Bishop, A. G.; Prenzler, P. D. Discrimination of storage conditions and freshness in virgin olive oil. *J. Agric. Food Chem.* **2006**, *54*, 7144–7151.

(16) Gandul-Rojas, B.; Roca-L.Cepero, M.; Mínguez-Mosquera, M. I. Use of chlorophyll and carotenoid pigment composition to determine authenticity of virgin olive oil. *J. Am. Oil Chem. Soc.* **2000**, *77*, 853–858.

(17) Roca, M.; Gandul-Rojas, B.; Gallardo-Guerrero, L.; Mínguez-Mosquera, M. I. Pigment parameters determining Spanish virgin olive oil authenticity: stability during storage. *J. Am. Oil Chem. Soc.* **2003**, *80*, 1237–1240.

(18) Psomiadou, E.; Tsimidou, M. Pigments in Greek virgin olive oils: occurrence and levels. *J. Sci. Food Agric.* **2001**, *81*, 640–647.

(19) Aparicio-Ruiz, R.; Gandul-Rojas, B.; Roca, M. Pigment profile in non-spanish olive varieties (*Olea europaea* L. var. Coratina, Frantoio, and Koroneiki). *J. Agric. Food Chem.* **2009**, *57*, 10831–10836.

(20) Roca, M.; Gallardo-Guerrero, L.; Mínguez-Mosquera, M. I.; Gandul-Rojas, B. Control of olive oil adulteration with copper-chlorophyll derivatives. *J. Agric. Food Chem.* **2010**, *58*, 51–56.

(21) Serani, A.; Piacenti, D. Sistema analitico per l'identificazione di oli deodorati in oli vergini di oliva. Nota 1 – Analisi dei pigmento clorofilliani in oli vergini di oliva. *Riv. Ital. Sostaze Grasse* **2001**, *78*, 459–463.

(22) Hornero-Méndez, D.; Gandul-Rojas, B.; Mínguez-Mosquera, M. I. Routine and sensitive SPE-HPLC method for quantitative determination of pheophytin *a* and pyropheophytin *a* in olive oils. *Food Res. Int.* **2005**, *38*, 1067–1072.

(23) Gertz, C.; Fiebig, H. J. Pyropheophytin a – determination of thermal degradation products of chlorophyll a in virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 1062–1065.

(24) Watanabe, T.; Hongu, A.; Honda, K.; Nakazato, M.; Konno, M.; Saithoh, S. Preparation of chlorophylls and pheophytins by isocratic liquid chromatography. *Anal. Chem.* **1984**, *56*, 251–256.

(25) Hynninen, P. H. Chemistry of chlorophyll modifications. In *Chlorophylls*; Scheer, H., Ed.; CRC Press: Boca Raton, FL, 1991; pp 145−210.
(26) Mínguez-Mosquera, M. I.; Gallardo-Guerrero, L. Anomalous transformation of chloroplastic pigments in Gordal variety olives during processing for table olives. *J. Food Prot.* **1995**, *58*, 1241−1248.
(27) Mínguez-Mosquera, M. I.; Gandul-Rojas, B.; Gallardo-Guerrero, L. Rapid method of quantification of chlorophylls and carotenoids in virgin olive oil by HPLC. *J. Agric. Food Chem.* **1992**, *40*, 60−63.
(28) Brown, M. B.; Forsythe, A. B. Robust tests for the equality of variances. *J. Am. Stat. Assoc.* **1974**, *69*, 364−367.
(29) Gandul-Rojas, B.; Mínguez-Mosquera, M. I. Chlorophyll and carotenoid composition in virgin olive oils from various Spanish olive varieties. *J. Sci. Food Agric.* **1996**, *72*, 31−39.
(30) Morello, J. R.; Motilva, M. J.; Ramo, T.; Romero, M. P. Effect of freeze injuries in olive fruit on virgin olive oil composition. *Food Chem.* **2003**, *81*, 547−553.
(31) Ranalli, A.; Pollastri, L.; Contento, S.; Iannucci, E.; Lucera, L. Effect of olive paste kneading process time on the overall quality of virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 57−67.
(32) Salvador, M. D.; Aranda, F.; Gomez-Alonso, S.; Fregapane, G. Influence of extraction system, production year and area on Cornicabra virgin olive oil: a study of five crop seasons. *Food Chem.* **2003**, *80*, 359−366.
(33) Roca, M.; Mínguez-Mosquera, M. I. Changes in chloroplast pigments of olive varieties during fruit ripening. *J. Agric. Food Chem.* **2001**, *49*, 832−839.
(34) Gallardo-Guerrero, L.; Roca, M.; Mínguez-Mosquera, M. I. Distribution of chlorophylls and carotenoids in ripening olives and between oil and alperujo when processed using a two-phase extraction system. *J. Am. Oil Chem. Soc.* **2002**, *79*, 105−109.
(35) Suzuki, T.; Shioi, Y. Re-examination of Mg-dechelation reaction in the degradation of chlorophylls using chlorophyllin a as a substrate. *Photosynth. Res.* **2002**, *74*, 217−223.
(36) Canjura, F. L.; Schwartz, S. J.; Nunes, R. V. Degradation kinetics of chlorophylls and chlorophyllides. *J. Food Sci.* **1991**, *56*, 1639−1643.
(37) Schwartz, S. J.; Lorenzo, T. Chlorophylls in food. *Crit. Rev. Food Sci.* **1990**, *29*, 1−17.
(38) Mínguez-Mosquera, M. I.; Gandul-Rojas, B.; Mínguez-Mosquera, J. Mechanism and kinetics of the degradation of chlorophylls during the processing of green table olives. *J. Agric. Food Chem.* **1994**, *42*, 1089−1095.
(39) Koca, N.; Karadeniz, F.; Burdurlu, H. S. Effect of pH on chlorophyll degradation and colour loss in blanched green peas. *Food Chem.* **2007**, *100*, 609−615.
(40) Gandul-Rojas, B.; Roca, M.; Mínguez-Mosquera, M. I. Chlorophyll and carotenoid pattern in virgin olive oil. Adulteration control. In *Proceedings of the 1st International Congress on Pigments in Food Technology (PFI)*; Mínguez-Mosquera, M. I., Jarén-Galán, M., Hornero-Méndez, H., Eds.; Seville, Spain, 1999; pp 381−386, PFI: ISBN 8469901850.