Effect of Various Compounds on Virgin Olive Oil Stability Measured by Rancimat

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Phenolic and orthodiphenolic compounds together with carotenoids, tocopherols, pigments, and fatty acids were tested for their antioxidant effect in 79 samples of virgin olive oils cv. Picual and Hojiblanca. A linear regression based on the oleic/linoleic ratio and the contents of phenols and tocopherols showed a good correlation (adjusted-$R^2 = 0.91$) with the stability measured by Rancimat, later verified with an external text set (adjusted-$R^2 = 0.95$). A tentative study on the percentage contribution of each chemical variable to stability is discussed. The contribution of phenolic and orthodiphenolic compounds was around 51%, the composition of fatty acids 24%, and in less percentage α-tocopherol, carotenoids, and chlorophylls. No effect, or very little, was shown by β- and γ-tocopherols.

Keywords: *Virgin olive oil; antioxidant activity; phenols; tocopherols; carotenoids; fatty acids; chemometrics*

INTRODUCTION

Oxidative stability is an important parameter in evaluating the quality of oils and fats, as it gives a good estimation of their susceptibility to oxidative degeneration, the main cause of their alteration. The greater or lesser stability of an oil means the conservation or not of the so-called dynamic parameters during the useful life of the product.

Decomposition of hydroperoxides has been established as being related to the number of double bonds in fatty acids. It is well-known that linoleate hydroperoxides decompose faster than oleate ones (Frankel, 1962). The oleate:linoleate:linolenate oxidation ratio has been reported to be of the order of 1:12:25, based on peroxide formation (Lea, 1952).

At the same time, any reaction preventing the propagation of peroxidation, or removing free radicals, plays an important role in the oxidation mechanism. Chain-breaking antioxidants, such as phenolic compounds, react with lipid radicals to form nonreactive radicals, stopping the propagation chain (Simic, 1992). In fact, these compounds are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation (Shahidi, 1992).

Carotenoids are pigments possessing conjugated hydrocarbons and have been found to be potent protectors against photosensitized oxidation, acting as singlet oxygen quenchers (Bradley, 1992). The physical quenching mechanism of carotenes is based on their low singlet energy state, which facilitates the acceptance of energy from the singlet oxygen.

The oxidative stability of olive oil is greatly affected by the presence of chlorophylls and their derivatives, especially in the presence of light (Interesse, 1971) as they have the ability to transfer energy from light into chemical molecules (Bradley, 1992). Endo (1984) reported the relative pro-oxidant activity of pheophorbides, pheophytins, and chlorophylls during methyl linoleate oxidation, while Usuki (1984) pointed out that pheophytins have high activity as photosensitizers.

Various researchers (Vázquez et al., 1973; Papadopoulos and Boskou, 1991; Baldioli et al., 1996; Angerosa et al., 1995) have studied the importance of the total or individual phenol contents with regard to virgin olive oil stability. The active phenols in the virgin olive oils are mainly o-diphenols such as hydroxytyrosol and its oleosidic forms, protocatechuic and caffeic acids (Baldioli et al, 1996; Montedoro et al., 1993; Papadopoulos and Boskou, 1991). The monophenol tyrosol and its oleosidic and derivative forms show, however, less antioxidant activity (Morales and Tsimidou, 1999; Morales and Pryzibylski, 1999). A few studies have been reported about the relationship between stability and other series of chemical compounds. Baldioli et al. (1996) studied the importance of total content of tocopherols. Gutiérrez et al. (1992) analyzed how the contents of chlorophyll and carotenoid pigments affect virgin olive oil stability, and Civantos et al. (1992) studied the great role of fatty acid composition in the stability of olive cultivars. However, very little is known about the percentage contribution of these compounds to the stability of virgin olive oil, which is the aim of the present study.

MATERIALS AND METHODS

Olive Oils. Virgin olive oil varieties Picual and Hojiblanca were selected for their different behaviors in terms of stability. The mean stability of the variety Picual is around 90 ± 2 h and that of Hojiblanca, 45 ± 1 h, due to their different composition in fatty acids and antioxidant compounds (Uceda et al., 1992). They are the main cultivars in Spain, with virgin olive oil from cv. Picual representing more than 18% of world production. Olive samples were collected during two successive crops (1995−1996 and 1996−1997) characterized by quite different weather conditions: the first was dry and the second wet. Thus, the sample set covers the effect of rainfall on virgin olive oil composition; e.g., the phenol content is lower in wet seasons. Olive oil was obtained in an industrial olive oil mill

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using a three-phase centrifugation system under the proper malaxing conditions of temperature and time for virgin olive oils.

The test and training sets, comprising 79 samples in total, were used for the statistical study. The samples of the training set (41) were of cv. Hojiblanca, while the samples of the test set (38) were of cv. Picual. A second test set comprising identical samples of virgin olive oil (79), but washed many times in order to remove almost all the hydrophilic compounds, was also used. This second test was used to check the contribution of these chemical compounds to virgin olive oil stability.

Analytical Methods. The peroxide index and the acidity, as oleic acid, were carried out following the analytical methods described in EC Regulations (EC, 1991). Fatty acids were measured as their methyl esters. Methyl esters were analyzed by gas chromatography on HP Inmovax column (30 m × 0.25 mm i.d.) following the EC Regulations (EC, 1992).

Chlorophyll and carotenoid pigments were quantified by spectrophotometry (Hewlett-Packard 8450 A) following the methodology described by Mínguez et al. (1991). Tocopherols were evaluated following the IUPAC Standard Method (IUPAC, 1992), but using δ-tocopherol as internal standard (Gutiérrez and Albi, unpublished). A solution of oil in hexane was analyzed by high performance liquid chromatography (LCD Analytical model 3200) on a silica gel column (Merck, Superspher Si 60, particle size 4 m, 250 mm × 4 mm i.d.), eluting with hexane-2-propanol 99.3:0.7 at a flow rate of 1 mL/min. A fluorescence detector (Jasco 821-FP) with excitation wavelength at 290 nm and emission wavelength at 330 nm was used.

All phenolic and orthodiphenolic compounds, including aglycones with OH-radical in σ-position, were isolated by extraction of a solution of oil in hexane with water−methanol 60:40 for three times. Folin−Cicalteu reagent and sodium molybdate 5% in ethanol 50% reagent (both Merck), respectively, were added to suitable aliquots of the combined extracts. The absorption of the solution at 725 nm (phenolic compounds) and 370 nm (orthodiphenolic compounds) was measured on a spectrophotometer (Hewlett-Packard 8450 A UV/vis). Results are given as mg/kg of caffeic acid (Vázquez et al., 1973).

The sensory analysis of samples were evaluated by an analytical panel of the Instituto de la Grasa, comprising 12 trained tasters, following Annex XII of Regulation EC/2568/91 of the Commission of the European Communities (EC, 1991). Outliers were removed following the method suggested by Albi and Gutiérrez (1991).

Stability was evaluated by measuring the oxidation induction time, with the use of the Rancimat apparatus (Metrohm CH 9100). A flow of air (10 L/h) was bubbled through the oil heated at 100 °C, and the volatile compounds were collected in cold water, increasing the water conductivity. The time taken to reach a fixed level of conductivity was recorded (Gutiérrez, 1989).

Statistical Analysis. All of the variables had an almost normal distribution, so that no transformation had to be applied to the data. The multivariate statistical procedures were applied under the strictest conditions.

Principal components analysis (PCA) was applied after normalization by autoscaling (column centering and column standardization). Cross-validation was repeated three times with different cancellation matrices, always detecting two significant components explaining 81.1% of the total variance. PCA was applied to detect possible outliers and to know the multi-colinearity between the chemical variables.

Stepwise linear regression analysis (SLRA) was applied to select the chemical variables (phenols, σ-diphenols, carotenoids, tocopherols, pigments, and oleic/linoleic ratio) that better explained the stability. SLRA was used with the ridge regression algorithm in order to diminish the effect of highly correlated independent variables (Ranzeboom, 1979). The backward stepwise algorithm was used to select the chemical variables as it initially assumes that all of chemical variables contribute to virgin olive oil stability. The test for selecting the variables was carried out using values of F-to-enter and F-to-remove, from the F-distribution statistical table (F:0.99) and according to the number of samples and chemical variables (Tabachnick and Fidell, 1983). Tolerance was defined as one minus the squared multiple regression of stability with all other independent variables in the multiple regression equation.

Once the chemical variables were selected, a piecewise linear regression analysis (with the quasi-Newton estimation method algorithm) was applied to these selected chemical variables to design the final regression equation.

Response surfaces were used to plot the fitted models of the chemical variables with respect stability. A quadratic smoothing algorithm was used to plot the figures.

RESULTS AND DISCUSSION

According to the limits set by the Commission of the European Communities for the classification of virgin olive oils, 68.4% of the studied samples of the variety Picual were of extra virgin quality, 18.4% virgin, 10.5% common, and 2.6% lampant. For the variety Hojiblanca, 61% of the samples were of extra virgin quality, 24.4% virgin, 7.3% common, and 7.3% lampant. The samples of the both varieties are well-balanced in terms of quality, although the number in the lampant category was higher in the Hojiblanca cultivar.

The values of oxidative stability for these samples, together with the minor compounds studied and the oleic/linoleic ratio, encompass a considerable range of stability. It is not easy to have samples of monovarietal oils covering the typical range of olive oil stability whose highest values correspond to oils of extra virgin quality. Generally, the quality parameters (EC, 1995) do not vary uniformly with stability, so that significantly rising or falling values are not often maintained. Stability, although is not a standard parameter of quality, is useful to provide information about the hypothetical shelf life of an oil. The lowest stability values provide information about the worst quality: e.g., greater acidity, higher peroxide values and extinction coefficients, and lower sensorial score.

While studying the production of flavor and off-flavors in virgin olive oil, Morales et al. (1997) reported that the content of polyunsaturated fatty acids diminished after 33 h of a thermoxidation process (100 °C and 22 L/h of oxygen stream). Linolenic acid practically disappeared (97.8%), while other acids were affected to a lesser extent, linoleic acid 7w (56%)—the most abundant polyunsaturated fatty acid—and even less the mono unsaturated oleic acid 9w (46%)—the most abundant fatty acid. The oxidation speeds of linoleic and oleic acids explain why stability is higher when the content of mono unsaturated oleic acid is high and the content of linoleic acid is low. Thus, the ratio oleic to linoleic has the most marked relationship with stability (Roda, 1997). On the other hand, this ratio is also useful in the characterization of olive cultivars (Aparicio, 1988; Aparicio et al., 1997; Civantos, 1992). The percentage of linoleic acid is lower for the variety Picual (2−5%) than for Hojiblanca (7−12%). This means a technical problem for the statistical methods, which have to minimize the influence of olive cultivars when building a regression equation for the stability.

Once the basic characteristics of the chemical compounds (phenols, tocopherols, and chlorophylls) were analyzed with regard to oxidative stability, the next step was to verify the results in the light of the conclusions reached by other authors. Table 1 shows the mean and range of the chemical variables analyzed in the samples

Table 1. Mean and Range Values of Chemical Parameters of Virgin Olive Oil Samples of cv. Hojiblanca and cv. Picual

	Hojiblanca		Picual	
chemical variable	mean	range	mean	range
stability (h)	45.41	10.80−78.65	77.43	22.60−180.90
oleic/linoleic ratio (%)	8.64	6.15−11.45	21.24	11.79−31.07
chlorophylls (mg/kg)	9.32	0.60−19.51	12.67	2.74−49.20
carotenoids (mg/kg)	6.78	2.60−18.18	8.93	4.09−26.40
α-tocopherols (mg/kg)	187.16	106.00−271.69	207.57	104.00−425.00
β-tocopherols (mg/kg)	1.47	0.98−1.95	1.49	0.86−2.64
γ-tocopherols (mg/kg)	10.94	8.70−14.20	14.49	4.80−20.70
total tocopherols (mg/kg)	199.54	114.70−283.19	223.54	114.20−432.44
phenols (mg/kg)	193.87	23.90−419.91	191.92	20.80−457.00
σ-diphenols (mg/kg)	12.97	0.1−31.81	13.99	0.58−42.90
peroxide index	7.24	1.4−16.4	8.65	3.00−18.70
overall grading (scale 1−9)	6.70	3.20−8.30	7.02	3.00−8.30
free acidity (% oleic acid)	0.44	0.14−2.66	0.42	0.11−2.62

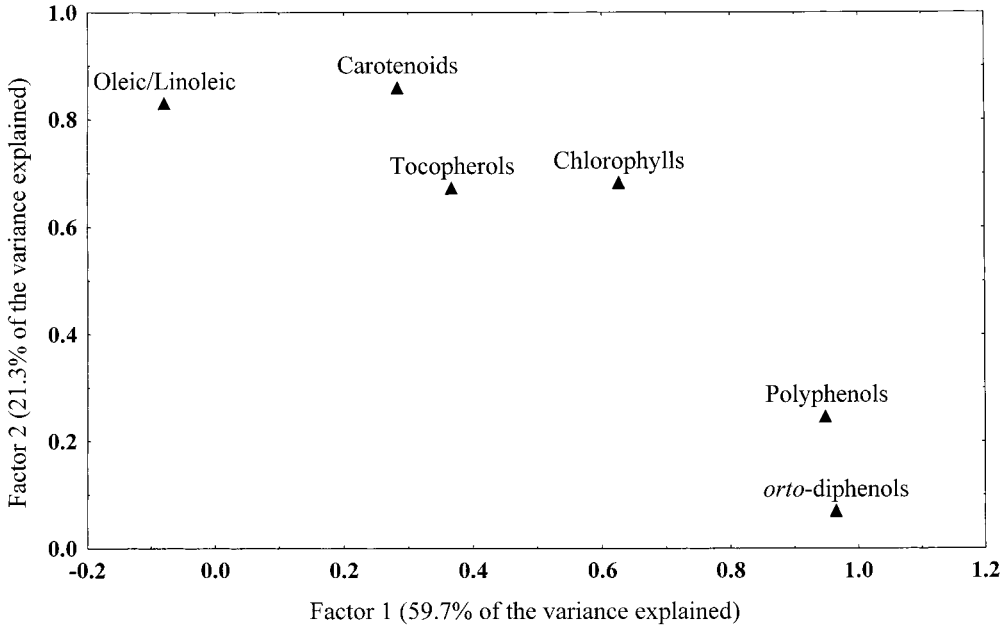

Figure 1. Results of principal components analysis applied to the training set. Varimax rotation was used to display the results.

of the two selected varieties, Hojiblanca and Picual. Univariate statistical analysis showed that the highest stability of Picual is mainly due to the oleic/linoleic ratio, as this variety has a lower content of polyunsaturated fatty acids. Univariate analysis of the two data sets (training and test sets) revealed a correlation between stability and the other chemical variables. The phenols ($R = 0.87$), σ-diphenols ($R = 0.77$), and the oleic/linoleic ratio ($R = 0.71$) had the highest values, followed by total tocopherols ($R = 0.65$), chlorophylls ($R = 0.68$), and carotenoids ($R = 0.59$). It is noteworthy that within tocopherols the greatest contribution was of α-tocopherol ($R = 0.66$), while γ-tocopherol showed no correlation ($R = -0.35$) with stability. Concerning the latter compound, Jung and Min (1991) had detected lower antioxidant activity of γ-tocopherol than of α- and β-tocopherols when worked in photosensitized oxidation. The different chemical behavior of γ-tocopherol can be explained by taking into account that the photosensitized oxidation involves singlet oxygen. Standard additions (from 50 to 200 mg/kg) of γ-tocopherol to a virgin olive oil of variety Picual did not show, however, a significant increase of stability ($\alpha = 0.95$) measured by Rancimat (Gutiérrez, unpublished).

From a sensory point of view, it would be expected that stability had a good positive correlation with the overall grading. High values of stability indirectly mean a low level of rancidity and hence low presence of the undesirable sensory descriptors such as rancidity (EC, 1995). A low level of correlation ($R = 0.59$) was found, however, between these parameters. This result corroborates the studies carried out by Morales et al. (1997) that detected that neither the peroxide index nor overall grading showed a good correlation with the oxidative stability measured by Rancimat. On the contrary, it is noteworthy that the overall grading has a high negative correlation ($R = -0.81$) with the free acidity, which is one of the quality indices (EC, 1991).

On the basis of this information, a stepwise linear regression analysis (SLRA) was applied under strict conditions (Tabachnick and Fidell, 1983), to avoid good results by chance. The training set only contains samples of cv. Hojiblanca, while the test set is exclusively constituted by samples of cv. Picual. The values of *F*-to-enter and *F*-to-remove statistical variables were selected from the *F*-table at 0.99. These *F*-values determine how significant the contribution of a variable (Table 1) to the regression has to be in order to be added to or removed from the equation. Under these conditions, SLRA selected the chemical variables: phenols, oleic/linoleic ratio, and tocopherols as having together the maximum correlation with stability. Principal components analysis confirmed the selection of these chemical variables. Figure 1 shows that there were three

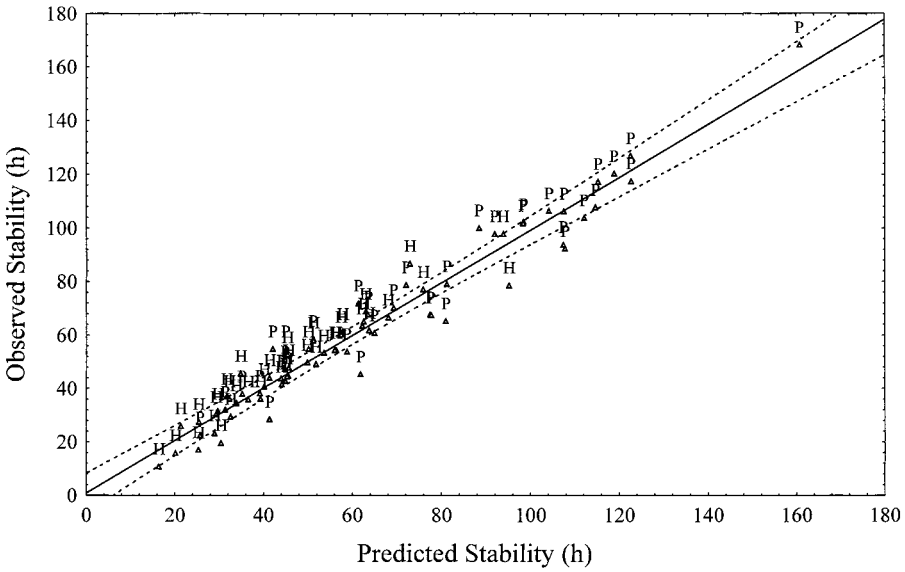

Figure 2. Results of applying the regression equation to all set of samples (training and test sets). Key: H, samples of cv. Hojiblanca; P, samples of cv. Picual.

groups: oleic/linoleic ratio; tocopherols, chlorophylls and carotenoids; and phenols and σ-diphenols. The high multi-colinearity between σ-diphenols and phenols ($R = 0.92$) prevented that the former was selected instead of the latter which had lower coefficient of variation.

The regression equation (adjusted-$R^2 = 0.91$) was $-18.52 + 0.13$*(total phenols) $+ 3.03$*(oleic/linoleic ratio) $+ 0.07$*(tocopherols).

The equation, built exclusively with the samples of the training set (cv. Hojiblanca), was applied to the samples of the test set (cv. Picual) to predict their stability. Despite the fact that the equation was verified with a test set made up of samples of an olive cultivar having quite different stability levels, the result was even better (adjusted-$R^2 = 0.95$). Figure 2 shows the results of SLRA with the two sets of samples (training set and test set).

From a mathematical point of view, the β-coefficients indicate the relative importance of the chemical variables in the regression equation. The β-coefficients are not, however, completely independent. They are affected by the other chemical variables of the regression equation. To minimize the effect of the other variables, the contribution coefficient of each chemical variable is calculated as the product of its β-coefficient and its independent correlation coefficient. The analysis of the β-coefficients of the selected variables showed that phenols had the highest standardized correlation coefficient ($R = 0.69$) vs only $R = 0.36$ for the oleic/linoleic ratio and $R = 0.14$ for tocopherols. These results could mean a certain disagreement with the values calculated by univariate statistics. However, SLRA is a multivariate procedure, and hence this apparent disagreement can be interpreted as the result of a certain synergy between the oleic/linoleic ratio and the other compounds. Figure 3 shows the response surface of the phenols and the oleic/linoleic ratio with respect to stability. The lowest value corresponds to the minimum of the two chemical variables and the highest value to their maximum. Thus, the slope of this surface can point out a synergy effect between the variables, oleic/linoleic ratio and phenols. Figure 4 shows the response surface but now using the total tocopherols. This figure shows that there is a certain reversion (no antioxidant activity)

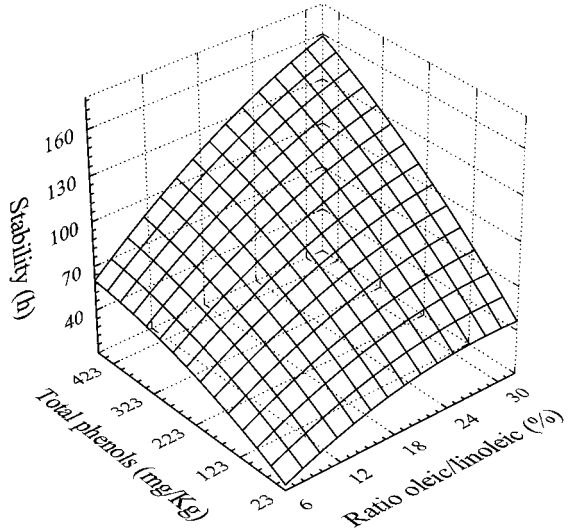

Figure 3. Surface response of virgin olive oil stability as a function of the content of phenols and the oleic/linoleic ratio.

when samples have a high tocopherol content with medium values of the oleic/linoleic ratio. The maximum antioxidant activity corresponds to the highest values of the oleic/linoleic ratio and the minimum content of tocopherol. This means that there is no synergy between these chemical variables but, on the contrary, an unknown antagonism under certain conditions. Similar studies were carried out with the other chemical variables.

Taking into account that the first regression equation has a high adjusted-R^2 value (0.95) for the test set, the contribution coefficients can be interpreted as the contribution to stability, with a correction factor of at least 0.95. From these results, the phenolic content would contribute around 50% of the stability of virgin olive oil, while the oleic/linoleic ratio would mean only 27%. Since a hypothetical synergy effect was detected between these chemical variables, it is better to conclude that 78% of the stability is due to the combined effect of both variables. Following an identical procedure, the contribution of total tocopherols was evaluated around 9%. There is a percentage of an unknown variable (13%).

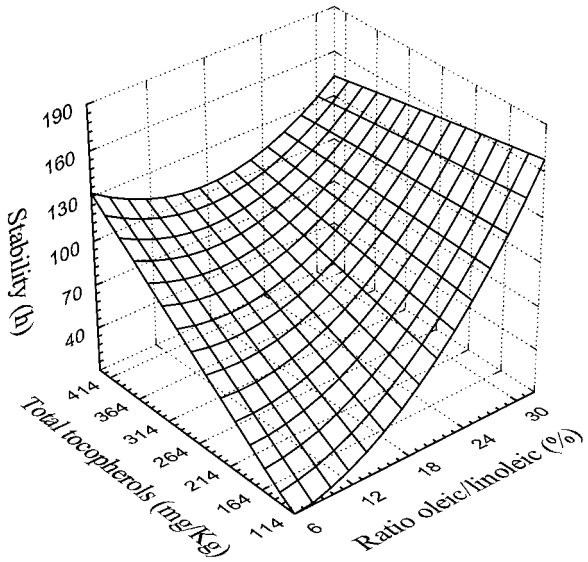

Figure 4. Surface response of virgin olive oil stability as a function of the content of tocopherols and the oleic/linoleic ratio.

Table 2. Contribution of Chemical Variables to Virgin Olive Oil Stability[a]

chemical variable	A (%)	B (%)
oleic/linoleic ratio	27	24
phenols	30	29
total tocopherols	9	
α-tocopherol		11
β-tocopherol		−1
γ-tocopherol		−1
chlorophylls		4
σ-diphenols	21	22
carotenoids		6
unknown	13	4

[a] Column A shows the initial contribution when olive oil was unwashed. Column B shows the tentative contribution of each chemical variable to virgin olive oil stability.

This percentage, perhaps, corresponds to the nonselected chemical variables (chlorophylls, carotenoids). Possibilities for the unknown variable include carbonyl groups (in the form of an aromatic ester, lactone, or flavone), that are considered essential molecular features required to achieve a high level of antioxidant activity (Dziedzic, 1984), and errors among others.

However, there was still the problem of the high correlation between phenols and *σ*-diphenols. SLRA was again used, but substituting the variable phenols by *σ*-diphenols. The new equation showed the *σ*-diphenol compounds as having a high antioxidant activity, although lower than that of phenols. Thus, the mathematical importance of phenols to stability should be corrected with this second equation. Taking into account the regression coefficients of these equations with stability (0.95 and 0.93) and their contribution coefficients, the contribution calculated was approximately 30% for phenols and 21% for *σ*-diphenols (Table 2, column A). Since the methodology for quantifying *σ*-diphenols also measures the oleosidic forms of 3,4-dihydroxy-phenyl-ethanol (the dialdehydic form of oleanolic acid linked to 3,4-dihydroxyphenylethanol-3,4-DHPEA-EDA and an isomer of oleuropenen aglycon-3,4-DHPEA-EA) and the mean concentration of the hydrophilic phenols in virgin olive oil samples is 99.4 mg/kg of *σ*-diphenols vs 96.7 mg/kg of monophenols (Baldioli et al., 1996), a simple mathematical operation shows that the hypothetical contribution of *σ*-diphenols and monophenols to virgin olive oil stability would be around 21 and 10%, respectively.

At the same time, the results seem to show that the other supposedly antioxidant compounds do not contribute to virgin olive oil stability in the presence of phenols, the main component protecting virgin olive oil against oxidation. After analyzing the data one by one, it is seen that the samples with the highest stability values also have the highest values of oleic/linoleic ratio, the highest contents of total chlorophylls, total carotenoids, and α-tocopherol. On the contrary, some low values of stability coincide with high values of *β*-tocopherol and *γ*-tocopherol.

The fact that, mathematically speaking, the contents of chlorophyll and carotenoid do not seem to contribute to virgin olive oil stability suggested removing the hydrophilic compounds. Samples were washed to remove as much as possible of the content of these hydrophilic compounds. Table 3 shows that, after washing the samples, the reduction in phenols content was around 90−95%, and that in *σ*-diphenols was even greater. The stability values diminished around 50%. Multiplying the initial contribution coefficient of phenols and *σ*-diphenols by their mean reduction percentage, the values (47−49%) correspond to the reduction of the initial stability. This result validates the contribution calculated for phenols using the unwashed samples.

Following an identical mathematical procedure, we detected that the small content of phenols in the washed samples showed a very low antioxidant activity but higher than expected, indicating that they are the most effective in protecting virgin olive oil. However, the process of removing the hydrophilic compounds reduced the correlation between phenols and *σ*-diphenols to 0.45. This meant re-correcting the initial contribution of phenols to virgin olive oil stability, since it was calculated without taking into account the possible contribution of total chlorophylls and carotenoids. Since only the

Table 3. Mean, Minimum, and Maximum Values of Chemical Compounds of Washed Virgin Olive Oils of cv. Hojiblanca and cv. Picual

	Hojiblanca		Picual	
chemical parameter	mean	range	mean	range
stability (h)	23.02	5.90−36.30	40.66	12.30−91.45
oleic/linoleic ratio (%)	8.64	6.15−11.45	21.24	11.79−31.07
chlorophylls (mg/kg)	8.93	0.60−18.69	11.77	2.08−48.50
carotenoids (mg/kg)	6.98	2.53−11.75	8.79	3.76−26.00
α-tocopherol (mg/kg)	184.43	103.40−267.41	204.59	105.60−432.20
β-tocopherol (mg/kg)	1.50	0.95−2.00	1.42	1.00−2.17
γ-tocopherol (mg/kg)	10.49	8.45−13.27	14.07	4.12−19.78
total tocopherols (mg/kg)	196.44	112.80−278.90	220.08	116.76−429.49
phenols (mg/kg)	11.23	0.92−20.99	14.92	1.24−43.70
σ-diphenols (mg/kg)	0.39	0.0−0.88	0.42	0.0−1.01

hydrophilic compounds had been (almost totally) removed, this experiment was used to assign the unknown contribution to stability (13%, Table 2 column A) partially to chlorophylls and carotenoids. Table 2, column B, shows the tentative results of assigning the contribution of α-, β-, and γ-tocopherols to stability. The total contribution of tocopherols is unchanged (except by decimals), since β- and γ-tocopherols seem to have no effect (percentages less than 1%) in terms of favoring virgin olive stability.

These results are only tentative, as we have not removed the chemical compounds one by one and then analyzed stability over time. Furthermore, the varieties have shown some differences apparently concerning certain chemical variables. Thus, the effect of chlorophylls in cv. Hojiblanca is quite different from that in cv. Picual, and this difference in behavior is increased with the synergy effect between chlorophylls and carotenoids.

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