JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Direct Spectrophotometric Determination of Bitterness in Virgin Olive Oil without Prior Isolation by pH Gradient

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Bitter taste, an organoleptic characteristic of virgin olive oil, has been related to phenolic compound composition. The usual method to assess this attribute is by a sensorial panel of tasters, while in the laboratory; methods based on physicochemical properties have been assayed as K_{225}, the most widely used one. However, a direct determination of bitterness in virgin olive oil is useful for quality-control purposes. The proposed method is supported by the observable spectral change undergone by the compounds responsible for bitterness as pH varied. This measurement was carried out directly in the oil, without prior isolation of bitter analytes. The difference of absorbance between alkaline and neutral medium showed a highly significant correlation ($r = 0.988$, $p < 0.0001$) with the conventional parameter (K_{225}). The method was rapid, required a small sample, allowed direct determination of bitterness in virgin olive oil, and could be easily automated.

KEYWORDS: Bitterness; phenolic compounds; virgin olive oil; spectrophotometric determination; pH gradient

INTRODUCTION

Olive oil is an integral ingredient of the Mediterranean diet and accumulating evidence of its health benefits includes the reduction of risk factors of coronary heart disease, the prevention of several types of cancers, and changes in the immune and inflammatory responses (*1*−*3*). In this respect, converging evidence indicates that such beneficial effects are related not only to the elevated oleic acid content but also to the high level of antioxidants in the nonsaponifiable fraction, including phenolic compounds, absent in seed oil (*4*−*6*).

Phenolic compounds have been related to the intensity of bitterness, an appreciated organoleptic characteristic of virgin olive oils (*7*−*9*), although a high intensity of this attribute can be rejected by the consumers even though the oil contains nutritional components or has a long shelf life. The intensity of the bitterness of olive oil has been linked to the presence of phenolic compounds derived from the hydrolysis of oleuropein, which during oil extraction leads to its aglycon, named secoiridoid derivatives of phenols (*10*).

The usual method to assess the bitter taste of olive oil is by a sensorial panel of tasters (*11*). However, an analytical panel is not likely to be available, because a permanent staff of trained tasters and a highly specialized panel chief are necessary. For this reason, methods for evaluating the bitterness level on the basis of physicochemical determinations would be useful for the industry. In this sense, using the relationship between these sensory attributes and phenolic compounds of olive oils, Gutiérrez et al. (*8*) proposed the use of the absorbance at 225 nm of the bitter-constituent fraction for the evaluation of the bitter taste. The bitter fraction was isolated from olive oil by solid-phase extraction (SPE) using C_{18}-phase cartridges and eluting with a mixture of methanol/water (30:70, v/v) and later analyzing by reversed-phase HPLC. A good relationship with the bitterness evaluated by an analytical panel was found. Later, a method to quantify the intensity of the bitterness of virgin olive oil was developed through isolation of the bitter extract by SPE using a C_{18} column and measurement of the absorbance at 225 nm, which showed a significant correlation with the intensity of bitterness that had been evaluated in a sensorial manner by a panel (*12*). This determination was converted into a routine analysis known as the K_{225} of olive oil. Moreover, this manual analytical determination has been automated by the use of a flow injection analysis system (*13*) and the use of robotized stations (*14*).

However, there is a constant need to improve these methods, to shorten the time required for the analysis, and to adapt it to routine analysis by direct measurement of bitterness without an isolation step, as occurs in the above determinations.

In this sense, the shift undergone by the spectral profile of phenolic compounds with pH gradient is well-known. The effect of pH on anthocyanin pigments (*15*−*19*) is well documented, as are those of other phenolic compounds such as phenol and various nitrophenols (*20*), benzoic and sorbic acids (*21*), vanillin and ethylvanillin (*22*), or gallic acid (*23*, *24*).

The aim of the present study was to establish a quick, accurate, and precise instrumental method to evaluate the bitterness of virgin olive oil without prior isolation of the bitter

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10.1021/jf051709y CCC: $30.25
Published on Web 11/05/2005

extract from virgin olive oil. Because oil solutions present acid−base properties that provide observable change in spectra with pH gradient, a direct analysis in oils and a significant relationship with bitterness were well established. The instrumental method requires only a small amount of virgin olive oil and is easily automated.

MATERIALS AND METHODS

Virgin Olive Oil (VOO) Samples. A total of 25 VOO were extracted from Picual olive variety from Spain in the laboratory using an Abencor oil mill (Abengoa, Spain), composed of a hammer mill, a thermobeater, and a vertical centrifuge. Olive paste was kneaded at 28 °C for 30 min (*25*), and the oil was collected by centrifugation of the paste. After decanting for 30 min, the oil samples were filtered and then stored at 4 °C until analysis. Six VOO of Picual and Arbequina varieties characterized by high and low stability, respectively, were used to validate the proposed method.

A lampante virgin olive oil (LVOO) with high acidity [4.32% expressed in oleic acid, according to quality criteria of International Olive Oil Council (*26*)] was used to evaluate the influence of free acidity of the oil.

Analytical Materials and Reagents. All solvents and reagents were of analytical grade unless otherwise stated, and doubly distilled water was used throughout.

Sodium hydroxide, 1-propanol, *n*-hexane, methanol, and phosphoric, acetic, and boric acid were from Panreac (Barcelona, Spain). SPE cartridges packed with diol phase (3 mL) were from Supelco (Bellefonte, PA), and SPE cartridges packed with C_{18} phase (6 mL) were from J. T. Baker (Phillipsburg, NJ).

Britton−Robinson acid buffer (BR-buffer) solution was prepared to provide a final concentration of 5×10^{-3} M phosphoric acid, 5×10^{-3} M boric acid, and 5×10^{-3} M acetic acid. The solution prepared in this way had a pH of 2.12. The BR-buffer of higher pH values was obtained by adding sodium hydroxide (0.6%) to acid BR-buffer (*27*). The pH of each buffer solution was measured with a CyberScan 2100 pH-meter (Eutech Instruments, Singapore).

Apparatus. Spectrophotometric measurements were made using a spectrophotometer system consisting of a deuterium−halogen light source (DH-2000-BAL, Mikropack, Ostfildern, Germany), an HR-200 spectrophotometer (Ocean Optics, Dunedin, FL), and a fiber optics transmission dip probe with 1 cm path length tips. The spectrophotometer was connected to a Pentium III equipped with OOIBase32 software. A Gilson Minipuls 3 peristaltic pump (Middleton, WI) was used as a propelling device.

Analytical Procedures. *Determination of K_{225}.* Bitterness was evaluated by the determination of K_{225} according to the method proposed by Gutiérrez et al. (*12*). A sample (1.00 ± 0.01 g) of VOO was dissolved in 5 mL of hexane and passed over the C_{18} column previously activated with methanol (6 mL) and washed with hexane (6 mL). After elution, 15 mL of hexane was passed to eliminate the fat, and then the retained compounds were eluted with methanol/water (1:1) to 25 mL in a volumetric beaker. The absorbance of the extract was measured at 225 nm against methanol/water (1:1) in a 1 cm cell. The results are expressed at 1% (w/v) concentration level (K_{225}).

pH Influence on Bitter Extract Spectra Obtained from VOO by SPE. Bitter extract from 1.00 g (± 0.01 g) of VOO was obtained by SPE with a C_{18} column according to the K_{225} method. Next, 2.5 mL of BR-buffer (5×10^{-3} M) at different pH values was added to 2.5 mL of bitter extract dissolved in methanol/water (1:1). The pH of the mixture as prepared was checked by a pH-meter, and later the spectra were recorded in the spectrophotometer against methanol/water (1:1)/BR-buffer (1:1) as a reference.

Lipid Matrix Free of Phenolic Compounds. Phenolic compounds were eliminated from VOO by SPE using a diol column. A diol-bonded phase cartridge was placed in a vacuum elution apparatus and conditioned by the consecutive passing of 6 mL of methanol and 6 mL of hexane. A solution of 2.5 g of the oil in 6 mL of *n*-hexane was poured into the column and, when the oil solution was drawn in, two 3 mL portions of hexane were passed. The combined fractions of hexane were evaporated in a rotary evaporator at room temperature under vacuum, and the oily residue was bubbled with a nitrogen stream. The absence of phenolic compound in the resulting lipid matrix was checked by SPE and HPLC analysis with a UV detector (*28*).

Direct Spectrophotometric Determination of Bitterness. In a reaction tube, four drops of VOO (~0.13 g of oil) were dissolved in 13.5 mL of 1-propanol. Then, 1.2 mL of BR-buffer (5×10^{-3} M) at pH 5.0 was added and shaken vigorously until a homogenate solution was obtained. The absorption spectra of each oily solution were recorded continually against a mixture of propanol/BR-buffer (13.5:1.2, v/v) as a blank, between 200 and 1000 nm. The peristaltic pump was used to generate the pH gradient by adding to the reaction tube an aqueous sodium hydroxide (1%) solution. The flow rate of the alkaline solution was 0.05 mL/min, and the mixture was under continuous magnetic stirring. The reaction was followed at 274 nm, where spectra profiles presented a maximum in alkaline medium. At $t = 0$ s the addition of alkaline solution was started. The reaction reached its maximum absorbance value at ~300 s. Simultaneously, the reaction was monitored at 800 nm to compensate for the small absorbance increment owing to the formation of a light turbidity. The final absorbance value at 274 nm was calculated by the following equation: $\Delta A_{274nm} = [A^*_{274nm}(300\ s) - A^*_{274nm}(0\ s)]$, where $A^*_{274nm}(300\ s) = A_{274} - A_{800}$ at 300 s and $A^*_{274nm}(0\ s) = A_{274} - A_{800}$ at 0 s. Results are expressed as means ± standard deviation (SD) of three determinations.

RESULTS AND DISCUSSION

Spectral and pH Behavior of Bitter Extract Obtained by SPE-C_{18}. Spectrophotometric evaluation of VOO bitter extract obtained by SPE using a C_{18} column at different pH values ranging from 2 to 13 showing acid−base properties provided observable changes in the spectra with pH gradient. At pH 2.0, the UV spectrum of the isolated bitter extract presented two major absorption peaks (**Figure 1A**), the first in the region located around 225 nm and the second around 280 nm. When the pH increased from acid to alkaline values, the first UV absorption peak for the isolated extract showed a bathochromic shift from 225 to 240 nm. On the other hand, the second UV absorption peak showed a hypsochromic shift from 280 to 274 nm. After the analysis of spectra evolution of the bitter VOO extract with a gradient pH, a wavelength of 274 nm appeared to be highly adequate to follow the evolution of bitter VOO extracts with pH variations.

Spectral and pH Behavior of VOO. The main objective of this work was to establish an accurate analysis of bitter taste without prior isolation of this fraction from VOO. In this sense, a clear VOO solution with a suitable pH value was important to obtain because the absorbance measurement could be affected by cloudy solutions. For setting the optimal composition of the VOO solution, several amounts of oil were dissolved in various solvents such as butanol, propanol, and tetrahydrofuran. Propanol was selected as VOO dissolvent at a ratio of four drops of oil (~0.13 g) in 13.5 mL of propanol because this provided a miscible mixture. Then, several amounts of BR-buffer at different pH values were added to the VOO alcoholic solution, 1.2 mL proving to be the best amount to obtain a clear solution.

Next, spectra of the VOO mixtures prepared in this way were recorded over the wavelength range of 200−400 nm against mixtures of propanol/BR-buffer without oil as the blank. The resulting spectra appear in **Figure 1B**, where the spectral behavior resembles that of the bitter VOO extract. Although the evolution corresponding to UV absorption peak located around 280 nm exhibited the same hypsochromic shift from 280 to 274 nm as described above in **Figure 1A** corresponding to the bitter VOO extract, small spectral shifts were observed around 225 nm, where a slight increase in the absorption intensity was registered. In view of the fact that the spectra indicate an important rise of absorbance at 274 nm

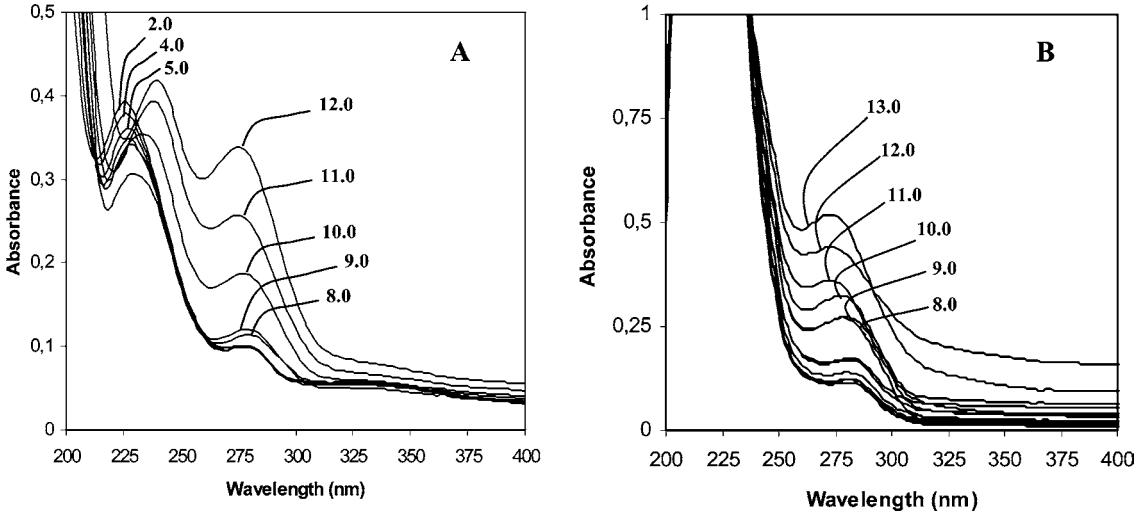

Figure 1. (**A**) UV–visible spectra of the bitter extract, recorded at different pH values ranging from 2.0 to 12.0; (**B**) UV–visible spectra of the virgin olive oil, recorded at different pH values ranging from 2.0 to 13.0.

when the pH approached alkaline values, this wavelength was selected to evaluate the bitterness of VOO. When the spectra ranging from pH 2.0 to pH 7.0 were evaluated, no significant change was detected in the absorbance intensity. For this reason, the alcoholic VOO solution was adjusted to pH 7.0 at the beginning of analysis in order to shorten the analysis time.

Optimization of the Analysis Procedure of Bitterness. The gradient pH was measured by a single channel with 0.5 mm i.d. PTFE tubing, which continuously added sodium hydroxide (by a Gilson Minipuls 3 peristaltic pump) to the reaction tube containing the alcoholic VOO reaction mixture. A very bitter VOO was used to set the final conditions of the analysis, and several parameters such as the sodium hydroxide concentration, BR-buffer concentration, and flow rate were well suited to achieve a reproducible pH gradient and provide absorbance values at 274 nm. All of the variables were optimized as a compromise between the analysis time and minimum turbidity by the univariant method, and the conditions evaluated for the sodium hydroxide concentration, flow rate, and BR-buffer concentration ranged from 1 to 5%, from 0.0125 to 0.2 mL/min, and from 0.005 to 0.0125 M, respectively. The optimal conditions to reach the highest absorbance value at 274 nm were 1% hydroxide concentration, 0.05 mL/min flow rate, and 0.005 M BR-buffer corresponding to 300 s of analysis time (data not shown). A major problem in spectrophotometric analysis is the cloudiness of solutions, because erroneous absorbance values can result. In this sense, a sodium hydroxide concentration of 1% was selected to avoid cloudy solutions promoted by salt insolubility. On the other hand, the BR-buffer was an important factor with regard to solubility of the VOO reaction mixture. In this regard, a low concentration of this buffer gave a clearer VOO solution. Finally, for a good analysis rate without sacrificing the precision of the method, a 0.05 mL/min flow rate was used.

The reaction was followed at 274 nm, where the spectral profile presented a maximum that increased with the pH gradient. The reaction reached the maximum absorbance value at ~300 s (**Figure 2**) corresponding to alkaline pH values. From this time on, the absorbance remained constant several minutes more, but the absorbance finally declined because of the oxidation of phenolic compounds in the alkaline medium. The mixture between the aqueous sodium hydroxide and oily alcoholic solution became slightly cloudy due to high amounts of NaOH added throughout the analysis despite the stirring. To

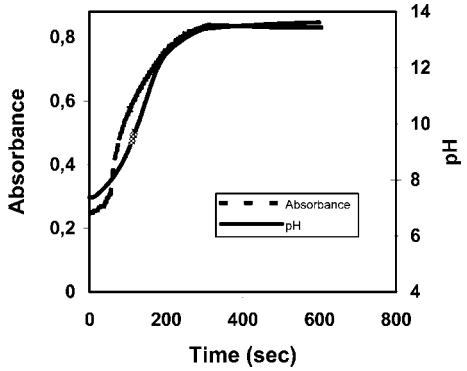

Figure 2. Evolution of absorbance at 274 nm and pH versus time corresponding to very bitter VOO.

subtract the small absorbance increase owing to this slight turbidity, a reference at 800 nm was selected to minimize the possible interference. In this way, the final absorbance value at 274 nm was calculated with the following equation: $\Delta A_{274nm} = [A^*_{274nm}\ (300\ s) - A^*_{274nm}\ (0\ s)]$, where A^*_{274nm} (300 s) corresponds to the difference of absorbance between 274 and 800 nm at 300 s, and A^*_{274nm} (0 s) signifies the difference in absorbance between 274 and 800 nm at 0 s.

Study of Interferences in the Spectrophotometric Analysis. *Behavior of Lipid Matrix without Phenolic Compounds.* To test the interaction of a lipid matrix without phenolic compounds in absorbance measurements at 274 nm, the lipid matrix was obtained from a VOO by purification through SPE using a diol column to remove phenolic compounds. The matrices were spectrophotometrically evaluated and ΔA_{274nm} values found. The result (0.158 ± 0.008) showed certain response values corresponding to the lipid matrix. This value agrees with the intercept of the linear regression between K_{225} and ΔA_{274nm} (0.17 ± 0.01, see below), and no interference was detected with the lipid matrix.

Effect of α*-Tocopherol.* Other parameters were studied to check that absorbance change at 274 nm corresponded only to bitter constituents of VOO. Also, α-tocopherol is an important minority component from VOO, with antioxidant properties owing to its phenolic group, and its possible interaction at 274 nm was evaluated. No other tocopherols were studied because α-tocopherol usually represents 95% of total tocopherol in VOO (*29*).

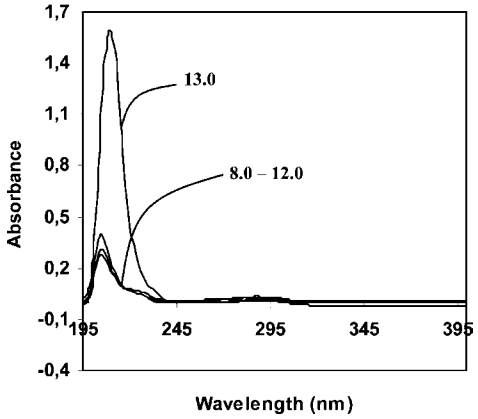

Figure 3. UV–visible spectra of α-tocopherol (3 ppm) in a mixture of propanol/BR buffer at different pH values ranging from 8.0 to 13.0.

Given that the usual tocopherol concentration in VOO ranges from 200 to 300 ppm and the sample concentrations assayed in the proposed method were below 1%, the actual tocopherol concentration in the reaction medium was ~2−3 ppm. In this sense, a solution of α-tocopherol in a mixture of propanol/BR-buffer at different pH values was prepared at 3 ppm, and its influence in the spectrophotometric measurement was evaluated. The spectra ranging from 200 to 400 nm at pH values from 8.0 to 13.0 are represented in **Figure 3** to evaluate possible interference of this compound with the spectrophotometric determination. As can be seen, no change occurred in absorbance values over the pH gradient at 274 nm, and therefore its influence can be considered to be negligible versus the absorbance corresponding to VOO bitterness at 274 nm.

Effect of Free Acidity in the Olive Oil. VOO are classified in four categories, according to their quality. This quality is evaluated, on the basis of several physicochemical parameters, by a panel of tasters. The free acidity (expressed as the percent of oleic acid) is the most important classification parameter. According to the free acidity, the virgin oils are classified as follows: ≤0.8%, extra virgin olive oil (EVOO); 0.8−2%, virgin olive oil (VOO); 2−3.3%, ordinary virgin olive oil (OVOO); and >3.3%, lampante virgin olive oil (LVOO). The latter is not suitable for direction consumption and must be refined before being used in food.

The evaluation of bitterness is of interest only in the case of oils with free acidity lower than 3.3%, as the rest of the oils are subjected to refining, a process that eliminates all of the sensorial attributes of virgin oils. Both EVOO and VOO present low acidity (≤0.8 and ≤2.0%, expressed in oleic acid, respectively), according to quality criteria of International Olive Oil Council (*26*).

Given that the basis for the method proposed is a change in the UV spectrum due to the variation in the mean pH, the presence of free fatty acids could interfere if these prevent the pH from reaching the desired pH value at the moment of final measurement (t = 300 s). For evaluation of the degree of interference of acidity in the spectrophotometric measurement at 274 nm, LVOO with a free-acidity value of 4.32% was selected to analyze its performance in the method proposed. The LVOO was dissolved in a mixture of propanol/BR-buffer and submitted to the overall procedure. The pH was monitored in comparison with that corresponding to an EVOO, and the results indicated a slightly slower rise in the pH between values of 7.0 and 11.0, as a consequence of the partial neutralization of the alkaline solution added to the reaction tube. Afterward (to $t \geq 280$ s), equivalent pH values were reached, regardless of the acidity of the oil studied, and therefore it could be determined that the acidity of the oil did not interfere with the determination.

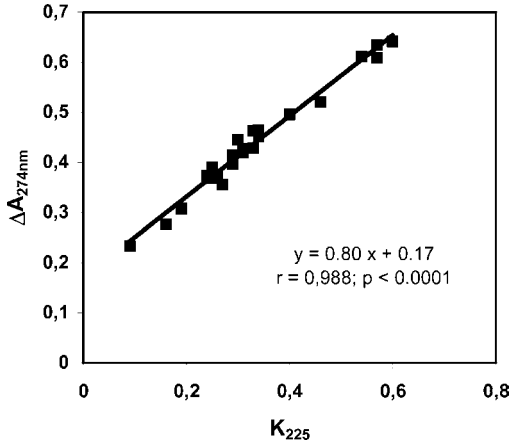

Figure 4. Linear regression analysis of data for the relationship between $\Delta A_{274nm} = [A_{274nm}\ (300\ s) - A_{274nm}\ (0\ s)]$ and K_{225} and for several types of virgin olive oils. The regression equation is $\Delta A_{274nm} = b(K_{225}) + a$, where b is the slope and a is the intercept; r represents the linear regression coefficient and P the probability that $r = 0$.

Table 1. Predicted and Reference Values of K_{225} for the Set of Samples Analyzed

sample	K_{225} (ref)[a]	ΔA_{274nm}	K_{225} (pred)[b]
1	0.25 ± 0.00	0.381 ± 0.002	0.26 ± 0.02
2	0.18 ± 0.01	0.302 ± 0.001	0.17 ± 0.01
3	0.31 ± 0.02	0.42 ± 0.01	0.31 ± 0.02
4	0.15 ± 0.01	0.29 ± 0.01	0.15 ± 0.02
5	0.47 ± 0.01	0.541 ± 0.002	0.46 ± 0.02
6	0.52 ± 0.02	0.582 ± 0.003	0.52 ± 0.02

[a] Reference K_{225}. [b] Predicted K_{225}.

On the other hand, different mixtures of LVOO with a very bitter VOO were prepared in the ratios of 3:1, 2:2, and 1:3 (LVOO/VOO), and their actual value of K_{225} was determined. These mixtures were submitted to the overall procedure, and the ΔA_{274nm} agreed with the one calculated from the linear regression between K_{225} and ΔA_{274nm} (see below) (data not shown). Again, it can be concluded that no influence in the absorbance values due to the pH gradient at 274 nm was observed in matrices with different ratios of acidity.

Validation. Because oils with different contents of bitter compounds present acid-based properties that provide observable changes in the spectra with the pH gradient, several VOO were submitted to the overall procedure, and the direct spectrophotometric measurement was made. The response of ΔA_{274nm} was checked by linear regression analysis in the K_{225} range assayed. The response was linear in the K_{225} range evaluated, from 0.09 to 0.60, giving an equation of $y = 0.80x + 0.17$ ($n = 24$), where y is ΔA_{274nm} and x is K_{225}. A linear regression coefficient of $r = 0.988$ ($p < 0.0001$) resulted (**Figure 4**). This equation was used to quantify K_{225} in VOO.

The precision corresponding to ΔA_{274nm}, expressed as the coefficient of variation (CV), ranged from 0.30 to 6.23% for the overall VOO analyzed, indicating that the analytical method is repeatable.

Six new VOO samples were submitted to the overall procedure, and the predicted and reference values of K_{225} obtained for the set of samples analyzed are summarized in **Table 1**. As can be seen, there is good agreement between the predicted and reference values of K_{225}.

In summary, the procedure described here allows direct spectrophotometric determination of the bitterness in virgin olive oils. The method is based on the absorbance rise observed at 274 nm when the pH of the solution increases toward an alkaline medium. The method proposed eliminates the need for prior isolation of the analytes from the lipid matrix, it requires less use of solvents and a lower quantity of sample, and it is quicker and easily automated, presenting satisfactory sensitivity, accuracy, and precision.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of Maite Ruano with the oil analyses.

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Received for review July 16, 2005. Revised manuscript received October 4, 2005. Accepted October 6, 2005.

JF051709Y