A Rapid Spectrophotometric Method for the Determination of Peroxide Value in Food Lipids with High Carotenoid Content

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ABSTRACT: A rapid and sensitive ultraviolet-visible spectrophotometric method for determination of peroxide value (PV) in foods with high carotenoid content (e.g., paprika oleoresin, paprika powder, red palm oil) has been developed. The proposed protocol [modified International Dairy Federation (IDF) method] was established from the IDF Fe(II)-oxidation-based spectrophotometric method, and the main one of the introduced modifications consisted of a clean-up extraction step of pigments before determining the PV by complexing Fe(III) ions with thiocyanate. Fe(II) oxidation time, reaction medium, and Fe(III)-thiocyanate complex formation time were optimized. The modified IDF method was compared with and was validated by iodometric AOAC official method with a good correlation (R^2 = 0.957) between data obtained by both analytical methods. The high sensitivity of the method allows the use of only about 0.010–0.015 g of sample, with a detection limit of 0.044 mequiv peroxide/kg of sample. Therefore, an improved spectrophotometric method for assessing PV in food lipids with high carotenoid content is now available and can be applied to any kind of sample, independent of oil and pigment content.

Paper no. J9982 in *JAOCS 78*, 1151–1155 (November 2001).

KEY WORDS: Carotenoid, iron thiocyanate complex, lipid oxidation, oil, oleoresin, paprika, peroxide value (PV), UV-visible spectroscopy.

Peroxidation of the lipids in fresh and processed foods is one of the main causes of deterioration, reduced stability, and formation of off-flavors that negatively affect quality and storage life of food product and its consumer acceptance (1–3). The radical species formed in the peroxidation process degrade fatty acids and other components of the lipid fraction, such as carotenoids, chlorophyll pigments, and tocopherols. When these natural antioxidants are present, peroxidation is delayed, so they (and other artificial ones) are commonly added to fats and oils to reduce alteration during processing and storage (4). Degradative processes are a problem to be avoided during the extraction, transport, and storage of fats and oils, and they are currently of concern with the increased popularity of polyunsaturated oils and margarines, which are more prone to such reactions (5). The peroxide palue (PV) is widely used as a measurement of the extent of these unwanted reactions not only in foodstuffs but also in biological samples, where such reactions are implicated in physiological processes related to the initiation of tumors and other degenerative diseases, alteration of the cell membrane structure, and the modification of DNA and proteins (6,7).

Spectrophotometric methods using the ultraviolet (UV)-visible range are currently those most widely used to determine PV in food lipids (such as oils, margarines, and butters), commonly using the oxidation of Fe(II) to Fe(III) ions that, once formed, can react with various reagents producing colored complexes. These complexes include the Fe(III)-thiocyanate in the International Dairy Federation (IDF) method and Fe(III)-xylenol orange in the ferrous oxidation-xylenol orange (FOX) methods (8–11). Such complexes absorb in the 400–600 nm wavelength range, and measurement is normally performed at wavelengths close to the absorption maximum. These methods, together with others using detection in the infrared region (12,13), are displacing classical volumetric methods such as iodometry (14). *A priori*, spectrophotometric determination has no disadvantages, except when the foodstuff contains compounds that absorb naturally in this measurement range, such as the carotenoid pigments.

In oils whose color is due to carotenoids (e.g., unrefined soybean and palm oils, and paprika oleoresin), in food lipids that contain carotenoids (e.g., butters or margarines enriched in β-carotene), and in other products derived from carotenoid-rich plants (e.g., concentrates and juices of tomato and carrot, pepper paste, and paprika), direct application of any of the above-mentioned methods generates an erroneous measurement, since none takes into account the presence and interference of pigments that absorb in the wavelength region used to determine PV. The color supplied by the carotenoids is the main quality index of the product. Thus, because the carotenoid fraction may also be degraded *via* lipid peroxidation processes (15,16), the determination of PV in fat-rich foods containing carotenoids may also provide information about color stability.

The present study is a response to the need to assess PV in food lipids that are rich in natural colored substances. Its main aim is the development of a method for the determination of PV, particularly for material with a high content of carotenoid pigments. The starting point is the standard method of the IDF (8), which uses ammonium thiocyanate as Fe(III)-complexing agent. Several modifications are introduced to enable the determination

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to be carried out without interference from the carotenoid fraction. The proposed method can be applied to all types of food lipids (liquids and solids), independently of their color.

MATERIALS AND METHODS

Samples. Paprika oleoresins and paprika powders from different sources were used for the present study as they are oily matrices with very high carotenoid contents. Other vegetable oils (corn, sunflower, olive, palm, and soybean) with lower carotenoid content were also used to develop the method. A set of 25 different olive oil samples was used for validation of the method against the standard iodometric AOAC method (14).

Sample preparation. Oleoresins and oils were used directly. For paprika powders and any other solid sample, oil was extracted by means of Soxhlet extraction; 10–15 g of sample was extracted with 75 mL of *n*-hexane for 4 h, followed by solvent removal under reduced pressure at 30°C (rotary evaporator).

Iodometric determination of PV. The iodometric AOAC method 965.33 (14) was used for the determination of PV of the olive oil samples used for validation. For the analysis, 0.3 g of oil was used and titrated with 0.001 N sodium thiosulfate solution.

IDF modified method for determination of PV. The proposed method was developed as a modification of the IDF standard method (8,9) that is based on the co-oxidation of Fe(II) to Fe(III) by hydroperoxides and the formation of the reddish Fe(III)-thiocyanate complex for spectrophotometric determination of PV.

To quantify the PV, the sample (0.01–0.05 g) was placed in a 10-mL screw-capped test tube and dissolved in 1 mL of chloroform/acetic acid (2:3), with addition of 100 μL Fe(II) solution, mixed for 15 s on a Vortex mixer (Heidolph, Schwabach, Germany), and left in the dark for 10 min. Deionized water (2 mL) (MilliQ®) was added, and 4 mL of diethyl ether [containing *ca.* 7 ppm butylated hydroxytoluene (BHT)] was used for pigment and oil extraction. Organic phase was discarded, and remaining ether in the aqueous phase was removed under N_2 current for a few seconds. To determine Fe(III), 1 mL of the aqueous phase was transferred to a disposable plastic microfuge tube and mixed with 100 μL of saturated ammonium thiocyanate solution. After 10 min, absorbance at 470 nm was measured against a water blank (correction of the spectrum baseline at 670 nm was performed). A reaction blank containing all the reagents, except the sample, was also performed, and the resulting absorbance value was subtracted from that of the sample.

Fe(II) stock solution was prepared by gently mixing a solution of 0.4 g of $BaCl_2 \cdot 2H_2O$ in 50 mL deionized water with a solution of 0.5 g of $FeSO_4 \cdot 7H_2O$ in 50 mL deionized water. Concentrated hydrochloride acid (2 mL) was added to the resulting solution, which was filtered and stored under cover. It is strongly recommended to prepare this solution as fresh as possible and check its stage before use by addition of a few drops of thiocyanate solution. Solution should be discarded if a pale pink color appears.

Fe(III) calibration. A working solution of Fe(III) 10.4 μg/mL was prepared from a standard stock solution [1040 μg Fe(III)/mL with 1% HCl, obtained from Sigma Ref. I-9011 (St. Louis, MO)] by dilution with chloroform/acetic acid (2:3). For calibration, a set of solutions of increasing Fe(III) concentration in the range 0–10 μg/mL was prepared by successive dilutions of the working solution. The modified IDF method was applied to 1 mL of each solution. The calibration curve was obtained by plotting absorbance (Abs 470–670 nm) vs. Fe(III) concentration.

Apparatus. UV-visible spectra were collected using a Hewlett-Packard UV-vis diode array spectrophotometer model 8452A (Palo Alto, CA) and a 1-cm path quartz cuvette. Data acquisition was carried out with UV-Visible ChemStation Rev. A.02.05 (Hewlett-Packard).

Statistical analysis. All determinations were run on triplicate samples, except to obtain repeatability (six runs). Statistical tests (mean, standard errors) were performed with a significance level $P < 0.05$ using Statistic software version 5.1 for Windows (StatSoft, Inc., Tulsa, OK). Method validation was carried out using the Passing and Bablok regression (17) which was run using the Method Validator software version 1.1.9.0 for Windows (Dr. P. Marquis, Metz, France).

RESULTS AND DISCUSSION

Figure 1A shows the overlap of the Fe(III)-thiocyanate complex and paprika carotenoid fraction spectra, which is the main reason why current spectrophotometric methods (IDF, FOX, modified FOX) (8,9,11) cannot be adequately employed when analyzing samples with high carotenoid content in the lipid matrix. This problem makes it necessary to modify an existing method so that PV can be determined in those samples that are initially outside the normal range of application.

The original approach was to modify one of the spectrophotometric methods, as these are quick and precise and require a small amount of sample. The IDF method (8) was chosen as the starting point. This method uses chloroform/methanol (7:3) as reaction medium in which the sample is dissolved and the oxidation of Fe(II) verified. However, as the complexes of the ferric ion are more stable in acid medium, modification of the reaction medium composition was studied to provide a lipophilic medium allowing solution of the sample while supplying an acid medium for the reaction. After various tests, the mixture chloroform/acetic acid (2:3), also used in the iodometric evaluation of PV (14), was found appropriate. Sensitivity of the method increased *ca.* 2.5-fold when the Fe(II) oxidation reaction was carried out in acid medium.

In the case of pigment-rich sources such as paprika oleoresins, an initial attempt was made to avoid their interference in the measurement using a blank containing the sample as reference and then applying the method and determining the PV. Even so, the resulting spectrum of the Fe(III)-thiocyanate complex still had interference and was mostly saturated (Fig. 1B). This meant that the measurement would have to be carried out at wavelengths far from the absorption maximum, with a consequent loss of sensitivity.

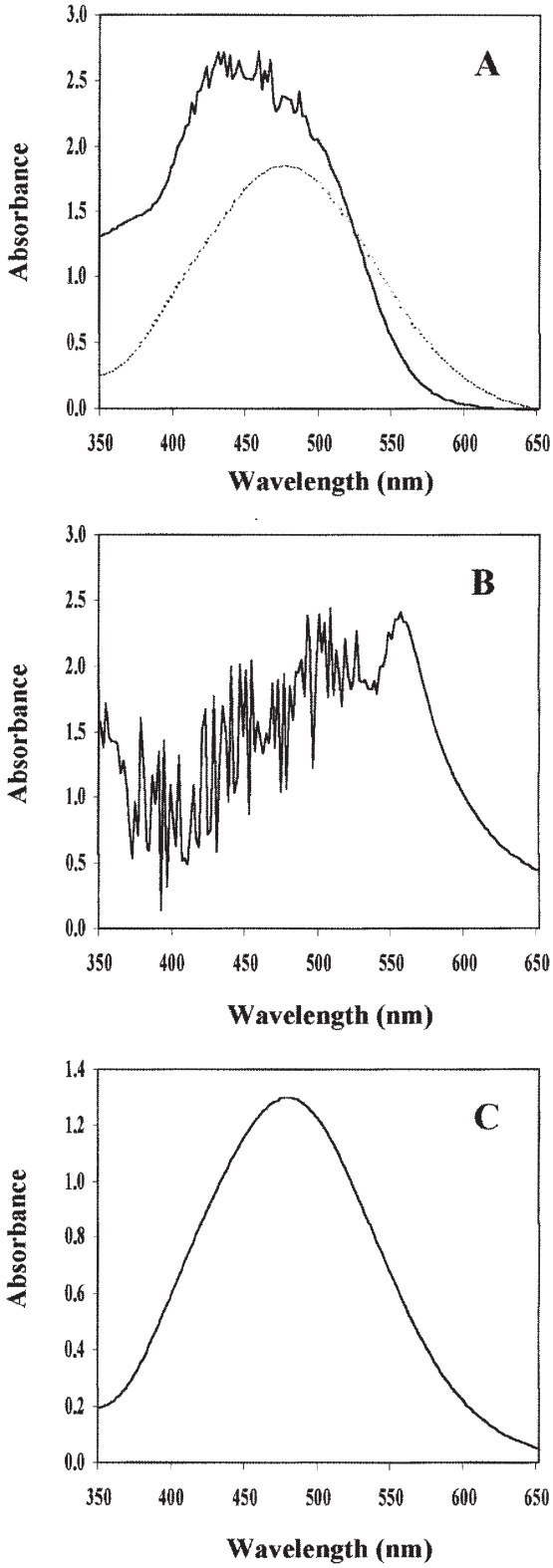

FIG. 1. Ultraviolet-visible spectra of (A) paprika oleoresin sample and Fe(III)-thiocyanate complex (dotted line) in chloroform/acetic acid (2:3); (B) Fe(III)-thiocyanate complex added to a paprika oleoresin sample vs. a blank consisting of the same sample in chloroform/acetic acid (2:3); (C) Fe(III)-thiocyanate complex in the modified International Dairy Foundation (IDF) aqueous phase after elimination of the paprika oleoresin carotenoids.

Because the carotenoid content formed the main interference, extraction of the pigments with organic solvent before performing the spectrophotometric measurement of PV was necessary. The strategy was to carry out the Fe(II) oxidation in the presence of the sample dissolved in chloroform/acetic acid (2:3), following separation and discarding the organic phase containing the coloration due to the carotenoid pigments, and finally evaluating in the aqueous phase the Fe(III) formed as Fe(III)-thiocyanate complex. The reaction volume was 1.1 mL, consisting of 1 mL of chloroform/acetic acid (2:3) containing the sample (0.010–0.015 g) and 100 μL of fresh aqueous Fe(II) solution. This volume ratio allows complete miscibility of the Fe solution and chloroform. Once the oxidation reaction is finished, the pigment cleanup process is performed. Among common organic solvents used for pigment cleanup, diethyl ether was chosen on the basis of its excellent color-extracting capacity and low miscibility with water. BHT was added (7–10 ppm) to prevent the collateral formation of peroxides. Two milliliters of water were added to the reaction medium together with *ca.* 4–5 mL of diethyl ether. That extraction solvent amount was sufficient for the cleanup in samples of low or moderate pigment concentration, while samples of paprika oleoresins required up to three or four successive extractions. A similar number of extractions for complete color removal should be applicable for other samples containing high levels of carotenoids. As the Fe(III)-thiocyanate complex is soluble in ether, its formation must be verified when the extraction is completed. Complexing reaction was performed by adding 100 μL of saturated solution of ammonium thiocyanate to 1 mL of the aqueous phase. The absorption spectrum of the Fe(III)-thiocyanate complex formed is now free of interference from the lipid matrix and presents an absorption maximum around 470 nm (Fig. 1C). The reaction time for effective oxidation of Fe(II) in contact with the peroxides of the sample was fixed at 10 min, similar to that of the IDF and FOX methods (8,10). Longer reaction times were tested, with no significant differences being found in the estimated PV. Time-course of the Fe(III)-thiocyanate complex reaction was also investigated (Fig. 2), and the optimal time for complexing during routine analyses was fixed at 10 min.

As already mentioned in the Materials and Methods section, samples of oils and oleoresins do not need the pre-analysis preparation required in the case of paprika and other solid samples. This preparation is basically the extraction of the lipid fraction in which PV is subsequently determined. Maceration extraction was tested with various solvents: *n*-hexane, diethyl ether, chloroform/acetic acid (2:3), chloroform/ methanol (7:3), and others (9). In all cases, the subsequent determination of PV in the extracted lipid phase gave unsatisfactory results, probably due to incomplete extraction of the peroxides. Finally, a most vigorous extraction procedure was chosen, using *n*-hexane in Soxhlet for 4 h, starting from *ca.* 10 g of sample, as standard procedure to obtain an oily concentrate (oleoresin) for PV determination. It was checked that this extraction procedure did not induce peroxide formation in the sample.

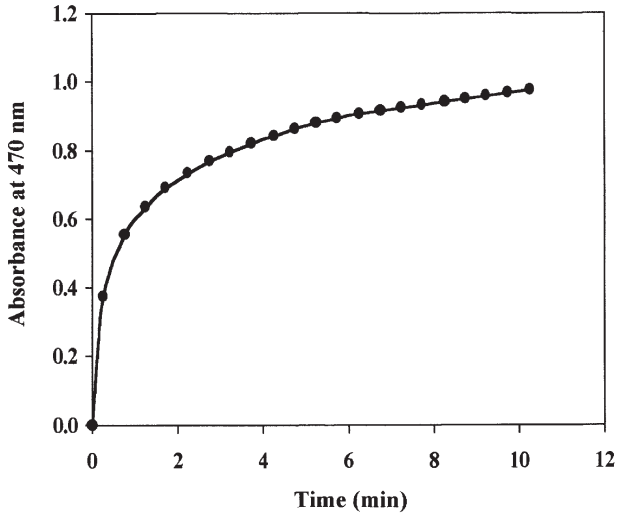

FIG. 2. Time-course of the Fe(III)-thiocyanate complex formation.

Figure 3 shows the calibration plot with a slope (m) of 0.0881 and a correlation coefficient of $R^2 = 0.9942$, which shows good linearity over the concentration range used. The m value must be taken into account for obtaining the PV of the sample using Equation 1:

$$\text{PV (mequiv peroxide/kg of sample)} = \frac{(A_{sm} - A_{bl})}{55.84 \times 2 \times m \times W_{sm}} \quad [1]$$

where A_{sm} is the absorbance of the sample at 470 nm; A_{bl} is the absorbance of the blank at 470 nm (both absorbances were corrected subtracting absorbance at 670 nm); m is the slope of the Fe(III) calibration plot; 55.84 is the atomic weight of Fe; 2 is the factor to convert milliequivalents (mequiv) of Fe to mequiv of peroxide; and W_{sm} is the sample weight in grams. In the case of solid samples, both the weight of sample extracted initially by Soxhlet and the weight of oil obtained must be taken into account. If a high PV is expected, the sample should be diluted appropriately in chloroform/acetic acid (2:3); this dilution factor is introduced into the sample absorbance term (A_{sm}). Although the linearity of the calibration in the concentration range used has been checked, the problem samples should be analyzed together with a standard sample of Fe(III) of intermediate concentration (5 μg/mL) and the m value recalculated in each case to minimize variations in the results due to the measurement conditions, state of the reagents, etc.

For the calculation of the limit of detection, six determinations without sample (blanks) were performed to obtain a mean absorbance of the blank (A_{bl}) and its standard deviation. The limit of detection was established as 0.044 mequiv peroxide/kg of sample, defined as the amount of analyte originating a value of absorbance equal to that of the blank plus three times its standard deviation (18).

The modified IDF method was validated against the AOAC official method (14). Validation was performed using 25 olive oil samples obtained from different olive varieties and with different storage times in order to get PV over a wide range. Olive oil samples were chosen because their generally low carotenoid content allows application of the iodometric method, which is not possible in the case of oleoresin and paprika. Samples were analyzed using both methods, and data were correlated using the Passing and Bablok agreement test (17). Figure 4 shows the mean PV values for each sample obtained by the two methods.

The following regression equation (Eq. 2), with a correlation coefficient of 0.957 and a standard deviation of 0.017, was obtained:

$$PV_{\text{modified IDF}} = 1.027 \times PV_{AOAC} - 3.568 \quad [2]$$

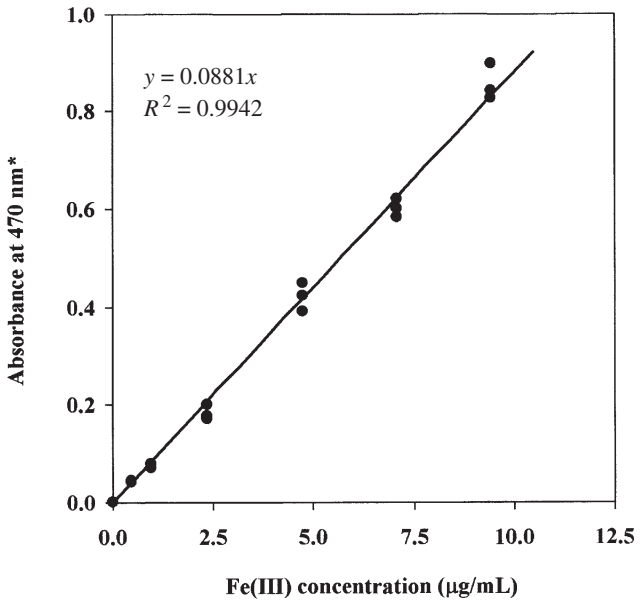

FIG. 3. Standard Fe(III) calibration plot for the quantification of peroxide value (PV) by the modified IDF method. See Figure 1 for abbreviation. *Absorbances were corrected by subtracting absorbance at 670 nm.

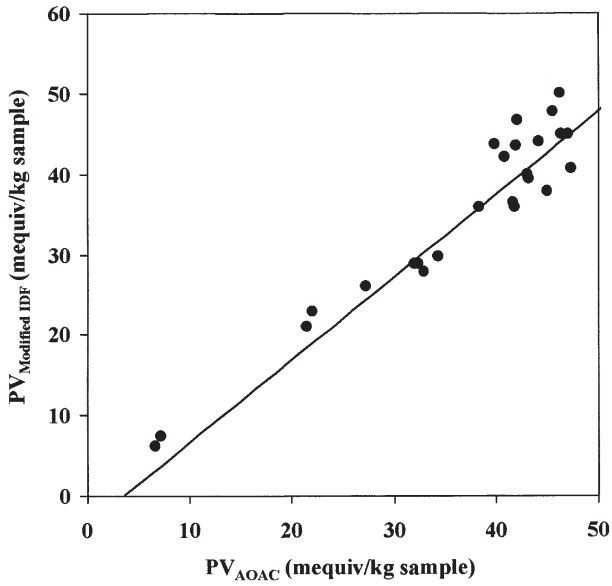

FIG. 4. Validation plot of modified IDF-method PV vs. AOAC official-method PV. The regression curve was obtained using the Passing and Bablok (17) agreement test. See Figures 1 and 3 for abbreviations.

TABLE 1
Peroxide Value (PV) of Paprika and Paprika Oleoresin Samples Determined by the Modified IDF Method

Sample	PV[a] (mequiv peroxide/kg)
Paprika 1	1.06 ± 0.19
Paprika 2	1.18 ± 0.20
Paprika 3	0.83 ± 0.19
Paprika 4	0.51 ± 0.08
Oleoresin 1	2.89 ± 0.51
Oleoresin 2	2.34 ± 0.39
Oleoresin 3	2.74 ± 1.30
Oleoresin 4	0.66 ± 0.49

[a]Mean value ± standard deviation ($n = 3$, $P < 0.05$). IDF, International Dairy Federation; mequiv, milliequivalent.

As defined in the method of Passing and Bablok (17), if the confidence intervals (for $P < 0.05$) of the intercept and slope contain the values 0 and 1, respectively, the data originated by the two methods are comparable and analogous. That is so in the present case.

Table 1 gives the PV values obtained for different samples of paprika and oleoresin. It can be observed that PV is higher in the oleoresins than in the paprikas, although lower than in the oil samples (Fig. 4). As a complementary experiment, different samples of vegetable oils were stored for 30 d at 25°C, and PV was determined at the start and end of the experiment. Table 2 shows the results obtained. In all cases, PV increased significantly, particularly in the paprika oleoresin. As shown in previous studies (19), lipid fraction in paprika is rich in polyunsaturated fatty acids which together with the presence of carotenoids susceptible to peroxidation could be the factors determining this greater increase in the PV of the oleoresin. This tendency toward lipid peroxidation denotes the need for a method applicable to such samples, which until now has not been adequately studied. The proposed method is quick, sensitive, and reliable, and is readily available and applicable to all types of fat samples, independently of their pigment content.

ACKNOWLEDGMENT

We express our sincere gratitude to MCYT (Ministerio de Ciencia y Tecnología) for the financial support of this research work (Project AGL2000-0699).

TABLE 2
PV of Different Oils. Initial Values and After 30 Storage Days at 25°C

Oil sample	PV[a] (mequiv peroxide/kg) 0 d	30 d
Olive	9.18 ± 0.74	15.47 ± 0.38
Soybean	9.25 ± 0.77	12.62 ± 0.26
Corn	22.79 ± 2.49	24.65 ± 1.61
Sunflower	18.21 ± 0.65	23.47 ± 1.65
Paprika oleoresin	0.29 ± 0.02	6.79 ± 0.84

[a]Mean value ± standard deviation ($n = 3$, $P < 0.05$). See Table 1 for abbreviations.

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[Received May 17, 2001; accepted August 24, 2001]