Direct Measurement of Lipid Peroxidation in Oil-in-Water Emulsions Using Multiwavelength Derivative UV-Spectroscopy

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Using second-derivative multiwavelength UV-spectral data (200−300 nm), it was possible to measure lipid peroxidation in oil-in-water lipid emulsions using a multivariate calibration method (partial least squares regression). For a linoleic acid emulsion (0.42 mM in phosphate buffer, pH 5.5−7.0), with metmyoglobin or ferrylmyoglobin as initiator, conjugated dienes could not be quantified by direct single-wavelength absorption spectroscopy (234, 244, or 280 nm) when compared to a reference method based on ether extraction and second-derivative spectroscopy at 234 nm. Direct multiwavelength absorption (200−300 nm) and second-derivative methods (234, 244, 280 nm, and 200−300 nm) provided superior results with high correlation coefficients (r ranged from 0.78 to 0.98) when compared to the standard extraction method. The multiwavelength second-derivative method (200−300 nm) gave the highest correlation and is recommended for on-line control of lipid peroxidation in heterogeneous systems.

Keywords: *Lipid peroxidation; on-line detection; second derivative spectroscopy; multivariate calibration*

INTRODUCTION

Lipid peroxidation is responsible for food quality deterioration, and increasing efforts have been devoted to development of methods to detect and quantify lipid peroxidation in model systems as well as in food systems. Today, numerous methods are available but only a few give the direct information about initial processes in lipid peroxidation, which is highly relevant for shelf-life prediction. Determination of thiobarbituric acid reactive substances (TBARS) (Yagi, 1982), determination of lipid peroxides (Asakawa and Matsushita, 1980; Jiang *et al.*, 1992), fluorescence measurement (Chio and Tappel, 1969), and detection of volatile compounds (e.g., hexanal) (Frankel and Tappel, 1991; Frankel *et al.*, 1994) represent the most widely used methods in measurement of lipid peroxidation. Most of these methods measure secondary oxidation products and give little information about initial lipid peroxidation. Measurement of oxygen consumption (Le Tutour, 1990; Le Tutour and Guedon, 1992) has also been extensively used, but this method is not always appropriate due to possible interfering oxygen consumption or production in complex systems (Ozhogina and Kasaikina, 1995). Measurement of hydroperoxides by simple iodometric titration (AOCS, 1992), by spectrophotometry (Asakawa and Matsushita, 1980; Jiang *et al.*, 1991; Jiang *et al.*, 1992), or by chromatographic separation using HPLC (Yamamoto *et al.*, 1980) is the most direct approach for measurement of initial lipid peroxidation. However, these methods do not allow continuous measurement in reaction mixtures or in food, and their use in quality control is thus somewhat restricted. Alternatively, formation of conjugated dienes can be followed by measuring absorption at 234 nm (Recknagel and Glende, 1984), provided other absorbing species are not interfering. This requirement is, however, often not met in complex systems, thus use of this method is limited. Mikkelsen *et al.* (1992) tried to solve this problem using the ketone diene absorption at 280 nm to quantify lipid peroxidation, as interfering absorptions are limited in this area. The use of derivative spectral techniques permit minimization or even elimination of interfering absorption, but surprisingly, this technique has received little attention for the measurement of initial peroxidation processes, presumably because of the complexity in generating derivative spectra. However, photodiode detection of full spectra combined with current computer facilities and the numerous advantages offered by the technique of derivative spectroscopy (background elimination, enhanced resolution, matrix suppression, discrimination against broad band, and scatter elimination) should ensure that derivative spectroscopy receive increasing applications in complex systems in which interfering absorption occurs. Corongiu and Milia (1983) introduced the use of second derivative spectroscopy for determination of conjugated dienes at 234 nm. Their results confirmed that more information is revealed using this method compared to reading simple UV absorption spectra due to suppression of the interference from absorption of non-oxidized lipids. This has subsequently been supported by other studies (Sergent *et al.*, 1993).

The purpose of the present study was to further develop a direct method to determine initial lipid peroxidation in complex systems. The system used for the development of the method consisted of an oil-in-water emulsion of linoleic acid in phosphate buffer at different pH's (5.5−7.0), with either metmyoglobin (MbFe(III)) or ferrylmyoglobin (MbFe(IV)═O) as initiators, as this model system presents special challenges to the numerical evaluation due to interfering absorption which changes during oxidation. Two types of spectrophotometric measurements were performed to follow lipid peroxidation. References values were ob-

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tained after extraction of the lipid fraction from the reaction mixtures, and subsequently, spectra (from 200 to 300 nm) were recorded directly on samples taken from the reaction mixture. The two sets of data were compared, and calibration models were set up to avoid the time-consuming extraction and purification procedures in future use. A chemometric method, PLS (partial least squares regression), was used to generate calibration models, in order to enable determination of the concentration of conjugated dienes in unknown samples by direct spectrophotometric measurement on the bulk solution.

MATERIALS AND METHODS

Chemicals. Equine metmyoglobin (Type III), linoleic acid, chelating resin (Chelex-100), and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Analytical grade chemicals and double-deionized water were used throughout. Buffers were passed through a chelating resin column to remove any free metal ions (Dunn *et al.*, 1980).

Preparation of Linoleic Acid Emulsion. Linoleic acid emulsions were prepared in 10 mL volumetric flasks by mixing Tween 20 (0.012 g) and linoleic acid (21 mM) with phosphate buffer (5.0 mM, pH = 6.5) as described by Mikkelsen *et al.* (1992). The pH was adjusted to approximately 9.0 in order to provide the highest possible stability of the emulsion. Fresh emulsions were prepared daily.

Preparations of MbFe(III) and MbFe(IV)═O. Metmyoglobin was dissolved in 5.0 mM phosphate buffer (pH 6.5), centrifuged (20000*g*, 10 min), passed through a chelating resin column (Dunn *et al.*, 1980) and the concentration adjusted to 0.2 or 0.4 mM using ϵ_{525} = 7700 L mol^{-1} cm^{-1} (Andersen and Skibsted, 1992). MbFe(III) solutions were stored at 5.0 °C in the dark. MbFe(IV)═O was prepared by mixing equal volumes of 0.4 mM MbFe(III) in phosphate buffer (pH 6.5) and 1.2 mM H_2O_2 and allowed to react for 10 min in order to ensure complete conversion of MbFe(III) to MbFe(IV)═O.

Reactions of Linoleic Acid with Myoglobin Species. Initiation of oxidation of linoleic acid by MbFe(III) was studied after mixing air-saturated phosphate buffer (0.15 M, pH 5.5−7.0) incubated in a temperature controlled waterbath at 25.0 °C with linoleic acid emulsion (21 mM) and MbFe(III) solution (0.2 mM). Initiation of oxidation by MbFe(IV)═O was studied using the same procedure, substituting MbFe(III) for MbFe(IV)═O. The reaction mixture was prepared directly in a 1 cm quartz cuvette placed in a temperature controlled cell compartment (25.0 °C) of an HP8452 UV−vis diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). Spectra ($200 \leq \lambda \leq 300$ nm) were recorded immediately at time intervals of 1 min for 10 min, and direct absorption data or second-derivate data at 234, 244, and 280 nm and from 200 to 300 nm were collected and used for setting up calibration models.

Determination of Conjugated Dienes (Reference Values). Conjugated dienes were determined by extraction of 1.00 mL reaction mixture with 4.00 mL of cold diethyl ether at time intervals of 1 min for 10 min (11 time points). The ether phase was collected and evaporated under N_2. The residue was dissolved in 1.00 mL of ethanol, and the absorption spectrum was recorded ($200 \leq \lambda \leq 300$ nm). The relative amount of conjugated dienes for each sample was obtained by reading the value after calculation of the second derivative at 234 nm (Corongiu and Milia, 1983), and the values obtained were used as reference values.

Multivariate Data Analysis. The multivariate calibration method, PLS, was used to obtain calibration models (Martens and Næs, 1989). The strategy of this method is to relate different blocks of independent and dependent data to each other. Multivariate analysis has several advantages: interferences can be incorporated into the model eliminating the need for the purification step, the noise is reduced by the use of more variables, and finally it is possible to detect outliers during calibration and prediction (Esbensen *et al.*, 1994). For calibration, six independent experiments in triplicate (198 data points) were included using the two types of measurements. Triplicates were averaged corresponding to 66 data points for each type of measurement. The independent variables (calibration data) were arranged in an **X** matrix in which each row corresponded to a specific time for each experiment and each column to a specific wavelength (234, 244, 280 nm or 200−300 nm). The dependent variables (references values) were arranged in a **Y** vector with the number of elements equal to the number of rows (66 samples) in **X**. The outcome of the PLS algorithm is a model of **X** given by a (low) number of latent variables, each of them being characterized by a score vector, a loading vector, and a model relating the scores to the dependent variables. The score and the loading vectors give important information about the relationship between samples and variables. To estimate if a new sample is appropriate for calibration, leverages and residuals were used (Martens and Næs, 1989). A high leverage value indicates that the sample is different from the calibration samples, presumably due to higher concentration of analytes or presence of unmodeled interferences. A high residual indicates interferences in the sample. The spectral data were preprocessed by mean-centering, and cross validation was performed by leaving out one experiment (11 data points) at a time. This step was necessary to validate the model and to prove it gave an accurate estimate of the concentration of dienes in unknown samples in comparison to the traditional extraction procedure. All calculations were performed using Unscrambler (CAMO A/S, V. 5.03, Trondheim, Norway) except for calculations of derivatives which were made using MATLAB (Mathworks, Inc.) with a Savitzky−Golay algorithm (Savitzky and Golay, 1964).

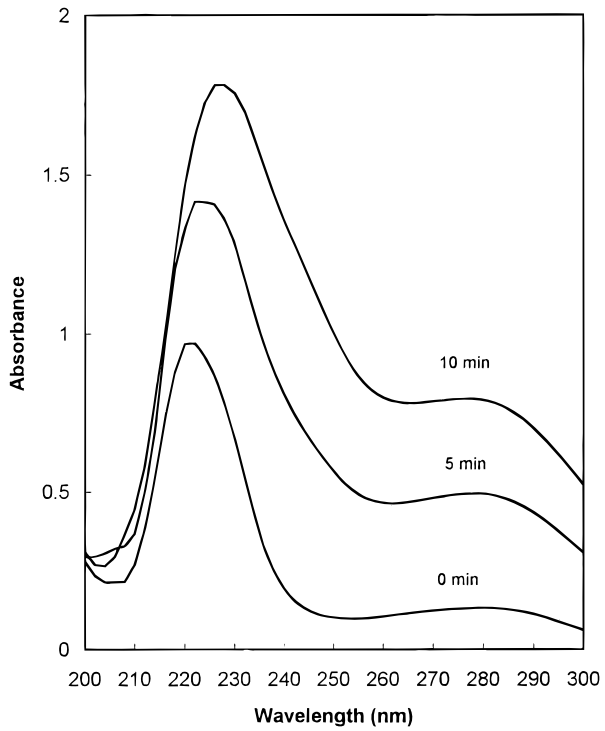

Figure 1. Typical absorption changes during peroxidation induced by MbFe(III) (4 μM) in linoleic acid oil-in-water emulsion (0.42 mM) after 0, 5, and 10 min incubation.

RESULTS AND DISCUSSION

Measurement of early stage lipid oxidation using conjugated dienes absorption at 234 nm has been extensively used during the last decades (Beuge and Aust, 1978; Hogg *et al.*, 1994; Recknagel and Glende, 1984). However, this method entails direct absorption measurement and is not always appropriate in food systems due to matrix interferences. Figure 1 presents

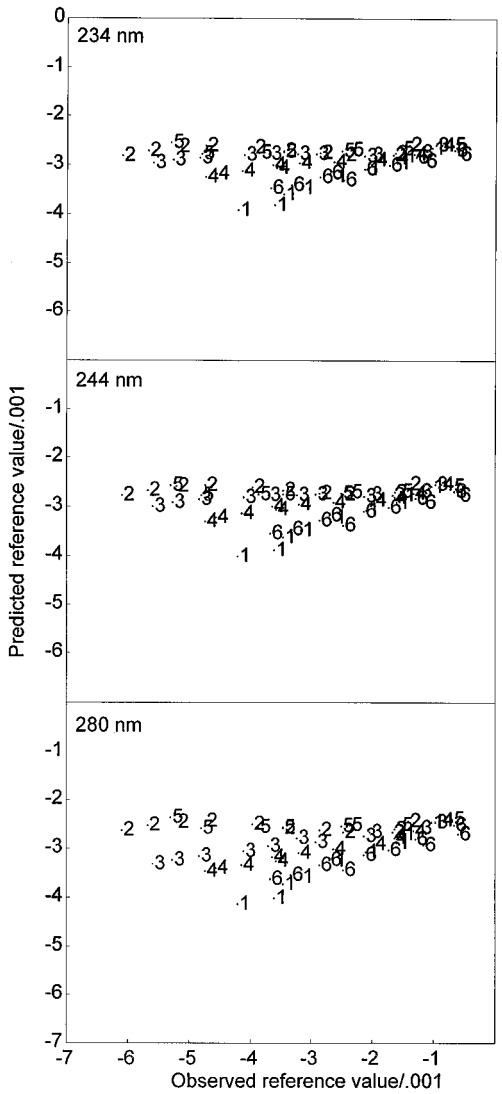

Figure 2. Correlations between conjugated dienes reference values and predicted values, obtained after cross validation from the model using single absorption wavelengths (280, 244, and 234 nm). The numbers 1−6 represent the different experiments. (1) pH 5.5, MbFe(III); (2) pH 7.0, MbFe(III); (3) pH 6.5, MbFe(III); (4) pH 6.0, MbFe(III); (5) pH 7.0, MbFe(IV)═O; (6) pH 5.5, MbFe(IV)═O. Initial concentrations of the prooxidative species and of the linoleic acid oil-in-water emulsion were 0.2 and 21 mM, respectively.

Table 1. Correlation Coefficients (*r*) and Root Mean Squared Error of Cross Validation (RMSECV) for Tested Calibration Models

	r		RMSECV	
calibration data	absorbance	2nd derivative	absorbance	2nd derivative
280 nm	0.02	0.83	1.5×10^{-3}	7.9×10^{-4}
244 nm	−0.01	0.78	1.5×10^{-3}	9.8×10^{-4}
234 nm	−0.03	0.87	1.5×10^{-3}	7.0×10^{-4}
200−300 nm[a]	0.97	0.98	4.0×10^{-4}	3.2×10^{-4}

[a] 51 individual wavelengths.

typical changes in UV absorption spectra during MbFe(III)-induced peroxidation of linoleic acid in an emulsion system. The large increase in turbidity with time, together with absorption caused by non-oxidized lipids, prevents absorption of the conjugated dienes to appear as a narrow and well-resolved absorption band. Measurement of lipid peroxidation in such complex systems using direct absorption at 234 nm is almost impossible due to interferences. To overcome such problems,

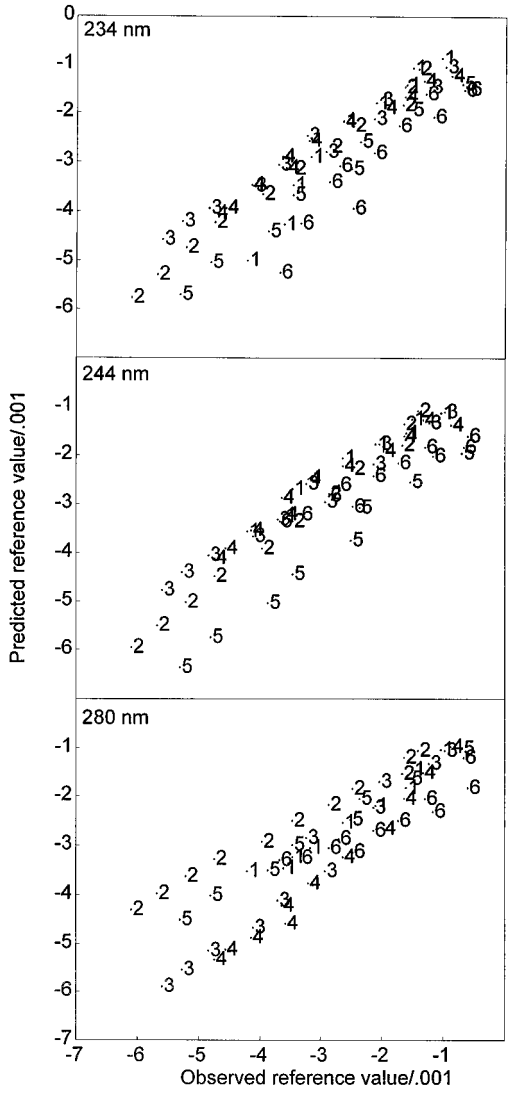

Figure 3. Correlations between conjugated dienes reference values and predicted values, obtained after cross validation from the model using second-derivative data (280, 244 and 234 nm). The numbers 1−6 represent the different experiments (see Figure 2 legend for more detail).

extraction of the lipid with an organic solvent and subsequent quantification of the conjugated dienes using the second-derivative spectra at 234 nm was shown to be a promising and accurate method by Corongiu and Milia (1983). This method is, however, troublesome, time consuming, and of little interest for on-line production control. In the present study, alternatives to lipid extraction were tested using mathematical modeling after direct measurement on the bulk solution.

For correlation of the direct spectroscopic measurements on the bulk solution to the reference data (obtained by extraction), calibration curves were generated using multivariate data analysis in six independent experiments. Absorption and second-derivative absorption data at 234, 244, and 280 nm, which represent *trans*,*trans*-conjugated dienes, *cis*,*trans*-conjugated dienes, and ketone dienes absorption, respectively, were tested, as well as the whole wavelength range from 200 to 300 nm (at every second nm). These data were subsequently used to predict the conjugated dienes values using PLS, and the predicted values were compared to the values obtained by the extraction procedure (reference data in each case).

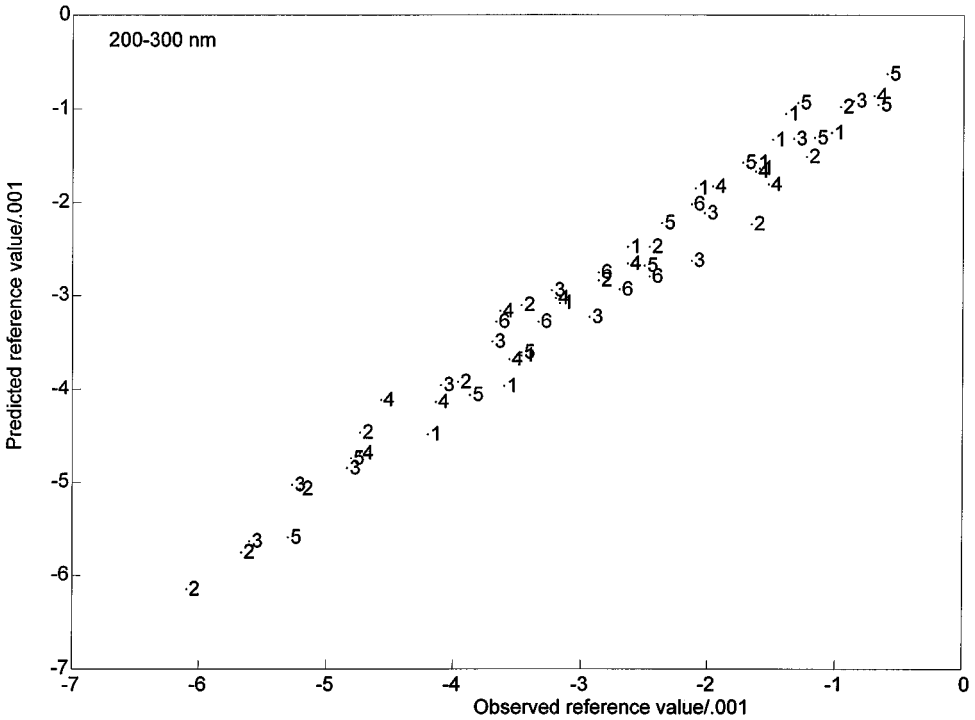

Figure 4. Correlation between conjugated dienes reference values and predicted values, obtained after cross validation from the model using second-derivative data from 200 to 300 nm (a total of 51 individual wavelengths). The numbers 1−6 represent the different experiments (see Figure 2 legend for more detail).

A plot of predicted conjugated dienes values obtained using direct absorption at 234, 244, and 280 nm versus reference values is presented in Figure 2. The observed patterns indicate a poor correlation for each experiment independent of the wavelength used. Moreover, the systematic deviations between different experiments strongly indicate that the prediction model cannot compensate for variations in pH and/or the nature of initiating species. This is also reflected in the correlation coefficients between the direct measurement and analysis based on extraction and in the root mean squared error of cross validation (RMSECV) (Table 1).

The use of second-derivative values instead of direct absorbance at 234, 244, or 280 nm to predict conjugated dienes values provided a better correlation between data for the present model system, as can be seen in Figure 3. The correlation coefficients and the RMSECV presented in Table 1 show, however, that correlations are not acceptable and that the mean errors are still high in prediction of unknown samples.

Incorporation of more than one wavelength to predict values for conjugated dienes is expected to result in improvements of the predictions as more information (possible interferences, differences between samples) is included in the calibration model. Additionally, inclusion of extra wavelengths may reduce noise, and outlier detection will be more effective. The wavelength range from 200 to 300 nm (a total of 51 wavelengths) was chosen as this area includes spectral contributions from non-oxidized lipid (210 nm), the different isomers of conjugated dienes (*trans,trans* and *cis,trans* isomers, respectively, at 234 and 244 nm), and ketone dienes (280 nm). Use of the whole spectral range resulted in a correlation coefficient ($r = 0.97$) which is surprisingly high compared to the correlation coefficient obtained for individual wavelengths. This significant improvement may be explained by the fact that the wavelength range used (200−300 nm) resulted in incorporation and correction of interferences in the calibration model. The number of latent variables is an indication of the complexity of the data. If only one latent variable is necessary, only one underlying phenomenon describes the variation in the data. In this model the number of latent variables was found to be 7, indicating that at least 7 phenomena, i.e., independent spectral variations, are responsible for the data variation. Further improvement was expected by the use of second derivative for the whole spectral range, and the plot of reference values against predicted values using second-derivative spectra between 200 and 300 nm is presented in Figure 4. The correlation coefficient ($r = 0.98$) is high and the RMSECV (Table 1) is low, which together indicate a good prediction model. The difference between the results obtained using the direct absorption or second-derivative data is small with respect to prediction; however, second-derivative data give clearly the most satisfactory result. The model based on second-derivative data is simpler, as only four latent variables were found to be necessary, in contrast to seven latent variables using the model based on raw data (nonderivatized). The simplest model should always be preferred, as a simpler model is more robust and interpretable, and in the present case the model based on second-derivative data was used. The equation giving the predicted value $\hat{y}$ using the latter prediction model is

$$\hat{y} = \sum_{\lambda=200}^{300} A_\lambda b_\lambda + b_0$$

where A_λ equals the second-derivative value at the specific wavelength λ, λ has a step width of 2 nm, b_λ is the calculated values given by the regression vector at the specific wavelength λ, and b_0 is a constant.

The results clearly demonstrate that continuous spectrophotometric measurement of lipid peroxidation is possible even in heterogeneous complex systems without

introduction of initial extraction and purification steps with the help of PLS. More than one wavelength is, however, necessary in setting up a calibration model to predict initial lipid peroxidation, and second-derivative spectra seem more suitable in complex systems due to suppression of interferences. The calibration model permits a quick and easy determination of lipid peroxidation in complex systems and can also provide, by the use of standard curves (second derivatives versus absorbances), quantitative information.

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