Quantitation of Heme Proteins from Spectra of Mixtures

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The Heme Proteins Spectra Analysis Program (HPSAP), a full-spectrum method, has been developed to quantify heme protein concentrations from spectra of mixtures. It utilizes the multiple-regression capabilities of a Lotus 1-2-3 spreadsheet. Micromolar extinction coefficients at 5-nm intervals from 500 to 640 nm are preprogrammed into the HPSAP spreadsheet for hemoglobin; oxyhemoglobinn; methemoglobin; ferrylhemoglobin; hemichrome; combined cytochromes of the mitochondria, both reduced and oxidized; and combined cytochromes of the microsomes, both reduced and oxidized. The analyst inserts experimental absorbance values at the same wavelengths into a reserved column in the spreadsheet. Multiple regression is performed to determine heme protein concentrations using the extinction coefficients of the heme proteins as independent variables and the experimental absorbance values as the dependent variable. A number of spectra have been analyzed using HPSAP with good results. Its versatility makes it a powerful method for the analysis of the complex spectra presented by oxidation in biological samples.

INTRODUCTION

To understand biological oxidative processes, it is necessary to quantify damage to proteins, lipids, and other cellular components. Existing methods of quantitation generally measure secondary irreversible oxidation products. The need also exists for an index of early reversible oxidation damage. The method described here, the Heme Proteins Spectra Analysis Program (HPSAP), provides such an index through measurement of both reversible and irreversible oxidative changes to heme proteins using visible light absorbance spectroscopy and multiple-regression analysis.

Currently, quantitation of heme proteins in simple systems, such as solutions containing fewer than four or five components, is generally accomplished using visible light absorbance spectroscopy and the use of N simultaneous equations derived from measurements at N wavelengths to solve for N unknown concentrations. For example, Williams (1964) introduced the use of simultaneous equations for calculating the concentrations of four cytochromes in mitochondrial suspensions from difference spectra, and Winterbourn (1990) used a similar approach to derive equations for the determination of concentrations of oxyhemoglobin, methemoglobin, and hemichrome in solution. While this "N-in-N" method is adequate for many simple systems, it is generally not suitable for more complex systems due to its sensitivity to choice of wavelength, instrumental fluctuation and round-off error, and interference from other absorbing species (Cappas et al., 1991; Berry and Trumpower, 1987).

A more recent development in multicomponent analysis is the use of "full spectrum" methods such as classical least squares, partial least squares, and inverse least squares which use measurements at a number of wavelengths greater than the number of unknown concentrations (Cappas et al., 1991; Berry and Trumpower, 1987; Thomas and Haaland, 1990). This redundancy of information enables these methods to overcome many of the problems associated with the N-in-N method, and they are currently in wide use. However, none of these methods has yet been successfully applied to the complex spectra presented by oxidative reactions of heme proteins in tissue slices or homogenates. HPSAP supplants the difficult and time-consuming preparation of multiple wet standards required by most multicomponent full-spectrum spectrophotometer–computer systems because HPSAP utilizes the spectra from the literature as standards. Wet standard mixtures of heme proteins in various redox states are difficult to prepare. Wet standard preparations of the many cytochromes of mitochondria and microsomes cannot practically be prepared and maintained.

HPSAP has been developed to simplify the application of classical least-squares analysis to the problem of heme protein oxidation. It uses the multiple-regression capabilities of a Lotus 1-2-3 spreadsheet to determine the concentrations of heme proteins in a mixture. HPSAP has been applied to spectra of mixtures of heme proteins from the literature and to laboratory preparations of these mixtures, including the complex spectra presented by tissue slices and homogenates, with equally good results. This method is a powerful and versatile tool for analyzing heme proteins and their oxidative products.

This method can have many applications in food chemistry and biochemistry including studies of chemical changes in meats during storage. Other large areas of application include oxidative damage to animal tissues induced by toxic oxidants (Chen et al., 1993a) and prevention of oxidative damage to tissues by nutrient antioxidants such as vitamin E, β-carotene, and selenium (Andersen et al., 1993; Chen et al., 1993b).

MATERIALS AND METHODS

Determination of Absorbance Values for Spectra of Heme Protein Mixtures from the Literature. The literature was searched for spectra of heme protein mixtures that could be analyzed with HPSAP, and a number of diverse spectra were obtained (Winterbourn et al., 1976; Mahoney et al., 1977; Bunn and Forget, 1986; Rachmilewitz et al., 1971; Wittenberg, 1979; Dawson et al., 1980; Wodick and Luebbers, 1974). Photocopies of the spectra were enlarged, and scales for both the ordinate (absorbance) and abscissa (wavelength) were drafted from the

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information given for each spectrum. Absorptivity values at 5-nm intervals from 500 to 640 nm were determined and used in the HPSAP to analyze each spectrum. Accuracy of absorptivity values was estimated at ±0.01 absorbance unit.

Preparation of Mitochondrial Fraction from Rat Liver. The mitochondrial fraction from rat liver was obtained using a modification of the procedure described by Hogeboom (1955). Eighteen milliliters of cold 0.25 M sucrose was added to 2 g of fresh liver, and the tissue was homogenized for 2 min; 10 mL of the homogenate was layered over 10 mL of 0.34 M sucrose and centrifuged for 10 min at 2000 rpm. The supernatant was removed and centrifuged for 20 min at 5000 rpm. The supernatant was removed and discarded. The precipitate was washed with 0.34 M sucrose and centrifuged for an additional 20 min at 5000 rpm. The supernatant was removed and discarded, and the resulting precipitate, or pellet, was assayed for protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Spectrophotometric Measurement of Mitochondrial Fraction from Rat Liver. Approximately 50 mg of the mitochondrial pellet was weighed to 0.1 mg on an analytical balance and transferred to a small spectrophotometer cell with a light path of 2.0 mm. A few milligrams of sodium dithionite (Fisher Scientific, Fair Lawn, NJ) was added and mixed with the mitochondria to reduce the cytochromes of the mitochondria. The cell was sealed with a microscope cover glass and mounted in the cell compartment of a Beckman DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) near the photoreceptor to reduce light scattering caused by the tissue. The sample was scanned from 500 to 640 nm against a background of four layers of Parafilm used to simulate turbidity. The absorbance was recorded at 5-nm intervals by a scan program in the spectrophotometer.

A stock hemoglobin solution of 1.05 mg/0.1 mL was prepared from hemoglobin from rat blood (Sigma Chemical Co., St. Louis, MO) in 0.1 M sodium phosphate buffer, pH 7.4. A second approximately 50-mg sample of the mitrochondrial pellet was weighed to 0.1 mg on an analytical balance and transferred to a spectrophotometer cell. Five microliters of the hemoglobin solution was added to the mitochondria in the cell and mixed well. The mixture was reduced with sodium dithionite, sealed, mounted in the spectrometer, and scanned from 500 to 640 nm as before.

Preparation of Chicken Kidney Homogenate and Oxidative Reaction. Kidneys from five male white leghorn chicks raised on a vitamin E adequate diet were pooled and homogenized with 9 volumes of oxygenated ice-cold 1.15% KCl/0.01 M phosphate buffer, pH 7.4, in a motorized glass and Teflon homogenizer at low speed. An EDTA–Fe^{2+} complex was prepared by mixing 1 part $FeSO_4$ with 3 parts EDTA, as described by Halliwell (1978). Fenton reagent was added to a sample of the homogenate by addition of the EDTA–Fe^{2+} complex to a final concentration of 50 μM Fe^{2+}, followed by the addition of hydrogen peroxide to a final concentration of 50 μM. EDTA was added to a second sample of the homogenate to a final concentration of 150 μM. Aliquots (500 μL) of the samples were removed for zero time spectrophotometric measurements. The samples were incubated for 180 min at 37 °C in a Gyratory water shaker bath with continuous shaking (180 cycles/min). After incubation, 500-μL aliquots were removed from the samples for spectrophotometric measurement.

Spectrophotometric Measurement of Chicken Kidney Homogenates. Aliquots removed for spectrophotometric measurement were mixed immediately upon removal with 1 volume of ice-cold glycerol to suspend the homogenate and to reduce turbidity. The mixtures were transferred to 1-cm silica cells and placed in the cell compartment of a Beckman DU-50 spectrophotometer (Beckman Instruments) near the photoreceptor to reduce light scattering. These were scanned from 500 to 640 nm against a background of four layers of Parafilm used to simulate turbidity. We determined that Parafilm has the same spectrum as does idealized turbidity. The absorbance was recorded at 5-nm intervals by a scan program in the spectrophotometer.

Analysis of Absorbance Spectra with HPSAP. Micromolar extinction coefficients at 5-nm intervals from 500 to 640 nm were determined from spectra in the literature for hemoglobin, oxyhemoglobin, methemoglobin, ferrylhemoglobin, and hemichromes (Winterbourn, 1990; Van Assendelft, 1970; King and Winfield, 1963; Yonetani and Schleyer, 1967; Rachmilewitz, 1969). Since all work described here is at neutral pH, variation of spectra with pH is not a problem. Comparisons of drafted values to available tabulated values indicated accuracy to approximately ±0.002 absorbance unit. Micromolar extinction coefficients for reduced and oxidized mitochondria and microsomes were calculated over the same range by determining micromolar extinction coefficients of the major mitochondrial and microsomal cytochromes from spectra in the literature (Wainio, 1970; Margoliash and Frohwirt, 1959; Sato et al., 1969) and summing their contributions to total absorbance based on their mole percents in specific tissues (Estabrook and Cohen, 1969; Williams, 1969). Thus, for rat liver the extinction coefficient for the mitochondrial cytochromes at each wavelength is based upon 1 μM total cytochromes and is the sum of 29.0% cytochrome aa_3, 30.1% cytochrome *b*, 18.3% cytochrome c_1, and 22.6% cytochrome *c*; for microsomal cytochromes the extinction coefficient is the sum of 39.8% cytochrome *b* and 60.2% cytochrome P-450. Extinction coefficients were entered as values into a block of cells in a Lotus 1-2-3 spreadsheet (Lotus Development Corp., Cambridge, MA), each heme species occupying one column (Boyle, 1993).

To analyze an experimental spectrum, the absorbance values of the spectrum at 5-nm intervals from 500 to 640 nm were entered into a reserved column of cells. Multiple regression was performed using the extinction coefficients of the heme species as independent variables and the experimental spectrum as the dependent variable. In cases in which turbidity was likely to be present, a column of wavelength values at 5-nm intervals from 500 to 640 nm was also used as an independent variable to derive a slope for a slanted baseline correction. Regression analysis determined the concentrations of the heme species and their standard errors; the slope and constant of a corrected baseline, if applicable, and their errors; and the coefficient of determination (r^2). The analysis was repeated as necessary using appropriate combinations of heme proteins. HPSAP is available on microfilm (Boyle, 1993).

RESULTS

Comparison of HPSAP Results to Spectra of Heme Protein Mixtures from the Literature. Figure 1A shows absorbance values from a spectrum of methemoglobin after exposure to superoxide (Winterbourn et al., 1976) and a model spectrum calculated with HPSAP based on its determination of heme protein concentrations for the system. Correlation between the HPSAP calculated spectrum and the original experimental spectrum is excellent ($r^2 = 1.0$). The HPSAP calculated concentration of oxyhemoglobin was 8.2 μM, and the concentration of methemoglobin was 28 μM. These values compare well with values of 6.4 and 27 μM for oxyhemoglobin and methemoglobin, respectively, calculated with the Winterbourn equations (Winterbourn, 1990).

Parts B and C of Figure 1 show absorbance values from spectra of the same sample of hemoglobin A in two different states of oxygenation (Mahoney et al., 1977) and the model spectra calculated with HPSAP based on its determination of heme protein concentrations. Correlation between the calculated and experimental spectra is excellent ($r^2 = 1.0$ in both cases). Calculations with HPSAP determined the percent oxygenation of parts B and C of Figure 1 to be 36% and 72%, respectively. These values compare well to published values of 33% and 75%. HPSAP calculated the concentration of hemoglobin A to be approximately 2.3 mM compared to a published value of 2.74 mM. This calculation was hampered by uncertainty regarding the path length in Mahoney's thin-layer optical cell but still shows good agreement for biological studies.

Figure 1D shows absorbance values from a spectrum of whole hemolysate from a patient with hemoglobin

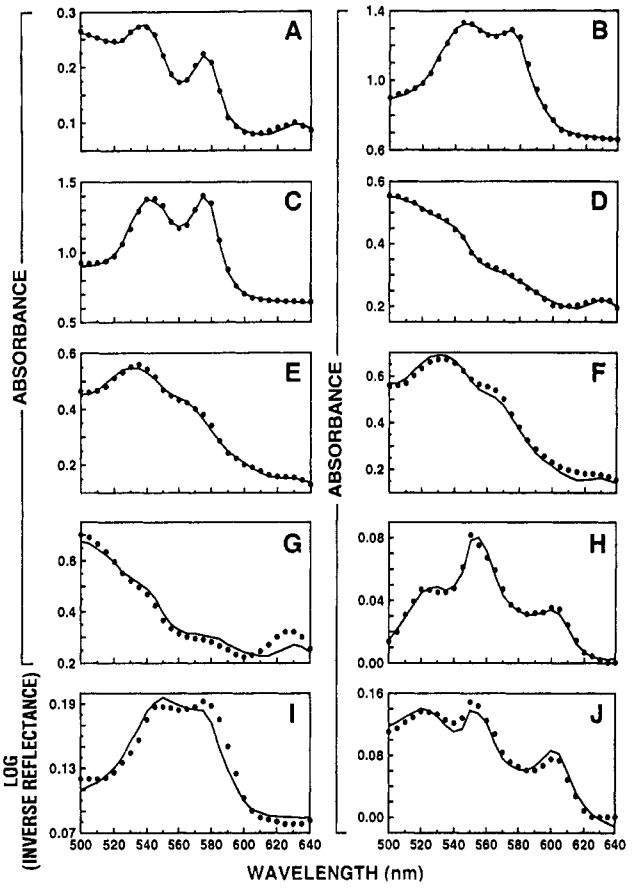

Figure 1. Analyses of absorbance spectra of heme protein mixtures from the literature with HPSAP, as described under Materials and Methods. Sources are cited under Results. (···) Values determined from spectra from the literature; (—) model spectra calculated with HPSAP.

Freiburg (β 23 Val → 0) 48 h after oxidation with ferricyanide (Bunn and Forget, 1986) and the model spectrum calculated with HPSAP based on its determination of heme protein concentrations. Figure 1E shows absorbance values from a spectrum of purified hemoglobin Freiburg from the same patient 48 h after the same oxidation (Bunn and Forget, 1986) and the HPSAP calculated spectrum. Correlation between the calculated and experimental spectra is excellent in both cases (r^2 = 0.998 for Figure 1D; r^2 = 0.997 for Figure 1E). For Figure 1D, HPSAP calculated the mole percents of methemoglobin and hemichrome to be 88% and 12%, respectively. For Figure 1E, HPSAP calculated the mole percents of methemoglobin and hemichrome to be 32% and 67%, respectively. Because the original spectra were published as qualitative examples of hemichrome formation in unstable hemoglobin variants, these calculations cannot be verified quantitatively, but they support the published qualitative descriptions of the spectra and are in accord with what is expected of unstable hemoglobin oxidation.

Parts F and G of Figure 1 show absorbance values from spectra of oxidized hemoglobin A and oxidized a subunits, respectively (Rachmilewitz et al., 1971) and the model spectra calculated with HPSAP. Correlation between the calculated and experimental spectra is very good in both cases (r^2 = 0.990 for Figure 1F; r^2 = 0.966 for Figure 1G). HPSAP calculated the concentration of whole globin in Figure 1F to be 7.3×10^{-5} M, compared to a published value of 7.4×10^{-5} M. It calculated the concentration of a subunits to be 2.9×10^{-4} M, compared to a published value of 2.5×10^{-4} M. Both calculated concentrations are in very good agreement with the published values.

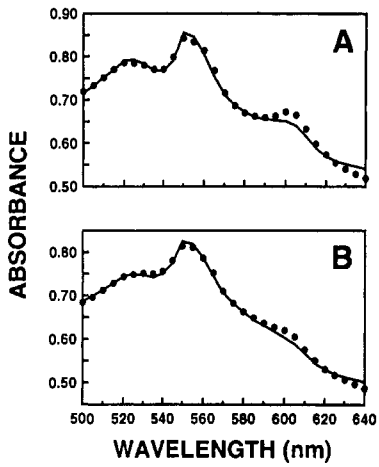

Figure 2. Analyses of absorbance spectra of the mitochondrial fraction from rat liver with HPSAP, as described under Materials and Methods. (A) Without added hemoglobin; (B) with added hemoglobin; (···) experimental absorbance values; (—) model spectra calculated with HPSAP.

Figure 1H shows absorbance values from a spectrum of isolated adult rat heart cells under anaerobic conditions (Wittenberg, 1979) and the model spectrum calculated with HPSAP. Because the published spectrum provided only relative measurement of absorbance, HPSAP could only be used to provide qualitative information. HPSAP matched the experimental spectrum with a high degree of correlation (r^2 = 0.988) using deoxymyoglobin and reduced cytochromes of the mitochondria in the proportions expected in rat heart (Williams, 1968). The HPSAP calculation supports the published qualitative description of the spectra and is in accord with the expected biochemistry.

Figure 1I shows values for the logarithm of the inverse of reflectance (LIR) of Caucasian skin (Dawson et al., 1980) and the model spectrum calculated with HPSAP. HPSAP matched the experimental spectrum using oxyhemoglobin, deoxyhemoglobin, and a slanted baseline to approximate the absorbance from melanin (r^2 = 0.951). Because LIR only approximates absorbance, HPSAP could not be used quantitatively in this example, but the results support the published qualitative explanation of the experimental spectrum.

Figure 1J shows absorbance values from a reflectance spectrum of blood-free perfused guinea pig brain under anoxic conditions (Wodick and Luebbers, 1974) and the model spectrum calculated with HPSAP. HPSAP matched the experimental spectrum with a high degree of correlation (r^2 = 0.974) using reduced cytochromes of the mitochondria in the proportions expected in guinea pig brain (Williams, 1968). Again, a quantitative comparison cannot be made, but the results support the published qualitative explanation of the experimental spectrum.

HPSAP Analysis of Mitochondrial Fraction from Rat Liver. Parts A and B of Figure 2 show absorbance values from spectrophotometric measurement of the mitochondrial fraction from rat liver without and with added hemoglobin, respectively, and the model spectra calculated with HPSAP. Correlation between the calculated and experimental spectra is very good in both cases (r^2 = 0.987 for Figure 2A; r^2 = 0.994 for Figure 2B). HPSAP fit both spectra using deoxyhemoglobin and reduced cytochromes of the mitochondria in the proportions expected in rat liver (Williams, 1968). The presence of a small amount of deoxyhemoglobin in the first spectrum can be accounted for as the result of incomplete washing

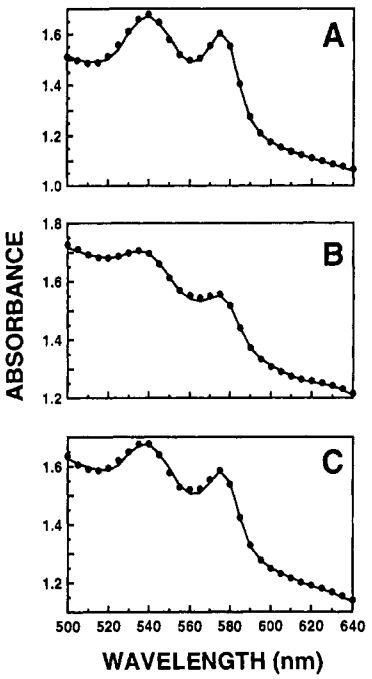

Figure 3. Analyses of absorbance spectra of chicken kidney homogenates with HPSAP, as described under Materials and Methods. (A) Without incubation; (B) after 180 min of incubation with Fenton reagent; (C) after 180 min of incubation without Fenton reagent; (···) experimental absorbance values; (—) model spectra calculated with HPSAP.

of the mitochondrial pellet. The difference between the deoxyhemoglobin concentrations of the two samples as calculated with HPSAP is in agreement with the concentration expected from the addition of hemoglobin solution (0.02 mM).

HPSAP Analysis of Chicken Kidney Homogenates. Figure 3A shows representative absorbance values for zero time spectrophotometric measurement of chicken kidney homogenate and the model spectrum calculated with HPSAP. Parts A and B of Figure 3 show absorbance values of chicken kidney homogenate after oxidation with and without Fenton reagent, respectively, and the HPSAP calculated spectra. Correlation between the calculated and experimental spectra is excellent (r^2 = 0.999 in all cases). The mole percents of each heme compound used by HPSAP to construct the model spectra in Figure 3 are as follows. At zero time the predominant heme compound is oxyhemoglobin (89%) with a small amount of deoxyhemoglobin also being measured (11%). Although small amounts of cytochromes of the mitochondria were expected to be present, they were not in amounts large enough to be included in these quantitative results. After oxidation with Fenton reagent, the predominant heme compound is methemoglobin (57%). Oxyhemoglobin is also present (26%), as are hemichromes (17%). The presence of hemichromes indicates a strong oxidative reaction, as is expected in the presence of Fenton reagent. The sample that was oxidized without Fenton reagent shows a much milder oxidation, which is also to be expected. In this case oxyhemoglobin is the predominant heme compound (73%), with methemoglobin comprising a smaller percentage (27%).

DISCUSSION

The results presented are representative of the wide variety of spectra that can be analyzed with HPSAP. It is this versatility that makes HPSAP a powerful method for analyzing spectra of mixtures of heme proteins. However, as with any analytical technique, it is most useful when its underlying assumptions are well understood.

First are the assumptions that heme proteins obey Beer's law and that their contribution to total absorbance is additive. These assumptions are well accepted for heme proteins within the concentration ranges presented and, most importantly to the expected applications of this method, within physiologically relevant concentrations. It should also be noted that these assumptions are used in other methods for determining concentrations of heme proteins, such as the Winterbourn equations (Winterbourn, 1990).

Next are the assumptions that sufficient spectral information is given within the wavelength range 500–640 nm to accurately analyze an experimental spectrum and that turbidity, which is known to be an exponential function, can be approximated as a straight line within this range. These assumptions are presented together because they are somewhat opposed. That is, a wider wavelength range would give more spectral information which should lead to an analysis with less error, but it would also decrease the validity of assuming turbidity is a straight line, thereby introducing more error. The wavelength range used by HPSAP then is a compromise. It is a modest wavelength range which includes all of the distinctive peaks of the expected heme proteins in the visible light range, excluding the Soret peaks. The Soret peaks were excluded because their absorbance is too strong to be determined in the same biological samples as the 500–640-nm range.

Another assumption is that the cytochromes of the mitochondrial electron-transfer chain and the microsomes exist either fully oxidized or fully reduced. This assumption is made for two reasons. First, the analysis of biological samples would be extremely difficult without this simplifying assumption; each cytochrome of the mitochondria and microsomes would need to be analyzed individually. This is mathematically undesirable since increasing the number of independent variables in regression analysis can drive the coefficient of determination (r^2) to one with no real improvement in fit (Freund et al., 1986) and it is more information than is needed for most, if not all, applications of this method. Second, the error this assumption introduces is small. For example, assuming cytochrome *c* to be fully reduced vs 50% oxidized and 50% reduced produces differences in micromolar extinction coefficients of less than 0.022 absorbance unit. The error would be larger when all of the cytochromes of the mitochondria are considered, but it would still be acceptably small.

A final assumption is that the micromolar extinction coefficients from the literature accurately represent the heme proteins in a given sample. An obvious case in which this would not be true is at different pHs. The extinction coefficients used in HPSAP are those at neutral pH. At pHs much more acidic or basic it would be necessary to change the extinction coefficient in the program to those appropriate for the experimental pH. Another case in which this would not be true is if widely differing slit widths were used when the extinction coefficients and the experimental spectrum were measured. Unfortunately, slit widths are often not reported with spectral data so they are difficult to take into account. Should it be necessary, changing the extinction coefficients in HPSAP to improve their accuracy under a given set of experimental conditions is a simple procedure.

The quantitation of mixtures of heme proteins in biological systems has long been a problem. This is

evidenced by the many spectra presented in the literature without quantitation, as seen under Results. Because quantitation has been so difficult, data interpretation is often given only in qualitative terms. The availability of the HPSAP method should allow data interpretation of these mixtures at the quantitative level, and it is proving to be of value in providing scientists with an index of early reversible oxidative damage (Chen et al., 1993b; Andersen et al., 1993). This method can be applicable in food chemistry and in the biochemistry of meat pigments. The studies of Chow (1991) and Chen et al. (1992) are examples. This method is applicable to investigations of oxidative damage to animal tissues by toxic oxidants (Chen, 1993a). HPSAP is also useful in studies of the protective effects of nutrient antioxidants against oxidative damage to tissues (Andersen et al., 1993; Chen et al., 1993b).

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