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Analytical Methods Section

Fluorimetric flow-injection determination of hydroperoxides in foodstuffs

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A reliable and sensitive stopped-flow injection method is described for the determination of hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide. It is based on the oxidation of leuco-phloxin to the fluorescent phloxin by hydroperoxide and haematin. Linear calibration graphs were obtained between 4×10^{-6} and 8×10^{-5} M, with a sampling-rate of 25 samples h^{-1}. The detection limit, defined as three standard deviations of the reagent blank, was $1{\cdot}5 \times 10^{-7}$ M. The usefulness of the method was tested in the determination of lipohydroperoxides in six commercial oil samples and of hydrogen peroxide in six milk samples of different levels of skimming. The results agreed closely with those obtained by the iodometric method.

INTRODUCTION

Hydrogen peroxide and organic peroxides are often the focus of clinical, environmental and biological studies. Hydrogen peroxide is used in many industrial and related processes as an oxidising, bleaching and sterilising agent. Lipid hydroperoxides have gained increasing attention in the food and clinical fields, because lipid peroxidation products are believed to be involved in the process of ageing, mutagenicity and many kinds of diseases.

The classical technique for the determination of hydroperoxides is based on the liberation of iodine by peroxides, measuring the resulting tri-iodide complex titrimetrically (AOAC, 1980) or spectrophotometrically (Hicks & Gebicki, 1979). Today, the enzymatic and non-enzymatic catalytic reduction of hydroperoxides is a common assay for the determination of lipid peroxides and of hydrogen peroxide. Leuco-dyes are usually introduced as the hydrogen donor and the colour (Yagi *et al.*, 1986) or the fluorescence (Black & Brandt, 1974; Cathcart *et al.*, 1983) of the oxidised form is measured.

Flow injection analysis (FIA) has proved to be a very useful and versatile automated technique (Ruzicka & Hansen, 1988; Valcarcel & Luque de Castro, 1987). Some papers refer to the determination by FIA of hydrogen peroxide (Ruzicka & Hansen, 1988), but none has been found dealing with the determination of organic peroxides.

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The reduction of xanthene dyes through their photochemical reaction with ethylenediaminetetra-acetic acid has been described (Koizumi & Usui, 1972; Pérez-Ruiz *et al.*, 1991). In this work, the analytical applications of the reduced form of phloxin in FIA were studied extensively for the determination of hydrogen peroxide and organic hydroperoxides. The new method is based on the oxidation of leucophloxin to the fluorescent phloxin by hydroperoxides and haematin. The procedure has been applied to the determination of lipohydroperoxides in oil samples and of hydrogen peroxide in milk.

MATERIALS AND METHODS

Apparatus

A Perkin-Elmer Model 3000 spectrofluorimeter connected to a Linseis 6512 recorder, a Hellma 176.052 QS flow cell (inner volume, 25 μl), a Gilson Minipuls HP4 peristaltic pump and an Omnifit injection valve were used. PTFE tubing of 0·5 mm i.d. was used for the mixing coil and for all connections.

Manifold

The flow-injection configuration used is outlined in Fig. 1; it consists of two pump lines. Leucophloxin solution and haematin solution are pumped at a flow-rate of 1·4 ml min^{-1} and merge in a Y-piece. The mixing of the two streams is stimulated in a coil (50 cm). The sample solution is introduced with the aid of an injection valve with a loop of 85 μl. All solutions must be de-aerated by bubbling nitrogen through them. Except for the pump tubing (Tygon), PTFE tubing (0·5 mm i.d.) was

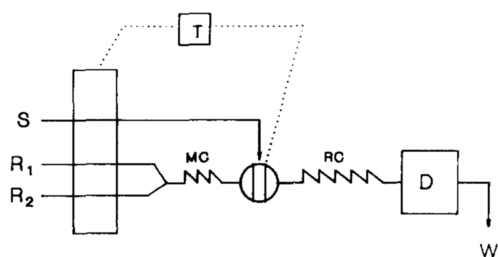


Fig. 1. Schematic diagram of the manifold used for the determination of hydroperoxides. S, sample; R_1, 5×10^{-5} M leucophloxin/0·04 M borax buffer pH 8·5; R_2, 5 mg litre^{-1} haematin/0·04 M borax buffer pH 8·5; MC, mixing coil; RC, reactor coil; D, detector; W, waste; T, timer. Dashed lines represent synchronous control line.

used throughout the manifold. A timer, synchronised to the injection system, allows the reagent stream to be stopped at any prearranged time as well as for any length of time.

Reagents

All chemicals were of analytical-reagent grade and were used without further purification. Doubly distilled water was used throughout.

Leucophloxin solution, 10^{-4} M, was prepared by mixing 100 ml of 2×10^{-4} M phloxin (Colour Index 45410), 50 ml of 0·1 M EDTA (disodium salt) and 50 ml of 0·15 M borax buffer pH 8·5. After bubbling pure nitrogen through the solution for 5 min, it was irradiated with a 300 W tungsten lamp until decoloured (*c.* 15 min). The solution must be kept in an oxygen-free environment.

Haematin solution, 5 mg litre^{-1}, was prepared by dissolving 1 mg of haematin (Sigma) in 0·5 ml of 0·1 M NaOH and then adding 50 ml of 0·15 M borax buffer and diluting to 200 ml with doubly distilled water; this solution must be made fresh each day.

Standard solutions of hydrogen peroxide and cumene and tert-butyl hydroperoxides, *c.* 0·01 M, were prepared by appropriate dilution of the commercial reagents with doubly distilled water. The actual concentrations of hydroperoxides in these standards were determined iodometrically. Working standard solutions were prepared from the stock solutions by dilution with doubly distilled water and used within 30 min of preparation.

Procedures

Determination of hydrogen peroxide and cumene and tert-butyl hydroperoxides

The samples containing between 4×10^{-6} and 8×10^{-5} M peroxide were sucked into the sample loop (85 μl) of the injection valve by means of the peristaltic pump and injected into the FIA system. The timer was programmed so that 8 s after the injection of the sample, the flow was stopped for 60 s and then the pump started again. The fluorescence intensity was measured using an excitation wavelength of 538 nm and an emission wavelength of 550 nm and the concentration of each peroxide was evaluated from the peak height by using a calibration graph prepared from freshly prepared standards.

Determination of lipohydroperoxides in oil samples

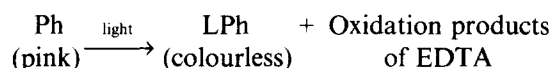
A known volume of oil sample (< 0·40 g) was placed in a 10 ml glass-stoppered centrifuge tube and diluted to 1·0 ml with ethanol. Methanolic sodium hydroxide solution (2 ml of 2 M) was added and the tube was shaken vigorously for 5 min. If the solution is turbid at this stage it must be centrifuged. A 1 ml aliquot was used to determine lipohydroperoxides following the above described procedure.

Determination of hydrogen peroxide in milk

The samples (10 ml) were previously spiked with hydrogen peroxide and after dilution with doubly distilled water (5 ml). Trichloroacetic acid (2 ml of 2 M) was then added to the samples and these were left to stand for 5 min. The curdled milk samples were then gravity-filtered and the pH of the filtrates adjusted to 8·5 with 2 M sodium hydroxide before being accurately diluted to 25 ml. Aliquots (85 μl) of these samples were injected into the flow system and analysed following the above described procedure.

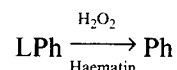
RESULTS AND DISCUSSION

Leucophloxin (LPh) is not commercially available, but can be obtained through the photochemical reaction between phloxin (Ph) and ethylenediaminetetra-acetic acid (EDTA). When a solution containing Ph and EDTA (disodium salt), at a pH over the range 5–10 and in the absence of oxygen, is illuminated with white light, photoreduction of the dye occurs and the pink colour disappears:



$$\underset{\text{(pink)}}{\text{Ph}} \xrightarrow{\text{light}} \underset{\text{(colourless)}}{\text{LPh}} + \underset{\text{of EDTA}}{\text{Oxidation products}}$$

LPh is oxidised to the fluorescent Ph by hydrogen peroxide and by cumene and tert-butyl hydroperoxides in the presence of haematin. The oxidation of the hydrogen donor LPh by these peroxides can be schematised by



$$\text{LPh} \xrightarrow[\text{Haematin}]{H_2O_2} \text{Ph}$$

From our data, each hydroperoxide generated a limit of approximately 1 mol of Ph/ml of peroxide. Haematin was required for this reaction as shown in Fig. 2(D). It has been observed that haematin has peroxidase activity. The most likely mechanism of haematin catalysis is one in which haematin forms a haematin–peroxide complex, which degrades to a ferryl-oxo and hydroxyl radical, both of which are capable of oxidising LPh (Dunford, 1982; Cathcart *et al.*, 1983).

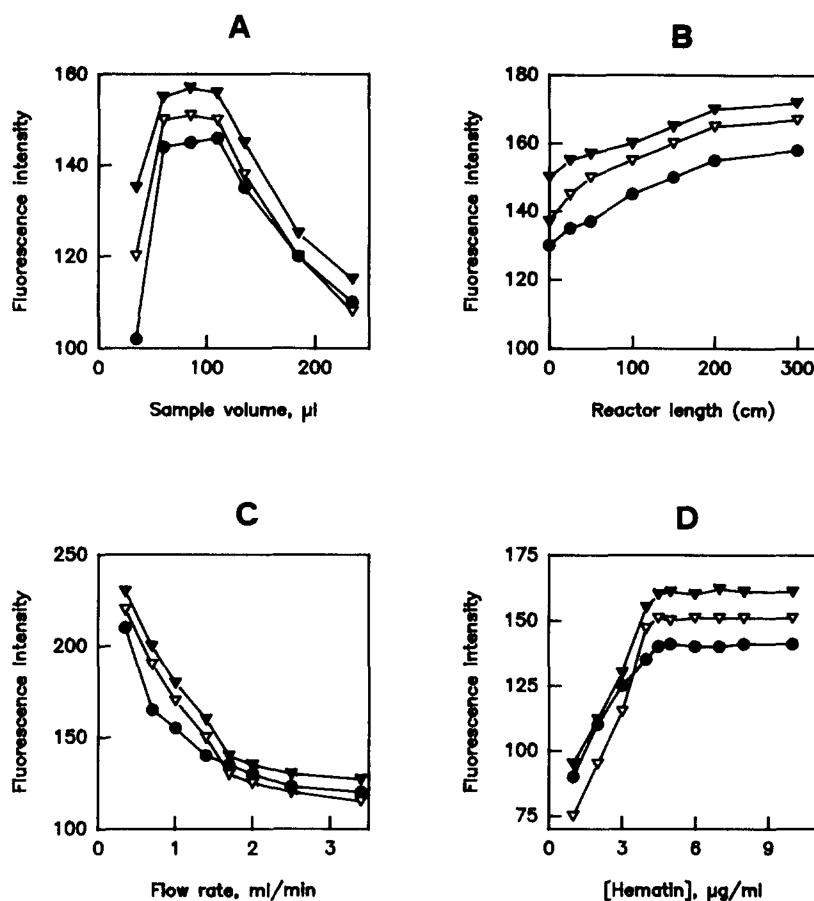


Fig. 2. Influence of variables on the analytical signal: (A) Sample volume; (B) reactor length; (C) flow rate; (D) haematin concentration. Hydrogen peroxide (●), cumene hydroperoxide (▲) and tert-butyl hydroperoxide (Δ). (For conditions see text.)

Excitation and emission spectra

The oxidation product of LPh by hydroperoxides, Ph, has a strong absorption band centred at about 534 nm, whereas the analyte and the reagents show no appreciable absorbance above 425 nm. Ph exhibits a bright orange fluorescence with the emission band centred at a wavelength of 550 nm. For further studies, the excitation monochromator was set at 534 nm with a 10 nm spectral bandpass and the emission monochromator was adjusted to 550 nm with a 10 nm spectral bandpass.

Flow injection system variables

The variables studied were sample volume, length of the reactor, flow rate, delay time and stop time. The concentrations used in these experiments were as follows: reagent solution, 2×10^{-5} M LPH/0·04 M borax buffer pH 8·5; haematin solution, 9 mg litre^{-1}; sample solution (hydrogen peroxide, cumene hydroperoxide or tert-butyl hydroperoxide), $1{\cdot}0 \times 10^{-5}$ M. The manifold parameters used in the optimisation procedure were a reactor length of 200 cm with an inner diameter of 0·5 mm and a stop time of 40 s.

The sample volume was varied between 35 and 240 μl. The volume of each peroxide injected in the range 60–110 μl yielded an almost constant signal, which decreased outside this range (Fig. 2(A)); a volume of 85 μl was chosen for further experiments.

The length of the reaction coil was examined over the range 0–300 cm (Fig. 2(B)). A slight increase of signal was observed with increase in reactor length up to 200 cm, above which it remained virtually constant. The length chosen for the reactor was 200 cm.

Increasing flow rates produced an exponential decrease in the signal (Fig. 2(C)), because of a corresponding increase in the dispersion. A flow rate of 1·4 ml min^{-1} was chosen as a compromise between the sampling frequency and sensitivity.

The best results were obtained if the pump was stopped when the sample plug was located in the reaction coil. After a given period of time the pump was triggered by a timer and the sample zone was directed towards the detector. A stop time of 60 s was chosen as a compromise between analytical signal and sampling rate.

Chemical variables

Reagents which directly influence formation of the fluorescent species were evaluated to determine optimum concentrations that provide high fluorescence intensity without utilisation of a large excess of reagents. Various concentrations of LPh and haematin and pH were evaluated. Although the flow system used separate reagent lines to evaluate these effects, results are presented as related to the system illustrated in Fig. 1. When LPh and haematin concentrations are increased, the fluorescence intensity also increases.

Dramatic changes are observed initially followed by more gradual increases. Haematin gave no increase above 4·5 mg litre^{-1}, while concentration increases for LPh from 4 × 10^{-5} to 10^{-4} M gave increases of 10%. The pH has an important effect on the development of the analytical signal because of its influence on LPh oxidation and fluorescence production. The rate of oxidation of LPh by hydroperoxides reached its maximum value at a pH greater than 8, while the fluorescence of phloxin showed maximum intensity at a pH greater than 5. The reagents used to obtain analytical results consisted of 5 × 10^{-5} M LPH/0·04 M borax buffer pH 8·5 and 5 mg litre^{-1} haematin/0·04 M borax buffer pH 8·5.

Calibration and sensitivity

The calibration curve for hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide under the recommended experimental conditions is linear for a peroxide concentration between 4 × 10^{-6} and 8 × 10^{-5} M. The detection limit of this technique is 1·5 × 10^{-7} M, based on a signal-to-noise ratio of 3. However, the fluorescence intensity begins to deviate from a linear response to the peroxide concentration above 10^{-4} M. Regression analysis of the linear portion of the calibration curve gives a standard deviation of 1% for the slope and a correlation coefficient of 0·9995.

The reproducibility was tested by injecting 10 samples of each analyte at two concentration levels. The relative standard deviations were 1·9%, 1·8% and 1·6% at the 4 × 10^{-6} M level and 0·47%, 0·37% and 0·43% at the 3 × 10^{-5} M level for hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide, respectively.

Interference study

The potential interference of various ions or substances in the determination of these three peroxides was examined (Table 1). Solutions of hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide and each species tested were mixed to obtain samples containing 0·06 μmol ml^{-1} of the peroxide. The tolerance ratio of each foreign species was taken as the largest amount yielding an error less than ±3% in the assay.

Any antioxidant present, such as the tocopherol usually existing in oil samples, may interfere in any analytical method based on a redox reaction for hydroperoxide, unless separated beforehand, for example, by high-pressure liquid chromatography. The influence of antioxidants in the present method was studied by using antioxidised oil and fresh oil. The influence on the slope of the calibration line was negligible if the amount of oil sample was restricted to that given in the procedure, but the slope decreased if a relatively large amount of fresh oil was used. It is therefore preferable to determine low hydroperoxide contents in oil samples by the standard-addition method.

Table 1. Influence of other substances on the determination of hydrogen peroxide (2·43 μg ml^{-1})

Species added	Limiting ratio of added species to hydrogen peroxide
NO_3^-, SO_4^{2-}, Cl^-, glucose	500[a]
Br^-	200
Ca(II), Mg(II), HPO_4^{2-}	30
Cu(II), Zn(II)	10
Ni(II), Co(II), Pb(II)	1
Mo(VI), Fe(III)	0·01

[a] Maximum molar ratio tested.

Table 2. Determination of hydroperoxides in oil samples

Oil	Hydroperoxides content (μeq/g)	
	FIA method[a]	Iodometric method[a]
Olive 1	10·47 ± 0·10	10·68 ± 0·17
Olive 2	7·51 ± 0·14	7·42 ± 0·12
Sunflower	35·28 ± 0·23	35·19 ± 0·15
Seeds	5·82 ± 0·09	5·93 ± 0·07
Maize	6·13 ± 0·13	6·08 ± 0·10

[a] Average of three determinations ± SD.

Analysis of real samples

The proposed flow-injection analysis in the stopped-flow mode has a great potential for the sensitive determination of hydroperoxides in real samples. This is confirmed by the results obtained in the determination of hydroperoxides in oil samples and of hydrogen peroxide in milk.

Commercially available oils were analysed and the results obtained are summarised in Table 2. As can be seen for all samples the assay results were in good agreement with the iodometric method.

The data found in the determination of hydrogen peroxide in milk are shown in Table 3. For all kinds of milk, the results obtained agree with the amount added to each sample.

CONCLUSIONS

The results presented in this work clearly demonstrate that leucophloxin, generated by the photochemical reaction between phloxin and EDTA, can be used as an excellent hydrogen donor for the fluorimetric determination of peroxides using the proposed flow-injection configuration in the stopped-flow mode. The method

Table 3. Determination of hydrogen peroxide in milk samples

	H_2O_2 added (μg ml^{-1})	H_2O_2 found (μg ml^{-1} ± SD)	Recovery (%)
Sample 1 (full cream)	3·21	3·14 ± 0·07	97·8
Sample 1 (full cream)	4·82	4·75 ± 0·11	98·6
Sample 2 (full cream)	3·21	3·12 ± 0·09	97·2
Sample 2 (full cream)	4·82	4·76 ± 0·07	98·7
Sample 3 (low fat)	3·21	3·15 ± 0·06	98·1
Sample 3 (low fat)	4·82	4·72 ± 0·09	97·9

has a good sampling frequency, low sample and reagent consumption.

We wish to emphasise that this newly devised method is simple, sensitive and reliable and is applicable for the measurement of the lipohydroperoxide content in commercial oil samples and of hydrogen peroxide in milk.

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REFERENCES

AOAC (1980). *Official Methods of Analysis.* Association of Official Analytical Chemists, Washington, DC, pp. 440–1.

Black, M. J. & Brandt, R. B. (1974). Spectrofluorimetric analysis of hydrogen peroxide. *Anal. Biochem.*, **58**, 246–54.

Cathcart, R., Schwiers, E. & Ames, B. N. (1983). Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal. Biochem.*, **134**, 111–16.

Dunford, H. B. (1982). In *Advances in Inorganic Biochemistry Vol. 4*, ed. G. L. Eichhorn & L. G. Marzilli, Elsevier, New York.

Hicks, M. & Gebicki, J. M. (1979). A spectrophotometric method for the determination of lipid hydroperoxides. *Anal. Biochem.*, **99**, 249–53.

Koizumi, M. & Usui, Y. (1972). Fundamental aspects of the oxidative and reductive photobleaching of xanthene and thiazine dyes. *Mol. Photochem.*, **4**, 57–92.

Pérez-Ruiz, T., Martínez-Lozano, C., Tomás, V. & López-Balsera, C. (1991). A simple photokinetic method for the determination of nitroprusside in biological and pharmaceutical samples. *J. Pharm. & Biom. Anal.*, **9**, 123–8.

Ruzicka, J. & Hansen, E. H. (1988). *Flow Injected Analysis.* John Wiley, New York.

Valcarcel, M. & Luque de Castro, M. D. (1987). *Flow Injection Analysis: Principles and Applications.* Ellis Horwood, Chichester, UK.

Yagi, K., Kiuchi, K., Saito, Y., Miike, A., Kayahara, N., Tatano, T. & Ohishi, N. (1986). Use of a new methylene blue derivative for determination of lipid peroxides in foods. *Biochem. In.*, **12**, 367–71.