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Determination of Benzoyl Peroxide and Benzoic Acid Levels by HPLC during Wheat Flour Bleaching Process

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Freshly milled wheat flour has a pale yellow color due to its carotenoids content. Benzoyl peroxide is a bleaching agent typically used to give such flour a better appearance. This free-radical initiator promotes carotenoids oxidation, thereby producing less colored compounds, and benzoic acid is a main final product. Samples of wheat flour were treated with 150 ppm of benzoyl peroxide to begin a bleaching process, and then subjected to ethyl ether extraction at different intervals of time. Benzoyl peroxide and benzoic acid levels in these extracts were monitored by means of HPLC in individual experiences. The resulting concentration of benzoyl peroxide after 9 days of contact with the bleaching agent was 11 ppm, dropping afterward to nondetectable levels. A maximum value for benzoic acid of 16 ppm was found after 12 h of bleaching. Subsequently this level decreased continuously until reaching a residual value of 6 ppm after 3 months.

Keywords: *Wheat flour; benzoyl peroxide; benzoic acid; bleaching reaction*

INTRODUCTION

Freshly milled wheat flour has a pale yellow tint and yields a sticky dough. This results in bakery products of a color that is less commercially acceptable, and dough made from this flour does not handle or bake well.

During flour storage some reactions take place between flour components and oxygen, an overall process known as aging or maturing. As a result, flour gradually turns into a less-colored product and, at the same time, renders a dough with improved bakery properties (Fennema, 1985). It has been well established that these improvements on rheological properties of dough, and bread quality such as loaf-volume, texture, and freshness retention (Pomeranz, 1985), as well as the resulting whiteness of the naturally matured flour, are a direct consequence of oxidative reactions.

Carotenoid pigments are responsible for the majority of the yellow and orange colors in the plant kingdom. Ripening grains of wheat contain these pigments, especially in the bran. When the endosperm is milled and made into flour the bran is separated. However, only a partial mechanical separation is possible because these closely adhering structures have different physical properties (Pomeranz, 1985). As a consequence, the resulting freshly milled flour has enough of the pigment remaining to cause the typical yellowish color. The majority of carotenoids can be viewed as joining together 8 isoprene (C_5H_8) units forming aliphatic-alicyclic structures. The bright color of these pigments comes from the chain of conjugated double bonds (Emodi, 1978).

Lutein is the xanthophyll present in the highest quantity in wheat seeds (Sims and Lepage, 1968). One kilogram of wheat flour contains ≈ 3 mg of carotenoids, mainly lutein (Farre-Rivora and Costes, 1974), and the

bread crumb color results from these natural yellowish pigments remaining in flour (Gélinas et al. 1998).

Oxidizing agents are normally added to flour in order to accelerate its natural maturing. It is known that their dough-improving action is because of oxidation of sulfhydryl groups present in flour gluten proteins. These agents may participate in bleaching only, in both bleaching and dough improvement, or in dough improvement only.

Flour bleaching involves primarily a disruption of the conjugated double bond system of carotenoids to a less-conjugated colorless system. Benzoyl peroxide is among the most common oxidizing agents used for wheat flour, and it exhibits a bleaching or decolorizing action without influence on baking properties (Fennema, 1985). Although it is used in various countries, regulations do not always exist concerning its acceptable quantities and use conditions. Benzoyl peroxide is a free radical initiator, and it produces carotenoid oxidation by a typical free radical mechanism (Fennema, 1985). Products of reaction between specific peroxy radicals and carotenoids have been studied in a variety of chemical systems, but not all products of the reaction have been identified (Kennedy and Liebler, 1991, 1992; Yamauchi et al., 1993).

The objective of this study was to follow the flour bleaching reaction by quantifying benzoyl peroxide and benzoic acid at various time intervals after treating wheat flour with a bleaching agent.

MATERIALS AND METHODS

Flour. The test material was a commercial flour, 000 quality, without additives. The content of ash, moisture, and protein were $0.589\% \pm 0.185$, $12.5\% \pm 0.10$, and $12.01\% \pm 0.10$ ($N \times 5.7$) respectively, as determined in accordance with the AOAC methods (AOAC, 1990).

Solvents and Reagents. Solvents used for HPLC were HPLC grade (EM Science, Gibbstown, NJ). Benzoic acid and diethyl ether were purchased from Merck Química Argentina

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SAIC (Buenos Aires, Argentina). Benzoyl peroxide was obtained from Sigma Chemical Co. (St. Louis, MO). Double-distilled water was used. All the other chemicals were of the highest grade available.

The bleaching additive was benzoyl peroxide 30% p/p: 30 mg of benzoyl peroxide was mixed with 70 mg of calcium carbonate (used as support), and the mixture was passed through a sieve in order to obtain a homogeneous powder.

Sample Preparation and Bleaching Reaction. Separating procedures were carried out by individual experiments in order to quantify benzoyl peroxide and benzoic acid.

The sample was prepared by blending 50 g of flour with bleaching additive, which represented 150 ppm of benzoyl peroxide in the flour. To obtain a homogeneous system, the mixture was passed through a polyester sieve (400 mesh cm^{-2}) four times, and held in the dark. The bleaching reaction occurred at room temperature and was monitored at specific time intervals. The products of reaction were extracted from the whole sample every 4 h for a period of 24 h, and daily for 3 months thereafter. The samples were analyzed by HPLC.

Calibration Graph for Benzoyl Peroxide. A working standard solution of benzoyl peroxide (80 mg L^{-1}) was dissolved in diethyl ether. Appropriate volumes of this working solution were diluted with the same solvent, and ppm of benzoyl peroxide in the solutions were correlated with ppm benzoyl peroxide in flour according to the extraction procedure, to make the standard curve like area vs ppm of benzoyl peroxide in flour.

Calibration Graph for Benzoic Acid. Stock solution was prepared by dissolving 122.12 mg of benzoic acid in 100 mL of methanol to get a concentration of $1 \times 10^{-2} \text{ M}$. A working standard was prepared by dilution of stock solution with methanol in order to yield a final concentration of $1 \times 10^{-3} \text{ M}$. Appropriate volumes of this working solution were diluted with the same solvent, and ppm of benzoic acid in the solutions were correlated with ppm of benzoic acid in flour, according to the extraction procedure, to make the standard curve like area vs ppm of benzoic acid in flour.

Extraction Procedure For Benzoyl Peroxide and Benzoic Acid. The standard procedure, which was carried out at room temperature, was developed in a flask supplied with a grinding stopper. The method involved the addition of 100 mL of diethyl ether to 50 g of flour that had been previously mixed and sifted with the bleaching additive. The mixture was shaken in a magnetic stirrer for 10 min and then it was left in repose for 15 min more.

An aliquot of diethyl ether (upper layer) containing the extracted products of reaction was withdrawn with a pipet, transferred into a 1.7-mL polypropylene tube, and held into ice until the moment of injection.

HPLC Method. HPLC was conducted on a Konik liquid chromatograph, equipped with KNK 500 G quaternary pumps and a variable-wavelength UV-vis 200 detector, a Rheodyne 7125 injector supplied with a 20-mL loop, and a Spectra Physics Analytical SP 4600 integrator. The ether extract was separated and analyzed (5 μL aliquots) using a $250 \times 4.6 \text{ mm}$ Alltech Econosphere C18 ($10 \mu\text{m}$) column, at room temperature. Different mobile phases were used to quantify benzoyl peroxide and benzoic acid. Benzoyl peroxide was well solved using methanol/water (80:20, v/v), but the peak of benzoic acid occurred too early with this mobile phase and overlapped with peaks of solvent. For the quantification of benzoic acid, phosphate buffer (0.03 M, pH 6.5)/methanol (95:5) was used. The flow rate was set at 1.0 mL/min. Peaks of benzoyl peroxide and benzoic acid were measured at a wavelength of 227 nm. Chromatographic peaks were identified by comparing retention times and spectra against known standards.

RESULTS AND DISCUSSION

Results shown in Figure 1 compare UV absorbance of a standard preparation of benzoyl peroxide, benzoic acid, and a sample of flour treated with bleaching agent.

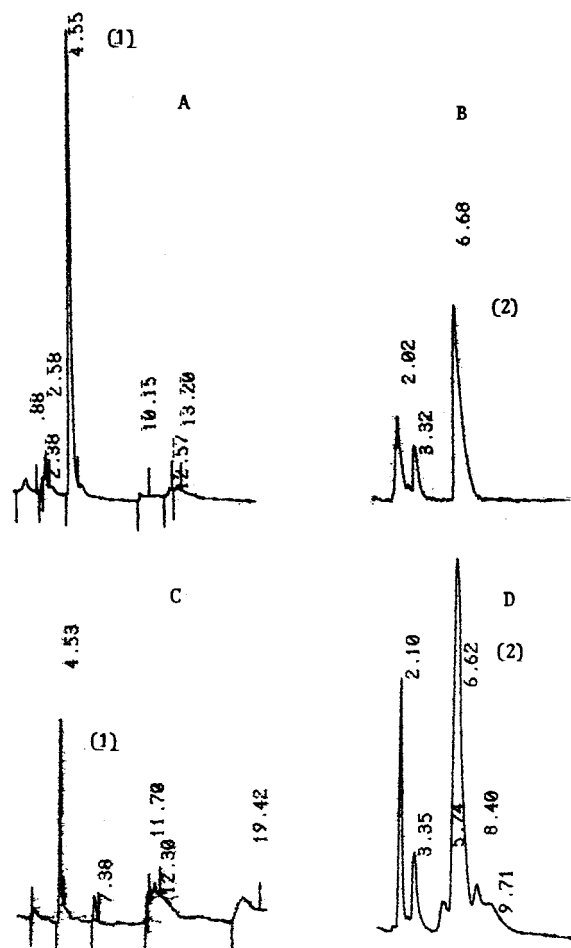


Figure 1. Typical chromatograms for the elution of a standard preparation of benzoic acid and benzoyl peroxide (A and B) and a sample of flour during bleaching reaction (C and D). Peak 1 is benzoic acid and peak 2 is benzoyl peroxide.

Comparison of retention times and spectra with those of pure standards established the identities of the peaks. The calibration curves of peak-area (A) ratio against the concentration (C) for benzoyl peroxide ($A = 4.67579 + 1.4488C$, $r = 0.9991$, $SD = 4.24669$, $n = 6$) and for benzoic acid ($A = -0.2018 + 1.31677C$, $r = 0.9997$, $SD = 1.08703$, $n = 6$) gave a linear response over a wide range of concentrations (Figure 2).

Precision. Three experiments of bleaching reaction were performed using the same reagents and apparatus to evaluate the method precision. Benzoyl peroxide (150 ppm, the amount used usually as bleaching additive) was spiked to the sample of flour, and determinations of benzoic acid and benzoyl peroxide remaining were carried out at different periods of time. Results in Table 1 show that a relative standard deviation (RSD) of less than 10% was obtained for benzoyl peroxide for all the times assayed. Table 2 shows the results for benzoic acid. RSD was slightly higher than 12% at the beginning of reaction. Then, most of the other values were below 10%. These results were satisfactory according to the Horwitz formula for intralaboratory studies (Horwitz, 1982).

Recovery. Recovery was tested by the standard addition procedure in order to demonstrate the accuracy of analysis. Known amounts of benzoyl peroxide and benzoic acid were added to the flour. Three determinations with three spiking levels were carried out

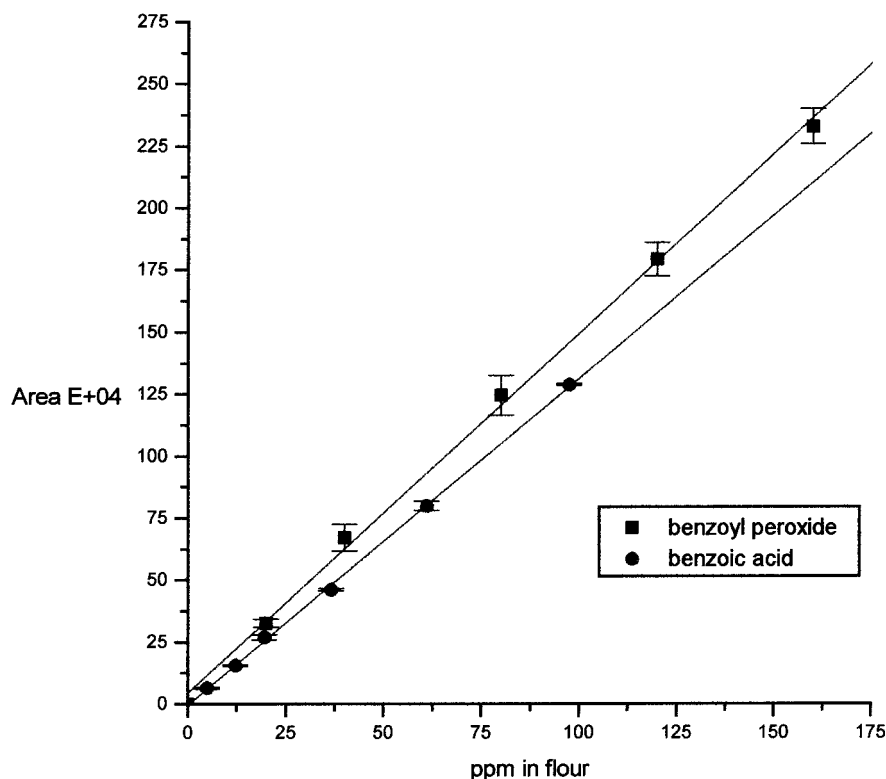


Figure 2. Typical standard curves for benzoic acid and benzoyl peroxide. Values represents means \pm SD for triplicate analyses.

Table 1. Precision of the Method for Determination of Benzoic Acid in the Flour at Various Time Intervals During Bleaching

time (h)	$\bar{x} \pm \text{SD}^a$	RSD, ^b %
0	4.71 \pm 0.67	14.25
2	7.41 \pm 0.87	11.60
4	7.99 \pm 0.18	2.23
8	11.52 \pm 0.84	7.33
12	16.39 \pm 0.71	4.32
16	11.55 \pm 0.81	7.01
20	10.75 \pm 0.69	6.42
24	10.21 \pm 0.51	5.01
30	9.61 \pm 0.26	2.71
36	9.01 \pm 0.39	4.37
48	9.07 \pm 0.14	1.53
60	9.29 \pm 0.22	2.39
72	9.77 \pm 0.65	6.63
96	9.10 \pm 0.52	5.75
120	9.08 \pm 1.80	19.82
144	9.20 \pm 0.87	9.51
168	8.59 \pm 1.01	11.75
192	8.22 \pm 0.80	9.84
216	8.12 \pm 0.38	4.69
240	6.80 \pm 0.53	7.78
360	6.23 \pm 0.19	3.14

^a Mean \pm standard deviation in milligrams per kilogram ($n = 3$). ^b RSD, relative standard deviation.

(Table 3). Recovery of benzoic acid from flour was satisfactory, because it was higher than 80% when the level spiking was 10 ppm and more than 90% when the concentration of standard added was higher. The mean recovery for benzoyl peroxide was $\sim 79\%$. The minor recovery obtained in comparison with benzoic acid was attributable to the reaction between carotenoids compounds and benzoyl peroxide during extraction procedure.

Benzoyl Peroxide and Benzoic Acid Contents during the Flour Bleaching Reaction. Concentrations of benzoyl peroxide and benzoic acid (in ppm) during the bleaching reaction are shown in Figure 3a

Table 2. Precision of the Method for Determination of Benzoyl Peroxide in the Flour at Various Time Intervals During the Bleaching

time (h)	$\bar{x} \pm \text{SD}^a$	RSD, ^b %
0	151.06 \pm 2.60	1.72
1	143.26 \pm 0.88	0.61
2	119.99 \pm 3.09	2.57
6	104.23 \pm 4.61	4.42
8	97.40 \pm 1.62	1.66
12	81.07 \pm 1.14	1.40
18	67.97 \pm 4.41	6.49
24	57.10 \pm 1.66	2.91
48	40.10 \pm 2.44	6.08
72	12.52 \pm 1.04	8.31
96	12.22 \pm 0.49	4.01
120	11.89 \pm 0.15	1.26
216	11.05 \pm 0.70	6.33

^a Mean \pm standard deviation in milligrams per kilogram ($n = 3$). ^b RSD, relative standard deviation.

and b, respectively. Benzoyl peroxide concentration decreased from 150 ppm at 0 time to 11 ppm after 9 days of contact. The active bleaching agent was not detected afterward. Benzoic acid concentration exhibited a maximum value of 16 ppm, 12 h after the beginning of reaction. In the following 30 h this concentration decreased slowly to 11 ppm and the remaining concentration was approximately constant during 9 days. Final concentration attained a value around 6 ppm after 3 months.

Data obtained showed that the main stable metabolite in the decomposition of benzoyl peroxide was benzoic acid. Concentration values reached for this substance indicated low levels through the bleaching process indicating no risk for health according to levels permitted when it is used as preservative agent in foods.

On the other hand, results indicated that benzoyl peroxide was not completely decomposed to benzoic acid. Under the chromatographic conditions used it was not

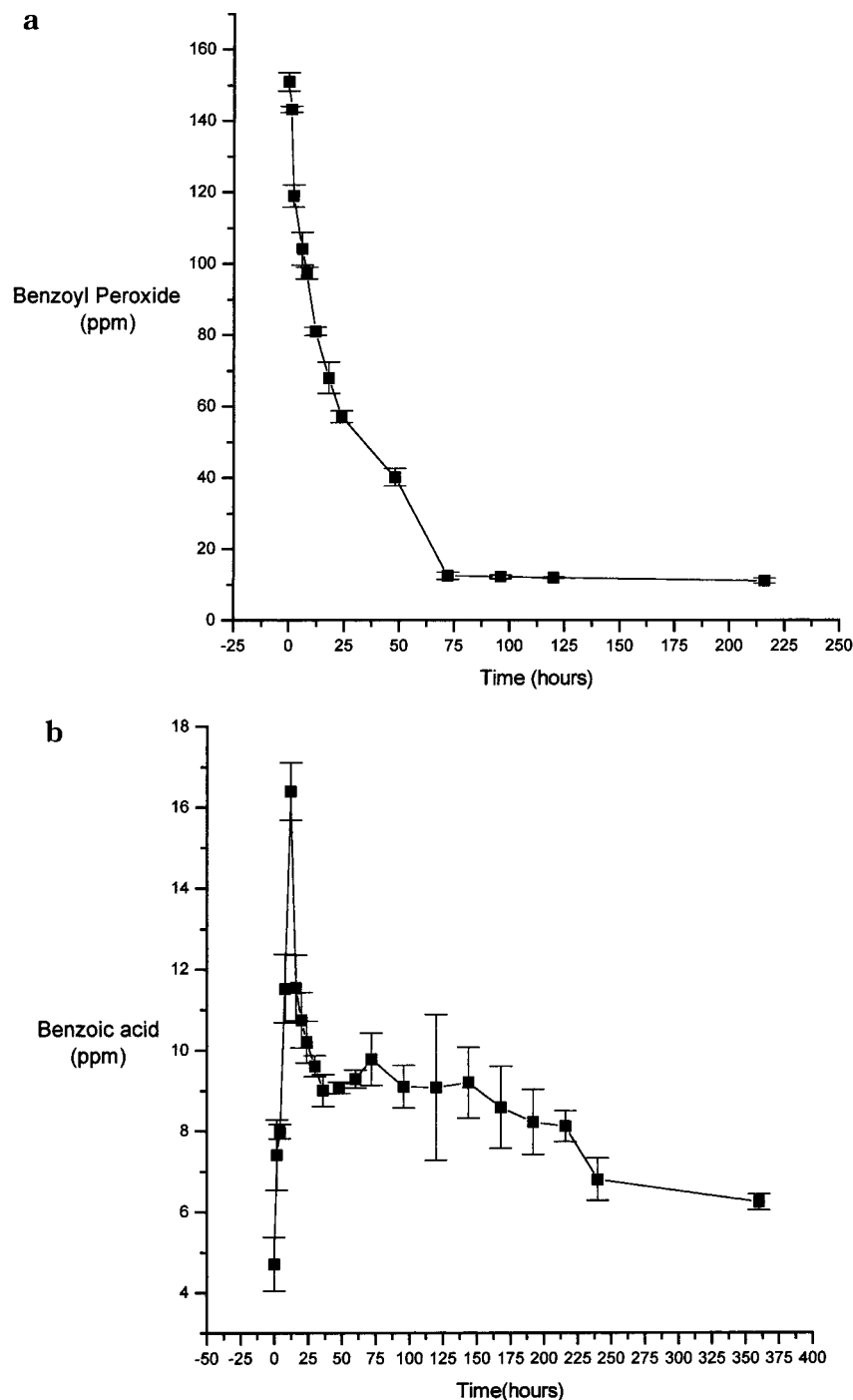


Figure 3. Panels a and b, levels (ppm) of benzoic acid and benzoyl peroxide in flour during increasing period of contact with bleaching additive. Values represent means \pm SD for triplicate analysis.

Table 3. Recovery of the Method for Determination of Benzoic Acid and Benzoyl Peroxide in Flour

	flour initial contents (ppm)	level I (10 ppm)	level II (25 ppm)	level III (50 ppm)	mean recovery, %
benzoic acid	ND	8.3 \pm 0.21	22.85 \pm 0.085	48.1 \pm 1.07	90.21
benzoyl peroxide	ND	6.06 \pm 0.94	20.25 \pm 0.45	47.3 \pm 2.14	78.96

possible to observe another product of reaction, but it is probable that a similar β -carotene–benzoyl peroxide mechanism occurs. It has been suggested that the chain-propagating peroxy radical is trapped by addition to the conjugated polyene system of β -carotene forming a resonance-stabilized, carbon-centered β -carotene radical (Burton and Ingold, 1984; Yamauchi et al., 1993). According to these mechanisms, benzoyl peroxide can react with flour carotenoids (mainly lutein) forming a

resonance stabilized peroxy radical carotenoid, although it too could be trapped generating a stable adduct (Pinzino et al., 1999).

These results are in accordance with the hypothesis that, following a free radical oxidative attack, part of benzoyl peroxide is transformed into benzoic acid and part could react with carotenoids by means of trapped peroxy radical in polyene systems or the adduct formations.

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