A Basis for Measuring the Intensity of Wheat Flour Pigments[1]

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ABSTRACT

The extraction method for measuring content of wheat flour pigment was re-examined, and recommendations for improving the method were made. For relative measurement of p.p.m. carotene, absorbance at 440 mμ and absorptivities of 1.9165 in naphtha-alcohol solvent (N/A) and 1.6632 in water-saturated butanol (WSB) are retained. Recommended changes are: the use of 7% absolute ethanol in n-hexane (v./v.) as solvent, and routine instrument checks with standard solutions of potassium chromate in 0.05*N* aqueous KOH. For absolute values, expressed as mg./100 ml. solution, the true pigments of wheat flour, lutein and its esters, must be used. Absorptivities were determined for the pigments at peak wave lengths in both N/A and WSB; again N/A is the preferred solvent. On the basis of knowledge of the composition of pigments in Thatcher and Mindum wheats, absorptivities were calculated for each flour type.

In the 5th edition of *Cereal Laboratory Methods* (1), absorptivities for carotene at 435.8 mμ in both naphtha-alcohol and water-saturated butanol are listed, and an equation is given for relating pigment concentration as determined by naphtha-alcohol extraction to intensity as found by extraction with water-saturated n-butanol. Later editions carry a simplified version of the procedure. The wave length and absorptivities used in the latest (7th) edition, however, are still inconsistent with the physics and chemistry of wheat flour pigments. Moreover, calibration of colorimeters or spectrophotometers against absolute absorbance standards is not recommended. Lepage and Sims (2) have identified the pigments of Canadian hard red and amber durum wheats and studied their spectral characteristics. This information further increases the need to re-examine the measurement of wheat flour pigments by the extraction method.

In this paper, the absorption spectra of typical wheat flour extracts obtained with both naphtha-alcohol and water-saturated n-butanol are reported. These spectra are compared with those of pure beta-carotene and all-*trans* lutein (xanthophyll) and its esters. Quantitative data are used to show the magnitude of the differences that result from the use of different standard materials for calibration, and the quality of the data obtained by use of colorimeters is compared with that obtained with spectrophotometers. Suggestions for improving the method for measuring wheat flour pigment concentration by the extraction method are offered.

MATERIALS AND METHODS

The following reference substances were used: pure beta-carotene (Eastman Kodak, Rochester, N. Y.) recently recrystallized from a chloroform: methanol mixture, and all-*trans* lutein, either from Eastman Kodak or prepared chromatographically from crude material supplied by Fluka, A.G. 9470

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Bucks. 5.6, Switzerland. The true lipid material extracted anaerobically from Thatcher (hard red spring) and Mindum (amber durum) wheat flours with n-butanol saturated with water at 15°C. (WSB) and with a 7% (v./v.) solution of absolute ethanol in hexane (N/A) served as sources of wheat flour pigments. The carotene and lutein were dissolved in chloroform, cyclohexane, WSB, and N/A, and the flour pigments in cyclohexane, to make solutions of known concentration for quantitative studies. Pigment was weighed to 0.001 mg., and solvent, equilibrated at 20°C., was weighed into volumetric flasks. From measured densities at 20°C., volumes of solvent were calculated. To determine the effect of water content on the absorption spectrum of carotene, n-butanol was equilibrated with distilled water at 1°, 15°, and 25°C. and used as solvent for carotene. Potassium chromate (N.B.S. Primary Standard) dissolved in 0.05*N* potassium hydroxide solution was used as the absorbance standard (3). The photometric accuracy of the spectrophotometers was further checked by use of neutral filters prepared by the Division of Applied Physics, National Research Council, Ottawa, Canada. In addition, wheat flours milled recently and 12 months previously were extracted with WSB and N/A; to simulate regular practice, "colorless naphtha" was used instead of hexane to prepare the N/A.

When absorbance through the 5-cm. colorimeter cells was being compared with that through the 1-cm. spectrophotometer cells, paired solutions were used in all cases, one member of each pair being five times as strong as the other. Flour:solvent ratios were also increased fivefold when necessary to accommodate the shorter light path of the spectrophotometer.

For qualitative analysis, a Bausch & Lomb 502 recording spectrophotometer was used, with a scanning rate of 2 and a time constant of 0.1. For quantitative work, a Photovolt Lumetron colorimeter with M440 and M420 filters and 5-cm. cells was used, as well as a Beckman DU spectrophotometer. Absolute absorptivities were calculated from data obtained with a calibrated Beckman DU spectrophotometer and calibrated glassware.

The pigment-containing fractions obtained by the silicic acid column chromatography of hexane and WSB extracts of Thatcher and Mindum flours (2) were analyzed spectrophotometrically. The fractions were taken to dryness under a stream of nitrogen, dissolved in appropriate volumes of cyclohexane, and scanned in a Bausch & Lomb 502 spectrophotometer.

RESULTS

Absorption of Flour Extracts in the Visible Region of the Spectrum

Recent and old extracts of both old and recently milled flour were compared, and the influence of solvent type on absorption spectrum was investigated. The results showed that, whereas the color extracted with WSB was stable in this solvent for at least 4 months, the N/A extracts of the same flour and of the same age no longer absorbed in the visible region of the spectrum. Because of this fading, only fresh extracts were used. When distilled technical-grade hexane was used instead of commercial naphtha, however, the pigment did not fade.

To determine the effect of the water content of WSB on the absorption

spectrum of carotene and its absorptivities, n-butanol was equilibrated with water at 1°, 15°, and 25°C. Samples of each solvent were taken, allowed to come to room temperature, and used to make quantitative, volumetric solutions of beta-carotene which had absorptivities of 1.596, 1.493, and 1.453 respectively. For this reason, WSB was prepared and used at one temperature, 15°C.

Quantitative Studies

Solutions of carotene, lutein, and flour pigment extracts in both N/A and WSB, as well as potassium chromate in aqueous alkali, were tested for adherence to Beer's Law in both the spectrophotometer and the colorimeter. All solutions gave linear plots of absorbance *vs.* concentration up to the limits tested: 0.5 mg./100 ml. for carotene and lutein; 60 mg./100 ml. for potassium chromate.

With the use of measured absorbances of solutions of known concentration, absorptivities were calculated for beta-carotene in cyclohexane, N/A, and WSB, lutein in WSB, and potassium chromate in 0.05*M* KOH (Table I).

TABLE I

ABSORPTIVITIES OF STANDARD MATERIALS, CORRECTED FOR INSTRUMENT EFFICIENCY[a]

Reference Material	Beckman D.U.				Lumetron Colorimeter			
	440 mμ		420 mμ		440 mμ		420 mμ	
	N/A	WSB	N/A	WSB	N/A	WSB	N/A	WSB
K_2CrO_4 in aq. KOH	GV[b] 0.0134		GV 0.0315		GV 0.0134		GV 0.0315	
	FV[b] 0.0133		FV 0.0294		FV 0.0115		FV 0.0289	
All beta-carotene	1.91	1.56	1.50	1.20	1.77	1.10	1.32	0.733
All-*trans* lutein	....	2.00	....	1.53	....	....	....	....

[a] 1 cm.; 1 mg./100 ml.
[b] GV = given value; FV = found value.

The marked differences in values between solvents and between instruments strongly indicate the necessity of stating conditions fully when flour color intensity is reported. Without exception, the N/A solutions had higher absorptivities than corresponding WSB solutions.

Varietal Differences in Pigments

The spectra of volumetric solutions of Thatcher and Mindum flour pigments plus lipids were recorded (Fig. 1). The spectra resemble each other and that of lutein but not that of carotene. From these curves, absorptivities were calculated. These values are only about 1/5,000 as large as the absorptivity of lutein, but the absorptivities of the Mindum pigments were almost twice those of Thatcher, and for each flour type the absorptivity of the N/A extract was larger than that of the WSB extract.

Hexane extracts of the flours containing the "free" lipid and associated pigment, and WSB extracts containing total lipid and pigment were fractionated on a silicic acid column to determine at what point the pigments appeared (2). Thatcher pigments were associated almost exclusively with the "free" lipid, whereas with Mindum flour, the pigments accompanied the

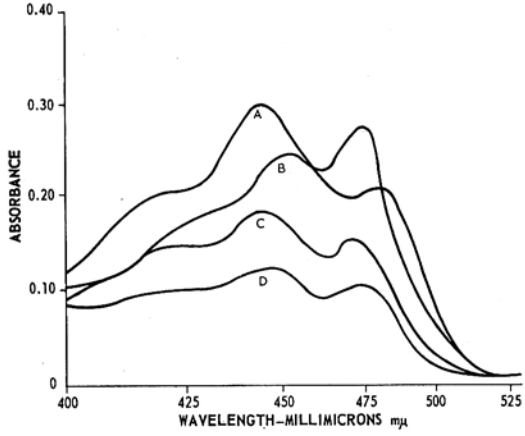

Fig. 1. Spectra of reference compounds and wheat flour pigments plus lipid in cyclohexane: A, *trans* lutein; B, beta-carotene; C, N/A extract of Mindum flour; D, WSB extract of Thatcher flour.

bound lipid. The pigments were eluted from the column in the following order: Thatcher pigment mostly preceded the triglyceride fraction, although some accompanied the unesterified sterol fraction; Mindum pigments were almost completely associated with the unesterified sterol fraction. The absorption spectra of each of the colored fractions were measured and compared. All of the spectra were quite similar; none agreed with that of beta-carotene, but all resembled the spectrum of all-*trans* lutein.

Absolute Absorbance of Lutein

Standard solutions of approximately 1.250 mg. in 500.0 ml. solvent were made up in chloroform, cyclohexane, WSB, and N/A. Absorbances were measured at peak wave length with a Beckman D.U. spectrophotometer, calibrated with a mercury lamp for wave-length accuracy and with neutral filters for photometric accuracy. For calculation of absorptivities, the cell length of 1.004 cm. was used (Table II).

TABLE II

ABSORPTIVITIES FOR CALCULATING ABSOLUTE PIGMENT CONCENTRATIONS[a]

	WSB (449 mμ)	N/A (445 mμ)	CYCLOHEXANE (449 mμ)	CHLOROFORM (456 mμ)
Lutein	2.336	2.377	2.117	2.116
Lutein monoester	1.546	1.571	1.399	1.399
Lutein diester	1.156	1.177	1.048	1.047
Thatcher	1.599	1.626	1.448	1.447
Mindum	2.191	2.230	1.986	1.985

[a] 1 cm.; 1 mg./100 ml.

DISCUSSION

The amount of pigment reported to be in a flour examined by the extraction method depends on the nature of the pigments present, the wave length

of the incident light, the absorptivity used to calculate the concentration, the liquid used as extractant and solvent for the pigment, and the photometric accuracy of the measuring instrument. In the last-mentioned case, it is assumed that there is no error due to imbalance at 0 or 100% transmission and that the wave-length scale and the monochromator are in adjustment; or, with a filter instrument, that the filter transmits the proper light.

Lutein has peak absorption at 449 mμ in WSB and 445 mμ in N/A, and at these wave lengths the tentative absorptivities are 2.336 and 2.377 respectively. Lutein mono- and diesters absorb at the same wave lengths of the parent compound, but their absorptivities are reduced by the increased molecular size of the compounds. Average molecular weights have been calculated for these compounds (2), and the following average absorptivities have been tentatively assigned: lutein monoesters, 1.546 (WSB), 1.571 (N/A); lutein diesters, 1.156 (WSB), 1.177 (N/A). Knowledge of the composition of the pigments in Thatcher and Mindum flours (2) permits calculation of the following absorptivities for the flours: Thatcher, 1.599 (WSB), 1.626 (N/A); Mindum, 2.191 (WSB), 2.230 (N/A). Thus not only does the amber durum wheat contain more pigment on a percent by weight basis (2) than the hard red spring wheat, but its average absorptivity is higher.

For research purposes, particularly plant breeding, knowledge of the true amount of pigment is important, and absorptivities appropriate to the nature of the pigments should be used (Table II). For control work and the screening of a large number of samples of similar flours, however, relative values and "p.p.m. carotene" calculated with a fictitious absorptivity generally suffice. For this type of work any value will do, and that prescribed in *Cereal Laboratory Methods* (1) is accurate for WSB at 435.8 mμ. The choice of solvent and wave length will be discussed later.

If hexane is used to make up the "naphtha-alcohol" solvent, instead of "commercial naphtha," the extracted pigments do not fade, and advantage can be taken of the useful properties of this solvent: higher absorptivity, reduced dependence of peak wave length on solvent, greater pigment solvent power, and freedom from need to prepare the solvent at a fixed temperature. In addition, this solvent is cheaper than n-butanol. It presents a fire hazard, but its fumes are not as obnoxious as those of butanol.

Whenever naphtha-alcohol and water-saturated butanol are considered as pigment solvents, an equation is derived for converting pigment concentration as measured in one solvent into a corresponding concentration for the other. Although this practice is sound mathematically provided absorbance and not transmittance is used, its chemical validity can be questioned. As shown here, the luteins absorb more strongly in N/A than in WSB, yet "butanol" color values are invariably higher, for a given flour, than "naphtha-alcohol" values. This invites the deduction that WSB extracts more pigment than N/A, yet the absorptivities of the extracts dissolved in cyclohexane indicate that the material extracted by the two solvents differs both in quality and quantity. Conventional interconversion, based solely on quantity difference, is therefore of doubtful value.

The intercept of the interconversion equation (1) might be considered

to take care of this problem. However, not only is it an average value but the intercept itself disappears when absorbance and not transmittance is plotted. The use of one solvent for flour pigment determination is, therefore, sound practice.

The amount of pigment reported in a sample can be a function of the instrument used and the wave length selected for reading the absorbance. Wave lengths of 420 and 440 mμ are not recommended, because the curve for lutein (Fig. 1) slopes sharply at these wave lengths. The peak wave length, around 447 mμ, depending on the solvent used, is preferable for this reason and because of the higher absorptivity at this wave length. Now that colorimeters and spectrophotometers with monochromators are ubiquitous, there is no longer any need to measure at traditional wave lengths. The use of the peak wave length is further indicated by the fact that the lutein peak is sufficiently broad so that small errors, due to an incorrect wave length scale or scale calibration, are unimportant.

A pair of Beckman D.U. spectrophotometers, a pair of Bausch & Lomb 502 spectrophotometers, and a Bausch & Lomb Spectronic 20, when tested for photometric accuracy with four solutions of potassium chromate of increasing absorbance, all showed apparent photometric inaccuracy which varied with wave length. Departures from calculated absorbances as large as 10% were noted, suggesting that standard solutions or neutral filters be used to calibrate the instrument. Even if only relative values are required, an instrument efficiency check has value in that it will reveal changes in behavior of the instrument. When absolute values are required, such a check is essential. Similar information is shown by Booth (4).

Procedure

On the basis of the data reported here, the following procedure for measuring intensity of wheat flour pigment is proposed:

Step 1. Preparation of Standard Graph. a. Filter-type colorimeter. Select a filter with peak transmission at 440 mμ. For relative values of "carotene" use a = 1.9165 in N/A and a = 1.6632 in WSB (1,4). Calculate the absorbance of solutions of definite pigment concentrations, using the formula, A = a.C.b. Plot A *vs.* C.

b. Instruments with monochromators. For relative values of "carotene" set the instrument at 440 mμ and follow the above procedure. For absolute values, set the wave length to the peak of lutein in the extraction solvent and use the values appropriate to the solvent and the flour type. Calculate A, and plot A *vs.* C.

Step 2. Determination of Colorimeter Efficiency. Prepare four solutions of potassium chromate in 0.05*N* aqueous potassium hydroxide, ranging in absorbance from 0.1 to 0.8 in cells of 1-cm. light path. These solutions obey Beer's Law. (Dilute them fivefold if 5-cm. cells are to be used.) Measure their absorbance in the instrument to be calibrated, calculate the absorptivity, and express it as a percentage of the absolute absorptivity of potassium chromate in 0.05*N* aqueous KOH at this wave length (3).

Step 3. Correction of Measured Absorbance for Colorimeter Efficiency. Apply the efficiency factor calculated in step 2 to the measured absorbances to correct them for instrument error. Use this corrected absorbance as ordinate in the graph prepared in step 1 and read the pigment concentration.

Step 4. Check on Colorimeter Efficiency. Check the colorimeter efficiency from time to time, with the standard potassium chromate solution. Apply the new correction factors whenever changed efficiency requires it.

Notes: 1. The above suggested method has the advantage that its reference standard is a potassium chromate solution that is stable for at least 2 years (3).

2. Use of a standard solution that also provides a check on colorimeter efficiency will permit more ready comparison of results between laboratories.

3. Although the absorptivity of carotene or lutein is used in the calculations, standard solutions of the pigments need never be prepared, and a standard absorptivity can be selected by a committee if so desired.

Literature Cited

1. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. Cereal laboratory methods. The Association: St. Paul, Minn. (5th ed., 1947; 7th ed., 1962).
2. LEPAGE, M., and SIMS, R. P. A. Carotenoids of wheat flour: their identification and composition. Cereal Chem. 45: 600–604 (1968).
3. NATIONAL BUREAU OF STANDARDS. Circular 484. U.S. Dept. of Commerce, Washington, D.C. (1949).
4. BOOTH, V. H. Carotene, its determination in biological materials, p. 41. W. Heffer and Sons Ltd.: Cambridge, England (1957).

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