Systematic Identification of Artificial Food Colors Permitted in the United States

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A new comprehensive procedure has been evolved from published methods for the analysis of artificial dyes in food. The procedure utilizes wool dyeing for screening, column adsorption on alumina for isolation, and paper chromatography for separation and identification. Semiquantitative estimations of the amount of dye present may be made by either reflectance or absorption spectrophotometry. In addition, the scheme offers the advantages of speed and certainty of separation of the food dyes which are permitted in the United States.

Although Federal regulations require that an application for the listing of a color additive include a method for its determination in food, no comprehensive procedure has yet been published which is suitable for the routine examination of food products for the presence of artificial colors. Existing methods are cumbersome and slow.

In the development of the following scheme, suitable for the routine isolation, separation, identification, and quantitative determination of the U. S. food colors, many methods presented in the literature were tested and evaluated (*1–38*).

For the isolation of artificial color from a food, alumina columns (*10*) and wool-dyeing procedures (*3, 4, 8, 12, 18, 31*) are preferred to solvent extraction (*2, 5, 17, 24, 25*), which discolors several of the permitted dyes.

Separation of the isolated colors is best accomplished by paper chromatography (*1, 2, 6, 7, 11–13, 18, 19, 22, 26–29, 31–34, 36–38*) which, under proper conditions, gives sharper separations than do techniques employing electrophoresis (*1, 23*), electrochromatography (*16*), column chromatography (*15, 20, 30, 32*), or solvent partition (*5*).

Color reactions with various reagents, when observed visually (*4, 36*) or spectrophotometrically (*5*), may be used for identification of the separated colors. R_f values of the chromatographed dyes, used in conjunction with color reactions (*13, 36*), provide further verification of their identities.

No satisfactory method of identification of unseparated dyes has been found. Though the spectrophotometer will respond to any and all dyes, it may not distinguish natural food colors from coal-tar dyes, nor does it permit the investigator to segregate from a mixture of colors those particular absorbances which are attributable to a specific dye.

The wool-dyeing procedure, in addition to its value as a tool for isolation, is one of the few techniques applicable to qualitative testing for the presence of artificial color. Lichen colors such as cudbear and litmus are the only common vegetable colors known to give false positives with this test. Ease and rapidity of performance make it ideal as a screening procedure.

Spectrophotometric methods (*14, 20*) are best suited to quantitative estimations of the dyes isolated from foods. In the development of the following procedure, methods yielding semiquantitative recoveries were incorporated so that the scheme would also be applicable to estimation of the amount of dye present in the food sample.

Table I lists dyes that are currently or were formerly certified for use in foods in the United States, together with their common synonymous designations

Apparatus

The only specialized apparatus used was a 20- × 300-mm. glass tube fitted with a stopcock and arranged for column chromatography. The tube was filled to a depth of 150 mm. with activated alumina (150 to 250 mesh, prepared by heating at 400° C. for 1 hour) which was held in place by glass-wool plugs above and below. The packing was washed with dilute HCl (1 + 9) to remove fines and to compact and acidify the column.

Table I. Dyes Currently or Formerly Certified for Use in Foods in the United States

FDC Designation	Common Name	1st Ed. Colour Index (9) No.	2nd Ed. Colour Index (10) No.	2nd Ed. Colour Index (10) Food Designation
Red 1[a]	Ponceau 3R	80	16155	Red 6
Red 2	Amaranth	184	16185	Red 9
Red 3	Erythrosine	773	45430	Red 14
Red 4	Ponceau SX	...	14700	Red 1
Red 32[a,b]	Oil Red OX	73	12140	...
Orange 1[a]	Orange I	150	14600	...
Orange 2[a,b]	Orange SS	...	12100	...
Yellow 1[a]	Naphthol Yellow S (Sodium salt)	10	10316	Yellow 1
Yellow 2[a]	Naphthol Yellow S (Potassium salt)	10	10316	Yellow 1
Yellow 3[a,b]	Yellow AB	22	11380	Yellow 10
Yellow 4[a,b]	Yellow OB	61	11390	Yellow 11
Yellow 5	Tartrazine	640	19140	Yellow 4
Yellow 6	Sunset Yellow FCF	...	15985	Yellow 3
Violet 1	Wool Violet 5 BN	697	42640	Violet 2
Blue 1	Brilliant Blue FCF	...	42090	Blue 2
Blue 2	Indigotine	1180	73015	Blue 1
Green 1	Guinea Green B	666	42085	Green 1
Green 2	Light Green SF Yellowish	670	42095	Green 2
Green 3	Fast Green FCF	...	42053	Green 3
Citrus Red 2[b]	1-(2,5-Dimethoxyphenylazo)-2-naphthol	...	...	...

[a] Delisted since 1955. [b] Oil-soluble dyes; all others are water soluble.

This process may be hastened by means of a pressure differential.

Solvent Systems

On the basis of experimental evaluation, three chromatographic solvent systems were selected for their ability to separate an aqueous mixture of all 14 of the recently-permitted, water-soluble food colors. Methods for their preparation are given below.

Pyridine – Ethyl Acetate – Water (1:2:2). Best for multicomponent mixtures and mixtures of yellow and orange dyes (*7*). One volume of pyridine was mixed with two volumes each of ethyl acetate and water; the mixture was shaken in a separatory funnel and allowed to separate. The lower phase was discarded.

3.9*N* Ammonium Hydroxide in Isobutyl Alcohol (IBA–ammonia). Best for mixtures of blue and green dyes (*13*). Equal volumes of concentrated ammonium hydroxide and isobutyl alcohol were shaken together in a separatory funnel and allowed to separate. After the lower phase was discarded, 5 ml. of the upper phase was pipetted into 100 ml. of water and titrated to the methyl red end point, using 1*N* HCl. On the basis of this titration, the remainder of the upper phase was adjusted to exactly 3.9*N* using isobutyl alcohol.

Isoamyl Alcohol-95% Ethanol–Concentrated Ammonium Hydroxide–Water (4:4:1:2). Best for mixtures of red dyes (*37*). The four liquids were mixed in the volumetric ratio 4:4:1:2, and were dispersed by shaking.

Procedure

Sample Preparation. In each case the sample should contain about 1 mg. of each color present. Normally, a 50-gram food sample will provide adequate color for subsequent determination.

Dry Gelatin Desserts. The sample was shaken with 100 ml. of 95% ethyl alcohol and decanted through a Büchner funnel under vacuum. This extraction was repeated until most of the color was removed; then the sample was shaken with 100 ml. of 1% aqueous ammonia and rapidly filtered under vacuum. The filtrates were combined and adjusted with glacial acetic acid until strongly acid to pH indicator paper.

Water-Soluble Foods (including candy, soda water, water ices). Solid samples were dissolved in a minimum volume of 1% aqueous ammonia; liquid samples were used undiluted. The solutions were filtered and made strongly acid with glacial acetic acid.

Oil-Soluble Foods. The sample was dissolved in a minimum volume of ethyl ether, and, without filtering, the water-soluble dyes were extracted with 1% aqueous ammonia. The aqueous extract was acidified with glacial acetic acid.

Foods Insoluble in Water or Fat Solvents. Meat samples were covered with 80% ethyl alcohol containing 1% ammonia, ground in a food blender, and filtered under vacuum using Celite as a filter aid. This process was repeated until no more color was extracted.

Alimentary pastes and bakery goods were treated in the same manner except that the ammoniacal alcohol solution was heated to boiling prior to blending.

In both cases, the alcoholic solution was then extracted with successive portions of *n*-pentane until no further color was extracted. The *n*-pentane portion was retained for testing for oil-soluble dyes. The alcoholic portion was diluted with water to about 50% ethyl alcohol, and the solution was acidified with glacial acetic acid.

Separation and Identification of Oil-Soluble Colors. Although oil-soluble food colors are no longer permitted in the United States, it is obviously necessary to screen for them. Silk's procedure (*35*), employing reversed-phase column chromatography for the isolation, separation, and identification of the oil-soluble dyes which formerly were permitted, has been found adequate.

Preliminary Qualitative Test for Artificial Color. A portion of the acidified aqueous solution, obtained as described under Sample Preparation, was boiled to remove the alcohol. A 2- × 2-cm. square of white, defatted, wool cloth was immersed in this solution and heated 15 minutes on the steam bath. The cloth was then removed, rinsed with water, covered with 1% aqueous ammonia, and heated again for 15 minutes on the steam bath. The wool was then discarded. The remaining solution was acidified to about pH 2 with acetic acid, and a new piece of white wool cloth was added. After heating 15 minutes on the steam bath, the cloth was removed and rinsed well with water. Presence of color in this fabric is indicative of an artificial dye. Lack of color on the cloth may be considered a negative test for acidic artificial dyes.

Isolation of the Color by Column Chromatography. The acidified aqueous solution obtained in the preparation of the sample was introduced on to the chromatographic column and its passage aided by pressure or vacuum. The column was washed with an equal volume of distilled water, and the adsorbed dye was eluted with aqueous ammonia (1 + 9). Two or three

Table II. Identifying Characteristics of Food Colors

(Figures in parentheses indicate secondary (weak) spots which may not be detectable)

FDC Designation	Pyridine–Ethyl Acetate R_f	IBA–Ammonia R_f	IAA–Ethyl Alcohol–Ammonia R_f	Neutral Color	Color after Exposure to: HCl fumes	Color after Exposure to: NH_3 fumes
Red 1	0.45	0.18	0.55 (0.62)	Rose pink	Rose pink	Rose pink
Red 2	0.06	0.01	0.24	Plum	Plum	Plum
Red 3	0.88	0.37	0.70	Pink	Orange	Pink
Red 4	0.50	0.04	0.36	Rose pink	Red	Orange
Yellow 1	0.59	0.22	0.59	Yellow	Decolorizes	Yellow
Yellow 5	0.08	0.00	0.17	Yellow	Yellow	Yellow
Yellow 6	0.43	0.10	0.52 (0.17)	Orange	Orange	Orange
Orange 1	0.71 (0.58)	0.23 (0.27)	0.60 (0.69)	Orange Orange-red	Purple Orange	Dark red Pale red
Violet 1	0.54 (0.50) (0.58) (0.72)	0.38[a] (0.21)[a]	0.76 (0.94)	Violet Blue	Yellow or decolorizes Decolorizes	Blue Blue
Blue 1	0.34 (0.43) (0.53)	0.20	0.59 (0.70)	Blue	Yellow	Blue
Blue 2	0.26 (0.38)	Disappears	0.30[b] (partially decomposes)	Blue	Blue	Blue
Green 1	0.59 (0.79)	0.38[c]	0.75 (0.92)	Aqua green	Orange or decolorizes	Decolorizes[d]
Green 2	0.40[c] (0.54)[c] (0.58)[c]	0.17[c] (0.12)[c] (0.29)[c] (0.40)[c]	0.68 (0.73) (0.78)	Aqua green	Orange or decolorizes	Decolorizes[d]
Green 3	0.42 (0.00)	0.09 (0.00) (0.18)	0.46 (0.62) (0.68)	Aqua green	Orange or decolorizes	Deep blue

[a] Permanently changes to blue. [b] May or may not be detectable, depending upon original concentration of Blue 2. [c] Colorless until dry. [d] Aqua green after ammonia evaporates.

Table III. Comparison of Recovery Values

FDC Designation	Pyridine–Ethyl Acetate, % Recovery		IBA–Ammonia, % Recovery		Amyl–Ethyl Alcohols, % Recovery	
	Elution	Reflectance	Elution	Reflectance	Elution	Reflectance
Red 1	68	90	82	105[a]	...	...
Red 2	71[a]	80[a]	69	87[a]	97	100
Red 3	62	72	77	76	71	68
Red 4	86	90	102	100	72	85
Yellow 1	60[a]	88[a]	90[a]	94[a]	68[a]	63[a]
Yellow 5	66[a]	100[a]	70[a]	81[a]	83	100
Yellow 6	59[a]	64[a]	75	100	...	...
Orange 1	43	46	...	...	...	...
Violet 1	36	80	...	...	...	...
Blue 1	...	...	69	77	...	...
Blue 2	72	87	...	...	...	...
Green 1	100[a]	89[a]	...	...	...	...
Green 2	...	...	54	76	...	...
Green 3	...	...	62	84	57	71

[a] Estimated from spectral curves of mixed spots.

column-volumes were necessary to elute all the color, but the total volume was kept as small as possible.

Separation of the Colors by Paper Chromatography. The color mixture eluted from the alumina column was separated by paper chromatography using the ascending-solvent method. Three separate paper chromatograms were made, one employing each of the three solvent systems listed above. Glass-stoppered graduated cylinders were used as chromatographic chambers so that the chromatograms could be run simultaneously. Volumes of column-eluate estimated to be equivalent to about 25 μg. of dye were chromatographed at room temperature on 1- × 12-inch strips of Whatman No. 1 or No. 31ET paper. The strips were either folded longitudinally to give them sufficient rigidity to stand alone, or secured at the top by the stopper with the end of the paper protruding from the cylinder. The latter method provided the better resolution of colors of low R_f value.

Identification of Colors. Upon removal of the paper strips from the cylinders, the positions and colors of the spots were noted. When the chromatograms were dry, the R_f value for each spot was calculated, and color changes in both HCl and NH_3 fumes were noted. Identification of the dyes was made by comparison of the R_f values and color changes with those of known dyes.

The R_f values given in Table II indicate the relative positions of the colored spots. However, these values are affected by so many variables that they are offered here only as a comparative guide. A trace of known dye, added to an aliquot of the sample before spotting, will provide an internal standard whose R_f will facilitate precise identification of the other colors. For example, erythrosine was so employed in chromatographing a mixture of all the food colors.

Quantitative Determination. Both absorbance and reflectance spectrophotometry were employed in estimating the quantities of dyes in the spots on the chromatograms. Reflectance measurements were made from the dry spots which had been cut from the paper strips. For absorbance measurement, the separate colors were eluted from the spots. In both cases, the readings were compared with comparable values of known dyes.

To elute the color, the spot was cut from the chromatogram, placed in a small beaker, covered with 1 to 2 ml. of 60% ethyl alcohol, and warmed on a steam bath until the paper was colorless. The cooled solution was transferred to a cuvette designed for small volume and long transmission. Cells of 2- and 5-cm. length and 4-mm. bore were quite satisfactory.

Results

The sequence of procedures presented here has been tested successfully in the State of California Food and Drug Laboratory over a period of 3 years in the identification of artificial colors in the following types of foods: dry gelatin desserts, candy, orange juice and orange beverages, soda water, flavoring syrups, water ices, alcoholic beverages, tomato catsup and puree, wines, and ground meat.

For quantitative application, representative samples of several types of products were examined to test the efficiency of the sample-preparation procedures. Extractions of 1 mg. of color added to 50 grams of ground meat ranged from 50 to 70% recovery. Twenty-five to 75% extractions were realized when 0.5 mg. of color was added to 10-gram samples of cookie dough, and the cookies were baked prior to extraction. Similar experiments with noodles and strawberry jam resulted in extraction percentages of 20 and 90%, respectively. When 1 mg. of a commercial lake (erythrosine) was blended with 25 grams of hydrogenated cottonseed oil and the sample extracted, a 95% recovery of the added dye was realized in the ammoniacal layer.

Thus, depending upon the type of food under consideration, the efficiency of sample preparation will vary from 20 (for baked goods) to 100% (for most water-soluble foods).

The alumina column was tested with 1-mg. portions of each of the colors under consideration. With this technique, recoveries varied from 80 to 95%, erythrosine and wool violet giving the lower recoveries because of incomplete elution by the ammonia solution.

The alumina column was also tested for its ability to retain natural food colors. When 50-gram samples of naturally colored orange concentrate, tomato catsup, carrot juice, wine, egg noodles, strawberry jam, and meat were subjected to the entire procedure, small amounts of yellow from the meat and orange color from the orange juice were eluted by the ammoniacal solution. Otherwise, little or none of the natural colors was passed by the alumina column.

The combined column and paper chromatographic techniques were then tested in the following manner. A mixture containing 1 mg. of each of the food dyes added to 7 ounces of a colorless, lemon-flavored soda water was subjected to the entire procedure. The color was eluted from the chromatograms using 1 ml. of 60% ethyl alcohol, and the quantity of color was estimated in the Cary Model 11 recording spectrophotometer using 20-mm. light-path cells with a 4-mm. bore. For purposes of comparison, Table III records these figures as well as recovery figures which were obtained from direct reflectance readings of the separated chromatogram spots using the Bausch & Lomb 505 recording spectrophotometer, the readings being evaluated on the basis of standard curves for each color.

Discussion

Under existing Federal law, no tolerances have been promulgated which would limit amounts of certified, water-soluble dyes in food. The regulatory laboratory need merely prove identity in the enforcement of this law. On this account, the proposed procedure is primarily a qualitative one. The recovery figures given are intended only as examples of the applicability of this procedure to quantitation.

With meat products, bakery products, and alimentary pastes, the dyes are bound tightly to the food. The freeing of this dye from the food may occupy many hours and still be far from quan-

titative. In this procedure, when it was found necessary to sacrifice higher quantitative recoveries for speed, this sacrifice was made. Thus, in the case of egg noodles, a procedure which would extract 40% of added tartrazine in 4 hours of shaking was replaced by one which would extract 20% in 10 minutes.

In general, sample preparation (with respect to quantitative determination of food dyes) is a most difficult part of any procedure—an aspect which has been largely neglected in studies of the subject.

It should be noted that wool violet and indigotine are unstable in alkaline solution. When the presence of either of these dyes is suspected, a minimum exposure to ammonium hydroxide is advisable. No breakdown of the other food dyes has been noted under the conditions of this procedure.

Since lakes have come into use in foods, it has become a routine necessary to render the extractants basic (as outlined under Sample Preparation) to ensure solution of the dye which has been adsorbed as a lake on a substrate. The extract is then acidified for testing by the procedures outlined above. The use of lakes as suspensions in oil-soluble materials makes it essential that a two-phase extraction system be used when dealing with fatty foods.

Of the methods tested, the adsorption column technique was found to be best suited to quantitative isolation. Weakly colored solutions may be adsorbed on the column and then eluted in a small volume, effecting a rapid concentration of colors. Natural colors are strongly adsorbed by alumina; little of these is eluted by the ammoniacal solution. However, since it has been demonstrated that some natural colors may be eluted from the column, the precaution of the wool-dyeing step takes on added importance.

In the selection of solvent systems for paper chromatography, more than 60 mixtures, described in the literature as effective, were examined. They were evaluated on the basis of their ability to separate a mixture of 20 μg. of each food color spotted on chromatograph paper. The pyridine–ethyl acetate mixture was the most successful, resolving all colors with the exception of FDC Blue 1, Green 2, and Green 3. These three dyes were effectively separated by the isobutyl alcohol–ammonia system. The isoamyl–ethyl alcohol mixture, while failing to separate as many of the dyes as does the pyridine–ethyl acetate system, is of sufficient qualitative resolving power for most routine applications, and possesses the advantages of ease of preparation and less objectionable odor.

The quantitative estimation of the dyes is complicated by overlap of the spots in some cases. Part of the problem may be resolved by cutting out the spots following their individual contours. Many of those which cannot be dealt with in this manner may be estimated by absorbance ratios, if the spectral peaks of the two dyes are sufficiently separated.

Because of variations of spot size and of relative saturation of the paper, the reflectance of dyes on paper fails to obey Beer's law. Thus, standard curves must be prepared to take into account both concentration and area. Consequently, quantitative estimates by the reflectance procedure may vary ±10% from the true value of the amount of dye present.

While quantitative recoveries are relatively low, the speed of the entire procedure recommends its use as a screening method for the detection of undeclared artificial color, nonpermitted color, and the gross quantitative misuse of permitted color. Once the identification of the colors present in a food sample has been made, direct spectrophotometric quantitative estimation of these colors may be a relatively simple task.

The preparation of the sample for paper chromatography requires approximately 30 minutes. Complete separation of the colors for quantitative determination will require 3 to 5 hours depending upon the solvent system and the type of paper used. With the technique described, chromatography may be run overnight, if desired. Qualitative identification of the common food colors may be accomplished with the pyridine–ethyl acetate solvent on Whatman No. 31ET paper within 30 minutes.

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