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Note

High-performance liquid chromatographic determination of subsidiary dyes, intermediates and side reaction products in erythrosine

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Erythrosine (Colour Index No. 45430) is a synthetic color permitted for use in foods in a number of countries, including Canada. A regulation under the Food and Drugs Act[1] limits the levels of permitted impurities in this additive. Subsidiary dyes, which are structural varients of the principal color, intermediates, which are compounds from which a color is directly or indirectly synthesized, and side reaction products, which are by-products formed during synthesis, are commonly recognized as impurities.

In the past, subsidiary colors were determined by the use of thin-layer chromatography (TLC)[2] or paper chromatography[3]. Cellulose column[4] or TLC procedures[5,6] were used to determine intermediates and reaction by-products. In recent years, high-performance liquid chromatography (HPLC) has been shown to be an improved and useful technique for the determination of impurities in synthetic colors[7–12]. Most of the methods work well when applied to azo-class colors, but some may not be applicable to erythrosine, a xanthene-class color.

Automated HPLC methods were developed for subsidiary dyes[10], and intermediates and side reaction products[13] in erythrosine. However, rather complex gradient programs were used and, as well, the latter method requires 0.2 *M* ammonium chloride in the mobile phase. For regulatory purposes, a simplified, yet accurate and sensitive, HPLC method, which could also be used in any laboratory with a fundamental HPLC capability was required.

A previously developed ion-pair HPLC method[14] was adapted for the determination of erythrosine and its subsidiary dyes. A second method using an ion-suppression reversed-phase system was developed for the determination of the intermediates and side reaction products; this system was also suitable for the major subsidiary dyes.

EXPERIMENTAL

Chemicals and reagents

Commercial samples of erythrosine were selected from samples submitted to the Branch for certification. Fluorescein and phthalic acid were obtained from Aldrich (Milwaukee, WI, U.S.A.) and 4',5'-diiodofluorescein was manufactured by East-

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man (Rochester, NY, U.S.A.). Hilton-Davis (Cincinnati, OH, U.S.A.) kindly provided limited quantities of the triiodofluorescein subsidiaries and resorcinol. Based on a previous study of brominated compounds[15], we synthesized two side reaction products in our laboratory; confirmation was by fast atom bombardment mass spectrometry, spectrophotometry and melting point. Monobasic potassium phosphate (KH_2PO_4) was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.) and all solvents were HPLC grade.

Standards of erythrosine, the side reaction products, 2-(2′,4′-dihydroxybenzoyl)benzoic acid (SR1) and 2-(2′,4′-dihydroxy-3′,5′-diiodobenzoyl)benzoic acid (SR2), the intermediates, resorcinol and phthalic acid, and the subsidiary dyes, fluorescein, 4′,5′-diiodofluorescein, 2′,4′,5′-triiodofluorescein and 2′,4′,7′-triiodofluorescein were utilized in the study.

Stock solutions of erythrosine were prepared at concentrations of 0.03–0.1% in methanol–water (1:1). Solutions for use with the ion-pair HPLC system contained 0.005 *M* tetra-*n*-butylammonium phosphate (TBAP). Similarly, subsidiary dye solutions were 0.02% in methanol–water (1:1) and resorcinol and SR1 were 0.015% in water. The concentration of phthalic acid stock was 5×10^{-4}% in water and SR2 was 0.015% in methanol. Purity of the standards was determined by spectrophotometry and this information was then utilized to obtain quantitative HPLC data for commercial samples of erythrosine.

Chromatography

The HPLC system consisted of two pumps (M45 and 6000A), a gradient programmer (M660) and a variable-wavelength detector (M450), all from Waters (Milford, MA, U.S.A.), a Rheodyne 7125 syringe loading injection port with a 20-μl loop, a Spectra-Physics (San Jose, CA, U.S.A.) SP4270 integrator and a Brownlee (Santa Clara, CA, U.S.A.) Spheri 10, RP-18 column (25 cm × 4.6 mm I.D.). Detector wavelengths of 500 nm and 249 nm were utilized for erythrosine and the subsidiary dyes and 230 nm was used for intermediates and side reaction products.

For ion-pair chromatography, the mobile phases (degassed) were methanol–water at ratios (v/v) of 40:60 in pump A and 70:30 in pump B, each containing 0.005 *M* TBAP.

Mobile phases (filtered and degassed) employed for reversed-phase chromatography of intermediates and side reaction products consisted of methanol–water at ratios (v/v) of 5:95 in pump A and 70:30 in pump B, each containing 0.02 *M* KH_2PO_4. Using diluted (1:1) phosphoric acid, the (70:30) mobile phase was adjusted to pH 4.6. Column flow-rate was 1.0 ml/min. The column was conditioned using A–B (1:1) (*i.e.* methanol–water, 38:62) for 30 min daily before samples were injected.

RESULTS AND DISCUSSION

Fig. 1 shows a gradient separation of the intermediates and side reaction products of erythrosine. The major subsidiaries of erythrosine are indicated as well. Fluorescein, 2′,4′,5′- and 2′,4′,7′-triiodofluorescein are completely resolved from the intermediates and side reaction products although 4′,5′-diiodofluorescein is eluted with erythrosine. This system is useful for screening a colour sample to identify qualitatively the components present. However, for accurate quantitation on a routine basis

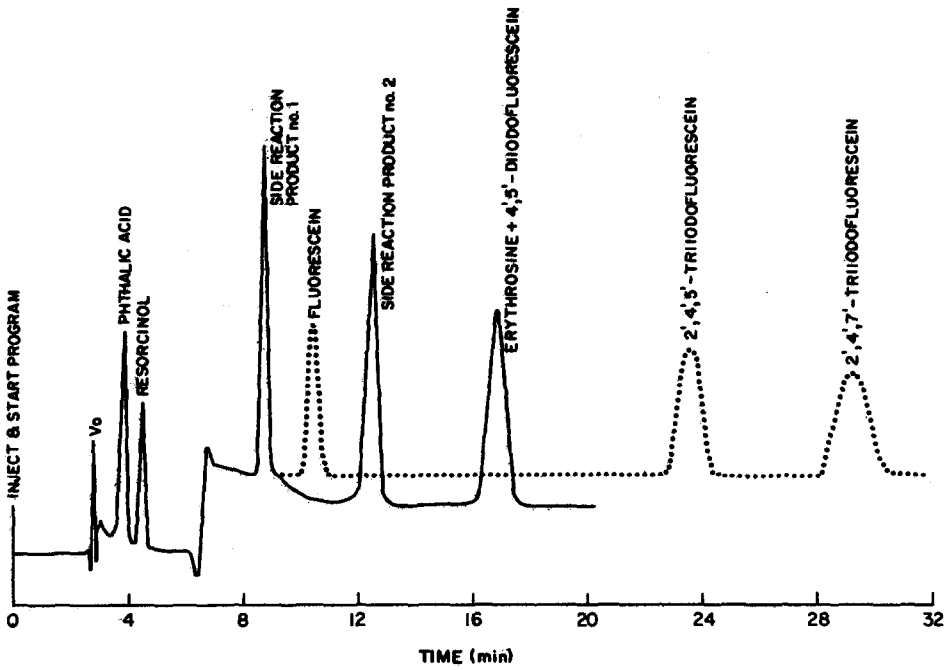

Fig. 1. Gradient chromatogram of the compounds studied. Initial mobile phase, (containing 0.02 *M* KH_2PO_4) methanol–water (36:64). Immediately after injection gradient was programmed to 64:36 in 3 min and held. Other conditions described in the text.

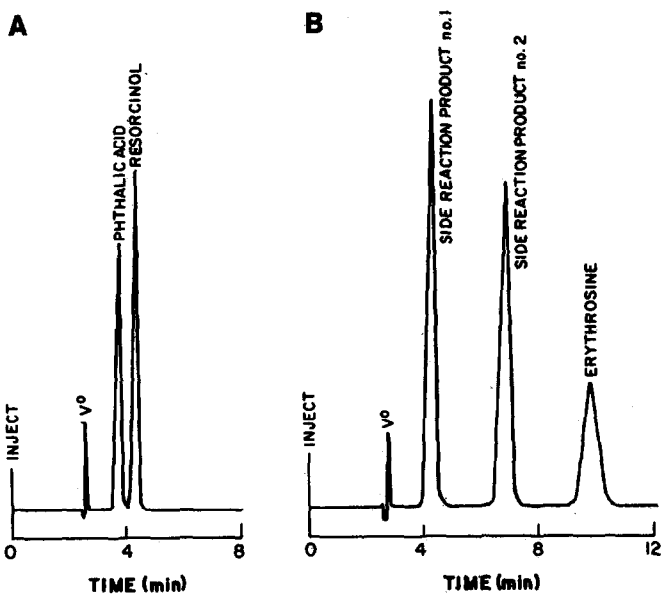

Fig. 2. Isocratic separation of (A) phthalic acid and resorcinol [mobile phase, methanol–water (36:64) containing 0.02 *M* KH_2PO_4] and (B) side reaction products 1 and 2 and erythrosine [mobile phase, methanol–water (64:36) containing 0.02 *M* KH_2PO_4]. Other conditions described in the text.

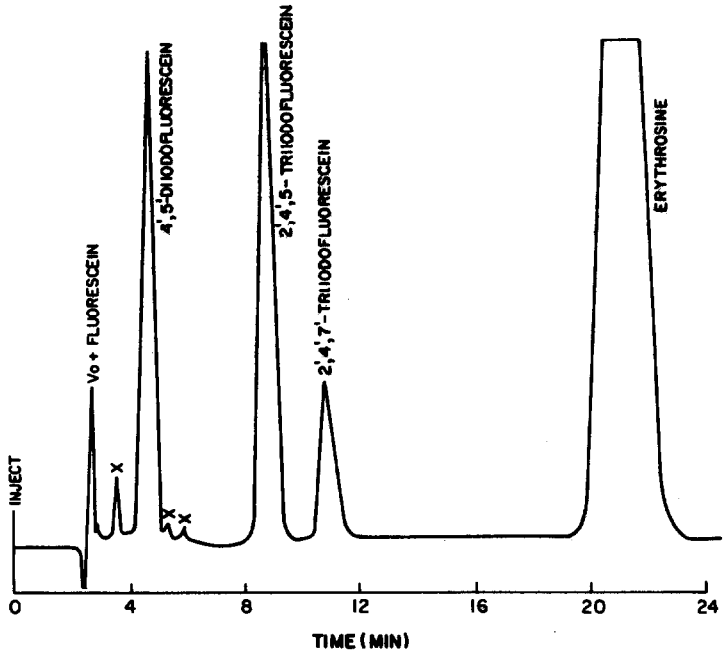

Fig. 3. Ion-pair chromatogram of erythrosine and subsidiaries. Mobile phase, methanol–water (64:36) containing 0.005 *M* TBAP. Other conditions as described in the text. X = impurities in 4',5'-diiodofluorescein.

isocratic systems were preferred. In order to separate 4',5'-diiodofluorescein from erythrosine, the latter part of the gradient was changed to methanol–water (60:40) (containing 0.02 *M* KH_2PO_4). This, however, extended the analysis time to about 50 min for 2',4',7'-triiodofluorescein.

Both phthalic acid and resorcinol were much less retained than the other compounds studied. Fig. 2 shows the isocratic separation of the two with a mobile phase composition of methanol–water (36:64) containing 0.02 *M* KH_2PO_4. Also, the figure shows the separation of the two side reaction products and erythrosine using a mobile phase composition of methanol–water (64:36) containing 0.02 *M* KH_2PO_4. Under the latter conditions, the two intermediates were unretained.

TABLE I

CONCENTRATION OF SUBSIDIARY DYES, INTERMEDIATES AND SIDE REACTION PRODUCTS IN COMMERCIAL SAMPLES OF ERYTHROSINE

4,5-DI = 4,5-diiodofluorescein; 2,4,5-TI = 2,4,5-triiodofluorescein; 2,4,7-TI = 2,4,7-triiodofluorescein.

Manufacturer	*4,5-DI (%)*	*2,4,5-TI (%)*	*2,4,7-TI (%)*	*Phthalic acid (%)*	*SR1 (%)*	*SR2 (%)*
A (U.K.)	–*	0.7	–	–	0.068	–
B (U.S.A.)	–	2.4	1.5	0.009	–	0.027
C (Japan)	–	0.8	2.7	<0.005	–	–

* Compound not detected.

With the reversed-phase system described above, the triiodo subsidiaries were eluted much later than erythrosine. Increasing the mobile phase strength to elute the two compounds more quickly resulted in the merging of 4',5'-diiodofluorescein with erythrosine, as previously mentioned. Ion-pair chromatography was very effective for separating the subsidiaries from erythrosine. Fig. 3 shows a separation of the three subsidiaries and erythrosine using a mobile phase of methanol–water (64:36) containing 0.005 *M* TBAP. It can be seen that the elution order has completely changed with all subsidiaries being eluted well before erythrosine. Since the subsidiaries can also be detected at 500 nm, no interferences from the intermediates and side reaction products would occur.

Commercial samples of erythrosine from the U.K., Japan and the U.S.A. were analyzed using the above methods (Table I) and all were found to have subsidiary dye, intermediate and side reaction product levels within allowable limits. Resorcinol, as expected[13], was not detected in any of the samples.

The analytical methods described above were used to study the thermal decomposition of erythrosine and fluorescein in sugar solutions, in a manner described previously for three azo class colors[16]. The detector wavelength was 500 nm. No decomposition of either compound was observed in sugar solutions at room temperature or after heating to 100°C. The recovery of erythrosine was 90.9% from a candy containing 90.8 ppm prepared at 150°C, while 92% of the fluorescein was recovered from a similar 150°C candy to which 3 ppm of that compound had been added. Sep-Pak procedures[14] were used for clean-up and 3 ml methanol–water (60:40) followed by 6 ml methanol were used to elute the compounds from the cartridge. No increase in the level of subsidiary dyes was observed.

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REFERENCES

1 *The Food and Drugs Act and Regulations, Division 6*, Dept. Nat. Health and Welfare, Ottawa.
2 M. Dolinsky, *J. Assoc. Off. Agric. Chem.*, 34 (1951) 411.
3 *Official Method FO-10*, Health Protection Branch, Ottawa, March, 1984.
4 W. B. Link, *J. Assoc. Off. Agric. Chem.*, 44 (1961) 43.
5 M. Kamikura, *Shokuhin Eiseigaku Zasshi*, 26 (1985) 243.
6 M. Kamikura, *Shokuhin Eiseigaku Zasshi*, 26 (1985) 643.
7 F. E. Lancaster and J. F. Lawrence, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 1305.
8 F. E. Lancaster and J. F. Lawrence, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 1424.
9 J. E. Bailey, *J. Assoc. Off. Anal. Chem.*, 63 (1980) 565.
10 R. J. Calvey and A. L. Goldberg, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 1080.
11 Y. Tonogai, Y. Ito and M. Iwaida, *Shokuhin Eiseigaku Zasshi*, 24 (1983) 275.
12 E. A. Cox, N. Richfield-Fratz, C. J. Bailey and R. H. Albert, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 240.
13 A. L. Goldberg and R. J. Calvey, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 103.
14 J. F. Lawrence, F. E. Lancaster and H. B. S. Conacher, *J. Chromatogr.*, 210 (1981) 168.
15 C. Graichen and J. C. Molitor, *J. Assoc. Off. Agric. Chem.*, 42 (1959) 149.
16 F. E. Lancaster and J. F. Lawrence, *J. Food Add. Contam.*, (1987) in press.