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APPLICATION OF DENSITOMETRY TO THE QUALITATIVE AND QUANTITATIVE EVALUATION OF PHARMACEUTICAL COLOURANTS

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SUMMARY

Samples from all the British manufacturers of food grade Amaranth and Sunset Yellow FCF were examined by a densitometric thin-layer chromatographic procedure which provides a rapid and convenient method for producing a qualitative and quantitative statement on the impurity profiles of dye materials. Wide variations in the impurities were found which could account for variations in toxicological results. The method could be incorporated into official specifications to control more precisely the quality of food grade dyes.

INTRODUCTION

As a result of the evidence of possible detrimental effects of food additives strict regulations are in force governing the use of colourants in foods and pharmaceuticals. The dyes permitted vary markedly from country to country and Table I indicates the international acceptability of ten common materials. Only Amaranth is acceptable in all 65 countries whose regulations were examined. Coloured impurities are usually present in food grade dyes and the nature and proportions of these are specified in the official standards. In certain cases these coloured subsidiaries are dyes in their own right, as defined by the Colour Index, and as such are controlled. For example Amaranth may contain four subsidiary dyes which are structurally related to the main component (Fig. 1). Crystal Ponceau, which is no longer acceptable in any of the countries whose regulations were examined, Ponceau 6R, which is acceptable in only 14, Fast Red E, which is acceptable in 24, and Ponceau 4R, which is acceptable in 45. Sunset Yellow FCF contains only one subsidiary, Orange II, which is a Colour Index dye. This is no longer permitted for use in any of the 65 countries examined, although it was previously used in the U.S. as D & C Orange No. 4.

TABLE I

RANKED LIST OF THE FREQUENCY OF ACCEPTANCE FOR USE BY COUNTRIES OF FOOD AND DRUG DYES

Regulations of 65 countries were examined.

Dye	*Colour Index (1956) No.*	*Colour*	*Number of countries accepting for use*
Amaranth	16185	red	65
Indigo Carmine	73015	blue	62
Tartrazine	19140	yellow	60
Erythrosine	45430	red	56
Sunset Yellow FCF	15985	orange	53
Ponceau 4R	16255	red	47
Azorubine	14720	red	39
Black PN	29440	blue	34
Patent Blue V	42051	blue	32
Fast Red E	16045	red	28

Pharmaceuticals are frequently coloured and mixtures of dyes are often used to obtain a particular shade. However, the differences in quantities of components used to produce a particular shade may differ by a hundredfold and therefore the situation can arise where the concentration of an impurity in a major component can

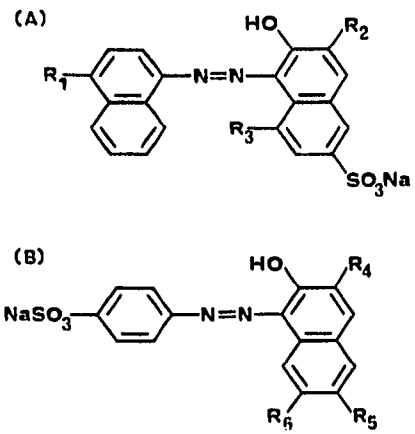


Fig. 1. Relationship between structures of (A) Amaranth and (B) Sunset Yellow FCF and their subsidiary dyes.

	R_1	R_2	R_3
Amaranth	$-SO_3Na$	$-SO_3Na$	$-H$
Ponceau 4R	$-SO_3Na$	$-H$	$-SO_3Na$
Fast Red E	$-SO_3Na$	$-H$	$-H$
Crystal Ponceau	$-H$	$-H$	$-SO_3Na$

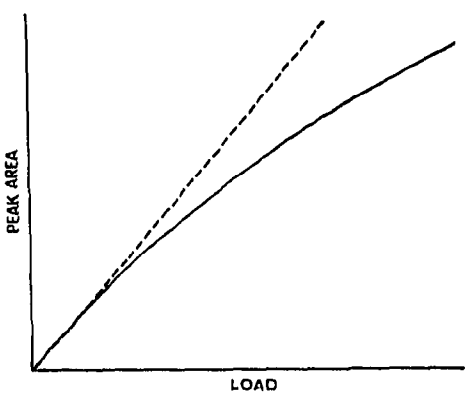
	R_4	R_5	R_6
Sunset Yellow FCF	$-H$	$-SO_3Na$	$-H$
Orange II	$-H$	$-H$	$-H$
R salt derivative	$-SO_3Na$	$-SO_3Na$	$-H$
G salt derivative	$-H$	$-SO_3Na$	$-SO_3Na$

exceed the total concentration of a minor component. If such an impurity is a dye which is specifically excluded in a particular country an interesting legal anomaly arises. If a naturally impure sample of the major dye is taken which contains the required proportion of minor subsidiaries to give the desired shade, then this is acceptable, provided that the level of subsidiaries is below the level of impurities laid down in the specification for the major dye. On the other hand, if the same mixture is artificially created by mixing clean major dye with the specific subsidiary dye required, then this is not acceptable, as the subsidiary dye must be considered as an individual component of the product rather than a minor impurity, and therefore this addition constitutes adulteration. This latter situation is further complicated by the variations in permitted dyes which can necessitate the use of different combinations to obtain the same hue for different countries. The main objective of this present investigation was to determine the quality and quantity of coloured impurities present in a range of samples of commercial dyes so that "reasonable" levels might be suggested to which subsidiary dyes could be reduced or, alternatively, levels to which they might be included.

Samples of the dyes Amaranth and Sunset Yellow FCF, permitted in Great Britain, were requested from all the British manufacturers of food grade dyestuffs, and in addition French National Standard samples of these were obtained from the Laboratoire National de la Santé Publique in Paris. France is the only country which will provide a national standard sample. Details of the test procedure and results are given below.

TEST PROCEDURE

We have previously reported briefly[1] a method for the determination of the impurity profiles of pharmaceutical colourants by densitometry of thin-layer separations. Although this gave a complete pattern of the distribution of material on the



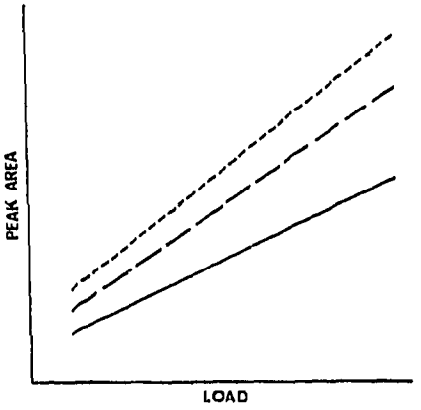


Fig. 2. Deviation of the absorption of light by Amaranth spots on cellulose plates from the Lambert–Beer relationship over the range 0–30 μg. – – –, Lambert–Beer relationship; ———, observed values.

Fig. 3. Response curves for Amaranth subsidiaries from 0.1–0.4 μg. ·····, Crystal Ponceau; – – –, Ponceau 4R; ———, Fast Red E.

chromatogram, it tended to overemphasise the proportions of impurities as, when the subsidiary peaks were sufficiently high to allow their estimation, the major component was oversaturated and therefore the Lambert–Beer law was not obeyed (Fig. 2). In addition there are variations in response factors between individual subsidiaries (Fig. 3). To obtain an accurate estimation of the levels of these impurities, it is therefore necessary to compare the test sample with standards of the subsidiary dyes of approximately equal, and accurately known, concentration, and to relate this determination to the load applied to indicate the percentage of each impurity.

$$\frac{\text{impurity peak area in test sample}}{\text{standard peak area}} \times \text{standard load} = \text{impurity load}$$

$$\frac{\text{impurity load in test sample}}{\text{total test load}} \times 100 = \text{percentage impurity}$$

1% (w/v) solutions of each of the twelve commercial British samples of Amaranth and of Amaranth LNSP, and 0.01% (w/v) and 0.02% (w/v) solutions of each of the three subsidiary dyes, Fast Red E, Crystal Ponceau, and Ponceau 4R (Solmedia, London, Great Britain), were prepared. The following volumes were applied to a 20 × 20-cm prepared thin-layer plate (MN CEL 300/254, Machery, Nagel & Co., Düren, G.F.R.) using Microcaps: for each subsidiary dye 3 × 1 μl of 0.01% (0.1 μg), 3 × 1 μl of 0.02% (0.2 μg), and 3 × 2 μl of 0.02% (0.4 μg), and for each of the 1% commercial Amaranth sample solutions, and for Amaranth LNSP, 1 × 2 μl (20 μg). Plates were prepared in triplicate, developed in the solvent system butanone–acetone–water (7:3:3)[2] for 10 cm, allowed to air dry, and scanned in transmission on a Vitatron TLD 100 flying spot densitometer in the log mode, with a scan speed of 1 cm/min, an aperture of 0.25 mm, a strike length of 14 mm, a filter of 525 nm, and a span of 970. Peak areas were estimated directly from the integrating recorder after correction for baseline drift.

Essentially the same procedure was used for the samples of Sunset Yellow FCF except that the plates were developed in butanone–acetone–ammonia (0.880)–water (7:3:0.02:3)[3] for 16 cm. Whilst the subsidiary Orange II was available (Solmedia) it was necessary to synthesise the higher sulphonated R and G salt derivatives[4].

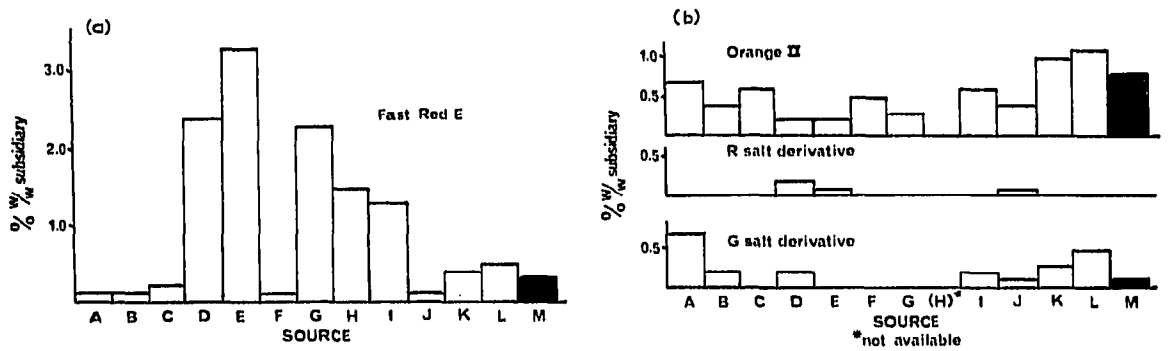


Fig. 4. Subsidiary dye content of commercial samples (A–L) and French National Standard Samples (M) of Amaranth (a) and of Sunset Yellow FCF (b).

RESULTS

The results for both Amaranth and Sunset Yellow FCF samples are given in Fig. 4. In the Amaranth samples the only subsidiary present in measurable quantities (greater than 0.05%, w/w) was Fast Red E. This appeared in all samples, being below 1% (w/w) in eight, between 1 and 2% in two, and above 2% in the last three. Traces of Crystal Ponceau were detected in three samples (B, H, and M) and of Ponceau 4R in one (E). Ponceau 6R was not detected in any samples in contrast to reports[5] of its presence in U.S. samples of Amaranth. Sunset Yellow FCF showed a more complex pattern of impurities in that whilst all contained Orange II and nine of the twelve samples the G salt derivative, the R salt derivative was found in only three cases. At the 0.5% (w/v) subsidiary level the reproducibility of the estimations was ± 7.8% of the mean value, *i.e.* 0.5 ± 0.04% (w/v). All the samples except specimen E of Amaranth fell within the 3% subsidiary dye limit set by the appropriate British Standard[2,3]. The samples designated M which were French National Standard samples contained considerable subsidiary dye.

DISCUSSION

There is clearly wide variation in the subsidiary dyes present in samples from different manufacturers of the same food grade dyes. In the case of Amaranth this is essentially a quantitative variation as Fast Red E is the only subsidiary present at appreciable concentration. With Sunset Yellow FCF more subsidiaries are found, although Orange II is always the major component. It is well recognised that it is impractical to produce a commercial dye of food grade which is 100% pure, but on the other hand the results from the Amaranth samples suggest that certain manufacturers do not find it too difficult to reduce subsidiaries to a low level.

Workers in different countries have often obtained different results in toxicological studies on colourants[6] and this present report emphasises the need for most careful characterisation of samples for toxicity testing[7], in particular with respect to their subsidiary dye content. It is unrealistic to test 100% pure dye as any results obtained will not apply to impure commercial material. Toxicity may well be due to the minor components and it must be remembered that it is usual to carry out toxicity testing at very high dose levels where the absolute level of subsidiaries is high relative to any normal intake pattern.

The procedure described here is a rapid and convenient method for making a qualitative and quantitative statement of the impurity profiles of dyes and it is suggested that it could be used to lay down standards where the subsidiary dye content was set at levels which prudent manufacturers did not find too onerous. We would tentatively suggest that perhaps 0.5% (w/w) of Fast Red E in Amaranth would be a reasonable example, although it must be emphasised that only samples from British manufacturers have been investigated and that there appear to be differences between countries[5] in the subsidiaries present, presumably due to alternative methods of manufacture. On the other hand, it could be argued that many of the impurities have not been satisfactorily proven to be toxic and that, since commercial samples containing relatively high concentrations of these have not produced obvious toxicity in use, a reasonable level would be that of the highest routinely found concentration.

Although this latter thesis is less satisfactory in some ways than the former low level designation, it could be developed to allow the use of synthetic mixtures, provided that the levels of subsidiaries in these mixtures did not exceed the maximum level specified.

There is certainly scope here for detailed coordinated studies on the toxicity and impurity profiles of different samples of the same food grade dyes.

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