DEGRADATION STUDIES OF SYNTHETIC FOOD COLOURING MATTERS

by

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CONTENTS

Summary

The heat and light stabilities of the 16 UK-permitted synthetic food colouring matters were investigated in aqueous solution. A specially constructed light box was used to provide exaggerated lighting conditions with fan-cooling of the samples. Comparison of stability has been made on the basis of the shape of the entire decay curve rather than by simple comparison of loss after a fixed degradation period. Indigo carmine and erythrosine showed little light stability, while ponceau 4R, chocolate brown HT and brown FK all showed fairly mediocre stability. The thermal stabilities of chocolate brown HT, ponceau 4R and, most especially, indigo carmine, all proved poor.

A new polarographic peak which appeared during the thermal degradation of red 2G was shown to be due to the deacetylation product, red 10B. Identification was made by polarographic and chromatographic (hplc) comparison with a red 10B standard. A method for the analysis of red 2G and red 10B present in the same solution was developed using differential pulse polarography. This method included the use of tetraphenylphosphonium chloride to produce a differential shift in the $E_{\frac{1}{2}}$ values of the respective colouring matters, and so increase the resolution of their polarographic peaks.

Hplc analysis was conducted using reversed-phase ion-pair chromatography. A temperature control system was devised for the hplc column (water jacket) which was shown to improve the resolution and reproducibility of retention of the chromatographic peaks. An eluent comprising 63/37/0.25 v/v/w methanol/water/cetrimide, an SAS-Hypersil packing and thermostatting at 40°C were found to be suitable for most of the thermal degradation solutions.

A series of 13 chromatograms of thermally degraded solutions were produced under these conditions for comparative purposes. An unexpectedly

large number of decay product chromatographic peaks were observed for some food colours. Whereas thermally degraded ponceau 4R only showed four peaks, most colours showed up to about ten peaks, and a few, e.g. amaranth, green S and sunset yellow FCF showed a dozen or more. Tentative identifications were made of some products from azo colours by reference to standard phenols and aromatic amines. Growth curves were plotted by hplc for a number of the products from the thermal degradation of yellow 2G.

Polarographic and/or chromatographic evidence clearly showed that the thermal degradations of yellow 2G and of red 2G were not single step, single pathway processes. The large number of products observed by hplc indicate that this is true of most of the colours. Moreover, thermal degradation pathways need not be the same as photodegradation pathways. This has been shown simply for ponceau 4R by comparison of UV spectra.

Qualitative analysis linked to hplc was also investigated. The construction of UV spectra of decay products by repeated chromatography at different detection wavelengths proved to be quite straightforward. It was found that the use of diazotisation as a pre-column reaction for the differentiation of aromatic amines from phenolic analogues was not suitable: the acidic and strongly ionic conditions employed for diazotisation disrupted the chromatographic ion-pair mechanism beyond correction. Use of fluorescamine proved rapid and simple for sulphanilic acid, but no significant reaction occurred in the case of naphthionic acid. A method was devised for the analysis of phenol-4-sulphonic acid and sulphanilic acid when chromatographically unresolved by comparison of peak absorbance at two wavelengths. Finally, preliminary work was carried out to find how the dependence of retention on eluent composition was affected by the presence of specific functional groups in the analyte.

This was seen as of particular application where functional group (e.g. carboxylate) analysis was required for decay products present in low concentration.

CHAPTER 1

The Use of Colouring Matters in Food

Historical Use of Food Colouring Matters

No one knows who it was who first added colouring matters to food, though the practice almost certainly pre-dates historical records. Diverse and widely separated peoples have independently adopted food colouring as a common feature of their cultures, making use of whatever was readily available, locally or by trade, derived from mineral, vegetable or animal origin. For instance, Egyptian tomb paintings from 1500 B.C. portray the manufacture of coloured confectionery[1], and it seems likely that the Toltecs in Central America were, as early as the 10th Century A.D., extracting cochineal (from the female of the Cocus cacti insect[2]) for use in food.[3]

In 1856, Mauve, the first artificial organic dyestuff was synthesised, quickly followed by many others.[1] Their commercial importance was not slow in being recognised. By 1860, a triphenylmethane dye, Fuchsine, is known to have been used in French wines.

In the present day, synthetic colouring matters have by no means wholly replaced the earlier substances derived from natural sources - indeed, old sources such as the once defunct Mexican cochineal industry have been revived[3], and new sources such as the production of beta-carotene from the fermentation of Blakeslea trispora fungus are being developed.[2]

By weight, easily the largest use of food colouring matter in the UK is of caramel (various types) - more than 98% in fact.[4a] However, in order to generate a wide range of colours and to provide them in sufficient

quantities, a significant amount of synthetic organic colouring matters are also incorporated into food.

Several directions are currently being taken to increase confidence in these synthetic colours. Where possible analogues of natural colours are used, e.g. synthetic carotenoids have become important since they were first marketed in 1954. Another approach has been taken by Dynapol Corporation in the U.S.A., who have pioneered colours that are not absorbed through the gut.[5a] These are the 'Polydyes', polymers of M.Wt. 2×10^4 to 1×10^6 based on, e.g. polyacrylate, with anthraquinone, azo, etc. chromophores attached by sulphonamido or amino linkages. In the U.K. however, the conventional synthetic colours of the azo, xanthene, triphenylmethane and indigoid types are still those used.

The Reasons for Colouring Food

No single answer explains why people add non-nutritive colouring materials to food. Perhaps early man first put colour in food eaten ritualistically to deepen its symbolic meaning, but, by the time the historical record began, it is clear that colour played an important role in enhancing the enjoyment of food (as with the Egyptian candy mentioned above). A desire to revel in splendour was surely responsible for the rococo style of the late middle ages, where 'swan with silvered body and gilt beak served on a green pastry pond' seems to have excited at least the visual appetites of those at the feast.[5b]

Today, economic reasoning, industrialisation, the need to convey products long distances to the consumer, and modern technological methods have combined to add other supports to the age old practice of colouring food. In fact, as 3 parts in 4 of all food is processed in some

manner prior to marketing in the U.K.[4b], and this usually involves loss of natural colour, much of present policy on the incorporation of artificial colouring matters into foodstuffs is dictated by the technical problems associated with such processing, and customer reaction to those problems.

There are three categories which may be distinguished:-

(a) where natural colour exists in a product but is weaker than a consumer assumes it should be, e.g. fruit yoghurts, pickles etc., or where the product would be virtually colourless without added colour, e.g. boiled sweets;

(b) when raw materials from different sources show variation in colour, colour may be added to give batch to batch uniformity; and

(c) where natural colour may be destroyed by processing (e.g. heat, chemicals, e.g. SO_2) or by storage (e.g. light), artificial colours are added to restore or maintain the appearance of the food.

A widely quoted example of consumer reaction to the omission of synthetic colouring matter in processed food concerns a trial made by a leading U.K. retailer a few years ago.[6] This firm ceased to add colour to

(i) canned garden peas (greenish-yellow or greenish-grey),

(ii) strawberry and raspberry jams (brownish-red initially, turning dull brown on storage) and

(iii) canned strawberries (straw coloured).

The trade in these goods fell to about half, and was only restored two years later when the synthetic colours were reintroduced, albeit at a lower level than originally.

This result demonstrates the importance that tradition and habit play in the acceptability of food. What it fails to show is whether, once food colours were prohibited by law so that no alternative manufacturer's products existed, consumers would grow accustomed to the new colour of processed food, or refuse it and go back to less convenient natural produce. Such a trial is scarcely likely to be performed in Britain, though in Eastern Europe where consumer choice is centrally limited, very little artificial colour is used in food.

Regulations in the U.K. and the E.E.C.

In a less technological age, British law on private commercial transactions could be attractively summed up in the well-known adage 'let the buyer beware'. Furthermore, the Middle Ages saw good self-policeing by the trade guilds as regards the honesty of their members and the quality of merchandise.[5b] During the 18th and 19th centuries, the old wisdom and institutions became unsuited to the new industrial society. Food adulteration was widespread, partly because of problems in supplying the rapidly expanding towns, and those who practised it must have been wholly ignorant of, or indifferent to, the dangers to the consumer. Food colouring was frequently part of such adulteration. Heavy metals and their salts such as red lead, mercury sulphide and even copper arsenite were detected by Accum and Hassall in various foods.[5b] They so alarmed the public that the Adulteration of Food and Drink Act (1860) followed by the Public Health Act (1875) were brought in, and indeed, the whole system of local authorities appointing public analysts resulted.

The introduction of the newly discovered organic dyes from 1860 was, in the light of past abuses, a distinct benefit. Soon, however, these organic colours were also suspected of toxicity. A committee set up

in the U.K. in 1923 by the Minister of Health recommended the drawing up of a list of colours considered safe for incorporation into food. The Government disagreed and only sanctioned (1925) the prohibition of colours known to be harmful. Besides metals and their salts such as cadmium, arsenic etc., the natural colour gamboge and certain coal-tar colours were banned. This action was belated and must have depended heavily on the good will of the food industry. In America from 1907 only colours of "known composition, examined physiologically and showing no unfavourable results"[1] were allowed onto a permitted list for use in food.

From 1925 onwards, the toxicological evidence mounted that some colours still in use were unsafe. The inadequacy of the U.K. position was obvious in that it was often colours of similar structure to those already prohibited that were suspected of toxic effects.[5b] Finally, in 1957 the U.K. went over to the practice of issuing a permitted list.[7] In addition, specifications for purity of permitted colours were made.[5b] There were revised regulations issued in 1966 and again in 1973.[8] A review of the 1973 regulations was published in 1979 by the Food Additives and Contaminants Committee on behalf of the Ministry of Agriculture, Fisheries and Food.[4]

The E.E.C. issued (23rd October 1962) a Council Directive on "the approximation of the rules of the Member States concerning the colouring matters authorised for use in foodstuffs intended for human consumption".[9] Seven amendments have since appeared, the latest being dated 20th January 1981.[10] The Directive operates on the basis that member states may include on their national permitted lists only those colouring matters that are on the E.E.C. list. When the U.K. joined the E.E.C. there were eight synthetic colours permitted in the U.K. which did not appear on

the E.E.C. list, while four colours were on the E.E.C. list that were banned in the U.K.[4c] Both the E.E.C. and the U.K. lists have been amended to rectify these discrepancies, and to admit more recent toxicological data. The U.K. amendments comprised:

(a) the Colouring Matter in Food (Amendment) Regulations 1975, which deleted orange RN, and

(b) the Colouring Matter in Food (Amendment) Regulations 1976, which deleted five colouring matters.

At present sixteen synthetic colouring matters (excluding inorganics and natural analogues) are permitted in the U.K. These are shown in Figure 1. Of these brilliant blue FCF, indigo carmine, sunset yellow FCF and tartrazine are regarded by the Food Additives and Contaminants Committee (1979) as acceptable; amaranth, black PN, brown FK, carmoisine, chocolate brown HT, erythrosine BS, green S, ponceau 4R, quinoline yellow, red 2G and red 10B are considered acceptable pending further specified toxicological testing; and patent blue V is believed to require further toxicological data before any adequate opinion can be formed.

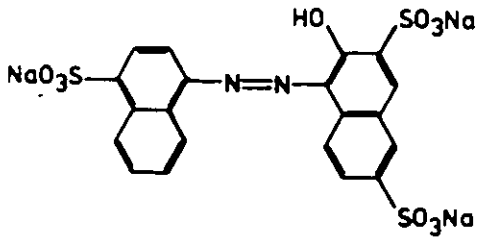
At present in the U.K. the amount of colouring matter added is not controlled since it is taken to be largely self-limiting - too much may make food unappetising.[4d] Extreme diets have been estimated,[4e] and reveal that present practice is unlikely to result in undue intake of particular colouring matters. Nevertheless it seems that in the future regulations could be introduced stating permissible levels, if only to further allay public disquiet, or to bring the law into line with that

dealing with other food additives, e.g. preservatives.

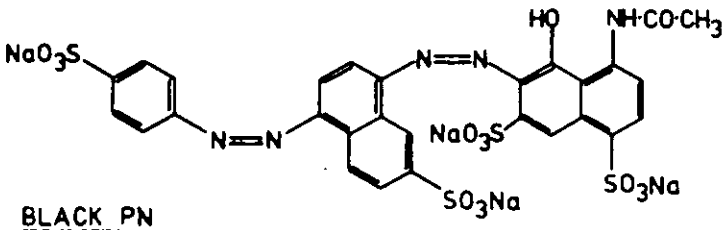
In summary, there is every likelihood that synthetic colouring matters will continue to be sanctioned given the commercial benefits, consumer preference and toxicological evidence supporting this, though polydyes and synthetic analogues of natural colours could play a larger role in the future.

The aim of the present work was to monitor the heat and light stability of the UK-permitted synthetic food colours by differential pulse polarography and then to study the decay products by hplc and other methods. Therefore an introduction to these techniques and a summary of previously published degradation studies is given in the following chapters.

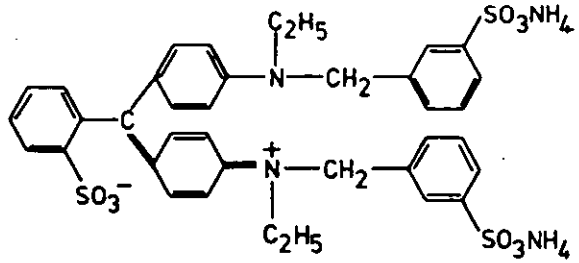
Structures of the U.K. permitted synthetic food colouring matters.



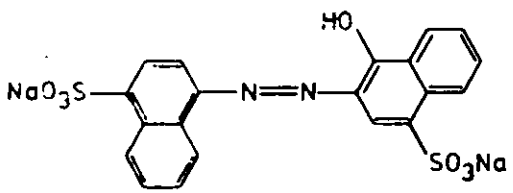
AMARANTH



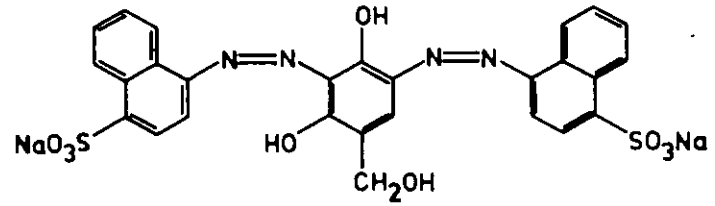
BLACK PN



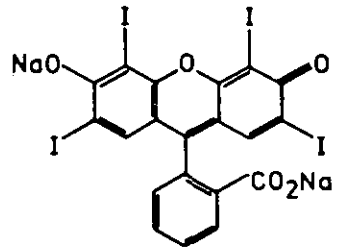
BRILLIANT BLUE FCF



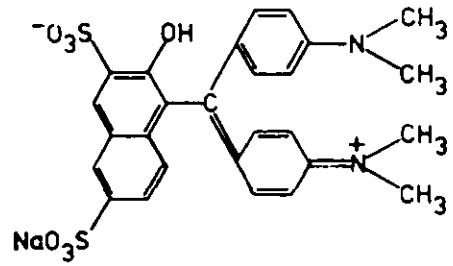
CARMOISINE



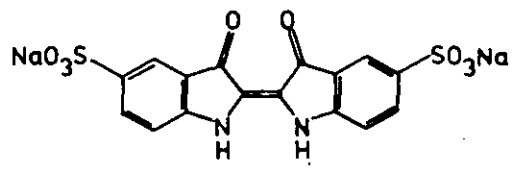
CHOCOLATE BROWN HT



ERYTHROSINE



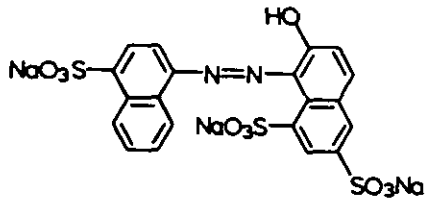
GREEN S



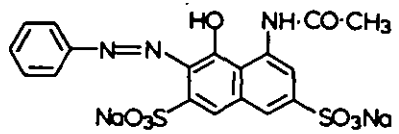
INDIGO CARMINE

Fig 1(a)

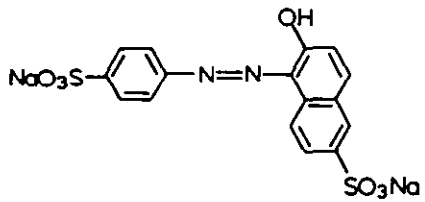
Structures of the U.K. permitted synthetic food colouring matters (cont'd)



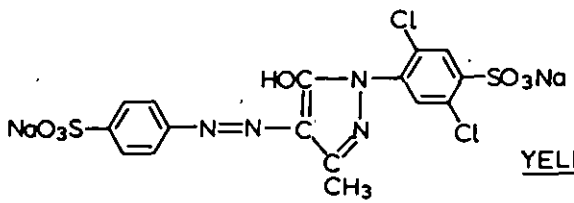
PONCEAU 4R



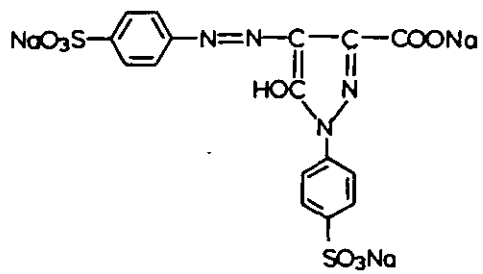
RED 2G



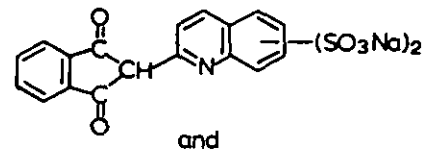
SUNSET YELLOW FCF



YELLOW 2G

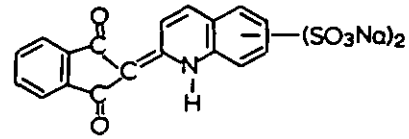


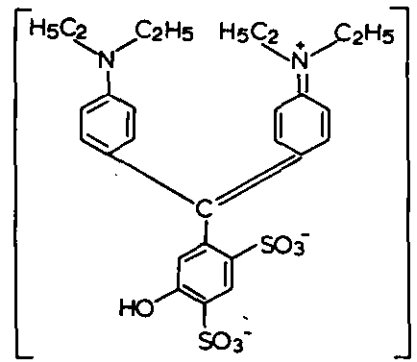
TARTRAZINE



and

QUINOLINE YELLOW





PATENT BLUE V

Fig. 1(b)

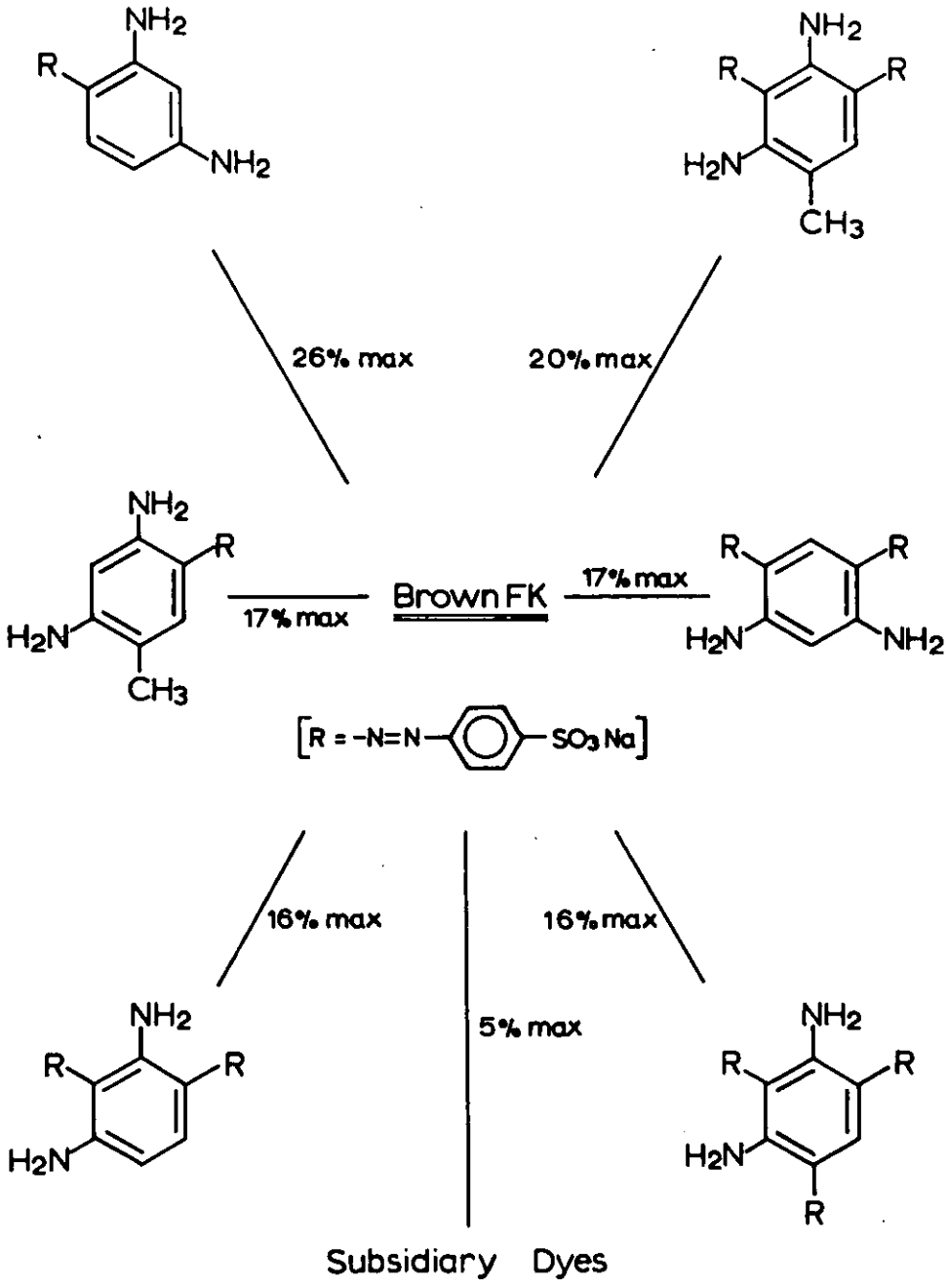


Fig. 1(c) Structures of the U.K.-permitted synthetic food colouring matters (cont'd).

CHAPTER 2

Stability of the Food Colours

Commercial food colours are not pure compounds, but consist rather of one or more (six in the case of brown FK) main dyes, together with intermediates used in their synthesis, and any side products formed.[4f] Published stability studies generally deal with these commercial samples, not with the purified dyes (except for Refs. 11 & 12), so that comparison of one study with another must allow for minor variations arising from differences in sample constitution, either between production batches, or between manufacturers, or indeed at different dates, as the trend has been toward stricter control of the impurities.

It is convenient to recognise three types of stability study:

(i) stability of food colours in aqueous solution;

(ii) stability of food colours during residence in the body (metabolic fate); and

(iii) stability of food colours incorporated into food or drugs.

While categories (iii) & (i) form the central subject matter of this section, brief mention will be made of (ii) also, as it provides a more general view against which the findings of category (iii) may be seen in better perspective.

(i) Tonogai et.al.[13] have examined the bacterial decomposition of some food colours which have been discharged in waste streams. They found that hardly any aerobic decomposition took place, but that various azo dyes, including tartrazine, sunset yellow FCF, amaranth, ponceau 4R

and others were readily and completely faded over 3 - 6 days under anaerobic conditions. [Such resistance to oxidative decomposition by bacteria had been noted previously by other workers]. Sulphanilic or naphthionic acid are among the possible products formed by reductive cleavage of the azo linkage in these dyes: about 50% of the expected amount of these products was detected, indicating that the remnant was possibly further decomposed, or else incorporated by microbial activation into the sludge employed.

Another possible cause of degradation in waste streams is exposure to sunlight. Porter[14] investigated this for a number of textile dyes including acid red 1, which doubles as the food dye red 2G. Although acid dyes were considered generally to show poor light fastness on textiles compared to disperse dyes, acid red 1 showed itself to be among the more light stable dyes tested in aqueous solution.

(ii) Various workers[15,16] have found that in the body (animal experiments) reduction of the azo group of food colours, e.g. amaranth, by azo reductase enzyme in the liver, is of minor significance, and that microorganisms in the intestines, such as Enterococci and Coli bacteria, account for most of the reductive action. Koether[17] describes this for carmoisine, amaranth and ponceau 4R (etc), and mentions the presence of naphthionic acid as a degradation product. Amaranth appears to be almost completely reductively cleaved in the small intestine.

(iii) Table 1* summarises the literature available on the stability of food colours either in simple media, especially aqueous solution, or in food

* p.20

or drug matrices, or in systems which model them. The comparative paucity[18] of references prior to about 1960 was seemingly due to a difference in attitude to food additives in general that then prevailed,[7] though specific contributory factors included:

(i) the former availability of the simple measure of substituting any dye that gave rise to noticeable stability problems with another that did not[19]- this widespread practice became increasingly difficult as various countries tightened up their regulations in the 1950's;

(ii) habitual reliance on published tables[20](such as that in reference 21) even though these tended not to take into account complicating factors[22](variation in matrix, synergistic effects of several additives, etc.);

(iii) the treatment of stability data as confidential when obtained by a company research laboratory[19];

(iv) a preference for qualitative testing in the absence of specific legal requirements.

The suggestion[19] that another major factor might have been an absence of standard equipment, at least in the case of light studies, seems plainly inapposite: the development of such apparatus for pharmaceutical purposes followed quickly once the need for it had been identified, and no aspect of this development would lead one to suppose that it could not have been carried out several decades earlier had the need arisen then. What has improved radically over the last forty years is not so much the means of promoting degradation, but rather the means of monitoring that degradation once it has occurred.

To sum up the pre-1960 attitudes of the food and drug industries: the food industry became concerned mainly when consumer acceptance was threatened, while the pharmaceutical industry was concerned that tablet identification (colour coding) should not be impaired. Since that time legislation, closer toxicological scrutiny and changes in the climate of public opinion have resulted in more workers publishing important stability data, and seeking means of reducing degradation where possible.

Sources of Stability Data

The stability of food or pharmaceutical colours lies within the range of interest of academic, food manufacturing and governmental research bodies. Table 2 shows which references stem from which type of organisation: it is clear that the three groups have made similar levels of contribution.

TABLE 2 : SOURCES OF PUBLICATIONS ON STABILITY OF FOOD AND PHARMACEUTICAL COLOURS

Academic Refs:	11, 12, 14, 18, 23, 26, 31, 32, 33, 36, 45, 46, 47, 58, 60.
Food Manufacturers, Industrial Research Associations and Dye Manufacturers Refs:	7, 19, 20, 21, 22, 25, 27, 38, 40, 41, 42, 61, 62, 63.
National/Local Government Agencies:	24, 29, 30, 34, 35, 37, 39, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 59.
Source Not Stated/Unclear:	28, 44, 64, 65, 66.

Distribution of Degradation Studies

The lack of data prior to about 1958 has already been discussed: since that date an average of about $2\frac{1}{3}$ papers per year has appeared.

The distribution of information about individual dyes varies very greatly, ranging from 25 references for tartrazine down to a single reference for brown FK. The factors which seem to have contributed to the inclusion of dyes in degradation studies are:-

(a) reds and yellows are used in greater amounts than other colours, so are regarded as more important;

(b) some colours are used frequently in pharmaceuticals, and

the pharmaceutical industry has shown particular interest in colour stability in tablets etc.;

(c) colours on the F, D & A permitted list receive more attention;

(d) colours notorious for instability are better referenced, esp. indigo carmine;

(e) colours only used in the U.K. (at present) naturally have fewer references than those used in a wider region.

Types of Study Conducted

In collating the information provided in the sources listed in Table 1, it seems important to recognise that the nature of all this information is by no means equivalent, and that comparison of results from different sources may be limited in some cases by this. It is possible to group the reference information as follows:-

(i) abstract only - it is not clear from the available material what the full extent of the evidence was upon which the conclusions presented have been based;
[References: 11, 64]

(ii) qualitative - author makes comparisons by means of verbal statements or simple number charts without giving exact % decompositions under defined conditions;
[References: 7, 21, 23, 24, 29, 31, 34, 36, 38, 41, 42, 47, 48, 50, 62, 63]

(iii) quantitative - exact % decomposition and operating conditions are recorded, but only one or two measurements are given per sample, i.e. insufficient for a kinetic decay curve to be drawn up;
[References: 12, 18, 22, 26, 31, 43, 50, 51, 57, 61]

(iv) graphical - as for group (iii) but with 3 or more measurements per sample, whether or not presented in the paper as a graph;

[References: 12, 19, 25, 27, 28, 30, 32, 40, 46, 49, 53, 55, 56]

(v) product identification - the degradation product is investigated and partial or full details reported of its structure.

[Partial Identification References: 7, 28, 37, 39, 52. Full Identification References: 33, 35, 45, 54, 59]

[Some references containing mixed material have been listed twice].

Stability Trends among the Food Colours

The results of the trials listed in Table 1 are given for the individual dyes in Appendix A. A number of the studies provide only qualitative or semiquantitative data designed to be used comparatively for selection within the range of permitted colouring matters available. Whereas this is suited to the needs of industry, there is a need for quantitative data to put such comparative results into perspective.

Two papers are summarised on the following pages which provide useful comparative data. One paper by Nursten and Williams[18] gives quantitative results, while the other by Jouffrey[23] only states the qualitative conclusions drawn from unspecified experimental work, and is basically a review article without references. There is good agreement between these papers on the good stability of amaranth, black PN, carmoisine and erythrosine towards heat in aqueous solution (or buffered at pH 7). What is apparent, however, is the difference in scope, purpose and presentation between the respective authors. When referring to Appendix A it is always necessary to take into account such important differences

when comparing references.

Nursten and Williams[18]

Heat stability in aqueous solution at pH 7 and pH 3 was determined, with the effect of ascorbic acid being observed. The conditions included heating at 250°F (121°C) for 20 minutes, with dye concentrations of 0.00083 - 0.0025% (suitable for spectrophotometry).

Results:-

(i) pH 7, no ascorbic acid:

< 5% loss:- Am, BFK, BPN, Ca, Er, R2G , SY, Ta

> 5% loss:- Y2G (9.9%), IC(11%), GS(14%), ChB(19%), P4R(55%).

(ii) pH 7 and 1 mg/ml ascorbic acid:

< 5% loss:- Am, Er, GS, P4R, SY, Ta, Y2G

> 5% loss:- R2G(5.2%), IC(5.4%), BFK(11%), ChB(18%; λ max. shift), Ca(35%), BPN(90%).

(iii) pH 3, no ascorbic acid:

< 5% loss:- Am, BFK, BPN, Ca, GS, R2G, SY, Ta, Y2G

>5 % loss:- IC(5.5%), BPN(1.9%[λ max shift] and 7.3%, two λ max measured), ChB(11%)

(iv) pH 3 and 1 mg/ml ascorbic acid:

< 5% loss:- GS, Ta, Y2G

> 5% loss:- IC(6.4%), SY(6.8%), R2G(7.7% and 8.9%, two λ max measured), P4R(8.2%), Am(8.3%), Ca(17%), ChB(49%; λ max shift), BFK(56%), BPN(87% and 64%, two λ max measured).

Jouffrey[23]

(1) Stability to natural light in aqueous solution [Scale 1 → 7]

IC (1 → 2:inadequate), Er(2:poor), Am(3:moderate)

BPN, Ca, PBV, QY ,SY and Ta (all 5:good stability).

(2) Stability to heat in aqueous solution

Am, BPN, Ca, Er, PBV all given as good, with no category given for the other dyes.

(3) Stability in oxidizing media

Am - limited stability, Ca - stable, PBV - turns green

P4R - weak stability, Er - little stability, IC - unstable

SY - stability very slight, QY - slight modification of the colour

BPN - variable sensitivity, bordering on colour change

Ta - doubtful stability

(4) Stability in reducing media

Am - unstable, Ca - stable, appearance impaired

PBV - yellows, P4R - unstable, Er - very little stability

IC - unstable, SY - unstable, QY - stable

BPN - variable sensitivity, bordering on colour change

Ta - discoloured by powerful reducing agents

TABLE 1 : SUMMARY OF PREVIOUS DEGRADATION STUDIES

Abbreviations Used

Am	Amaranth	IC	Indigo Carmine
BB	Brilliant Blue FCF	PBV	Patent Blue V
BFK	Brown FK	P4R	Ponceau 4R
BPN	Black PN	QY	Quinoline Yellow
Ca	Carmoisine	R2G	Red 2G
ChB	Chocolate Brown HT	SY	Sunset Yellow FCF
Er	Erythrosine	Ta	Tartrazine
GS	Green S	Y2G	Yellow 2G

Format

In order to condense the information in the table, the following connecting words are used: and, or, with/without. These are not used loosely (as in ordinary English) but indicate the number and range of variable factors employed, viz.

A or B and C with / without D

means that at least two factors were present, the first being chosen from the group A, B, the second from C. For some combinations, D was additionally present.

For a few cases where many choices are given, some combinations of factors may not have been reported. Nor have all the combinations been used necessarily for all the dyes listed.

[The abbreviation H^+ is used for acid, either separately or as part of a name, e.g. acetic acid = acetic H^+]

Reference Number	Factors Promoting the Degradations Investigated and Dye Matrix Used	Food Dyes Investigated which are currently UK Permitted
7.	[Part Review] (i) Processing-effect of H_2S or S cpds. liberated. (Fish paste) (ii) Light and Fe and citric H^+ (Lemon curd) (iii) Storage (Canned, aq. or in food)	BPN, Er, GS, P4R, PBV, SY, Ta, Y2G
11.	Heat with/without H_2SO_4 or fructose (Aq.)	Am, SY
12.	Heat with/without H_2SO_4 or fructose (Aq.)	SY
18.	Heat and pH3 or 7 with/without ascorbic H^+. (Aq.)	Am, BFK, BPN, Ca, ChB, Er, GS, IC, P4R, R2G, SY, Ta, Y2G
19.	Light (Tablets)	BB, Er, IC, QY, Ta
21.	Light or acetic H^+ (10% or glacial) or HCl (10% or 30%) or NaOH (10% or 30%) or NaCl or $FeSO_4$ or alum or oxidizing.	Am, BB, Er, IC, QY, SY, Ta
22.	Storage and heat and non-ionic surfactants (Aq.)	IC, Ta
23.	Natural Light (aq. or on cellulosic support) or pH or oxidative or reductive media or heat (Aq.)	Am, BPN, Ca, Er, IC, P4R, PBV, QY, SY, Ta
24.	Heat and processing (Cooked meat)	Am, Ca, Er, P4R, R2G
25.	(i) pH 6.6 and dextrose or mannitol or sorbitol or dextrose or lactose or sucrose and natural light, (ii) pH 6.6 and reducing catalyst (Hydroquinone or p-hydroxypropiophenone or hydroquinone monomethylether or nordihydroquaiaretic acid) and light (iii) pH 6.6 and sugar (as in (i)) and reducing catalyst (as in (ii)) and light. (iv) pH 6.6 only (control) (Aq.)	IC
26.	Gelatins (2 types) (Liquid or solid solutions)	Er (and others specified only in thesis)
27.	Light (Lakes or aq. incorporated into tablets).	BB, Ta.

Reference Number	Factors Promoting the Degradations Investigated and Dye Matrix Used	Food Dyes Investigated which are currently UK Permitted
28.	H^+ and iron or mild steel. Reduction partly reversed with air O_2. Hydrazo/aromatic amino products. Protective action of sorbic H^+. (Aq.)	Am
29.	Heat with/without Na_2SO_3 or ascorbic H^+ or H_2O_2. (Aq. or salmon or marmalade or anchovy paste).	Am, BPN, Er, IC, P4R, QY, SY, Ta.
30.	Baking with/without subsequent storage.	Ca, Er, IC, SY.
31.	[Review] Heat, light, pH, additives, sugars, surfactants, reducing or oxidising agents, H^+, OH^-. (Aq. or lakes).	Am, BB, BPN, Ca, Er, IC, P4R, PBV, QY, SY, Ta.
32.	Light and heat or ambient or frozen with/without pectin or ascorbic H^+ and/or citric acid. (Gelatin gels, 'soft' or 'firm').	Am.
33.	Aluminium A-5 or steel-3 or steel 20 and acetic H^+ or citric H^+ or tartaric H^+ or malic H^+ or HCl. (Aq.)	Am, SY.
34.	Storage and original processing (Tomato ketchup).	R2G, SY, Ta.
35.	(i) Storage or processing and presence of reducing sugars (ii) Modelled using sucrose and glucose (orange dyes formed). (Confectionery, dragées etc.)	BPN.
36.	[Partly review]. Refrigerated storage or ambient storage (dark) or ambient storage (diffuse light) and pH control (2-8). (Aq. or syrup(2 forms) or elixir).	Am, BB, Er, IC, SY, Ta.
37.	(i) Baking and sucrose or dextrose or baking powder ($CaHPO_4$, $NaHCO_3$, corn starch) or $NaHCO_3$ (plus some combinations).	Am.

Reference Number	Factors Promoting the Degradations Investigated and Dye Matrix Used	Food Dyes Investigated which are currently UK Permitted
	(Cakes-incorporated as aq. or lake). (ii) Processing (Biscuits, candy, ice-cream cones, puffed cereal).	
38.	Light	Am, BPN, Ca, Er, IC, P4R, PBV, QY, SY, Ta.
39.	SO_2 or model pickle system or ascorbic H^+ or H^+ or [glucose and fructose] or nitrite or dithionite or [SO_2 and light].	Am, ChB, Er, GS, SY, Ta.
40.	Heat with/without pH 3-7 buffer (Tablets).	BB, Ta.
41.	SO_2 (gelatin) [glycerin, methylparaben and propylparaben acceptable].	Am, BPN, Ca, IC, P4R, PBV, QY, Ta.
42.	Processing with/without subsequent storage. (Boiled sweets, fondants, toffees, fish paste, madeira cake, fresh sausages).	Am, BPN, Ca, Er, GS, IC, P4R, R2G, SY, Ta, Y2G.
43.	Light (Tablets).	Am, BB, Er, SY, Ta.
44.	Gamma radiation (Aq.)	Am and other azo dyes.
45.	[Model systems; mechanistic study]. H-donor (mandelic H^+ or acetone and flash photolysis. (Water and/or ethanol).	Ca, P4R, R2G, Y2G.
46.	U.V. Radiation (Dragée mass or aq.)	Am, P4R
47.	Storage (overlong or inappropriate). (Fish roe).	BPN
48.	Heat (action of S cpds). (Gravy browning, meat and potato pies).	P4R
49.	(i) citric H^+ or tartaric H^+ or KNO_3 and Fe or Sn. (ii) glucose or lactose or ascorbic H^+. (iii) Na_2SO_3 or $NaHSO_3$ or $NaNO_2$ (Aq.)	Am, Ca, Er, IC, P4R, SY, Ta
50.	Heat or light or H^+ or OH^- (Aq.)	IC, SY.

Reference Number	Factors Promoting the Degradations Investigated and Dye Matrix Used	Food Dyes Investigated which are currently UK permitted
51.	Manucol and direct or diffuse light or dark at 37° and storage. (Orange squash (i) synthetic or (ii) real, containing various sugars, ascorbic H^+, etc).	SY, Ta.
53.	Bacterial (Streptococcus lactis and cremoris) reduction. (Fermentation broth, aq.)	Ca.
54.	Bacterial reduction (as above). (Fermentation broth, aq.)	Ca.
55.	Ascorbic H^+ (reduction) with accompanying autocatalysis.	BPN
56.	Bacterial reduction in air or N_2 atmos. and in presence of lactic acid and lactose. (Fermentation broth, aq.)	BPN, SY, Ta.
57.	Light and interaction with Er.	Er, IC: all certified F, D & C affected except BB, Ta.
59.	Light (decomposition products examined).	IC.
61.	(i) lactose, conventional (ii) lactose, spray dried (iii) d-glucose (iv) d-galactose (Aq. buffered pH ~6.7).	BB, IC, Ta.
62.	Heat or storage and Sn and/or Fe or $SnCl_2$ with/without citric H^+ or malic H^+ or oxalic H^+. (Sucrose syrup or aq.)	Er.
63.	(i) Bacterial reduction (B. michaelisii) at 55° and pH<6. (ii) Zn dust. (Brine, i.e. canned peas).	Ta, Y2G.
64.	Light (Tablets).	BB, Er, IC, QY, Ta.
65.	Interaction of Er with other colours and in the presence of light and OH (Aq.)	Am, Er, SY, Ta.

CHAPTER 3

Polarography

Voltammetry is one of the major electroanalytical techniques. It may be defined generally as "the measurement of current-voltage relationships at an electrode immersed in a solution containing electroactive species".[67] Most commonly, the current flowing across an electrode-solution interface is determined when a controlled potential is applied. Polarography is the name given to voltammetry performed at a dropping mercury electrode (dme).[68a]

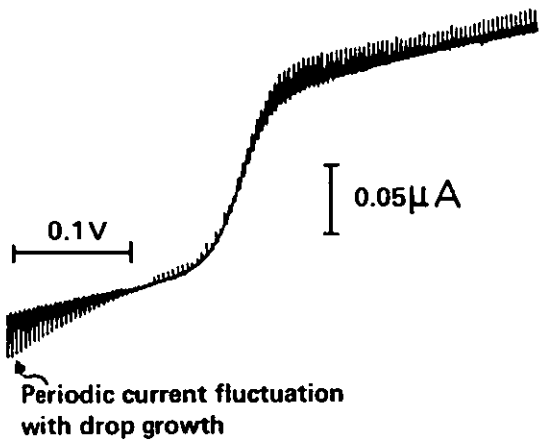
During polarographic analysis, electrolysis occurs giving rise to a faradaic current. Many complex factors are liable to influence the position and shape of the potential-current curve observed. However, if the electrode process is fast with respect to the 'time domain' of the polarographic technique being used[69a] in conventional dc work this is the drop time, usually 2-8s - then it is said to be reversible, and the position at which the polarographic wave appears is given by the Nernst equation, which is a thermodynamic description of the electrochemical system:

$$E = E^\circ - \frac{RT}{nF} \ln \frac{[Red]_\circ}{[Ox]_\circ}$$

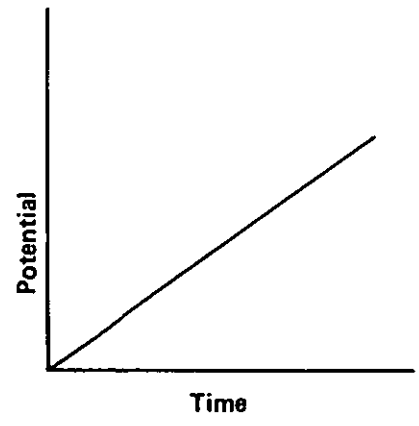
where $[Ox]_\circ$ and $[Red]_\circ$ represent the activities of the oxidised and reduced forms of the analyte at the electrode surface, respectively, E° is the standard redox potential and E is the potential applied.[70a] It is possible to show that for reversible electrode processes $E^\circ \approx E_{\frac{1}{2}}$, the half-wave potential, which may be determined directly from a polarogram (Figure 2).

For an electrode process which is both chemically and electrochemically reversible, the rate of electrolysis - and hence the current measured - may be limited by the formation of a layer depleted in analyte adjacent to the

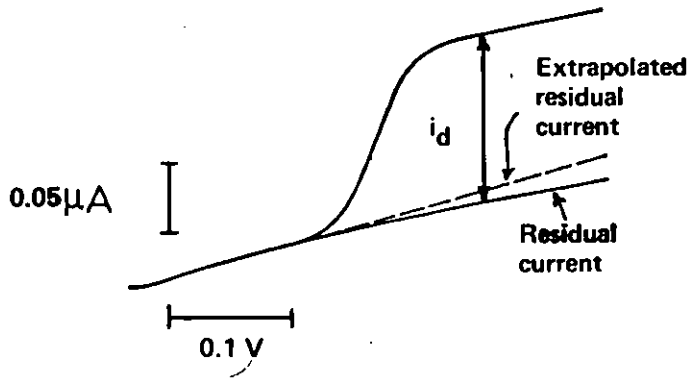
Features of a d.c. polarogram



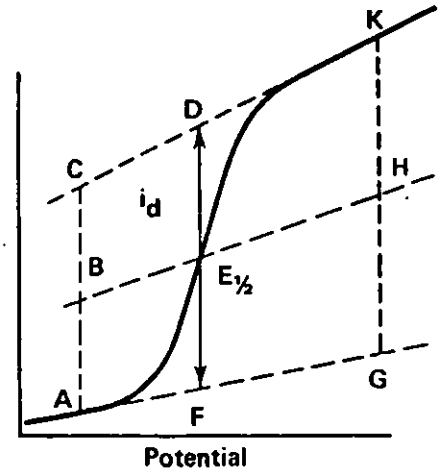
(a) Unfiltered polarogram



(b) Form of the applied potential



(c) Average current polarogram



(d) Graphical determination of diffusion current, i_d, and half wave potential $E_{1/2}$

Fig. 2

electrode surface.[68b] The faradaic current will then depend upon the rate at which diffusion processes transport fresh material from the bulk solution to the electrode surface, so that the polarographic wave observed is said to be diffusion controlled.

This diffusion contolled limiting current in dc polarography is given to a good approximation by the Ilkovic Equation[70b]

$$i_D = 0.732\ n\ F\ C\ D^{\frac{1}{2}}\ m^{\frac{2}{3}}\ t^{\frac{1}{6}}$$

where

i_D = diffusion-controlled current, A

n = number of electrons in the electrochemical process

F = Faraday constant (96500 coulombs)

C = concentration of electoactive species in the bulk solution, mol cm^{-3}

D = diffusion coefficient of the electroactive species in the supporting electrolyte, $cm^2\ sec^{-1}$

m = flow rate of mercury, $g\ sec^{-1}$

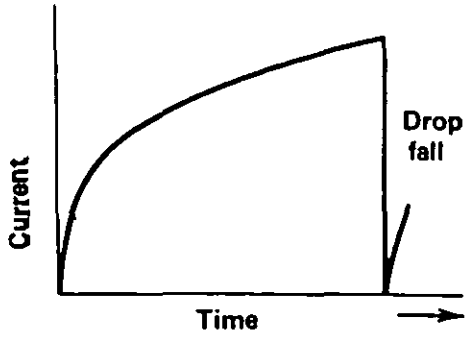
t = drop time, sec.

Figure 2 shows a dc polarogram with the form of the applied potential. The analytically important features are firstly the half-wave potential, $E_{\frac{1}{2}}$, as this is characteristic of the electroactive species, and secondly, the magnitude of the diffusion-limited current, i_D, because from the Ilkovic equation, $i_D \propto$ concentration (C). Calibration graphs may be constructed and the technique used quantitatively.

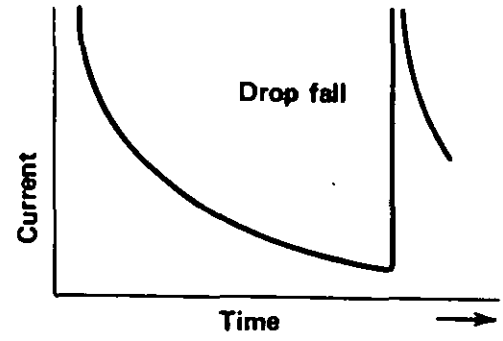
As well as the faradaic current, a second current exists

due to the build up of an electrical double layer at the solution-electrode interface. Because this charging current depends upon electrode area and applied potential, it produces short periodic fluctuations associated with the growth of individual mercury drops, and a baseline sloping upwards with increasing potential (see Figure 2).[68c] When the concentration of the electroactive species is below 10^{-4}-10^{-5}M the charging current becomes of comparable magnitude to the faradaic current, and so the limit of detection for standard dc polarography is reached at 10^{-5}-10^{-6}M.[69b]

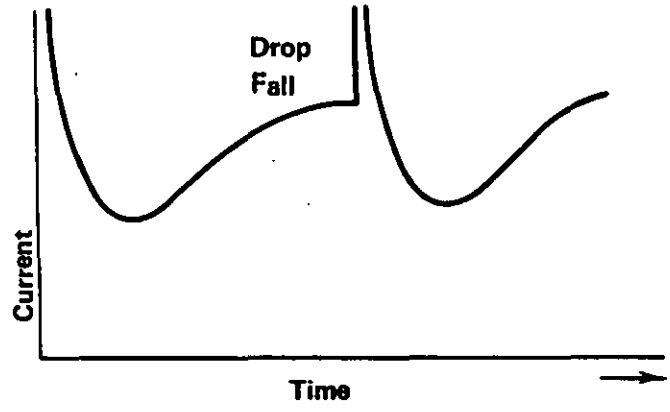
One method of lowering detection limits and of facilitating the interpretation of polarograms is to use current-sampled dc polarography [70c] (sometimes still referred to as "Tast Polarograph"[71]). At the start of the life of a mercury drop the charging current is at a maximum since drop surface area is changing at its greatest rate. The faradaic current, however, increases during drop growth reaching a maximum immediately before drop fall. This occurs because, as the mercury surface area increases, the volume of the depletion layer is spread more thinly around the drop, and the mercury surface progressively contacts higher concentrations of the electroactive species.[70b] Electrolysis competes unsuccessfully to widen the depletion zone. [The general expression for diffusion towards a growing dme is given by Fick's Second Law, the solution of which, incorporating appropriate concentration boundary conditions, is simply the Ilkovic equation stated above]. These factors determine that the most favourable faradaic : charging current ratio exists late in the drop life (see Figure 3i). In current sampled dc polarography, the current is only recorded during the final, e.g. 5-20 msec of a 5 sec drop life.[69c] Although the improvement in sensitivity is somewhat marginal because of the decreased measuring time, there is a major benefit in the form that the readout takes since the short periodic fluctuations are virtually eliminated, and smooth, stepped curves produced instead (see Figure 4). Drop time must be



(a) Faradaic current



(b) Charging current



(c) Total current

Fig. 3(i) Variation of Faradaic and Charging currents with drop life in d.c. polarography

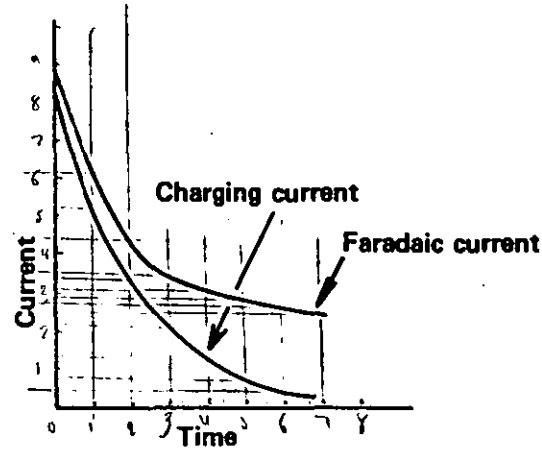
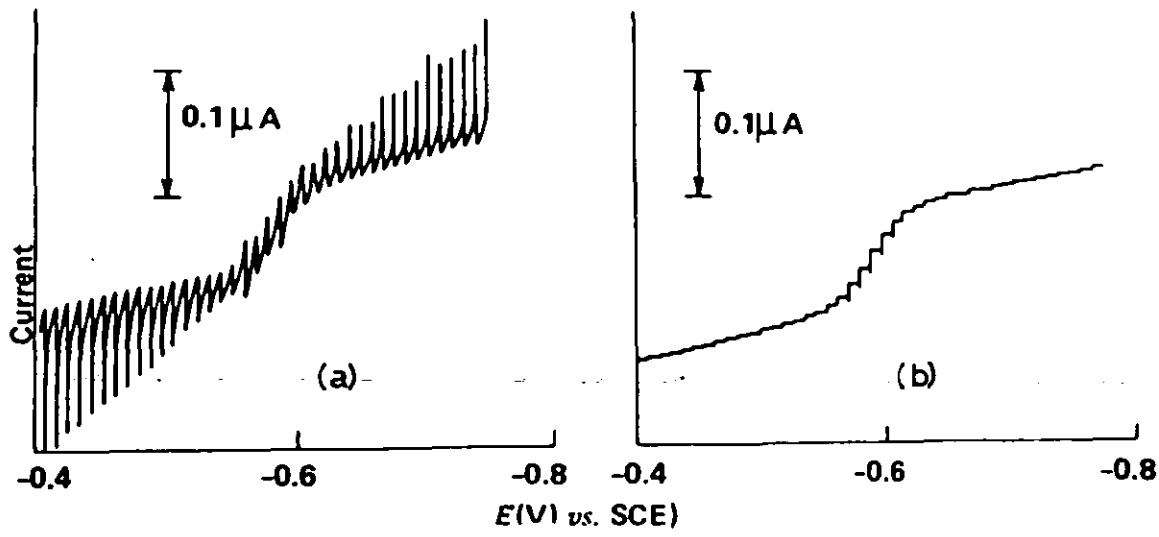


Fig. 3(ii) Variation of Faradaic and Charging currents with drop life in pulse polarography.

Comparison of waveforms in d.c. and current sampled d.c. polarography

(Polarograms for $10^{-5}M$ Cd^{2+} in $0.01M$ HCl)



(a) Conventional d.c. mode
(b) Current sampled d.c. mode

Fig. 4

controlled by mechanically dislodging the mercury at exact intervals using the timer that is linked also to the current sampling circuit.

Numerous approaches have been taken to overcome the relative lack of sensitivity of dc polarographic methods, and many of these are discussed in a recent text by A.M. Bond.[69] For the present, however, only two methods are widely known and routinely used, viz. normal (or integral) pulse polarography (n.p.p.), and differential pulse polarography (d.p.p.). In these methods the potential range is covered using square wave potential pulses[70d] as shown in Figure 5 for n.p.p. and in Figure 6 for d.p.p., together with typical polarographic waveforms obtained. From the diagrams it may also be noted that current sampling is incorporated in both pulse methods.[70c]

Normal Pulse Polarography

The sudden application of a jump in potential (e.g. of 50 msec. duration) when the mercury drop is near its maximum size and no depletion layer has previously formed generates a far larger faradaic current than is produced in dc polarography. N.p.p. is indeed the most sensitive of the four polarographic modes discussed here (cf. Figure 7). Provided that a scan is begun in a potential region where no faradaic process takes place, the Cottrell equation applies:[69d]

$$i_l = n\,F\,A\,D^{\frac{1}{2}}\,C\,\pi^{-\frac{1}{2}}\,t_m^{-\frac{1}{2}}$$

where i_l = normal pulse current on the diffusion plateau, Amp

t_m = interval between pulse application and measurement, sec

A = area of Hg surface, cm^2

and the other variables are as given above in the Ilkovic equation.

Note that the signal is again directly proportional to the analyte concentration. Dividing the Cottrell by the Ilkovic equation:

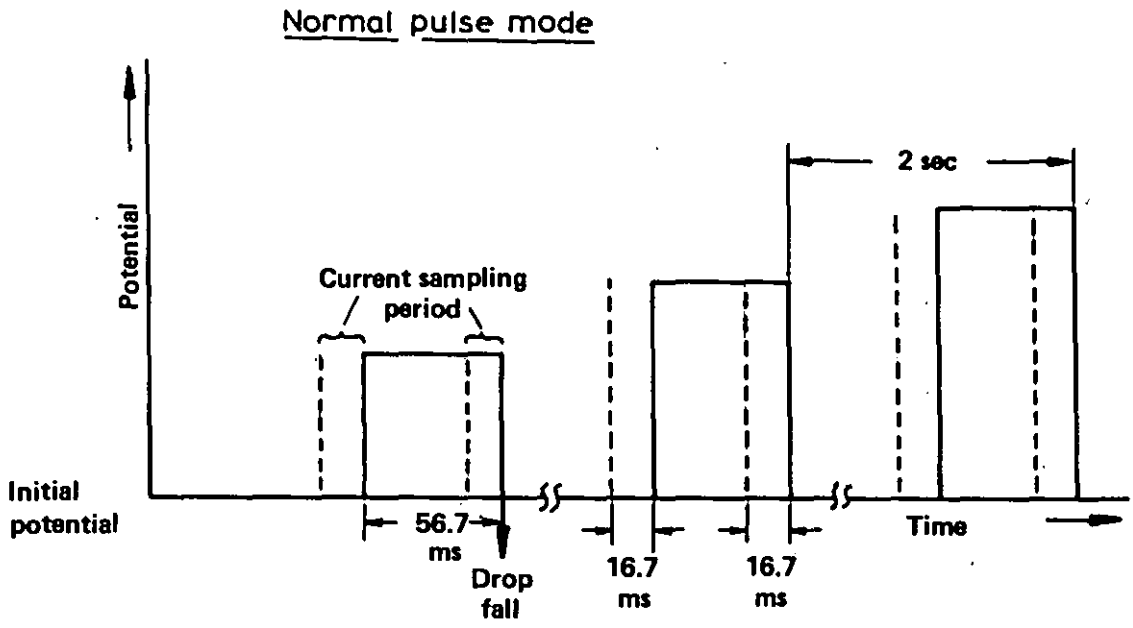


Fig.5 Format of the applied potential and current measurement in normal pulse polarography.

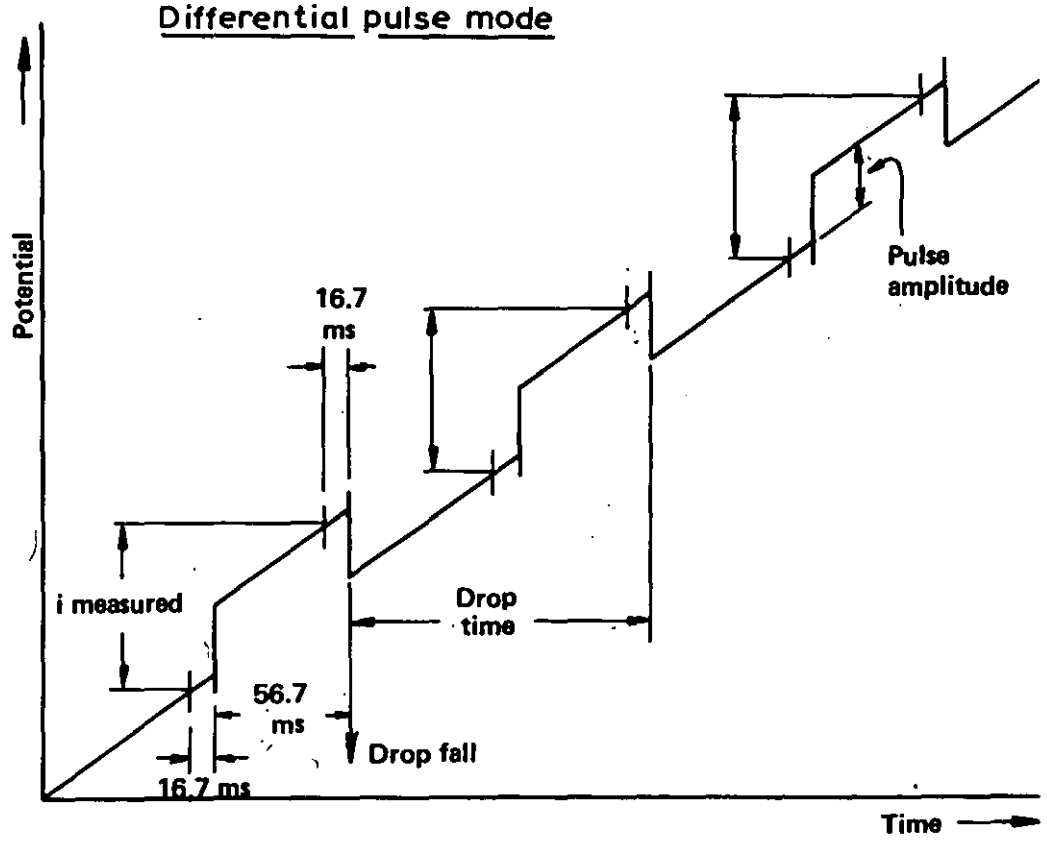
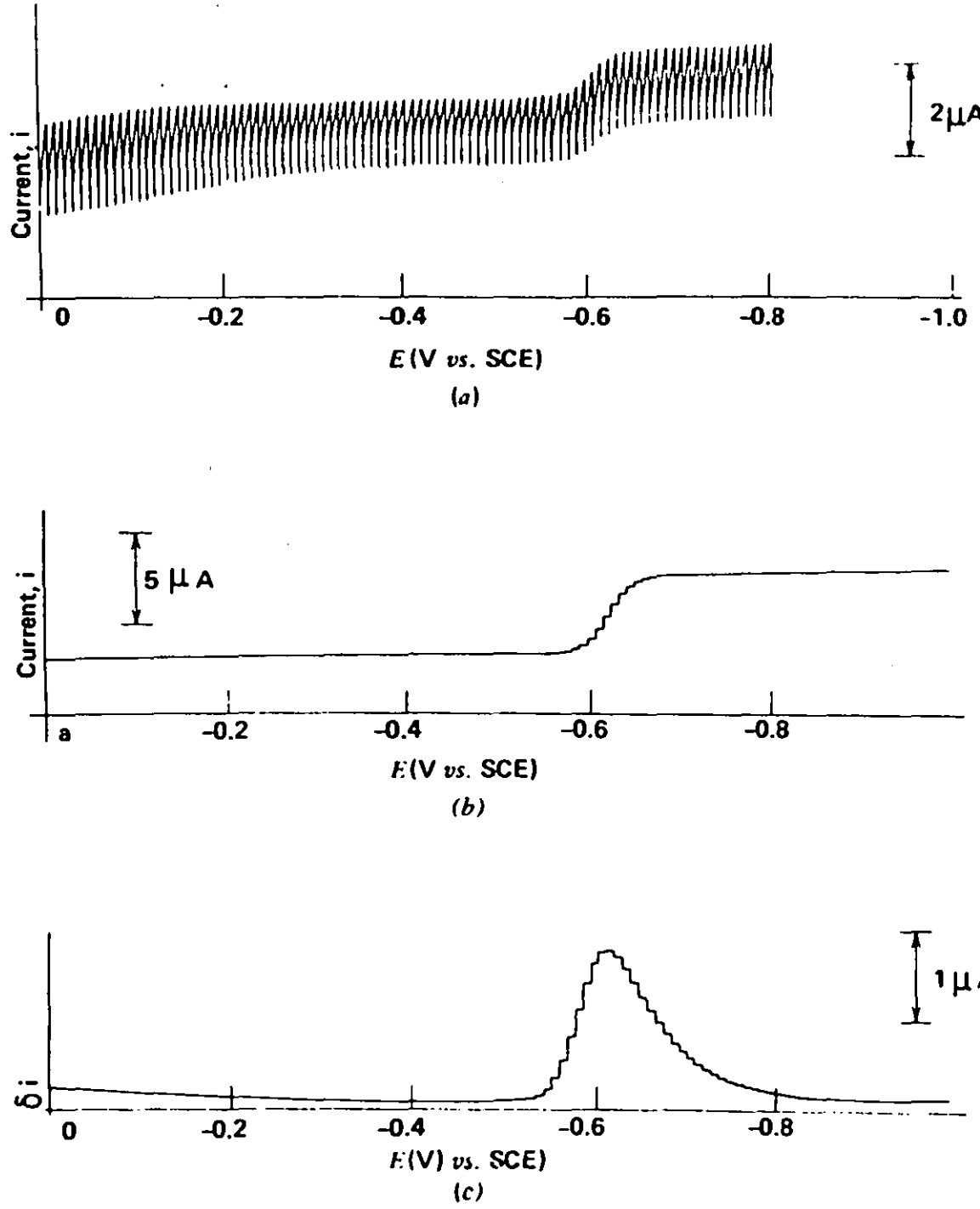


Fig. 6 Format of the applied potential and current measurement in differential pulse polarography.

Comparison of waveforms in d.c., normal pulse and differential pulse polarography.

(Polarograms of 10^{-3} *M* Fe^{3+} and 10^{-4} *M* Cd^{2+} in 0.1*M* HCl)



(a) Conventional d.c. mode
(b) Normal pulse mode, E_b = 0.2V *vs* SCE
(c) Differential pulse mode, ΔE = 50 mV

Fig. 7

$$\frac{i_l \text{ (n.p.p.)}}{i_d \text{ (d.c.)}} = \frac{t^{\frac{1}{6}}}{t_m^{\frac{1}{2}}} \cdot \sqrt{\frac{3}{7}}$$

Substitution of typical values gives the ratio as ca. 6-7, indicating the sort of enhancement in sensitivity produced by n.p.p. over d.c. polarography.

When the pulse is applied, a large charging current flows, though this decays exponentially with time, and significantly faster than does the faradaic current (see Figure 3b).[69e] Furthermore, drop areas at the pulse application and sampling times are identical for successive mercury drops, and the surge in charging current only increases slowly between pulses, owing to the increments in potential. Delaying pulse application until late in the drop life has the added benefit of minimizing the charging current that flows prior to pulsing and which is associated with drop growth.[69d] Some polarographs subtract this small component from the measured current.

Whereas the drop time governs the 'time domain' in dc. polarography, in n.p.p. the controlling factor is the pulse duration prior to measurement of the current, t_m (see Cottrell equation).[69f] Since $t_m \simeq \frac{t}{100}$, many electrode processes which are for practical purposes reversible when used in dc. polarography, are plainly not so when n.p.p. is applied. Though the slope of the sigmoidal wave may decrease in steepness, the limiting current in the plateau region is unaffected. In this n.p.p. is analogous to dc. polarography.

Differential Pulse Polarography

The limit of detection in n.p.p. is mainly set by the high slope of the baseline current due to the charging effects of the large amplitude pulses required to scan wide ranges of potential.[72] It becomes increasingly

difficult to measure small waves against this curving baseline.

In d.p.p. the potential range is scanned using pulses of uniform, moderate amplitude superimposed on a linear dc ramp (Figure 6).[69d] The current recorded is a 'difference current', viz., the current at a time, t_m, after the pulse minus the current immediately prior to the pulse, such that the polarographic wave is a stepped peak and not S-shaped as in n.p.p. and d.c. methods. As pulse amplitude is decreased from the high levels used in n.p.p., both faradaic and charging currents decline, though not necessarily at similar rates.[72] A range of pulse amplitudes may therefore be found for a particular analytical solution over which the faradaic:charging current ratio is increased markedly, so lowering the limit of detection for d.p.p., perhaps 10x below that for n.p.p. On the other hand, d.p.p. differs from n.p.p. in that the pulse basepoint shifts during scanning so that it must eventually enter a potential region (on the S-wave) where faradaic processes are occurring. Taken with this, it should be noted that no single pulse allows the full wave height (diffusion current) to be measured. One effect of this is a fall in sensitivity, i.e. measured signal (μA), with respect to n.p.p. (The PARC 174A polarograph amplifies the readout in the d.p.p. mode by x10 relative to other modes to offset this inconvenience). Rather more importantly, these factors render the d.p.p. wave critically dependent upon the electrode kinetics, a distinct disadvantage not shared by n.p.p. or the d.c. methods. This in turn means that even minor changes in sample matrix which influence the electrochemical reduction/oxidation rate may strongly affect peak height.

As stated above, peak height, i_p, in d.p.p. can never exceed, and is generally less than, wave height, i_l, in n.p.p. However, for a reversible process the two may be simply related by the approximate formula (cathodic waves)

$$\frac{i_p}{i_l} = \frac{\sigma-1}{\sigma+1} \qquad \text{where } \sigma = \exp(-nF\Delta E/2)$$

and where peak potential, $E_p = E_{\frac{1}{2}} - \Delta E/2$

and ΔE is the pulse amplitude, V.

One important consequence of this is that since $i_l \propto C$, so $i_p \propto C$, giving linear calibration plots in d.p.p. Another consequence is that the greater the number of electrons, n, involved in the electrode process, the more closely $(\sigma-1)/(\sigma+1) \rightarrow 1$, especially for smaller pulse amplitudes, and the greater the sensitivity obtained (Figure 8). For small pulse amplitudes, $i_p \propto n^2$.

Resolution

If two electroactive species are present, their respective d.c. polarographic waves may overlap, rendering the measurement of individual wave heights impossible by ordinary methods. Under favourable circumstances a mini-computer can be used to separate the component waves by curve fitting techniques.[73] Usually only one parameter need be measured for a fully resolved wave - the diffusion current, i_d. By measuring the current at many potentials sufficient data may be gathered for the curve fitting operation on unresolved waves. The equations describing each wave must be known (the Nernst equation given above is a thermodynamic description of the reversible electrode process $Ox + ne \rightleftarrows Red$; equations exist covering other thermodynamic or kinetic possibilities) and the two wave heights must be assumed to be additive. Thereafter the data gathered may be substituted into simultaneous equations to give the fraction of the combined diffusion current arising from each component wave. The method may also determine unknown $E_{\frac{1}{2}}$ values. Possibly the greatest limitation here might prove to be the frequency with which polarograms in practice show distortions from the ideal sigmoidal shapes as given by the curve equations.

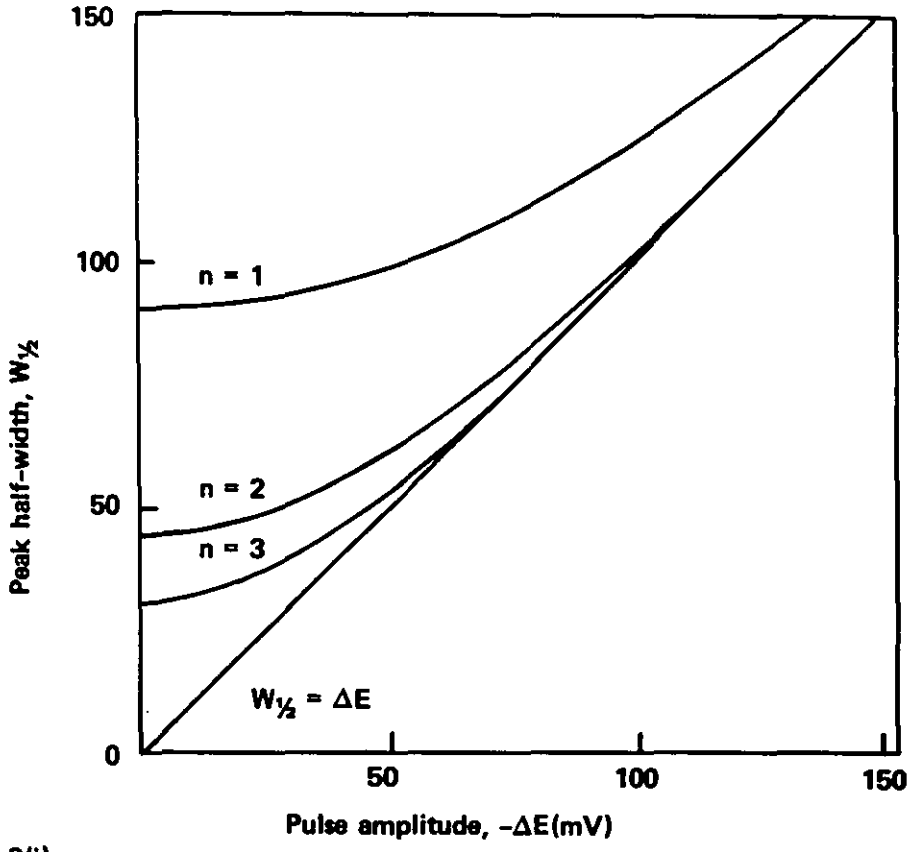


Fig. 8(i)
Variation of peak half width, $W_{½}$, with pulse amplitude, ΔE, for various values of n

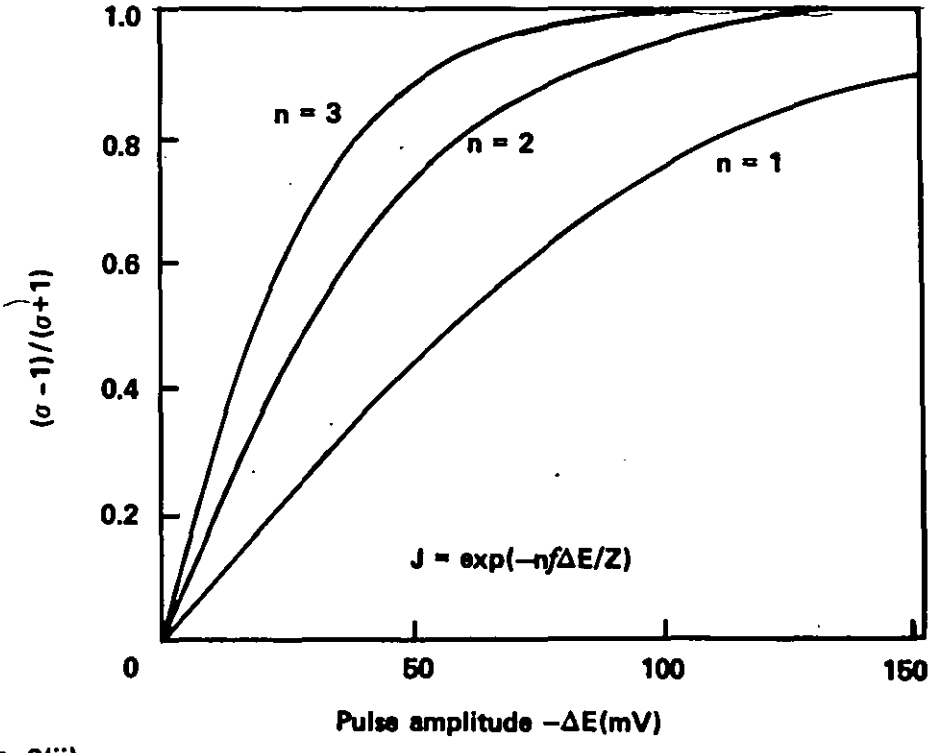


Fig. 8(ii)
Variation of $(\sigma - 1)/(\sigma + 1) = i_{DPP}/i_{NPP}$ with pulse amplitude ΔE, for various values of n.

Another means by which more of the available data can be utilized to provide greater resolution is by the generation of a derivative output (peak shaped).[69g] Derivative d.c. polarography has historically been troubled by difficulties in suppressing the charging current effects. Damping using filters gave rise to severe peak distortion. The application of the method to time-averaged data with the aid of modern parallel-T circuitry, or to current sampled data has proved superior. Unless scan-rate is very slow, peak height is influenced by scan rate and drop time, but this is acceptable when both samples and standards are run under near-identical solution conditions.

In d.p.p. the polarographic method itself generates an output similar to that obtained by taking the derivative of a d.c. signal. Indeed, for both techniques[69g,72] the peak half-width, $\Delta E_{P/2}$ (Figure 8) is about $^{90}/_{n}$ mV for a reversible process (only true for d.p.p. when using small pulse amplitudes). The value of $\Delta E_{P/2}$ increases for d.p.p. with larger pulse sizes until $\Delta E_{P/2}$ approaches the pulse amplitude for all values of n for very large pulses.[72] Some trade off may be required between increased sensitivity and better resolution. Because resolution is little improved below 20 mV pulse amplitude (Figure 8i), while sensitivity is considerably decreased, generally pulse amplitudes of 20-100 mV are chosen taking into account the need for a low limit of detection as described above.

Enhanced Waves

Various mechanisms exist by which the electroactive species may be regenerated after electron transfer.[74] This lessens the concentration gradient across the depletion layer and effectively results in some of the molecules close to the electrode surface being detected (oxidised/reduced) more than once. The measured current is therefore greater than would be so for the diffusion-limited case. The regenerative mechanism may involve catalysis or disproportionation. Regeneration must occur at a rate of the order of, or

faster than, the timescale of the polarographic experiment to give wave height enhancement.[69h] Where the supporting electrolyte exerts a large effect on the rate of regeneration, careful selection of the electrolyte can either maximise the gain in sensitivity from regeneration or suppress the enhancement entirely to avoid the increased likelihood of interference that arises wherever a chemical rate constant is involved.

Irreversible Waves

During the discussion so far it has been pointed out that certain central statements and equations only hold for reversible electrode reactions, i.e. those which conform to a thermodynamic description.[70e] This does not imply that quasi-reversible or irreversible systems are generally unsuitable for analytical application, though due care must be exercised.

Irreversible systems share with reversible ones the fact that electrolysis rate is ultimately controlled by diffusion processes. However, kinetic factors render the rate of the electrode reaction slow relative to the time scale of the polarographic technique employed, such that no wave appears until more negative potentials (for reduction processes) than are expected from purely thermodynamic considerations. A sufficiently irreversible process may give no wave at all over the polarographic range. For a shifted wave, the S-wave rises less steeply, but the wave height is unchanged compared to the reversible case.

Kinetic Waves

Besides electrode kinetics, chemical changes and surface effects may greatly influence the polarographic wave. The wave height may be modified (decreased), as well as the position of the wave altered, as diffusion processes play a secondary role to the rate of the controlling process. In his book[74] (1968), Mairanovski deals specifically with

catalytic and kinetic waves. Although kinetic currents are very widely encountered, for present purposes, only surface phenomena need be considered further.

Adsorption at the Mercury Electrode

A simple description of adsorption at the mercury-solution interface is provided by the Langmuir equation.[74a] This shows that for monomolecular covering by adsorbed species, the fraction of the possible adsorption sites occupied is given by the ratio $\frac{\beta c}{1+\beta c}$, where c is the concentration of the adsorbed compound in the bulk solution, and β is an adsorption coefficient. Langmuir gave the following expression for β : $\beta \propto \delta . e^{\frac{W}{RT}}$

where δ is the thickness of the surface layer (adsorbed),

W is the work required to remove one mole of adsorbed species from the mercury-solution interface.

R is the gas constant per mole, and

T is the absolute temperature.

Langmuir's equation does not account for the effects of interactions between adsorbed molecules such as are known to occur between large, polar organic molecules. Here an enhanced degree of adsorption has been observed, especially at intermediate values of the bulk concentration, c. The enhancement in adsorption decreases with rising temperatures, and various other factors may exert an influence.

For neutral species, adsorption usually is most pronounced at a potential, E_m, near the potential at which the mercury surface holds zero charge. This latter potential is also that at which[71b]

(i) the charging current fluctuations seen on a d.c. polarogram are smallest (cf. Figure 2),

(ii) the surface tension of the mercury is greatest, and

(iii) the natural drop time is longest.

The adsorption maximum may be understood in qualitative terms as follows.[74a] Adsorption of large organic molecules (which have a lower dielectric constant than water) reduces the capacitance of the mercury-solution interface, such that as the potential is set further from E_m, the more favoured the exchange of water for adsorbed organics becomes. This creates a dependence of the work function, W, on electrode potential (taken w.r.t. E_m), and thereby affects the adsorption coefficient, β.

Change in potential also influences β in that attraction of an adsorption site for a molecule varies with potential.

Dipolar (surfactant) molecules affect the potential difference across the mercury-solution interface when adsorbed. Frumkin related this to the adsorption coefficient, β:

$$\beta = \beta^\circ \exp. \{\frac{(C-C')\phi^2}{2RT\Gamma_\infty}\} = \beta^\circ \exp. \{-a\,\phi^2\}$$

where β° is the value of β at E_m;

C and C' are the capacities of the double layer (mercury-solution interface) in the absence of, and with full monolayer coverage of the dme surface, respectively;

ϕ is the electrode potential taking $E_m = 0$; and Γ_∞ is the quantity of adsorbed species for 1 cm^2 completely covered by a monolayer.

The value of C is dependent upon the ionic strength of the supporting electrolyte, whereas C' is virtually independent of this. Therefore, if other effects such as salting out are absent, addition of extra electrolyte

tends to decrease adsorption, more so the further the potential is set from E_m.

The presence of ionic species which adsorb at the dme surface may shift the value of E_m markedly. For anions, a positive shift in potential occurs; for cations it is negative.

A further factor that has influence on the value of E_m, is the structure of the adsorbed molecules, e.g. an aldehyde will give a more negative E_m than will a hydrocarbon, which in turn will have an E_m more negative than a (poly)phenol.

The above discussion has largely assumed that the adsorption equilibrium is already established. The limiting rate step appears to be the diffusion-controlled transport of adsorbing species to the electrode (up to several tens of minutes for equilibrium), rather than adsorption itself (typically $< 10^{-5}$ seconds for organics). The fraction of the equilibrium concentration per unit electrode area that is adsorbed after a particular time, t, will increase,

(i) approximately linearly with $t^{\frac{1}{2}}$ when t is small,

(ii) with the value of β,

(iii) with increasing concentration of the adsorbed compound in the solution, and

(iv) with the magnitude of D/Γ_∞ where D is the diffusion coefficient of the adsorbed molecule.

Polarographic Consequences of Adsorption

Two cases may be distinguished:[69i]

1. The electroactive species or the product of the electrode reaction is adsorbed. Here a second, 'adsorption' wave is generated. The shape of the wave in the

limiting current region may be altered by maxima, minima or other distortions. This first case will not be treated further.

2. A species not directly involved in the electrode reaction is adsorbed and thereby exerts an influence on the electrode reaction. The wave may be shifted, split or otherwise affected.

In the following discussion of this second case, the adsorbed species will be referred to as 'the surfactant'.

The original and best established use for sufactants in conventional dc polarography is in the elimination of maxima. Maxima arise when there is tangential motion of the mercury-solution interface, caused either by a gradient of interfacial tension (maxima of the 1st kind) or by the movement of mercury out of the capillary (2nd kind).[75] Adsorbability is the key factor in determining the efficacy of a surfactant that suppresses maxima of the 1st kind. It was noted inthe foregoing discussion that a given compound is only significantly adsorbed over a limited range of applied potential, and so particular maxima suppressors operate between fixed potential limits, e.g. gelatin will suppress the maximum of the Fe(III) reduction wave in 0.25 M H_2SO_4 at 0.2 V, but proves inactive for that of the reduction wave of Mn(III) in pyrophosphate at 0.3 V for which peptone may be used instead.[70f]

There are other effects which surfactants can produce, and these are relevant here. The adsorption of the surfactant may decrease the rate of electron-transfer causing a decline in peak current (in d.p.p.), a shift in the peak position to a more negative value (i.e. for reduction waves) and often a loss of rectilinearity to the calibration curve.[76] For this common result, the main benefit that may arise is that differential shifts may increase the resolution between two peaks.[77] Occasionally, the action of the surfactant is different, and is not always easily explained. Small shifts

to less cathodic potentials have been observed.[78] A single compound that gives rise to two unresolved polarographic peaks may have one peak selectively moved so as to merge with the first peak giving a better shaped single peak. There is, e.g., evidence that ion-pairing with a reduction intermediate may account for the effects of dodecyltrimethylammonium chloride on the polarographic behaviour of aromatic nitro compounds.[79] Most interestingly, whereas cationic surfactants have been shown to increase the peak height of an analyte by increasing the reversibility of the electrode process, at least one case is known where a non-ionic surfactant (Triton X-100) increases the peak height of an analyte (benzylpenicellenic acid) while simultaneously decreasing the electrode reversibility (as shown by an increase in peak half-width).[79] Furthermore, a possibly unique effect is found in that the ratio i_p/C increases with concentration in the absence of Triton X-100, but remains either constant or decreases depending on the concentration range with increased concentration. No explanation has so far been provided.

CHAPTER 4

Modern Column Liquid Chromatography

High performance liquid chromatography (hplc) is a collective term covering a range of separation techniques in the fields of Analytical and Preparative Chemistry. These techniques are grouped together largely on the basis of the small particle size and compactness of the column packings used, as it is these factors that necessitate elevated pressures to create an adequate flow of liquid mobile phase, and so determine the major design features of the equipment employed. More detailed knowledge quickly reveals that, at the molecular level, the individual methods clearly depend upon different mechanisms to achieve their varied separations. Table 3 differentiates the commoner modes of hplc and cites typical solutes to which the method might be applied to effect separations. The following discussion will focus largely upon the reverse-phase mode of hplc.

Components of the Chromatograph

In general a high performance liquid chromatograph comprises

(a) a high pressure pump feeding eluent from a reservoir to an injector head at constant pressure or flow rate,

(b) an injector/column assembly,

(c) a detector/recorder system,[80] and

(d) connective tubing.

Frequently other components are included, e.g. pre-column, reactor, peak integrator, thermostat, etc.

The variety to be found in commercial instrumentation in these

TABLE 3

HPLC Mode	Examples of Supports (S) and Stationary Phases(SP)	Examples of Mobile Phases and Ion-Pair Reagents (IP)
Adsorption	Silica or Alumina (S/SP) (often with trace of water as adsorption deactivator)	Hexane, halocarbons (often saturated with water)
Liquid-Liquid Partition	Silica (S) Polyethyleneglycol (SP)	Hexane (pre-column for saturation with SP)
Reversed Phase	Alkylsilyl-bonded silica	Methanol/water Acetonitrile/water
Ion-exchange	$-(CH_2)_nNR_3^+ X^-$ or $- C_6H_4-SO_3^- H^+$ bonded silica	Buffer (esp. pH 2-8)
Ion-Pair Partition	(i) Silica (S) + $HClO_4$ (SP) or NH_4OH (SP); or (ii) Alkylsilyl bonded SiO_2	(i) Butanol/chloroform; or (ii) Methanol/Water containing cetrimide, or acid or base
Size Exclusion	Controlled pore size glass or silica, optionally silanized to prevent adsorption	Toluene Buffer (not high pH)
Affinity	Specific binding ligand e.g. antibody, bonded to silica support	Buffer to prevent denaturing of biological samples (not high pH)

TABLE 3

HPLC Mode	Examples of Solutes Separated	Notes
Adsorption	Amines (on alumina S/SP) Phenols	Reproducibility often poor because of difficulties with trace water
Liquid-Liquid Partition	Ethylene oxide oligomers	Thermostatting essential. Least used hplc mode
Reversed Phase	Lipids, steroids	Most versatile mode of hplc
Ion-Exchange	Amino acids Nucleic acids	Often low efficiency of separation, though good optimised systems exist
Ion-Pair Partition	As for ion-exchange	Can sometimes choose counter ion that enhances detectability
Size exclusion	Polymers Large organic molecules	Often useful for initial clean up of biological samples
Affinity	Biological Materials, e.g. enzymes, viruses	Can show highest specificity of any mode

components is considerable.[81a] Also, hplc is often performed using equipment partly constructed or at least modified in laboratory workshops. The particular apparatus used in the present work is described in the experimental section.

THEORY OF CHROMATOGRAPHY

Introduction

Despite the differences in mechanism between the modes of modern liquid chromatography, the results obtained, viz., the chromatograms, may be assessed w.r.t. many parameters, e.g. efficiency of separation, resolution etc., that are largely applicable to all the modes. Further, some features such as band spreading have certain of their causes common to all chromatographic modes, though details will differ, e.g. band spreading due to dead space is a qualitatively common feature, but may differ slightly in degree depending on the diffusivity of a particular solute in a given eluent. However, only an insight into specific chromatographic mechanisms can help to explain many important factors, e.g. peak shape, dependence of peak retention on possible variables, etc., and so provide a measure of prediction suggesting ways to obtain desired separations and to improve performance.

To summarize, there are features in the equipment, in the assessment methods, and in part of the theory of the physical processes concerned that are common to all the chromatographic modes described in Table 3. This allows use of similar equipment and technique, cutting costs and making the chromatographer able to adapt his expertise relatively quickly to different modes.

But it is in the differences in chromatographic mechanism

that hplc provides its breadth of applicability,[82] allowing separations which previously could only be done with great difficulty, or not at all, to be performed rapidly and simply.[83a]

Resolution and Retention

An idealised chromatogram is given in Figure 9 labelled with the most basic parameters required in chromatogram assessment. The retention time of a peak, A, is the period elapsing between injection and detection and may be expressed as a time, t_A, (e.g. in minutes) or as a volume of eluent required to effect the elution, V_A, (e.g. in mls.) These are related[81b]: $V_A = f_V\, t_A$ where f_V is the volumetric flow rate (mls/min). It is convenient to define a quantity, K'_A, the phase capacity ratio of A: $K'_A = \frac{t_A - t_o}{t_o}$ (i) where t_o is the retention time of an unretained species, and usually measurable from a chromatogram from the refractive index peak of the sample solvent where this is not too dissimilar from the mobile phase (Figure 9).

The peak width, W_A, is measured between the intercepts on the baseline of tangents to the points of inflection of the Gaussian shaped peak.[84a] This width increases with the square root of the distance moved. By adaptation of an early theory concerned with the efficiency of a distillation column,[85] the band dispersion factor may be defined (called the height equivalent of a theoretical plate, H):[86] $H = \frac{L}{16}\left(\frac{W_A}{t_A}\right)^2$ ...(ii)

where L is the column length. The factor $16\left(\frac{t_A}{W_A}\right)^2$ is termed the number of theoretical plates, N, and is the standard measure of column efficiency. The resolution, R_S, of two adjacent peaks, B and C (Figure 9) increases as a function of N, and is a dimensionless quantity.

Basic chromatographic parameters. (Standard spectroscopic detection)

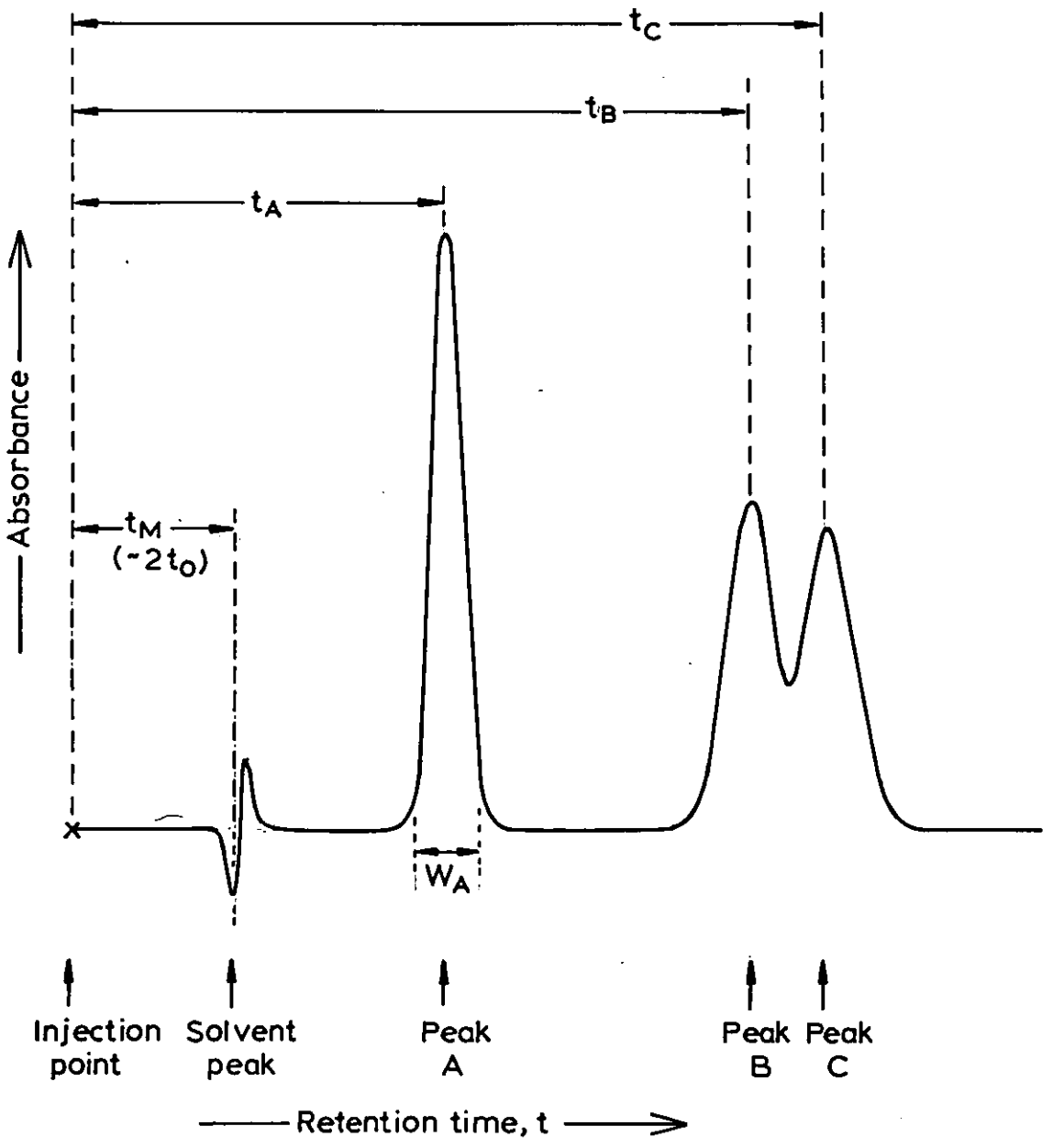


Fig. 9

The definition of R_s[83b] is: $R_s = \frac{t_C - t_B}{\frac{1}{2}(W_B + W_C)}$ ... (iii)

Now, $t_C = t_o(1 + K'_C)$ by rearrangement of equation (i).

So, $R_s = \frac{t_o(K'_C - K'_B)}{\frac{1}{2}(W_B + W_C)}$ ... (iv)

N is the same for both peaks B and C, and

$N = 16\left(\frac{t_C}{W_C}\right)^2$ or $\sqrt{N} = \frac{4t_o(1 + K'_C)}{W_C}$ ... (v)

Substitution of equation (v) in equation (iv) gives

$$R_s = \frac{(K'_C - K'_B).\sqrt{N}}{4\left(1 + \frac{K'_B + K'_C}{2}\right)} \quad \text{... (vi)}$$

Let $\frac{1}{2}(K'_B + K'_C) = \overline{K}'$ and define a separation factor, $\alpha = \frac{K'_C}{K'_B}$

Then $R_s = \frac{K'_C - K'_B}{(1 + \overline{K}')} \cdot \frac{\sqrt{N}}{4} = K'_C \frac{(\alpha - 1)}{(1 + \overline{K}')} \frac{\sqrt{N}}{4}$ ... (vii)

or since $K'_C \equiv \frac{2\overline{K}'}{\alpha+1}$ we have the final form that

$$R_s = \frac{1}{2} \frac{\alpha-1}{\alpha+1} \cdot \frac{\overline{K}'}{1+\overline{K}'} \cdot \sqrt{N} \quad \text{... (viii)}$$

Some authors make the reasonable simplification that $\alpha+1 \cong 2$.[83b,84b] A value for $R_s > 0.8$ is often taken as showing adequate resolution for individual quantitation of peaks B and C.

Equation (viii) is of practical importance to the chromatographer. The three factors, $\frac{\alpha-1}{\alpha+1}$, $\frac{\overline{K}'}{1+\overline{K}'}$ and $\sqrt{N}$ are largely determined by different chromatographic variables, so each may be optimised separately.[83b] For instance, the value of α may be varied by changing the composition of the

mobile or stationary phases. The value of $\overline{K}'$ may be changed by choosing a mobile phase of different solvent strength (discussed later), while the value of N depends on L (equations (ii) and (iii)) and upon H-- itself dependent on the quality of the column packing and its nature, and on other factors such as the linear flow velocity of the eluent, u, where

$$u = \frac{f_v}{a_m} \quad . \ . \ . \ (ix)$$

and a_m is the cross-sectional area of the eluent within the column.

The Chromatographic Process and On-Column Band Spreading

Consider the hypothetical model shown in Figure 10. Solvent X is held in a series of adjacent cells, while solvent Y, which is immiscible with X, moves without disturbance over the surface. Each cell containing Y can only communicate with its neighbours via solvent X. A solute is introduced at the inlet, and distributes between the two phases according to its differing affinity for each. Given that dynamic equilibrium is established at a rate that is fairly rapid w.r.t. the motion of the upper solvent past the lower, random diffusion processes will create a Gaussian density distribution across the solute band along the axis of travel ("longitudinal") i.e. a Gaussian shaped peak will be detected at the column end. In effect a series of sequential extractions has been performed in a neat and continuous manner. If two solutes are present with differing distribution coefficients between solvents X and Y, then, given a large enough number of cells, their respective bands will be separated.

Quite apart from the difficulty of physically stabilizing the system in this form, the separating power provided would, in general, be quite dismal, and a very high sample dilution would occur. This may be shown by comparing two of the band broadening processes operating:

Factors influencing chromatographic separation

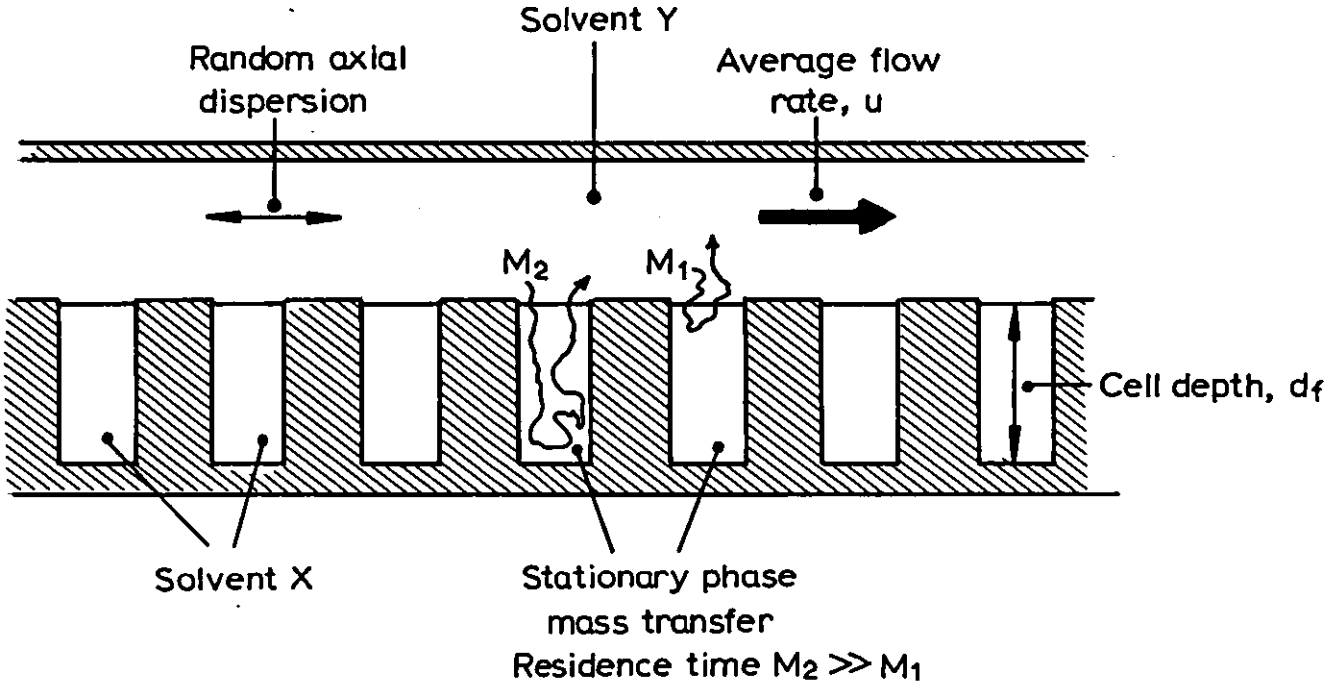


Fig. 10

(1) Longitudinal Diffusion[β3C] a solute will randomly diffuse in all directions at a rate $\propto D_m$, its diffusion coefficient in the mobile phase. The broadening increases with time, so the faster the transit from column inlet to the detector the better.

Broadening = $C_d \cdot {}^{Dm}/u$ where u is the linear flow rate of the mobile phase (X), and C_d is the coefficient of proportionality;

(2) Stationary-Phase Mass Transfer: a solute molecule that enters solvent Y to a small depth will on average return sooner to the mobile phase than another molecule that has gone to greater depth. Hence the two solute molecules will be separated and band broadening occurs. The smaller the cell depth, d_f, the less that scope for such broadening exists.

Broadening = $C_s \frac{d_f^2 u}{D_s}$ where D_s is the diffusion coefficient of the solute in the stationery phase (Y), and C_s is the coefficient of proportionality.

Comparison of the two broadening effects shows that one effect is $\propto 1/u$ while the other is $\propto u$. The values of D_m and D_s are constant irrespective of the dimensions of the apparatus, while the value of d_f would be very large for the macroscopic system shown. This reflects the fact that the distances travelled by solute molecules is so large relative to their inherent diffusion rates (determined by D_m and D_s), and that effective equilibriation is impossible between individual cells and the mobile phase unless the value of u is very low indeed - in which case band broadening by longitudinal diffusion becomes very great.

Qualitatively, equilibriation can be made more rapid by increasing the interfacial area between the two phases X and Y, and by decreasing the volume of individual cells and of the mobile phase adjacent to the cells. These considerations coupled with the practical requirement that the system should be physically stable are important factors in determining the general form of the packed column in liquid chromatography.

In column liquid chromatography, the cells in the above hypothetical model are embodied by particles of, for example, silica that are closely packed in a tube. In the classical form, mobile phase passes through the column by the action of gravity, while in modern chromatography some form of pump is required. The mechanism of interaction need not be liquid-liquid partition, and is the single most characteristic feature by which the different hplc modes are distinguished (see Table 3).[81b] The particles of packing material are general porous, increasing their surface area very greatly, but creating other (lesser) problems in communication between stationary and mobile phases.

There are now five main on-column band-broadening processes, as listed with their effect on plate height in Table 4.[83c,83d] Here longitudinal diffusion and stationary-phase mass transfer are essentially as for the macroscopic case, except that C_s and C_d are much smaller, and that the factor that determines residence time in the stationary phase may not simply be diffusion as in the liquid-liquid partition case.

The nature of the packed column itself introduces new band-broadening terms in Table 4. Eddy diffusion is a consequence of no longer having one channel through which mobile phase moves, but many thousands. Some flowpaths will be wider than others, and here flowrate

will be higher. Solute molecules in these flowpaths will advance further than their counterparts in narrower paths. Mobile phase mass transfer is the broadening due to differential flowrates between the centre of a given flowpath and its edges. Stagnant mobile phase mass transfer arises because mobile phase can enter the pores of the packing particles (and, indeed, must do so as the stationary phase will be contained, in general, within these pores). A solute particle that migrates deeper into the pores through this stagnant mobile phase will take longer on average to rejoin the mobile phase moving past the packing particles than one that penetrates only to a shallower depth.

One further, but largely avoidable, factor in on-column band spreading is that flow within about 30 particle diameters from the column wall will generally be faster than elsewhere because the packing is less ordered and looser.[81c] Solute particles reaching this wall region will contribute to band spread by their differential flow rate. For this reason central injection of samples is adopted, and minimum diameter to length ratios are observed when designing columns, to ensure elution before a significant percentage of solute can spread laterally into the wall region ("infinite diameter effect").

TABLE 4 [83d]

Band-broadening process	Contribution to plate height, H.
(i) Eddy diffusion	$C_e\, d_p$ } $= Au^{0.33}$
(ii) Mobile-phase mass transfer	$C_m\, d_p^2 .\ u/D_m$ }
(iii) Longitudinal diffusion	$C_d .\ D_m/u$ $= B/u$
(iv) Stagnant mobile-phase mass transfer	$C_{sm}\, d_p^2 .\ u/D_m$ $= Cu$
(v) Stationary-phase mass transfer	$C_s\, d_f^2 .\ u/D_s$ $= Du$

C_e, C_m, C_d, C_{sm}, and C_s are plate height coefficients.

d_p = diameter of packing particle

d_f = thickness of stationary-phase layer.

In Table 4 it may be seen that certain variables, e.g. d_p, are incorporated into the constants A, B, C, D. These are accordingly only valid as constants when considering the effect of the single variable, u, for an individual packed column. A simplified equation for plate height, H, is obtained:[83d]

$$H = Au^{0.33} + B/u + Cu + Du$$

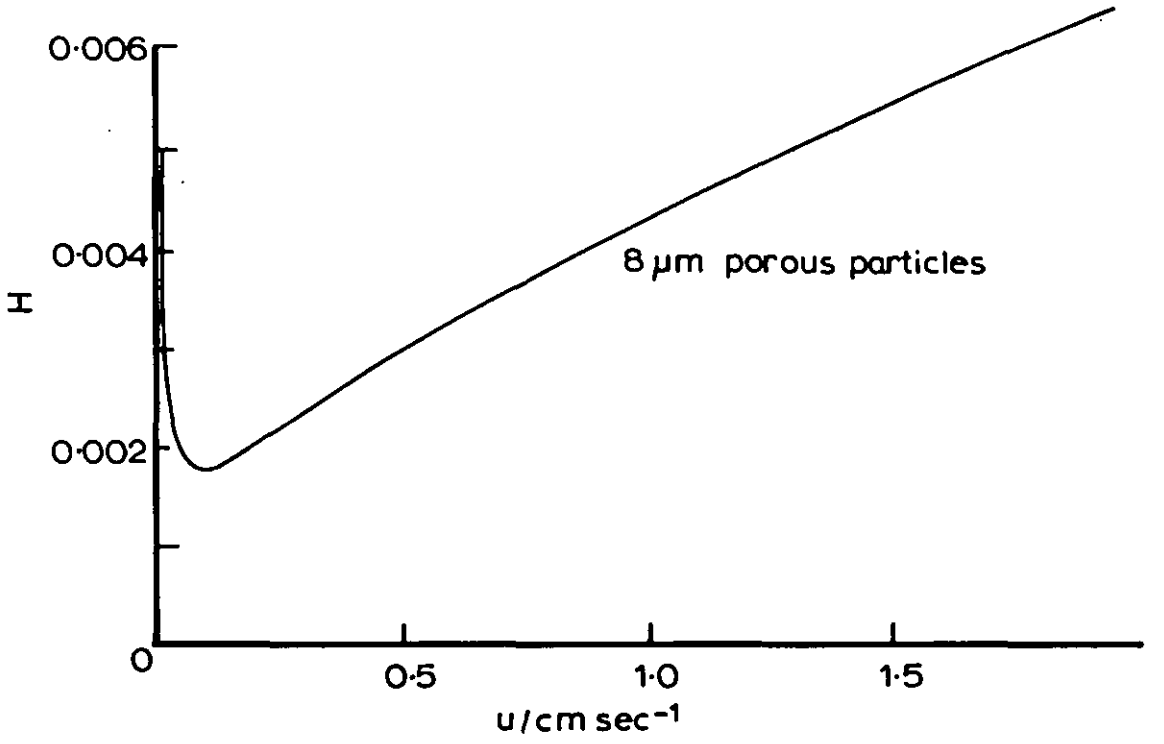
For modern, fully porous microparticulate packings (3-10 μm diameter) the stationary phase may comprise interactive sites at the pore surface (ion-exchange; adsorption; affinity) or else a monomolecular bonded layer (reverse-phase). In either case $d_f \rightarrow 0$ and so $D \rightarrow 0$. The equation is simplified:

$$H = Au^{0.33} + B/u + Cu$$

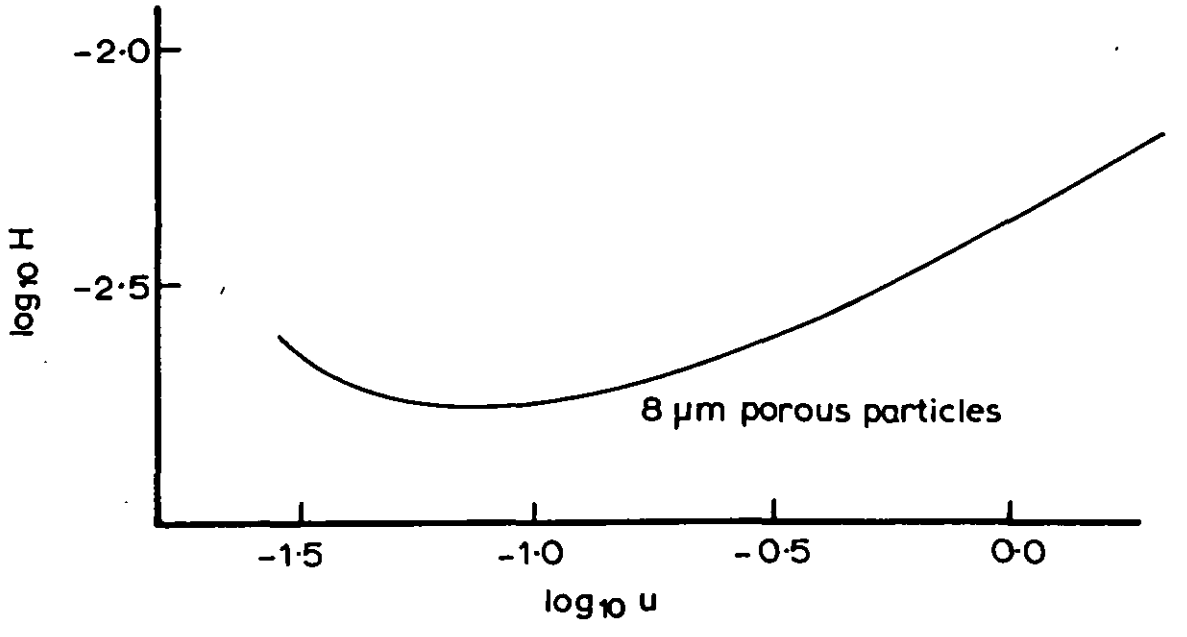
Figure 11 gives a plot showing the effect on column efficiency of variation of flow rate, u. It is apparent that an efficiency maximum (minimum in H) occurs. At flow rates below that for maximum efficiency, the term B/u dominates, while at flow rates above this the Cu factor becomes most important (i.e. slow equilibriation w.r.t. speed of mobile phase). At the point of maximum efficiency, the term $Au^{0.33}$ is at its greatest relative importance, and here the quality of the packing controls band spread.

In practice, the need for analyses to be performed quickly means that most chromatography involves sacrifice of a little efficiency in order to increase u above that giving minimum H. Microparticles minimize this loss.[84c]

Effect of flow rate, u, on column efficiency



(a) Flow rate vs. plate height



(b) $\log_{10}$ (flow rate) vs. $\log_{10}$ (plate height)

Fig. 11

Part and parcel of these ideas connecting column efficiency, analysis time, particle size and goodness of packing, are the effects of column pressure drop, Δp.[83b,81b]

$$\Delta p = \frac{\phi' \eta}{t_m} \left(\frac{L}{d_p} \right)^2$$

where ϕ' is a column resistance parameter (dimensionless),

t_m is the elution time of the eluent. The eluent in the column comprises the mobile phase plus the stagnant mobile phase within the porous packing particles, and

η is the eluent viscosity.

The value of ϕ' is ca. 1000 for irregular-porous microparticulate packings and ca. 500 for spherical-porous ones, making some chromatographers prefer the latter. This equation may be written in terms of t_o, the elution time of a solute confined to the mobile zone, rather than t_m, in which case t_o = 0.45 to 0.55 t_m. (Large polymers may elute at t_o preceding the solvent peak at t_m.)

Substitution of typical values ($\eta = 10^{-3}$ N s m^{-2}; L = 0.1 m; $d_p = 5 \times 10^{-6}$ m; t_m = 100 s) for a spherical-porous microparticulate column (ϕ' = 500) gives:

$$\Delta p = \frac{500 \times 10^{-3}}{10^2} \cdot \left(\frac{0.1}{5 \times 10^{-6}} \right)^2 = 2 \times 10^6 \text{ N m}^{-2}$$

which is equivalent to 290 psi.

Because $t_m = \frac{L}{u}$, in this example $u = 10^{-3}$ m s^{-1}. This does not mean, in practice, that application of 290 psi will give a linear flow rate of this value.[83b] During operation, filters (pump; column top; column bottom) may progressively gather debris increasing their flow resistance, while narrow bore tubing and other components will generate their own, relatively constant, contribution to the overall pressure

requirement. A poorly packed column may give an increased value for ϕ' and hence a higher pressure drop, Δp.

This completes the discussion of the circle of dependent quantities outlined at the start of this section on modern column liquid chromatography: the discussion has shown how high efficiency columns (H small) require microparticulate packings, and that this means operation at high pressure.

Total Band Spread

There are close physical and theoretical relationships[83b,86] between the statistical measure of dispersion, viz., standard deviation, and the chromatographic measure of dispersion (band spread), plate number, N (see Figure 12). When a chromatographic system is analysed for the component contributions to band spread, the form of the equation used is identical to that for the summation of the standard deviations of the variables in an overall process, viz.,

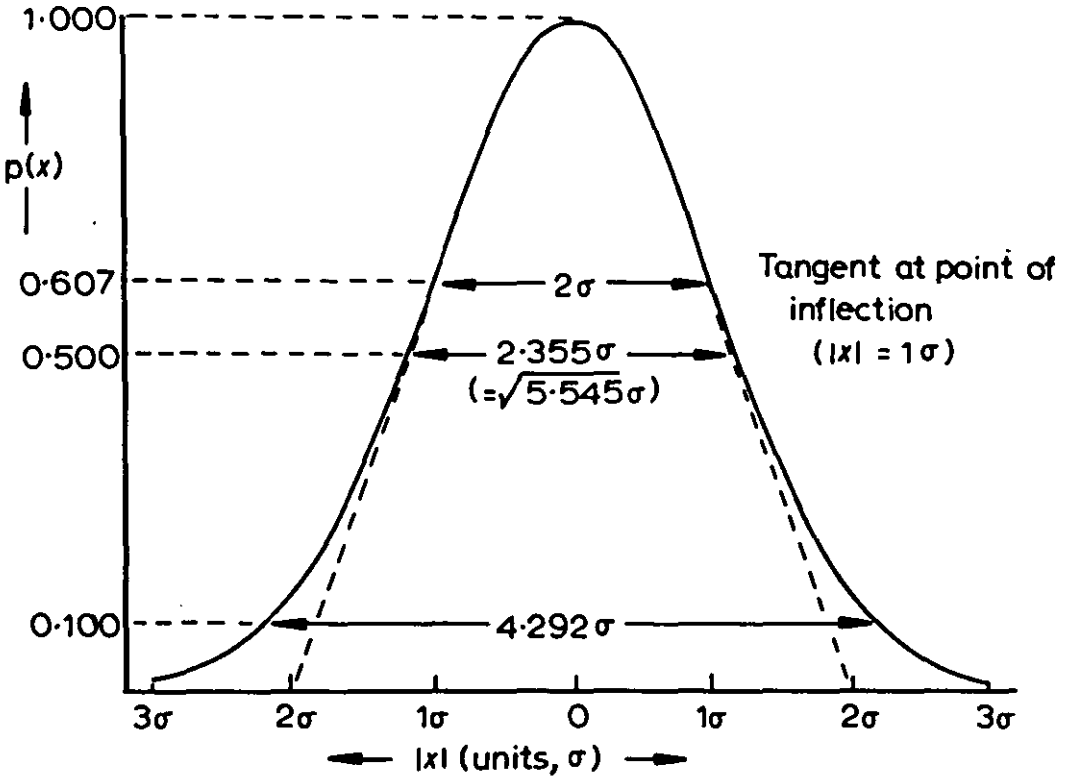
$$W^2_{total} = W^2_{inj} + W^2_{con} + W^2_{col} + W^2_{det} \quad [81c]$$

where W is the peak width due to the factor denoted by individual subscripts : inj = injection; con = connections; col = column; and det = detector. Discussion in the previous sections has centred on W_{col} because of its great importance. With good apparatus design it is possible to limit the peak width due to extra-column effects, W_{ec} ($W^2_{ec} = W^2_{inj} + W^2_{con} + W^2_{det}$) to about half that due to on-column effects, i.e., $W_{ec} = \frac{W_{col}}{2}$. In this case,

$$W^2_{total} = W^2_{ec} + W^2_{col} = 0.25\ W^2_{col} + W^2_{col}$$

$$W_{total} = \sqrt{1.25}\ W_{col}, \text{ and so } W_{total} = 1.12\ W_{col}.$$

Hence, only 12% extra total peak width is generated when extra-column peak



Gaussian Peak Shape

Shown as

$$p(x) = \exp\left\{-\frac{x^2}{2\sigma^2}\right\}$$

$$\int_{-\infty}^{\infty} p(x)dx = \sigma\sqrt{2\pi}$$

[To normalise

$$p(x) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left\{-\frac{x^2}{2\sigma^2}\right\}$$

]

Fig. 12

width, W_{ec}, is maintained at $\frac{1}{2} W_{col}$.

Retention Mechanisms

It is not feasible to design a stationary phase/support material which interacts in a single manner with all possible solutes. For instance, any hplc column packed with micro-porous silica (however modified) may act as a makeshift size exclusion column for polymers. A silica column as used for adsorption chromatography may show sufficient ion-exchange behaviour to base some separations on that principle.[87] And a reversed phase column may retain some adsorptive power due to incomplete coverage with bonded lipophilic groups.[88]

The operation, in this way, of more than one retention mechanism need not necessarily produce poor chromatographic results. However, band tailing may occur where there are only a few interaction sites available for one of the mechanisms (e.g. residual adsorption sites in reversed phase chromatography).[83e] These sites may be quickly overloaded so that the bulk of solute molecules are unretained by the given mechanism, and band tailing is caused.

Retention Mechanism in Reversed Phase Chromatography

It is a useful preliminary to a discussion of reversed phase ion-pair chromatography (RP-IPC) to first consider the basis for retention in the simple reversed phase mode. The most direct reason for this is that the stationary phase in RP-IPC, viz. the ion-pair reagent, must be retained by reversible interaction with the column similar to (but with essential differences from) that of a solute in reversed phase chromatography. More generally, similarities exist between the two modes that are most logically discussed first for the simple reversed phase case.

A range of mechanisms has been advanced including,

(a) the bonded organic molecules at the surface of the porous silica provide adsorption sites for lipophilic solutes;[89]

(b) the bonded organic molecules, together with associated molecules of eluent (or the more lipophilic component for mixed eluents), act as a stationary 'liquid' phase;[90] and

(c) where dimethylalkyl substituents are present in the bonded molecules, these may act as do liquid crystals in forming an ordered liquid phase.[91]

Mechanism (a) is analogous to adsorption chromatography, and was the earliest theory, while mechanism (b) is analogous to liquid-liquid partition chromatography, the hplc mode closest to the simple case of liquid-liquid extraction, and mechanism (c) is, of course, an obvious analogy to another much studied field.

One might wonder why such approaches are taken rather than a more direct assessment, given that the 'true mechanism' must present certain features that differentiate it from each of the above analogues. To be wholly satisfactory retention mechanisms should take in all the parameters necessary for a complete thermodynamic description of the chromatographic process. As this is scarcely feasible, mechanisms are simplified by omitting processes whose free energy changes are insubstantial relative to other processes; these latter then provide the framework for the mechanism. For instance, Snyder's simplified equations provide a useful model in the case of adsorption chromatography;[81d] here the solution energies of both solute and eluent molecules are ignored because they are several orders of magnitude less than the adsorption energies concerned. This approximation is, unfortunately, no longer valid in reversed phase operation where eluents are generally highly polar. A most useful outcome from a successful

simplified mechanism is its ability to predict eluent compositions with increased separating power for given solutes. Prediction of solute retention is also sought.

Aspects of some Postulated Retention Mechanisms

1. Locke (1974):[89] It is not valid to regard an adsorbed monolayer as a bulk phase displaying normal thermodynamic properties - even multilayers would be of too small dimension to permit this. Despite this acknowledged fact, Locke used an adsorption model as no better method seemed available. He stressed in this model that:

   (a) for similar solutes (homologues) elution order must be the inverse of their solubilities in the mobile phase [the reverse sequence to that expected in straight phase adsorption with active adsorption sites, or in liquid-liquid partition];

   (b) the nature of the organic bonded group determines solute functional group selectivity.

   Locke further envisaged that prediction of relative retention for members of those homologous series within the scope of his treatment (not, for example, ones where acidity shows substantial variation through the series) would prove possible, and that the dependence of retention on solute solubility in the mobile phase could be used indirectly to determine solubilities of sparingly soluble homologues given that of one member of the series.

Knox and Pryde (1975):[90] The switch from active adsorption sites to a column packing with a lipophilic monolayer coating is especially effective in improving peak shape for complex, polar solutes. This is possibly explained by the absence of the 'template effect' in the latter case.

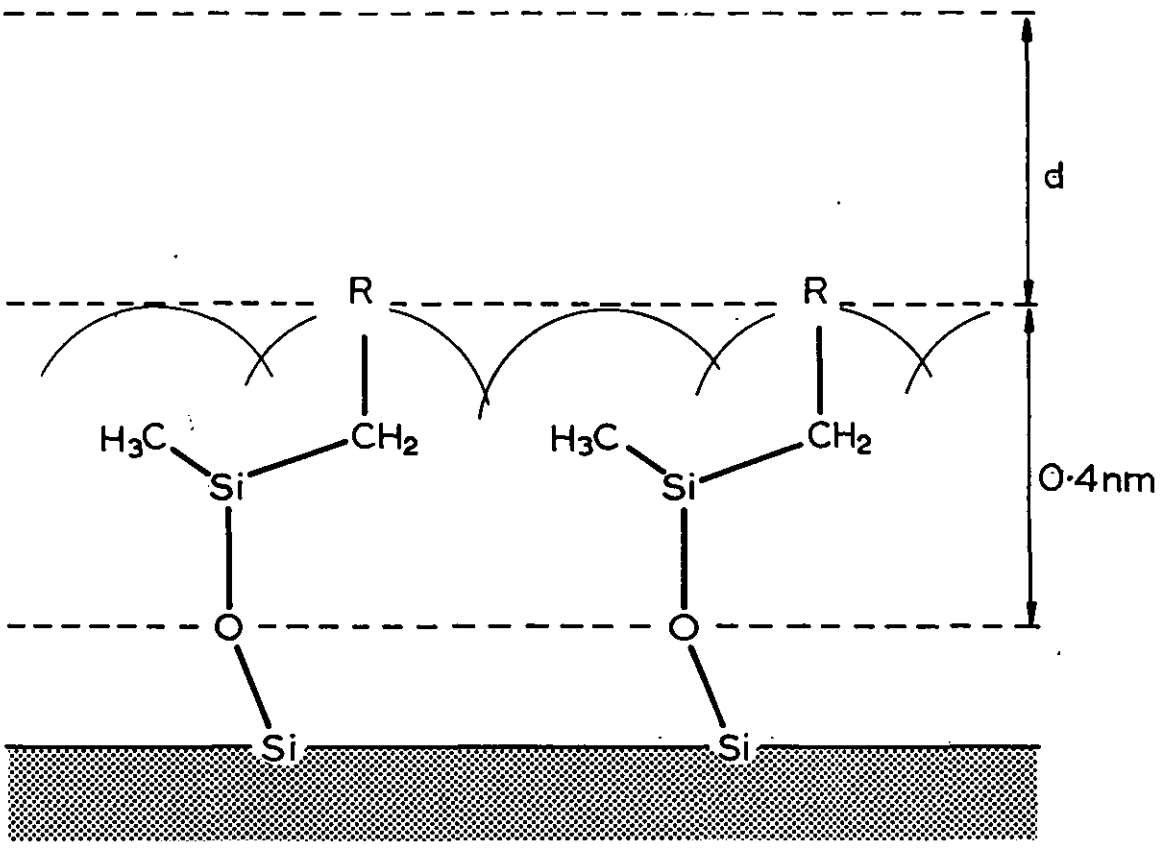
For an ordinary adsorbent which has been deactivated, e.g. by trace water in the mobile phase hydrogen-bonding to the more active sites, certain configurations of adsorption sites ('templates') favour maximum interaction with all the sterically available polar groups in the molecule, while others interact with only one such group. A form of mixed retention mechanism then operates because of the differential adsorption energies involved. For reversed phase operation the polar phase is a liquid, and no such permanent configurations can exist.

The mechanism that is closest to that which many chromatographers now favour as a general model is that provided by Knox and Pryde (1975). For many species they noted the elution order as corresponding to the inverse order to polarity, and in particular that short-alkylsilyl- (SAS-) silica seemed to act as a preferential absorbent for the less polar component(s) of the eluent, which in turn comprised a stationary liquid phase for the selective partition of solutes. Among the advantages over conventional liquid-liquid chromatography were greater physical stability and chromatographic versatility. This also was the same original theory accounting for reversed phase ion-pair separations (tetracyclines eluted by aqueous perchloric acid containing acetonitrile as the organic modifier, with SAS-silica packing).

In the same paper these authors referred to the difficulty of explaining the elution order of some benzodiazepines on octadecylsilyl- (ODS-)silica - i.e. elution order was not a simple inverse function of polarity. More recent papers have separated the concepts of hydrophobicity or, better, lipophilicity, from that of polarity, improving the accuracy of the retention mechanism but losing something of its simplicity.

Molnar and Horvath (1977):[92] Ionisation of a molecule in reversed phase by adjustment of pH decreases retention because of increased polar interaction with the mobile phase. Unbuffered eluents may provide conditions where equilibria exist between ionogenic groups and their ionised forms, in which cases the mixed retention will cause serious deterioration in peak symmetry. Molnar and Horvath looked at complex molecules (peptides) and found the analogous result that introduction of an extra polar amino acid residue decreased retention, while a hydrophobic amino acid residue increased it.

Kovats et.al. (1978):[91] The surface of acid leached glass capillaries was used as a model for that of fume silica in order to test the nature of the surface after successive reaction with alkyldimethylsilanols. The analogy between these surfaces was born out by comparing the surface density of bonded groups and their homogeneity on each surface after repeated silylation. By using capillary surfaces the specific free energy of immersion, ΔF_i^*, of the surface after saturation in the vapour of the wetting agent used was able to be estimated from the contact angle, θ, which in turn was derived from the capillary rise of the wetting agent as measured by a cathetometer. $\Delta F_i^* = -\gamma \cos\theta$ where γ is the surface tension of the liquid. The structure of the bonded surface was investigated. Closest to the surface of the silica dipolar forces exist. For trimethylsilyl-bonded surfaces, wetting angles were poorly reproducible and the surface was considered to be 'rough' on the molecular scale. For ethyl - and higher n - alkylsilyls, reproducibility improved. The density of silylation was found to be important in limiting dipolar forces. Above the silica, the dense dimethylsilyl layer exists, while above both the n-alkyl groups form a deep sparse layer (see Figure 13). It is with this upper layer that lipophilic liquids may associate. For the longer chain (C_{14}-C_{22}) alkyl-



(A second methyl group is eclipsed by each methyl shown)

Schematic diagram of n-alkyldimethylsilyl bonded silica surface

$d_{max} = 0{\cdot}126 \times (z - 1)$ nm

$d_{min} \simeq 0{\cdot}5\ d_{max}$

where z is the carbon number (e.g. 1 - 22) such that the n-alkyl group R is C_zH_{2z+1}

Formulae assume tetrahedral carbon bond angles, and that the C–C bond length is 0·154 nm

Fig. 13

silyls, a plot of cos θ vs. temperature showed a region where cos θ (and hence surface free energy) changed rapidly with temperature. This was interpreted as showing that the n-alkanes used as wetting agents were associating with the alkyl-silyl groups, forming mixed crystal-like surfaces. Below the 'transition temperatures' the surface was less wettable than above. Lowest transition temperatures were found where an n-alkane was 2 methylene groups shorter than the bonded long chain alkyl (and less). Values for transition temperatures from 10-60°C for C_{14}-C_{22} were found, and above these temperatures the surfaces behaved as though swollen with wetting agent.

It is useful to note from this study (among others) that

(a) differences in retention mechanism may occur according to the temperature of operation; and

(b) short chain alkyl bonded phases may interact with eluent and solutes rather differently from long chain alkyl, both because of the greater lipophilicity of the latter, and because of reduced shielding to the dipolar forces on the underlying silica surface.

Because most of the evidence collected by Kovats et.al. involved use of n-alkanes as wetting agents, care must be exercised in extending aspects of their findings to describe the retention mechanism in reversed phase chromatography (where it is usual to employ polar eluents and where polar solutes may also be used. Nonetheless, the strong evidence for surface association with lipophilic molecules is of interest in explaining aspects of reversed phase ion-pair chromatography.

Effects of Temperature on Peak Retention and Resolution in Reversed Phase Hplc.

In the past two or three years emphasis has been placed on column

temperature as a means of generating a small but significant improvement in efficiency.[93] Often, temperatures around 60° are quoted, but any increase above ambient will result in some benefit owing to[81b],

(a) ease of thermostatting to keep K' values constant,

(b) improved equilibriation rate between the phases - peak shape may become more symmetrical, and

(c) reduction in eluent viscosity resulting in shorter analysis time or else permitting decrease in mobile phase strength to give improved separation in the same analysis time.[94] Viscosity reduction is also the basis of (b), because of concomitant increase in mass transfer in both 'stagnant' and 'moving' mobile phases.

Retention in Reversed Phase Ion-Pair Chromatography

It may have become clear from previous sections that differences between ion-pair and other reversed phase separations are less pronounced than one might otherwise suppose.[90] Except where dispersive forces (entropy of mixing) alone account for the degree of solubility of a solute in a given liquid, then solvation produces some reduction in free energy, so stabilizing the solute. Therefore, any polar species, ionic or not, will attract the company of polar eluent molecules in loose association, though obviously the ionic species will do so more strongly. Likewise, if a component is added to the eluent that shows a tendency to ionize, the ions formed may reversibly bind not only oppositely charged solute ions, but also, to some extent, polar sites on solute molecules. [Many forms of reversible complexation may be made the principle of a chromatographic separation (e.g. 95)]. More specifically, it is possible to envisage, for example, a cationic detergent such as cetrimide (n-hexadecyltrimethylammonium bromide), reversibly associating with the bonded organic surface of the silica support by means of its long chain alkyl

segment such that its quaternary ammonium function protrudes into the partly aqueous eluent ready to 'hook' passing anionic solutes. By this device, solute ions that would be scarcely retained in the absence of the ion-pair reagent (detergent) may show substantial and useful retention. Alternatively, it is possible to envisage formation of the ion-pair in solution, which then associates reversibly with the stationary phase.

This simple model, though easy to visualize, falls well short of being a complete picture.[96] Firstly, it assumes that ion-ion attractions are responsible for the formation of ion-paired species. While such attractions undoubtedly do play a role, the major factor in prolonging the union beyond that of most ion-ion interactions in aqueous medium, is the hydrophobic nature of the ions involved. Aqueous eluents are highly structured, and this structure is least disturbed if large, hydrophobic species can be herded together into fewer, larger 'cavities'.[97] It is therefore the increased physical stability in the eluent that helps to counteract the dissociative tendency of the ion-pair.

A second refinement of the simple model may be arrived at through a consideration of two equilibria that may be postulated to be involved.[81e] The first assumes that only the ion-pair formed between the solute (an)ion and the ion-pair reagent (cat)ion is partitioned into the organic phase:

$$B^-_{aq} + P^+_{aq} \rightleftharpoons (B^-P^+)_{org} \quad - - - - \text{(I)}$$

The second also takes account of the ion-pair cation entering the organic phase with its original anion, C^-, in which case ion-exchange may take place between B^- and C^- while P^+ is retained by the organic phase:

$$P^+_{aq} + C^-_{aq} \rightleftharpoons (P^+C^-)_{org} \quad - - - - \text{(II)}$$

or, combining the two equilibria to show this ion-exchange:

$$(P^+C^-)_{org} + B^-_{aq} \rightleftharpoons (P^+B^-)_{org} + C^-_{aq} \quad - - - - \text{(III)}$$

Virtually always it is arranged that $[P^+]_{TOTAL} >> [B^-]_{TOTAL}$ so that $[P^+C^-]_{org}$ or $[P^+C^-]_{aq}$ or $[P^+]_{aq}$ is independent of $[B^-]_{aq}$. The relative contributions made by equilibria (I) and (II) depend upon the nature of P^+. Where P^+ is a large, hydrophobic, organic cation, then equilibrium (II) will become important. This may be taken to be the case for cetrimide.[98] For small organic (cat)ions, or inorganic ions, the hydrophobic effect contributed to the ion-pair P^+B^- by the solute ion, B^-, will be relatively more important, and equilibrium (I) must predominate.

Various additional equilibria may be added to the above where necessary, but the model is sufficient as it stands to predict the qualitative effect of change in the principal chromatographic parameters. Most importantly, it is apparent that the ratio $[P^+B^-]_{org}/[B^-]_{aq}$ will be decreased by the addition of organic modifier(s), e.g. acetonitrile, methanol etc., to the aqueous mobile phase. However it must be remembered that these same modifiers may be selectively extracted into the organic phase, thereby somewhat compensating for this effect. Difficulties in appreciating the manifold effects of changing single chromatographic parameters, e.g. ionic strength of the eluent, may still distort quantitative predictions, or even give confusing qualitative pictures.

In their review of ion-pair liquid chromatography, Tomlinson et. al.[96] considered the use of conditional extraction constants, E^*, in order to treat several related distribution processes simultaneously.[99] Let the stoichiometric extraction constant for equilibrium (I) be defined as,

$$E_{PB} = \frac{[P^+B^-]_{org}}{[P^+]_{aq}[B^-]_{aq}}$$

Then $E^*_{PB} = E_{PB} \cdot \frac{\alpha_{PB}}{\alpha_{p^+}\,\alpha_B}$

where the α_x are the Ringbom alpha coefficients.[100] These coefficients increase from unity as related processes, not accounted for by equation (I), e.g. dissociation of the ion-pair in the organic phase, exert their distorting effect on (I).

Considering the primary process simply as a distributive one between two phases, and avoiding any mechanistic descriptors that treat the organic phase as one with bulk properties, it is possible to treat the problem in a manner compatible with general chromatographic formulae. Firstly, a distribution coefficient for B^-,

$$D_B = E_{PB} \cdot [P^+]_{aq}$$

becomes upon correction by use of α-coefficients,

$$D_B = E^*_{PB} \cdot [P^+]_{aq}.$$

These may be related to the basic chromatographic parameters;

$$K'_B = D_B \cdot \frac{V_S}{V_M} = \frac{t_B - t_0}{t_0}$$

where t_B and t_0 are the retention times of B^- and of an unretained solute respectively. V_S is the volume of the stationary phase, and V_M that of the mobile phase. K' is the phase capacity ratio (c.f. section on Resolution and Retention).

Then $K'_B = \frac{V_S}{V_M} \cdot E^*_{PB} \cdot [P^+]$

It is possible, despite a lack of proper theoretical foundations, to go someway towards modelling the effect of change in pairing ion, P^+, or in organic phase by liquid extraction experiments.[96]

Bidlingmeyer et.al.[101] found that, in the case of sodium (C_5 - C_8) alkylsulphonates and of octylamine hydrochloride, ion-pair formation did not occur in the mobile phase (i.e. equilibrium (I) did not operate). For hydrocarbons, such as toluene, retention behaviour was

independent of ion-pair reagent concentration (0-20mM). There seems little reason to discount ion-pair formation as being involved in the mechanism in either phase, as do the authors of this paper, particularly as they replace this by an assortment of "not only electrostatic forces, but also forces that are eluophilic (showing affinity for the mobile phase), eluophobic, adsorbophilic and adsorbophobic". A reconsideration of reversed phase retention mechanisms (see previous section) will show all these forces as factors in one theory or another under other names.

Recent work by Knox and Hartwick[102,103] laid heavy emphasis upon the importance of equilibrium (III) and minimized the role played by equilibrium (I). They also found that, to a first approximation, the retention behaviour depended upon the surface density of charge functions belonging to adsorbed pairing reagent, and that variation in size of the hydrophobic segment of the pairing ion generated only a comparatively weak secondary influence.

Selection of an Hplc Method

Flowcharts are often provided in textbooks on hplc[81f] as well as in some research articles[104] to guide the hplc user in the selection of an appropriate chromatographic mode for a given application. Figure 14 comprises about one third of the schema given in Ref. 83f (redrawn), i.e. the portion which deals with water-soluble samples of moderate (< 2000) molecular weight. On this chart is shown the sequence of decisions that might lead to reversed-phase ion-pair chromatography being adopted for the separation of food dyes and related substances.

Leading authorities on hplc such as Knox have warned against

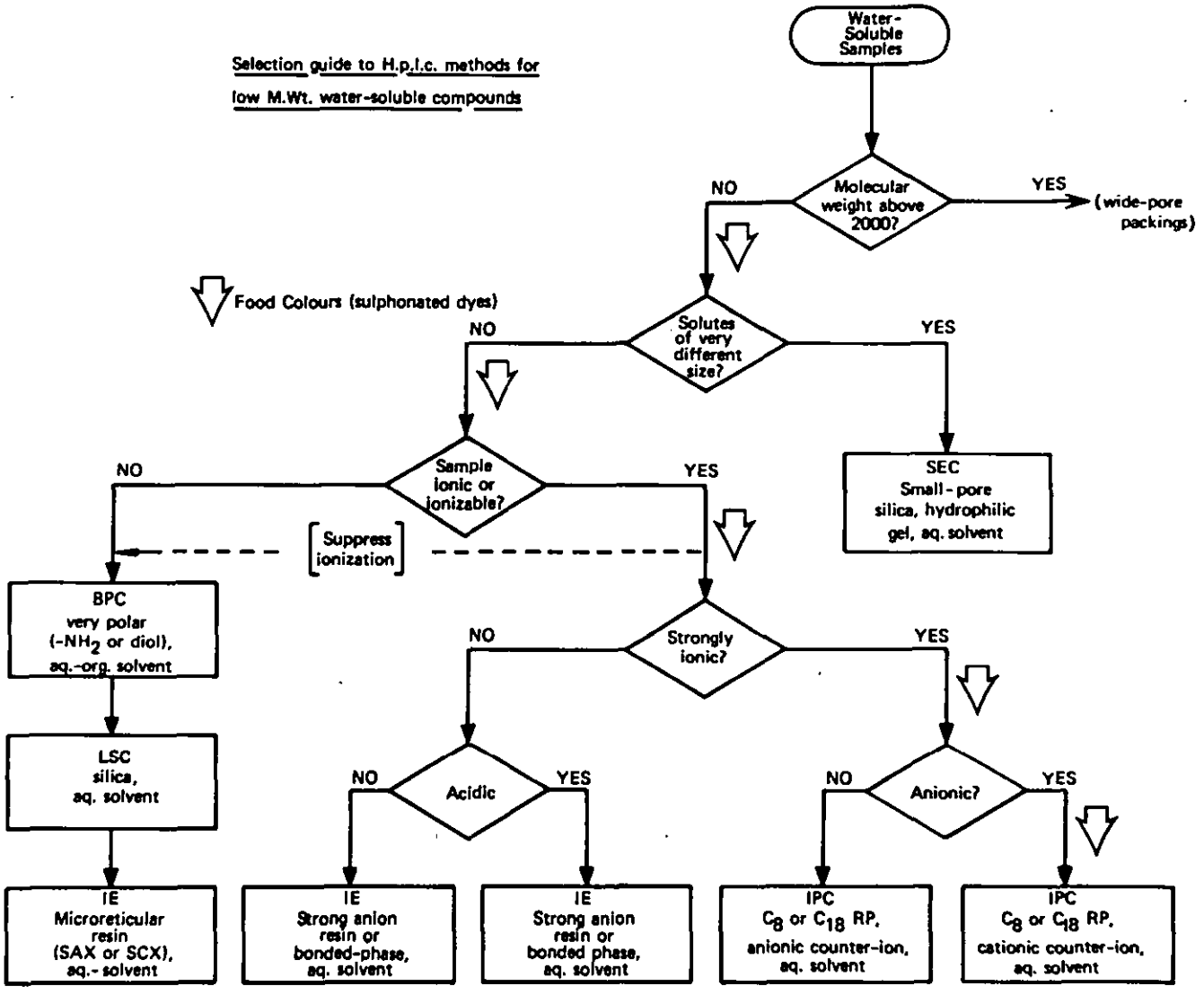


Fig. 14 Selection guide to h.p.l.c. methods for low m.wt. water soluble compounds.

unthinking adherence to such guides and cite examples where interesting separations may be obtained by less obvious approaches.[81f] However, in some respects the flowchart shown in Figure 14 may be considered hierarchical, the earlier decisions (highest up the chart) being safest to regard as the least flexible, the later ones being more amenable to variable interpretation.

The best method for selecting an approach, though by no means always available or necessarily infallible, is to consider what others have done in the same or related fields as published in the literature. Table 7 is a résumé of hplc methods published over the past decade dealing with food dyes, their intermediates and their assay in solution or in real food matrices. Only one reference[39] deals with food dye degradation products as such. Comparison with Figure 14 reveals that in practice the spread of methods is a little wider than might have been supposed. Although this does confirm the approximate nature of the guidance provided by such charts, it still ought not to lead to an overemphasis of this point: not all of the methods listed are equally appropriate for routine application; not all are suitable for adaptation to solving more exacting separation problems than those exemplified; some were trial methods, since largely discarded (e.g. NP-IPC); or still too novel[111] to predict their future use; while most of what remains is predicted by Figure 14.

Looking at the structure of the UK permitted synthetic food colours (Figure 1), all, with the single exception of erythrosine, are salts (sodium, ammonium, calcium, internal) of aromatic sulphonic acids. Most contain one to three sulphonate groups, though black PN contains as many as four. Erythrosine precipitates from aqueous solution at pH values below ~pH 4 because the carboxylate function is rendered in the H form. To render sulphonate groups in the H form requires acidities

beyond the range of the hydrolytic stability of reversed phase packings (pH 2 or lower).[115] Because sulphonates are therefore to all intents fully ionised and so fully open to interaction with ion-pair reagent (this maximises their retention and minimizes mixed retention mechanisms) there is no need to add buffer to the eluent for this purpose. Indeed, because some buffer ions may compete for ion-pairing reagent counter-ions (the sulphonated dyes may have greater affinity, but are present in relatively minute amounts) care must be taken when buffers are added for other purposes to an ion-pair separation system. It also follows that ion-suppression methods (see Figure 14) are not suited to work involving sulphonated compounds.

Roles played by buffers in Table 7 include:

(i) ionic displacement; when ion-exchange chromatography[84c] is employed, the exchanger is initially equilibriated with buffer counter-ions of lower affinity that the sulphonate groups of the dyes, e.g. borate. The eluent is then gradually enriched in a high affinity counter-ion, e.g. perchlorate, to elute the solutes.

(ii) optimising ionisation of other solutes;[115] if a weak acid is present, e.g. erythrosine, a buffer may be needed in ion-pair systems, for the reasons outlined above.

(iii) operation of a salt effect;[106] some buffers, e.g. ammonium acetate, probably prove effective in increasing retention of large sulphonated molecules by suppression of their solubility in the mobile phase. This reliance on increased hydrophobicity with higher ionic strength, limits the method to large molecules and makes it a poor choice for separating smaller sulphonated molecules, e.g. dye intermediates (or perhaps certain putative degradation products). The method may,

however, prove to have advantages in separating positional isomers.

(iv) generally; the buffering of ionic strength and of pH may help contribute to the reproducibility of complex chromatographic separations, and provide an added means of 'fine-tuning' these.

From Table 7 it may appear that ion-exchange chromatography is nearly as popular as is reversed-phase ion-pair chromatography. This is misleading: ion-exchange hplc has been used by the American Food and Drug Administration to separate and quantify various food colours in the presence of their intermediates and side products formed during manufacture. Exhaustive collaborative studies and detailed statistical analysis of the results[109,113,119] confirms the value of the method in this application. Nevertheless ion-pair chromatography is the method of choice for separating food colours one from another as Table 7 indicates, and in general it presents advantages over ion-exchange methods including;[83g]

(a) generally better column efficiencies - though this may be less true of highly optimised systems than of systems routinely varied during method development. Modern bonded phase ion-exchange packings can give good results due to their small particle size and incompressibility - nevertheless it is the older pellicular type packing of large particle size that is quoted in the references to Table 7.

(b) reversed phase packings generally show better stability than do ion-exchange packings: unfortunately the very techniques used to increase separation efficiency, e.g. operation at elevated temperatures (e.g. 60°C) or addition of small amounts (a few percent) of organic modifiers e.g. ethanol, have been found to accelerate the decay of ion-exchange materials.

(c) reversed phase materials tend to show substantially lower batch to batch variation than do ion-exchange materials.

(d) reversed phase materials do not suffer adsorption 'template effects' as do fixed exchange site packings.

TABLE 7: HPLC METHODS FOR FOOD COLOURS

Reference No.	Run Type	Buffers/Ion-Pair Reagent	Food Dyes Separated
39.	Isocratic	CTMAB (IP)	Am; ChB; Er; GS; SY; Ta.
50.	Isocratic	CTMAB (IP)	See Note 1
104.	Isocratic	CTMAB (IP)	Ca; Er; IC; PBV; P4R; QY; SY; Ta
105.	Isocratic	TBAC (IP)	P4R; SY
106.	Gradient	NH_4Acet (B)	IC
107.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	SY
108.	Gradient	Na Borate/$NaClO_4$ (B)	SY
109.	Gradient	Na Borate/$NaClO_4$ (B)	Am
110.	Isocratic	CTMAB (IP)	IC
111.	Isocratic	Crown ethers (cmplx.); KAcet (B)	PBV
112.	Isocratic	CTMAB (IP); HAcet (B)	See Note 1
113.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	SY
114.	Isocratic	Na phosphate (B)	SY
115.	Isocratic/ Gradient	TMAC; TBAC; CTMAC (IP) $NH_4H_2PO_4$ (B)	Am; Er; IC; Ta
116.	Gradient	NaH_2PO_4 (B)	BFK
117.	(i) Isocratic (ii) Isocratic	(i) CTMAB (IP) (ii) CTMAB (IP)	(i)Am; Ca; P4R; SY; Ta (ii) P4R; SY; Ta
118.	Isocratic	TBAP (IP)	Am; BBl; Er; IC; SY; Ta
119.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	SY
120.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	SY
121.	Gradient	KH_2PO_4 (B)	Am; BBl; Er; IC; SY; Ta
122.	Isocratic	TBAP (IP)	Am; BPN; Ca; IC; PBV; P4R; QY; SY; Ta
123.	Isocratic	?	Am; BPN; IC; SY; Ta
124.	Isocratic	TBAP (IP)	SY

Reference No.	Run Type	Buffers/Ion-Pair Reagent	Food Dyes Separated
125.	?	$(NH_4)_2CO_3$ (B)	Am; BBl; Er; IC
126.	(i) Gradient (ii) Isocratic	(i) TBAH (IP); phosphate (B) (ii) Pentanol (SP); TBAP (IP); phosphate (B)	Am; BPN; Ca; Er; GS; IC PBV; P4R; QY; SY; Ta
127.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	SY
128.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	Am
129.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	IC
130.	Gradient	$Na_2B_4O_7/NaClO_4$ (B)	SY
131.	Isocratic	TBAH; TDA; TEAH (IP) Formic Acid (B).	Ta

Reference No.	Chromatographic Mode	Packing Material	Particle Size**	Mobile Phase Bulk Components	Volume Ratio of those Components
39.	NP-IPC	SiO_2	5	PrOH/H_2O	3:1
50.	RP-IPC	BP-C_2	5	H_2O/MeOH	25→20:75→80
104.	RP-IPC	See Note 3	5	H_2O/MeOH	15:85
105.	RP-IPC	BP-C18	10	H_2O/Acetone	4:1
106.	RP	BP-C18	5	H_2O/MeCN	1:→1
107.	IE	P/C-NR_3^+	25-37*	H_2O	B. Grad.
108.	IE	P.C-NR_3^+	25-37*	H_2O	B. Grad.
109.	IE	P.C-NR_3^+	25-37*	H_2O	B. Grad.
110.	RP-IPC	BP-C2	5	H_2O/MeOH	22:78
111.	NP-cmplx.	SiO_2	8-10	CH_2Cl_2/MeOH	9:1
112.	RP-IPC	BP-C2	5	i-PrOH/H_2O	7:3
113.	IE	P/C-NR_3^+	25-37*	H_2O	B. Grad.
114.	RP	BP-C18/ C8/C2	5-10	H_2O/MeOH	10:3
115.	RP-IPC	BP-C18	10	H_2O/MeOH	80→1:20→99
116.	RP	BP-C18/ C_3NH_2	5	H_2O/MeCN	3:2, B. Grad.
117.	(i) NP-IPC (ii) RP-IPC	SiO_2 BP-C2	6.5 7	H_2O/PrOH (etc.) H_2O/PrOH	1:3 (etc.) 5:2
118.	RP-IPC	BP-C18	10	H_2O/MeOH	55→40:45→60
119.	IE	P/C-NR_3^+	25-37*	H_2O	B. Grad.
120.	RP-IPC	BP-C8	10	H_2O/MeOH	40→55: 60→45
121.	RP	BP-C8	10	H_2O(B)/MeOH	B. Grad, 9→1: 1→9
122.	RP-IPC	BP-C8	10	H_2O/MeOH	40→55: 60→45
123.	RP-IPC	BP-C2/C18	10*	MeOH/H_2O i-PrOH/H_2O	99→5:1→95 15:85
124.	RP-IPC	BP-C18	See Note 2	H_2O/MeOH	50:50
125.	RP	BP-C18	?	H_2O/MeOH	?

Reference No.	Chromatographic Mode	Packing Material	Particle Size**	Mobile Phase Bulk Components	Volume Ratio of those Components
126.	(i)RP-IPC (ii)RP-LLC	(i)BP-C18 (ii)BP-C18	10 10	(i)H_2O/MeOH (ii)H_2O	4→6:6→4
127.	IE	P/C-NR_3^+	25-37*	H_2O	B. Grad.
128.	IE	P.C-NR_3^+	25-37*	H_2O	B. Grad.
129.	IE	P/C-NR_3^+	25-37*	H_2O	B. Grad.
130.	IE	P.C-NR_3^+	25-37*	H_2O	B. Grad.
131.	RP-IPC	BP-C18	10	H_2O/MeOH	1:1

Key to Table 7

Note 1 - For ChB, 100% MeOH was used.

Note 2 - Authors state they used Bondapak C18 which is listed* as 37-75 μ particle size. This might be a misprint for μBondapak C18 which is 10 μ particle size.*

Note 3 - C-18 is stated in the introduction/abstract, while C-8 is stated in the experimental section.

Food Dye Abbreviations:

Am	Amaranth	GS	Green S
BPN	Black PN	PBV	Patent Blue V
BBl	Brilliant Blue FCF	P4R	Ponceau 4R
BFK	Brown FK	QY	Quinoline Yellow
Ca	Carmoisine	R2G	Red 2G
ChB	Chocolate Brown HT	SY	Sunset Yellow FCF
Er	Erythrosine	Ta	Tartrazine
IC	Indigo Carmine	Y2G	Yellow 2G

* Information partly obtained by reference to appendices 3-5 of A. Pryde and M.T. Gilbert, "Applications of HPLC" (Ref. 82).

** Particle sizes are given in microns, but in all but one or two cases the figure given is only a broad approximation as commercial packings are seldom sieved to close tolerances.

Other Abbreviations:

Acet	acetate
B	buffer
B. Grad.	buffer gradient
BP-C18	octadecylsilyl bonded SiO_2 (BP-C2 etc. analogously).

BP-C_3NH_2	aminopropyl bonded SiO_2
CTMAB	cetyltrimethyl ammonium bromide
CTMAC	cetyltrimethyl ammonium chloride
complx	neutral complexing agent
IE	ion-exhange
IP	ion-pair reagent
MeCN	acetonitrile
MeOH	methanol
NP-cmplx	normal phase + complx
NP-IPC	normal phase + IP
P/C-NR_3^+	polymer coated strong (quaternary ammonium) anion exchanger
Pr and i-Pr	propyl/iso-propyl
RP	reversed phase
RP-IPC	reversed phase + IP
RP-LLC	reversed phase liquid-liquid chromatography
SiO_2	silica gel
SP	stationary phase
TBAC	tetrabutylammonium chloride
TBAC	tetrabutylammonium phosphate
TBAH	tetrabutylammonium hydroxide

CHAPTER 5

General: Instrumentation, Chemicals and Samples

Polarograph

A Model 174A Princeton Applied Research (PAR) Polarographic Analyser was employed. The facilities provided by this instrument comprise:

(i) DC Polarography

(ii) Sampled DC Polarography

(iii) Normal Pulse Polarography

(iv) Differential Pulse Polarography

(v) Various Voltammetric Modes (sweep/stripping voltammetry)

(vi) Phase-sensitive AC Polarography (requires substantial added equipment).

Factors recommending this equipment include:

(a) high compliance potentiostat (±80 V, 18 mA)

(b) high electrometer input impedance (10^{11} ohms)

which together ensure accurate results over a wide range of solution conductivities.

Current flowing through the working electrode is measured, and output as a proportional voltage to the ordinate axis of the X-Y recorder, except that the polarograph amplifies the electrode current : output voltage ratio x 10 when operating in the differential pulse mode (X-axis is proportional to the scan potential).

The original model, the 174 PAR instrument (c. 1972), was confirmed as being suitable for polarographic measurements by Osteryoung et.al.[132], who nevertheless noted certain limitations including:

(a) scan rate, drop time and pulse amplitude must be held constant for peak current to be directly proportional to the concentration of the electroactive species;

(b) whereas an increase in scan rate from 0.5 mV/s to 2 mV/s speeds up analysis time by x 4, peak current decreases by about 20%;

(c) at faster scan speeds peak current will be increased by more than that expected theoretically by increasing the pulse amplitude, e.g. > 10 x increase by changing the pulse amplitude from 5 to 100 mV;

(d) at a fixed pulse amplitude, peak current is directly proportional to the drop area if a constant scan rate x drop time product is used.

In practice few problems arise because drop time, electrode area, scan rate and pulse amplitude need seldom be varied once a suitable combination has been selected. The limitations occur largely as a result of the use of long time constant circuitry in order to average out noise when operating at high dilution.

Light Apparatus

Sunlight is a notoriously irreproducible influence on degradation: weather conditions, geographic location and a host of other possible factors intervene. Consequently, the American drug industry has made use of 'fadeometers',[43] specially designed chambers with critically controlled environments for assessing the lightfastness of their products.

For the present series of experiments there was no need to employ quite so sophisticated and costly an apparatus: features such as controlled humidity (available for tablet experiments) and specimen rotators were considered to be superfluous. Exact thermostatting, while desirable, was also dispensed with: trials showed little thermal degradation even well above the operating temperature of the constructed apparatus (about

10 - 12°C above ambient).

The apparatus was set up as in Figure 15. Light intensity was increased and made more uniform by the reflective inner wall of the apparatus body. A constant forced draught reduced the temperature of sample solutions from c.80° (no draught) to c.30° with the fan on. A mesh shelf raised sample containers above the base of the apparatus to allow maximal air circulation around these.

Chromatographic Apparatus

The hplc equipment was arranged in conventional manner as shown in Figure 16. A modular set up such as this is preferred to a "one company, one box package" as,

(a) access to working parts is simpler, and

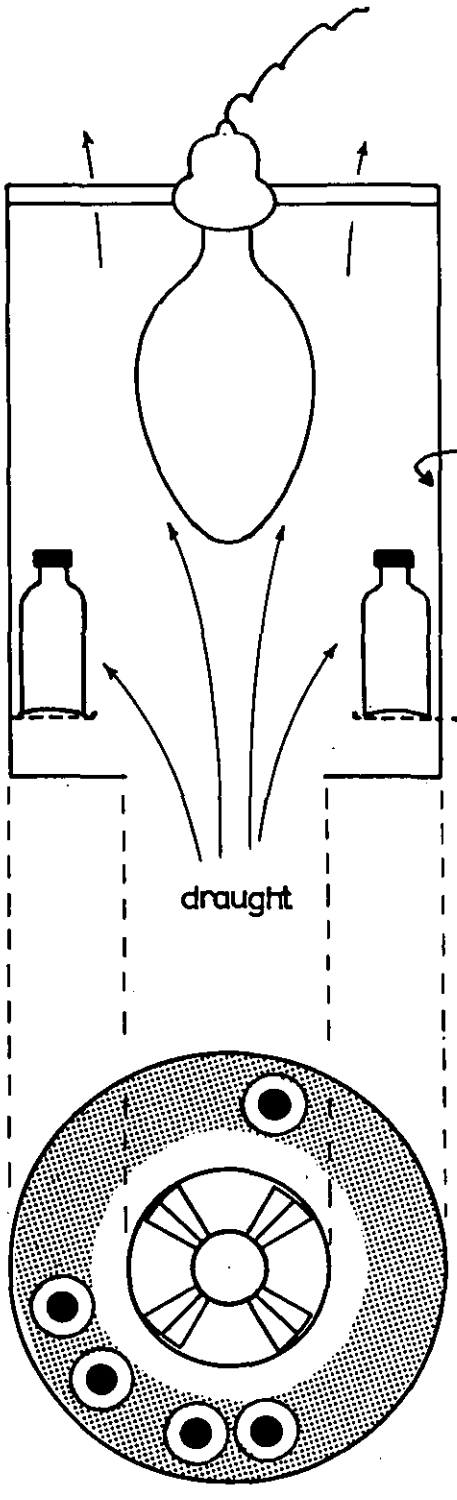
(b) substitution of components is very much easier.

System elements included:-

(i) Pye Unicam . LC-XPS pump - this single piston damped pump was of the constant flow variety with flow rate settable at 0.1 ml/min intervals. High and low pressure variable cut-outs were incorporated, as was a pressure monitor which was based on energy requirement for maintaining flow . For a single piston pump, pump noise was low, allowing acceptable baselines down to 0.04 - 0.02 AUFS detector scale. Operation up to c.3000 psi was routine, while above 4000 psi the pump laboured excessively. Reliability proved to be excellent.

(ii) 6 and 7 port valve injectors (Rheodyne, Inc., Type 50) - for the earlier part of the work a 6-port injector was used. This proved inconvenient because as much as 0.5 mls of sample solution could be required to avoid memory effects by washing out traces of previous samples. Replacement by the 7 port valve was a substantial benefit: fitted with a 20 µl variable load loop, this injector gave good reproducibility of sample size using a minimum of sample.

Photodegradation apparatus



View from above showing placement of the sample bottles.

Scale 1 : 5

Fig. 15

H.P.L.C. Apparatus

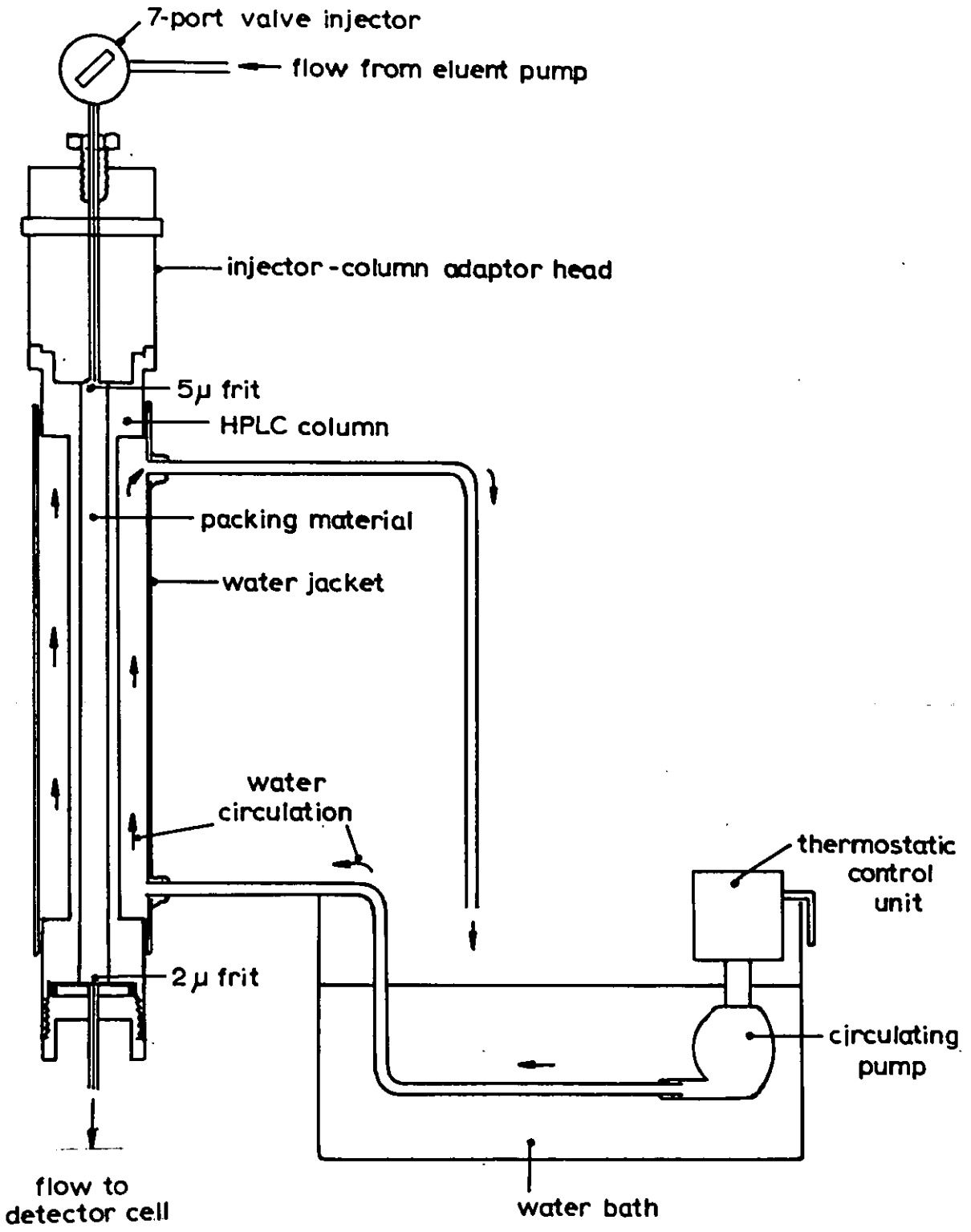


Fig. 16

(iii) Shandon Southern Scientific column and injector head - the 100 x 5 mm (internal length/diameter) column was of standard type 316 steel [composition: 16 - 18% Cr, 10 - 14% Ni, 2 - 3% Mo, max. 2% Mn, max. 1% Si, max. 0.08% C, max. 0.045% P and max. 0.030% S[133]]. The column bottom was covered by a 2 μ woven wire mesh frit, the column top by a 5 μ frit. PTFE washers created the high pressure seals.

(iv) Pye Unicam PU-4020 UV detector - most of the later work used this model. Incorporation of a deuterium lamp and variable wavelength control allowed operation in the range 190 - c.400 nm. This instrument contained a standard 8 μl silica flow cell. The attenuation range (1.28 AUFS to 0.05 AUFS) gave acceptable linearity up to 0.64 AUFS when tested. The 0.02 AUFS range constituted a practical limit with the part aqueous eluents involved.

(v) Linseis LS.4 chart recorder - 10 mV input. Originally, this recorder generated more noise than others substituted for it, but this fault was corrected by putting a 22 μF capacitor across the terminals. Sensitivity did not appear to be affected.

(vi) Steel microbore tubing - minimum lengths of 15 or 25 μ tubing were used to connect the injection valve to the column, and the column to the detector cell.

(vii) other tubing - wider bore steel tubing connected the pump to the injection valve: 1 - 2 m plastic tubing led from the detector cell to waste, so creating back pressure to avoid degassing in the cell.

Spectrophotometers

SP-800 (Pye Unicam Ltd., Cambridge): simple scanning spectrophotometer (700 - 190 nm) with single output range, viz., 2.0 absorbance

units full scale (AUFS).

SP8-100 (Pye Unicam Ltd.): scanning spectrophotometer (800 - 190 nm) with low stray light levels (quoted at 0.005% at 220 nm[134]) permitting multiple range selection from 2.0 down to 0.005 AUFS.

Column Packing

During the present study hplc columns have shown considerable variation in useful lifespan, ranging from a few days to about eight months. The longer lasting columns encourage the opinion that the presence of ion-pairing salts in the eluent do not necessarily render a column liable to accelerated deterioration as some workers have indicated.[83h] One long surviving column was that used at 40°C for most work. When columns required repacking, even when physical collapse was indicated, the packing material was discarded (not repacked) as gradual deterioration of bonded groups inevitably occurs to some degree also.

A number of empirical guidelines have been derived to help in selecting a technique:[81g]

(i) the particles must not agglomerate during packing;

(ii) the particles must impact the column bed being packed at high velocity;

(iii) sedimentation of packing particles must be minimized between slurry formation and commencement of the packing;

(iv) the bed must be packed under high compression.

Although these factors are not quite mutually independent (e.g. a high impact velocity is better obtained with a low viscosity slurrying liquid, while a slow sedimentation rate tends to require high density liquids), overall, good compromises can be arrived at by means of a wide range of equipment, technique and slurrying liquids. The methods adopted

are described below:

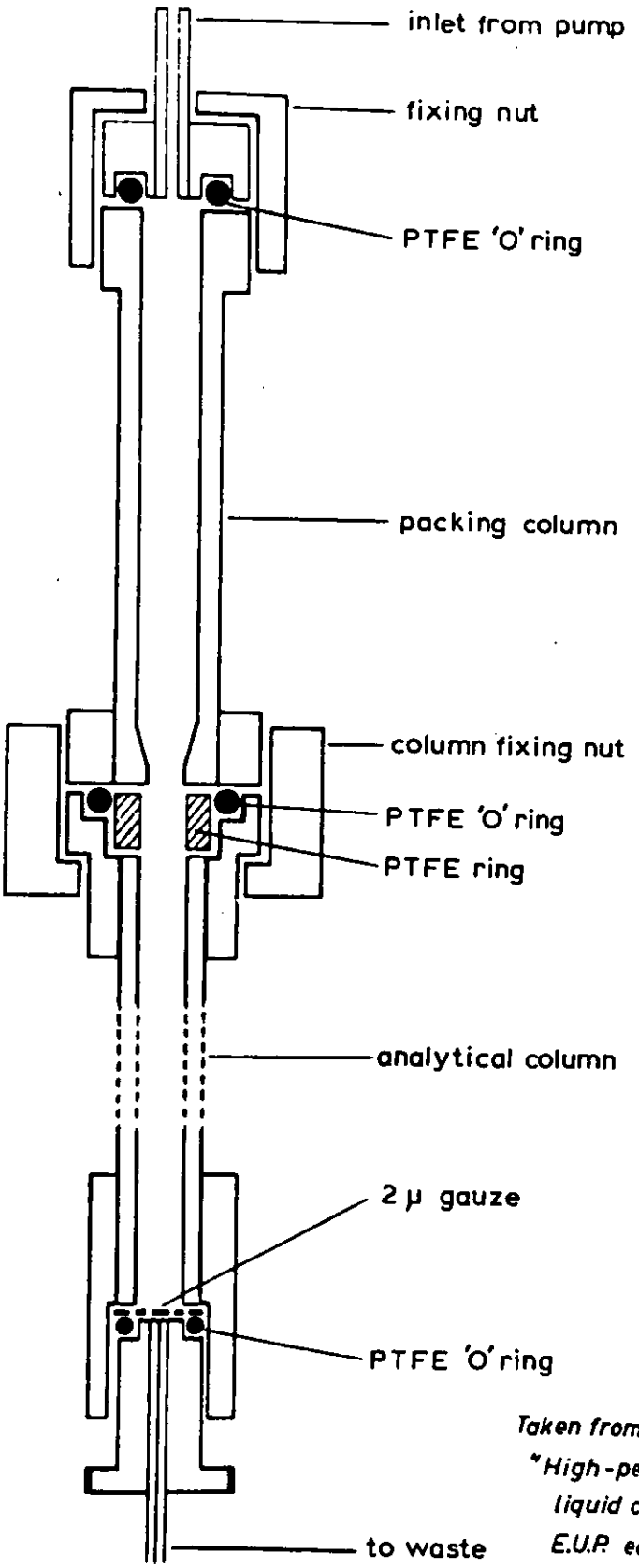
(a) the column was thoroughly cleaned with a nylon brush to present a smooth surface on the inside;

(b) a packing liquid was selected: acetone, propan-2-ol, methanol and methanol/water (70:30) were tried (all hplc grade). The last named proved unsatisfactory, possibly because fine agglomeration of the packing particles was increased in the presence of water. The other liquids tried all performed adequately — if any significant advantage was conferred by a decreased sedimentation rate using propan-2-ol, it was well offset by the lower viscosity of methanol. These packing liquids were pre-dried e.g. over anhydrous potassium carbonate for acetone or propan-2-ol, and over type 4A molecular sieves for methanol[135];

(c) alternative pre-columns were tried, viz., 11 ml and 33 ml capacity. About 1.7 g of SAS-hypersil (Shandon Southern Scientific) were weighed out (fume cupboard) and dispersed in 11 ml (or 33 ml) of packing liquid, either by constant shaking (5 - 10 minutes) or, more conveniently, by standing the mixture in an ultrasonic bath;

(d) the pre-column was attached vertically to the packing pump as shown in Figure 17, and the dispersed packing mixture poured in. The analytical column to be packed was immediately inverted and screwed onto the precolumn. 100 ml of packing liquid was pumped upwards driving the packing mixture into the analytical column, bedding the SAS-hypersil against the 2 μ retaining frit. Early in

H.P.L.C. column packing assembly



Taken from:
"High-performance liquid chromatography"
E.U.P. ed. J.H. Knox

Fig. 17

the study only about 4000 psi was used when doing this, but later it was preferred to apply the maximum pressure available with the packing pump, viz., 6000 + psi [Haskell air driven fluid pump : 11 ml cycle; 122/1 intensifier ratio]. The pump was turned off, the packing assembly inverted by 180°, then 50 ml more of packing liquid was pumped through. The pump was again turned off, and the assembly left for 45 minutes in order to allow pressure reequilibriation before removing the column;

(e) excess packing was carefully scraped out to allow the 5 μ top frit to fit squarely over the column top, and the column was then ready for fitting into the chromatographic system to be tested.

Britton Robinson Buffer

This buffer nominally comprises 0.04 M each of boric, acetic and phosphoric acid. The buffer was prepared by dissolving 9.9 g boric acid, 9.6 g glacial acetic acid and 17.8 g phosphoric acid (88%) in 1000 ml aliquots of distilled water, then combining these and making them up to 4 l. The resulting pH 1.9 - 2.0 solution was adjusted to the desired pH using 3 M NaOH solution (120 g/l). This wide-range buffer does, of course, show greatest buffer strength in the regions of the pKa values of the respective acids and acid salts, viz.:

acetic acid, $pK_1 = 4.76$

boric acid, $pK_1 = 9.24$

phosphoric acid, $pK_1 = 2.12$; $pK_2 = 7.21$; $pK_3 = 12.32$

Apart from its buffer range, B-R buffer was chosen as it gives an acceptable polarographic cut-off potential with no artefacts at less negative potentials.

Hplc Eluents and Reagents

The water used in the eluent mixtures was triply distilled on a glass column and collected in glass containers: this avoided the problem of slow accumulation of phthalate plasticiser on the column when water is stored prior to use in plastic containers.

Hplc grade methanol, n-hexadecyltrimethylammonium bromide ("cetrimide") and acetic acid were obtained from Fisons Chemicals Ltd.

Eluent mixtures were made up by measuring the volumes of the component liquids, methanol and water, separately; when these liquids are mixed a small volume reduction may occur depending on the liquid ratio used (e.g. 2½% reduction on mixing methanol [2 parts] to water [1 part] was observed).

Samples and Reagents

Food Colouring Matters

Samples of the sixteen permitted colours, together with Red 10B, were kindly provided by the Laboratory of the Government Chemist. Later work was carried out on samples generously donated by Pointings Ltd. These were all commercial grade compounds.

Phosphonium Salts

Tetraphenylphosphonium chloride was supplied by Cambrian Chemicals, Croydon. Tetraphenylarsonium chloride was obtained from Schuchardt, München, Germany. These were analytical grade reagents.

Nitrogen Gas Scrubbing Solution

White spot nitrogen, as supplied by British Oxygen Corporation, is virtually free of oxygen. To remove final traces, or to act as a safeguard, a vanadium (II) scrubbing solution was used when deoxygenating samples for polarographic analysis.

Approximately 2 g ammonium metavanadate was boiled with 25 ml concentrated hydrochloric acid. The solution was diluted to 200 ml [green], and shaken with a few grams of heavily amalgamated zinc [sky blue], more acid being added if any precipitate or turbidity formed.

This semi-reduced solution was then split between two 250 ml gas scrubbing bottles, whose inlet tubes were fitted with gas distribution devices, either sintered glass frits or multihole bulbs. Each bottle contained c.25 g heavily amalgamated zinc to re-reduce V(III) that formed in the presence of O_2. Prior to use nitrogen was bubbled through until themid-blue coloration changed to violet. Each few months, additional hydrochloric acid or amalgamated zinc was added to regenerate the solution, which was completely replaced each 9 - 12 months.

The two scrubbing bottles containing V(II) solution were used in series with a third containing water whose purpose was to limit carry over of acid fumes etc. Vanadium was preferred to chromium, despite the latter's greater efficiency, because of its greater ease of preparation and regeneration.

Sulphonic Acid Standards

A range of aromatic sulphonic acid derivatives was obtained from Fluka A.G. (Switzerland). Sulphanilic and naphthionic acids were obtained from BDH Chemicals Ltd., Poole, England.

Diiodofluorescein (C.I. 45425)

This compound, designed for use as an adsorption indicator, was supplied by BDH Chemicals Ltd., Poole, England.

Mercury

Trebly distilled mercury was supplied by Alexander Pickering & Co. Ltd., Slough, England.

Selection of Analytical Techniques

Two broad approaches to the investigation of food dye stability were:

(i) quantitative measurements of loss of the parent compounds;

(ii) qualitative observations of the degradation products.

These approaches were not mutually exclusive, nor was there any absolute reason why both types of measurement could not have been largely carried out by a single analytical method, viz., hplc. Nevertheless, good experience had previously been built up in this laboratory in the polarographic analysis of food dyes, and the use of this technique suggested several advantages (see Table 8), not least that it was based at the molecular level on a different principle to that of spectroscopy, and so might offer a degree of complementary information to that provided by hplc with UV detection. Standard UV - Visible spectroscopy served very much an ancillary role in these studies.

Polarographic Procedures

Originally, methods developed in this laboratory by D. Bhanot[136] and Dr. A.G. Fogg for the polarographic quantification of the permitted food colouring matters were used with no basic modifications. These methods made appropriate use of tetraphenyl-phosphonium chloride (tppc) where this had proved to be of benefit, and provided optimum pH values for the analyses. Later, however, it was judged to be a worthwhile precaution to dispense with the tppc except in the single case of the Red 2G/Red 10B system (not investigated by Bhanot) which had been looked at originally without tppc present.

TABLE 8: COMPARISON OF ANALYTICAL TECHNIQUES

UV-VIS SPECTROSCOPY	HPLC WITH UV DETECTION	DIFFERENTIAL PULSE POLAROGRAPHY
Adequate sensitivity Absorption peaks broad in the visible and unselective in the UV below 260 nm.	Less good sensitivity Highly selective through chromatographic separation, and able to utilise short wavelengths (230 - 250 nm) to detect wide range of compounds.	Adequate sensitivity Polarographic peaks quite narrow and selective.
Technique is simple and reliable. Each analysis may take, e.g. 10 mins.	The technique is simple and reliable, but the apparatus is less so, and requires more care in setting up, and more frequent maintenance.	Technique is reliable but more care is required than for UV-Vis spectroscopy to ensure this. Deoxygenation of the solution prior to analysis increases analysis time to 15 - 20 mins.
Linear range quite small, e.g. 0.05 - 1.0 absorbance units, i.e. less than 2 orders of magnitude change in analyte concentration.	As for UV-Vis spectroscopy.	Linear range large, e.g. 10^{-7} to 10^{-3} M analyte concentration.
Requires little method modification for food dyes.	Requires careful adjustment in methods to gain the potential selectivity available: highly flexible.	Method development can vary from straight forward to quite complicated.

The reason for this was that tppc interacts differently with different analytes: in some cases peaks may be enhanced in size or shape, or shifted in potential to a more useful position on a polarogram; in other cases peaks can be heavily suppressed, distorted or split, or shifted out of the available polarographic range. For the food colours, themselves, these effects have been well investigated by Bhanot and Fogg, and no new problems were presented: it was the possibility of new peaks being formed, and remaining unobserved owing to such effects as these, that argued against further use of tppc.

The procedures given are set out for the analysis of sample solutions of c.100 ppm $^{w}/v$ colouring matter. Where other concentrations were involved in a particular study, the polarographic range and/or the volume ratio of sample solution to other solution components were adjusted accordingly.

TABLE 9: CONTENTS OF POLAROGRAPHIC STUDIES

4 ml of 100 ppm $^{w}/_{v}$ colouring matter aqueous solution
20 ml of B-R buffer adjusted to desired pH
4 ml of 5000 ppm $^{w}/_{v}$ tppc solution (where used)
q.v. distilled water to 50 ml (volumetric flask).

Food Colour	Use of tppc	Polarographic Range	pH of buffer
Amaranth	No	5 µA fsd	9.0
Black PN	No	5 µA fsd	1.9
Brilliant Blue FCF	No	2 µA fsd	1.9 or 3.0
Brown FK	No	2-5 µA fsd	10.6
Carmoisine	No	10 µA fsd	1.9
Chocolate Brown HT	No	2 µA fsd	4.0
Erythrosine BS	No	2-5 µA fsd	4.0-6.6
Green S	No	5 µA fsd	4.0
Indigo Carmine (i)	Yes	1 µA fsd	9.0
Indigo Carmine (ii)	No	2 µA fsd	9.0
Patent Blue V	No	2-5 µA fsd	1.9
Ponceau 4R (i)	Yes	2 µA fsd	9.0
Ponceau 4R (ii)	No	10 µA fsd	9.0
Quinoline Yellow	No	5 µA fsd	1.9
Red 2G	No	2-5 µA fsd	9.0
Sunset Yellow FCF	No	10 µA fsd	1.9
Tartrazine (i)	Yes	5 µA fsd	9.0
Tartrazine (ii)	No	5 µA fsd	9.0
Yellow 2G (i)	Yes	5 µA fsd	9.0
Yellow 2G (ii)	No	5 µA fsd	9.0

CHAPTER 6

PHOTOSTABILITY OF THE FOOD COLOURS

Photolysis of Azoaromatics

Irradiation in the visible or ultraviolet region of the spectrum (or the application of heat) may lead to the geometrical isomerisation of the azo group without concomitant decomposition.[140a] The quantum yield is less than unity and depends upon temperature and wavelength, but not upon the solvent. Prolonged irradiation may randomise the isomers independently of their initial concentrations, with trans → cis being less favoured than cis → trans.

Prolonged irradiation of azobenzene in strong acid solution produces benzo[c]cinnoline by cyclodehydrogenation.

It has been shown that irradiation of solutions containing simple organic compounds (e.g. acetone, D,L-mandelic acid) produces either radicals or substances in excited states, which are capable of transferring their energy and effecting the reduction of the azo linkage as follows:[45]

$$Ar_1\text{-}N{=}N\text{-}Ar_2 + H^{\cdot} \xrightarrow{k_1} Ar_1\text{-}\overset{H}{N}\text{-}\dot{N}\text{-}Ar_2$$

$$2\ Ar_1\text{-}\overset{H}{N}\text{-}\dot{N}\text{-}Ar_2 \xrightarrow{k_2} Ar_1\text{-}NH\text{-}NH\text{-}Ar_2 + Ar_1\text{-}N{=}N\text{-}Ar_2$$

$$2\ Ar_1\text{-}NH\text{-}NH\text{-}Ar_2 \longrightarrow Ar_1\text{-}N{=}N\text{-}Ar_2 + Ar_1NH + Ar_2NH$$

Rate equations for k_1 and k_2 are

$$k_1 t = \frac{1}{C_o - H_o} \ln \frac{C}{C-(C_o-H)} \cdot \frac{H_o}{C_o}$$

$$2k_2 t = \frac{1}{2C - 2C_o.H_o} + \frac{1}{H_o}$$

where k_1 and k_2 are the rate constants for the respective processes, and H, C, C_o and H_o are present or original (subscript o) concentrations of the dye (C) or simple organic H-donor respectively.

In aqueous solution the final process is believed to take place

very rapidly indeed, giving only amines, without hydrazines, as products.

Cleavage of the azo linkage removes the chromophore such that this type of degradation destroys the colour of a dye. The syn-anti isomerisation, however, does not necessarily achieve this.[140b] The 'parent' compound, azobenzene, displays a 319 nm (near UV) maximum in its anti-configuration with a molar extinction coefficient of 22,000. The syn-form has its maximum shifted to 280 nm, with an extinction coefficient of 5,260. For other azoaromatics, such behaviour could conceivably result in erroneous measurements if incorrect assumptions are linked to an insufficiently selective analytical technique.

Photodegradation Studies

Studies under Natural Light

Initial sighting studies were undertaken in order to assess the effect of daylight before any work was commenced using the light apparatus (Figure 15). Firstly, selection was made of those dyes with least fastness toward sunlight in simple aqueous solution. Later, more detailed investigation of the selected dyes was undertaken, and some other parameters introduced.

Effect of Natural Light on a Range of Colours

Experimental:

A simple aqueous solution was made up containing 100 (±2) ppm $^{w}/_{v}$ for each colouring matter. Each solution was divided, half being stored in a stoppered flask in a dark cupboard, and half on an east facing window still exposed to daylight. The sample pairs were polarographically compared at 1 - 2 week intervals. The study was continued for 52 days from November to January.

Results and Discussion

Colours showing no decay over the period of the study

included amaranth, black PN, brilliant blue FCF, green S, ponceau 4R, quinoline yellow, tartrazine and yellow 2G. Colours showing only marginal loss (<10% w.r.t. dark stored solution) included brown FK (5½%), indigo carmine (6½%) and patent blue (3%). Chocolate brown HT suffered 11.1% degradation, and carmoisine 20.5%, while erythrosine was easily the most heavily degraded colour tested, showing 29% loss after only eight days, and complete loss after two months with little resemblance between the polarograms of sample and standard - though visible spectroscopy indicated 7% retention.

It did not prove feasible to construct kinetic decay curves from the data obtained because certain intermediate measurements in this study were discarded after an intermittent electrical fault, discovered on the polarograph, put them in doubt.

The major indication to emerge from this study was that of the excellent degree of light stability shown by most of the colouring matters tested under the moderate conditions and at the concentration used.

The least expected result to emerge was the low loss sustained by indigo carmine. Literature references have generally emphasized the susceptibility of this dye. For this reason it was decided to look further at indigo carmine, as well as at erythrosine, the only heavily degraded colouring matter.

Effect of Natural Light on Erythrosine

Experimental (1st Study)

Three further studies were made of erythrosine in diffuse sunlight. The first of these examined the degradation of largely deoxygenated solutions at different erythrosine concentrations. Solutions containing 16, 40 and 100 ppm $^{w}/_{v}$ erythrosine respectively (this ratio

of concentrations was chosen to be 1 : 2.5 : 2.5^2) were prepared in 20% strength pH 6.6 B-R buffer, and each was divided between two screw-top bottles, one for exposure and the other to be stored in the dark to serve as the standard. Deoxygenated nitrogen was bubbled for 5 - 8 minutes through each solution. Then the bottle tops were tightly secured, excluding as much air as possible, before exposure or storage in the dark. Paired samples were withdrawn each 5 - 8 days over a month and polarographically compared, then deoxygenated as before prior to being returned to their respective positions.

Results (1st Study)

Degradation curves were plotted from the data obtained (see Figure 18). These results are discussed with those from the other studies which follow.

A parallel study to the above conducted at pH 4.5 was discontinued when excessive precipitation occurred.

Experimental (2nd Study)

Five solutions were prepared containing 16, 26, 40, 66 and 100 ppm $^{w}/_{v}$ erythrosine respectively in 20% strength pH 6.5 B-R buffer. Half of each solution was exposed, as previously, and half stored in the dark for comparison. No deoxygenation was undertaken. Polarographic measurements were made after 5, 8 and 11 days upon the paired samples.

Results (2nd Study)

Degradation curves were plotted for the five solutions (Figure 19). A new peak was observed on the polarograms (Figure 20) and its growth plotted (Figure 21).

Photodegradation of erythrosine in natural light

(deoxygenated solution, pH 6·6)

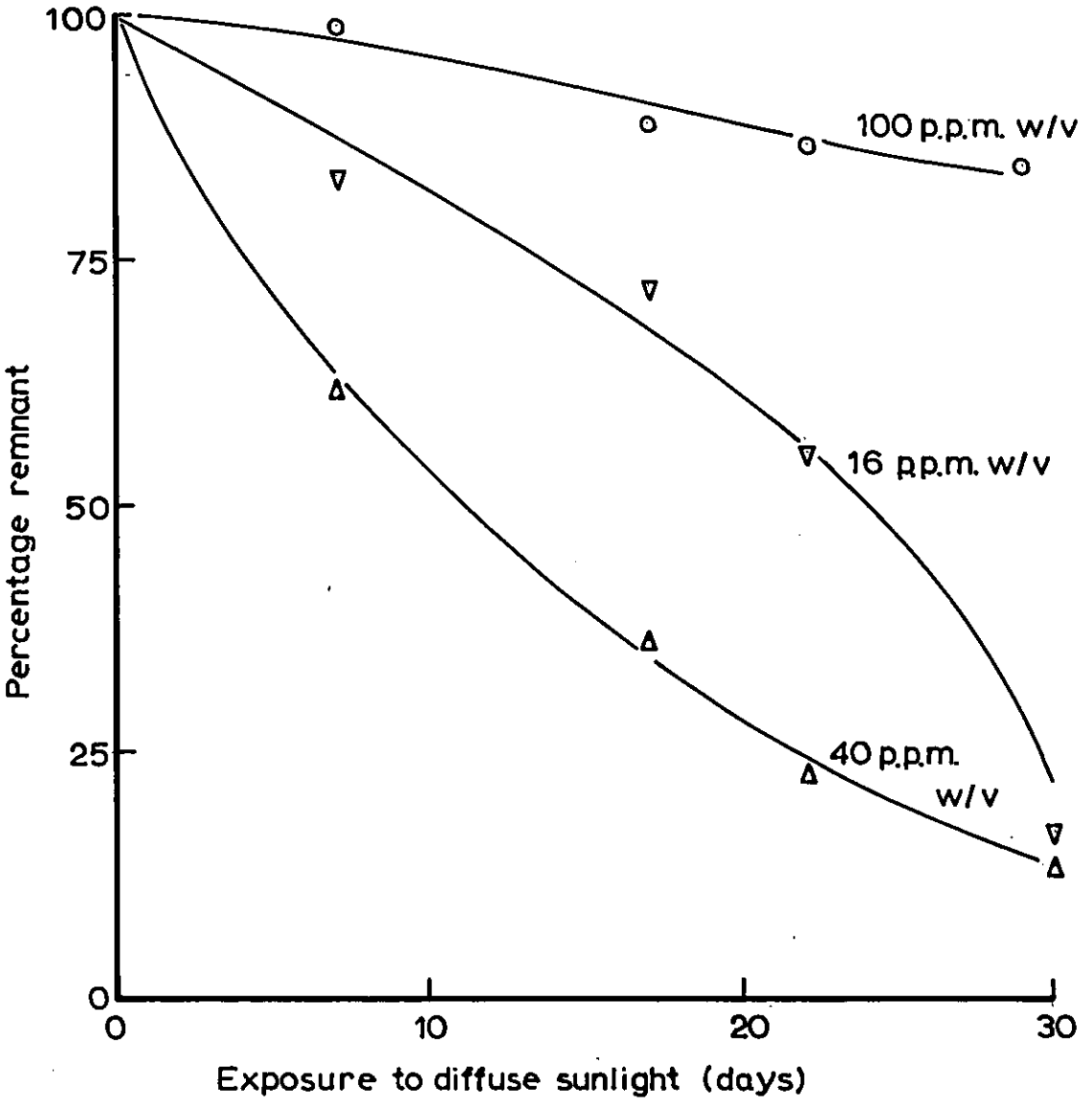


Fig. 18

Photodegradation of erythrosine in natural light, at pH 6·5.

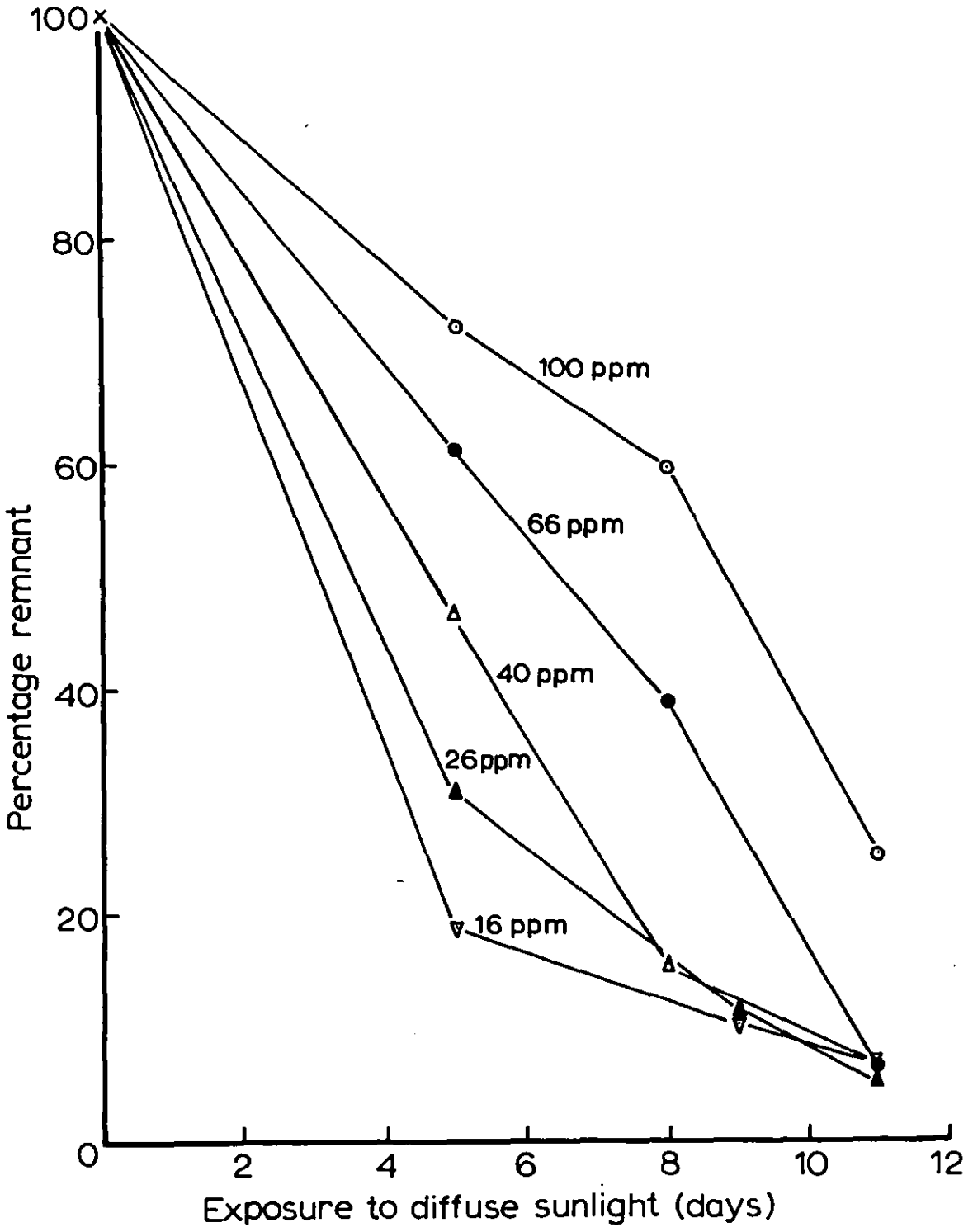


Fig. 19

Polarograms of erythrosine during photodegradation in natural light at pH 6·5

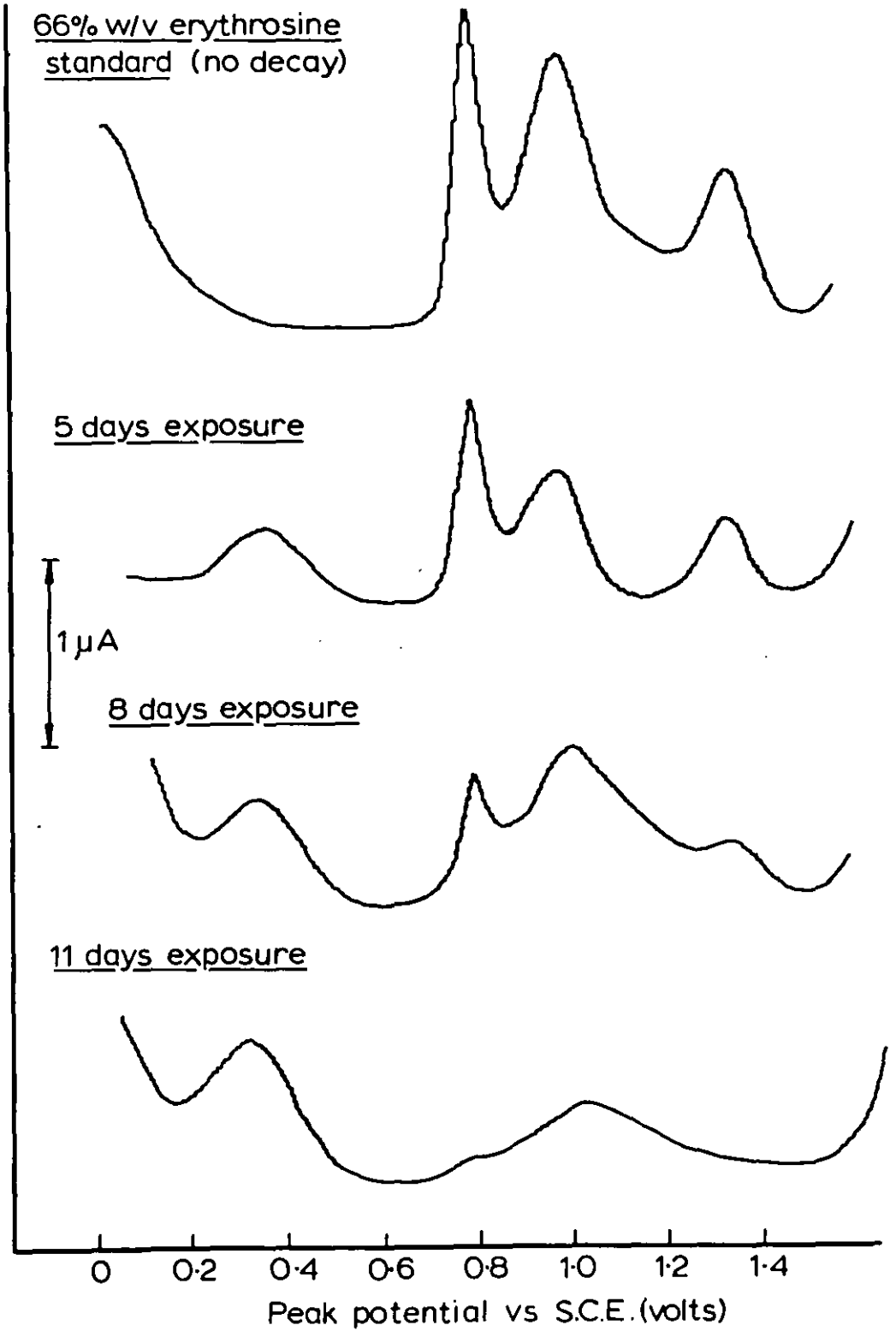


Fig. 20

Growth curve of new polarographic peak arising during erythrosine photodegradation

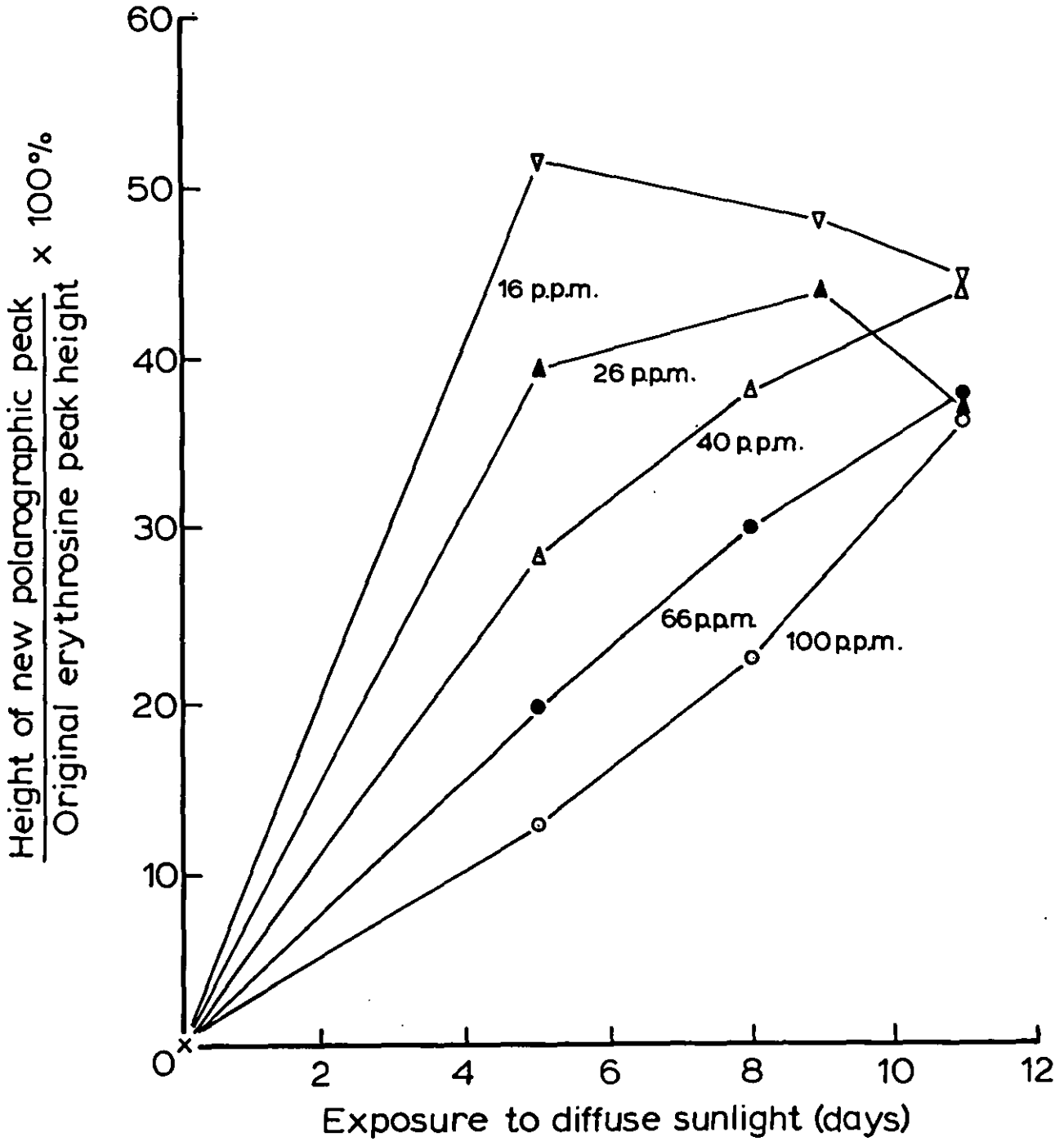


Fig. 21

Experimental (3rd Study)

Five solutions were prepared containing 40 ppm $^{w}/v$ erythrosine in 20% strength B-R buffer of pH 5.0, 5.5, 6.0, 6.5 (as 2nd study) and 7.0 respectively. The procedure then followed that of the 2nd erythrosine study above.

Results (3rd Study)

The degradation curves observed are given in Figure 22. The new polarographic peak was again measured. A spot test conducted at pH 10.0 showed complete loss of erythrosine (40 ppm $^{w}/v$) within two days.

Discussion

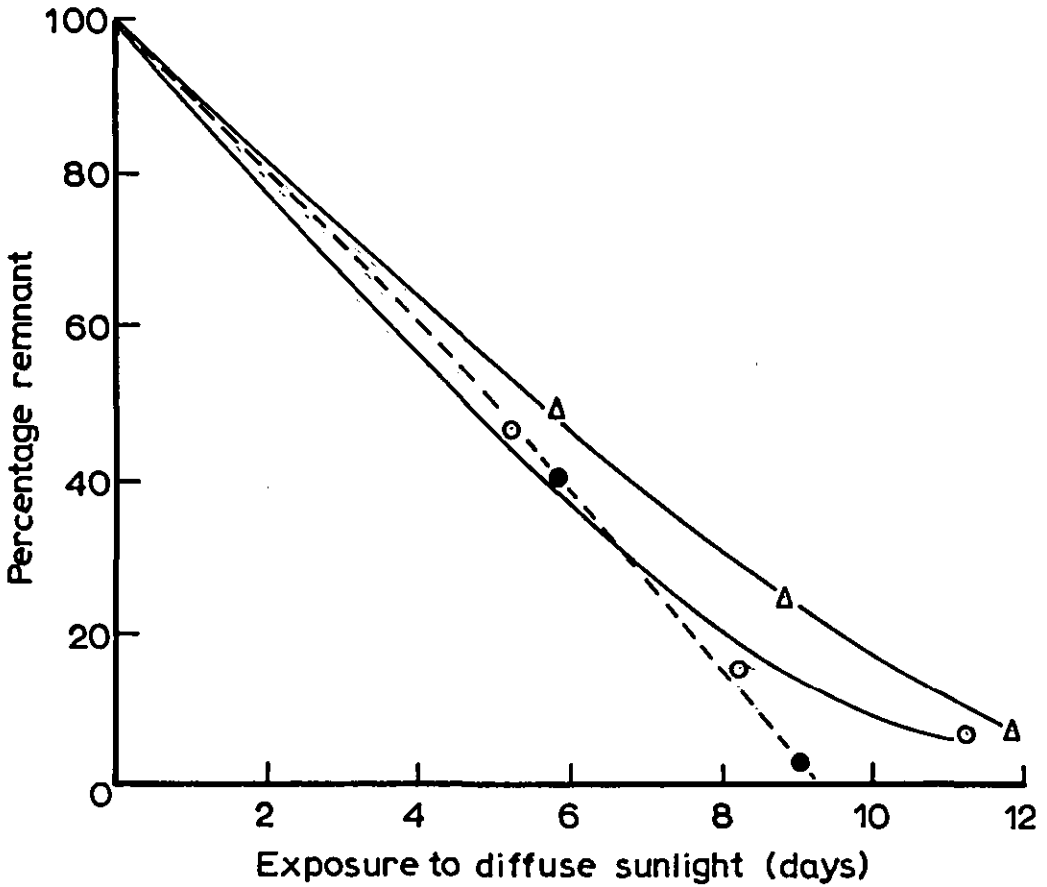
The results of the first experiment were unexpected in that no even trend occurred linking concentration with percentage decay rate. Rather the 40 ppm $^{w}/v$ solution was least stable (Figure 18). Though all three decay curves give moderately high linear correlation coefficients, viz., -0.97 for the 100 ppm and 40 ppm curves, and -0.96 for the 16 ppm curve, upon inspection, at least the latter two appear to be non-linear.

The main realisation that came from this first study was that more information was needed for comparison. The second study was conducted to provide data on a wider range of concentration. No deoxygenation was used in this case. As may be seen from Figure 19, in this study the percentage decay rate showed a correlation with concentration in the order of percentage decay, 16 ppm > 26 > 40 > 66 > 100, at least for moderate degradation.

Comparison with the first experiment shows one unmistakable result. All the degradation rates are much faster when oxygen is present than when it is exluded. Again in the second study the higher

Effect of pH on erythrosine photodegradation

(40 p.p.m. w/v aqueous solutions)



Δ pH 7·0

o pH 6·5

● (---) pH 5·0

Plots for pH 5·5 and pH 6·0 solutions were nearly identical to that for pH 5·0 except that at day 12 the pH 5·5 solution showed 1% retention and the pH 6·0 solution showed 3% retention.

Fig. 22

concentrations show best linearity. The linear regression lines for the 100 ppm erythrosine solutions in the respective studies give a quantitative measure of the effect of air-oxygen:

O_2 excluded (study 1): Remnant (%) = 100.7- 0.59 t (t = exposure, days)

Air present (study 2): Remnant (%) = 103.0 - 6.46 t

(correlation coefficient = -0.98)

And so, with no air-oxygen present the decay rate is ca. 0.6% per day, whereas with air-oxygen present it is 6% per day at 100 ppm. The influence of oxygen remains strong at lower concentrations, but it is less easy to obtain a quantitative measure.

Study 3 investigated the pH effect over a narrow range, viz., pH 5-7. The photodegradation seemed virtually unaffected for pH 5 - 6.5, and only slightly so for pH 7.0. [Few foods in which erythrosine is used are alkaline, or even neutral]. The precipitation observed in Study 1 below pH 4.5 limited the pH range on the acid side.

A novel feature was noted on one of the later polarograms obtained during Study 1: a new peak, or possibly a much enhanced minor peak previously present, was seen at -0.35 V. This had not been observed on most of the polarograms as these had been mainly commenced at c. -0.5V to shorten the analysis time. Therefore, when study 2 was commenced, the scan range was extended, the scan beginning at 0.0 V instead (i.e. scanning negative).

A growth curve for the new peak in study 2 is given in Figure 21. The growth can also be seen from the short sequence of polarograms reproduced in Figure 20. These show another important feature also: the shape of the three erythrosine peaks changes during the degradation. This appeared to be due to the growth of new polarographic peaks underneath the second and third erythrosine peaks (i.e. those at more negative

potential), though the first peak remained largely unaffected as could be judged by the fact that, after heavy degradation had occurred, this peak disappeared leaving no underlying peak. For this reason the first peak only was used to assess the amount of erythrosine present. Another reason for choosing this peak was its sharper form indicating good reversibility. The peak potential for this peak is given for comparison with that of the other peaks present in Table 10.

There is a possible indication from figure 21 that the new peak produced at c.-0.35 V (pH 6.5) is itself decaying at a rate not too dissimilar to the time scale of the experiment. Once the absolute decay rate of erythrosine (e.g. mmol/day) becomes small, two of the curves (16 ppm and 26 ppm) also start to fall.

TABLE 10

Potentials of erythrosine polarographic peaks in pH 6.5 B-R buffer:
-0.78 V : -0.97 V : -1.33 V (Height ratio of peaks: 1.0 : 0.69 : 0.44)
Potentials of photodegraded erythrosine polarographic peaks (pH 6.5):
-0.35 V : broad, possibly unresolved peak at c.-1.03 V.

Comparison of polarograms of erythrosine, diiodofluorescein and fluorescein

Experimental

Solutions were prepared containing c.15 ppm $^{w}/_{v}$ erythrosine, diiodofluorescein or fluorescein, 30% ethanol, 40% standard strength B-R buffer (pH 6.0) and q.v. distilled water. These were scanned polarographically over the range 0.0V to the cut-off at ca.-1.35 V, and the peak heights and positions measured.

Results

Colour	Concentration	Peak Positions (V) and [Heights (μA)]
Erythrosine	14.8 ppm $^{w}/_{v}$	$-0.01[1.2]$; $-0.56[2.3_2]$; $-0.81[1.7_2]$; $0.95[.94_5]$
Diodofluorescein	15.3 ppm $^{w}/_{v}$	$+0.01[.67_0]$; $-0.55[2.8_0]$; $-0.82[1.0_2]$; -1.2[E.C.O.
Fluorescein	15.5 ppm $^{w}/_{v}$	$-0.10[2.1_5]$; $-0.58[2.6_8]$; $-0.97[1.4_0]$

E.C.O. = on edge of cut off.

Discussion

Erythrosine has long been known to deiodinate under favourable conditions producing fluorescein. For instance, the action of a tin-iron couple in acidic solution on cherries coloured with precipitated erythrosine liberates fluorescein, whereas the boiling of erythrosine liberates iodine, but does not give fluorescein: it has been speculated that diiodofluorescein is the product.[62] In sulphur dioxide solution, storage has caused deiodination to an extent equivalent to 80% of the erythrosine losing one atom of iodine, though the product was not identified.[39]

In the present study of erythrosine photodegradation no greenish-yellow or other fluorescence could be detected by visual inspection which eliminates fluorescein as a degradation product. Spot tests indicate fluorescein to have sufficient light stability not to be lost as a transient daughter product.

The polarography of erythrosine, diiodofluorescein and fluorescein was originally attempted in simple aqueous 40% strength B-R buffer, but the diiodofluorescein proved too sparingly soluble for the method to work. Most of the light-degraded solutions had previously shown fine dispersions of an orange-red substance or a precipitate of this. This had originally been ascribed to the original erythrosine precipitating

on storage at higher pH values than other workers had noted, but upon finding the low solubility of diiodofluorescein, it was thought possible that some of the precipitate was this compound. Redissolving the precipitate was certainly far less easy than dissolving erythrosine. The colour of diiodofluorescein is very similar to that of erythrosine. Neither compound is significantly fluorescent unlike fluorescein.

Erythrosine, fluorescein and diiodofluorescein were all successfully dissolved in the ethanol-aqueous buffer mixture described. However, the three compounds all exhibited the sharp peak at -0.55 to -0.58 V (which is the peak at -0.71 V on the pH 6.0 erythrosine polarogram run without ethanol in solution), and the relative intensities of those peaks leaves no doubt that the new peak at c.-1.03 V (pH 6.5) is not due to either diiodofluorescein or fluorescein because the -0.78 V (pH 6.5) peak would not disappear were this to be so. Moreover, the precipitation of diiodofluorescein would be too complete to give the peak intensity observed in some polarograms.

Whereas the degradation products remain unidentified, it is possible to consider the shape of the decay curves from a kinetic view point. An authoritative kinetic treatment would naturally require knowledge of both the mechanism and the products, but in fact it is kinetic data that is often used to help to derive the mechanism, and certainly a decay curve can give some indication of the likely mechanism in simpler cases. The erythrosine case is reviewed in this way below, but first a consideration of the Beer-Lambert Law and of 1st order kinetics will be useful.

Beer-Lambert Law[137,138]

The Beer-Lambert law may be stated in the form,

$I/I_0 = 10^{-\varepsilon c l}$ where I = emergent light intensity

I_0 = incident light intensity

ε = molar absorptivity ($l\,mole^{-1}\,cm^{-1}$)

c = concentration (mole l^{-1})

l = path length (cm)

This simple formula only holds true under a limited range of conditions which include:

(i) solutions should be < 10^{-3} M for A to remain linear with C as this relies on the refractive index, n, staying constant. [$\epsilon n/(n+2)^2$ is the parameter that actually remains constant];

(ii) high absorbance readings, i.e. $I \ll I_0$, suffer distortions due to the increased influence of stray light reaching the detector;

(iii) low absorbance readings, i.e. $I \approx I_0$, suffer disproportionate uncertainty and so are imprecise (sets limit of detection);

(iv) analytes must not alter their chemical nature with dilution, e.g. dissociate;

(v) temperature should be held constant - increase in temperature tends to shift absorbance bands to longer wavelength.

Self-shielding in Solution

Were every interaction between a dye molecule and an incident photon to result in an irreversible chemical change destroying the chromophore, then the concentration of a solution would not affect the percentage decay rate of the dye, assuming physical shielding to be the only effect operating. In fact only a small fraction, ϕ ($0 < \phi < 1$) of such interaction results in decay: otherwise even the least light-fast dye would be still far less so. Other possibilities include:

(a) scatter (no absorption) - same wavelength

(b) re-emission (fluorescence) - longer wavelength generally

(c) deactivation (collisional, vibratory).

The Beer-Lambert law is non-qualitative: it does not describe the fate of molecules absorbing photons, only the extent of such absorption. Scatter and re-emission largely occur at angles quite dissimilar to 180° to the incident angle and so the incident radiation will decrease for molecules shielded by intervening molecules.

Expected Result for Photodegradation when 1st Order Kinetics Operate

Ordinary 1st order kinetics[139] are frequently used to describe decomposition reactions, viz., A → B + C. If the initial concentration of A is a, and after a time t, x moles per litre of A have degraded, then,

$$\frac{dx}{dt} = k_1 (a - x) \qquad \text{where } k_1 \text{ is the 1st order rate constant.}$$

Integration gives $- \ln (a - x) = k_1 t + \text{constant}$

If $x = 0$ at $t = 0$ the constant of integration is $-\ln a$.

Hence, $$\ln\left(\frac{a}{a-x}\right) = k_1 t$$

or $$\underline{\underline{x = a (1 - e^{-k_1 t})}}$$

The usual test of whether ordinary 1st order kinetics are applicable is a plot of $\ln(\frac{a}{a-x})$ vs. t. A linear curve indicates 1st order kinetics, the gradient being k_1.

In dilute solutions where the light intensity I_0 was approximately constant at every path length, l, through the solution, 1st order kinetics might be expected.

Kinetics of Photodegradation

The rate of reaction, in the simplest case, depends upon the

number of photons absorbed (in a suitable frequency range) per unit time. Let the photon yield be ϕ and the incident light intensity be I_0:- then

$$\frac{dx}{dt} = k\ I_0\ \phi\ (1 - 10^{-\varepsilon C l}),\ \text{or, as}\ \frac{dx}{dt} = -\frac{dC}{dt},$$

$$\frac{dC}{1 - \exp(-2.303\ \varepsilon C l)} = -kI_0\phi\ .dt,\ \text{or,}$$

$$\frac{\exp(2.303\varepsilon C l).dC}{\exp(2.303\ \varepsilon C l)-1} = -kI_0\phi\ t + \text{constant}$$

From the rule that $\int \frac{du/dt}{u}\ .\ dt = \log_e u$ it follows that

$$-kI_0\phi\ t = \log_e\ \{\exp(2.303\ \varepsilon C l)-1\}\ .\ \frac{0.4343}{\varepsilon l} + \text{constant.}$$

When $t = 0$, constant $= -\ \log_e\ \{\exp(2.3030\ \varepsilon C_0 l)-1\}\ .\ \frac{0.4343}{\varepsilon l}$

or, $kI_0\phi t = \log_e\ \{\frac{\exp(2.303\varepsilon C_0 l)-1}{\exp(2.303\varepsilon C l)-1}\}\ .\ \frac{0.4343}{\varepsilon l}$

When there is <u>high</u> shielding, i.e. $2.303\varepsilon C l > 5$ (of course, $C_0 > C$) this expression reduces to $kI_0\phi t = \log_e\ \{\frac{\exp(2.303\varepsilon C_0 l)}{\exp(2.303\varepsilon C l)}\}\ .\frac{0.4343}{\varepsilon l}$

$$kI_0\phi t = \log_e\{\exp(2.303\varepsilon l.(C_0-C))\}\ .\ \frac{0.4343}{\varepsilon l}$$

or, $2.303\ kI_0\phi\varepsilon l t = 2.3030\varepsilon l.\ (C_0-C)$

So $\frac{t}{C_0 - C} = \text{constant} = \frac{1}{kI_0\phi}$

(i.e. a linear decay curve persists until $2.303\ \varepsilon C l < 5$).

For <u>low</u> shielding, $2.303\varepsilon C l < 0.05$

because $e^x = 1 + x + x^2/2! \ldots$, $e^x \to 1 + x$ for small x

So, $kI_0\phi t = \log_e\ \{\frac{\exp\ (2.303\varepsilon C_0 l)-1}{\exp\ (2.303\varepsilon C l)\ -1}\}\ \ .\frac{0.4343}{\varepsilon l}$

The right hand side tends to

$$\log_e\ \{\frac{2.303\varepsilon C_0 l}{2.303\varepsilon C l}\}\ \ .\ \frac{0.4343}{\varepsilon l}$$

or $kI_0\phi t = \frac{0.4343}{\varepsilon l}\ .\ \log_e\ (C_0/C)$

Therefore, for dilute solutions first order kinetics come into operation, i.e. rate $\propto$ concentration

$$(^{dx}/_{dt} \propto C).$$

Between the linear and exponential limits of behaviour, in the region of rapidly changing shielding with changing concentration, intermediate behaviour is found.

Photodegradation: erythrosine

Because glass containers were employed, and the light had already passed through a glass window, there was probably little ultraviolet in the light reaching the trial solutions. Measurement of the visible spectrum of a commerical sample of erythrosine showed a molar extinction coefficient of 76,000 1 mol^{-1} cm^{-1} at the peak maximum of 528 nm. The value of 48 - 49,000 1 mol^{-1} cm^{-1} is more typical of the peak as a whole (integrated average ε). For a 16 ppm $^{w}/_{v}$ solution, a path length of 4 cms and with erythrosine having a molecular weight of c. 880, then $\frac{\varepsilon C l}{0.4343}$ = 8.2. However during degradation the value would fall rapidly below 5. A plot of this and other concentrations up to 100 ppm is shown in Figure 23.

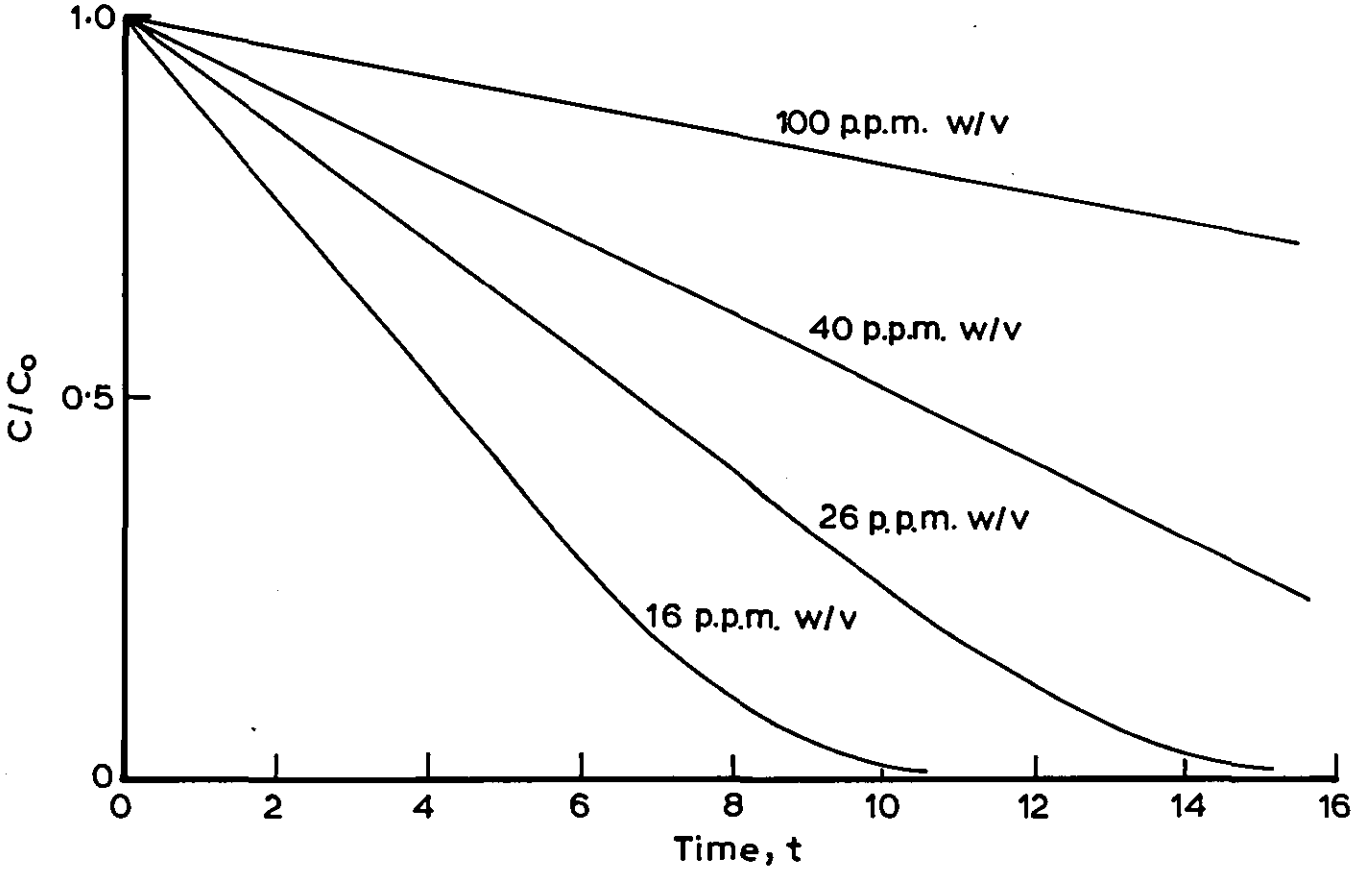
Comparison may be made with the decay and growth curves, Figures 21 and 19. In Figure 19, the initial fall off rates are linear for the 40, 66 and 100 ppm $^{w}/_{v}$ solutions. The gradients derived from the t = 0, 5, 8 day points are 4.8%/day (100 ppm), 7.6%/day (66 ppm) and 10.6%/day (40 ppm). If the theory that concentrated solutions, giving linear decay curves over a portion of their decay, are related by the equation

$$t \propto C_0 - C$$

is correct, the 100, 66 and 40 ppm solutions should give gradients of 1 : 1.52 : 2.5 respectively. In fact the ratio found is 1 : 1.58 : 2.21.

Theoretical decay curves for erythrosine

Plot of $t = \log_e \left\{ \frac{\exp\left(\frac{\varepsilon C_0 l}{0{\cdot}4343}\right) - 1}{\exp\left(\frac{\varepsilon C l}{0{\cdot}4343}\right) - 1} \right\}$



ε = 49,000 l mol^{-1}cm^{-1}

l = 4 cm

$C_0 = \frac{\text{conc}^{n}.\text{(p.p.m.)} \times 10^3}{880}$

Fig. 23

For the growth curves (Figure 21) the linearity is less good because there is evidently secondary degradation coming into play, at least for the more dilute solutions. However comparison of the 5 day exposed solutions show the 100, 66 and 40 ppm $^{w}/_{v}$ solutions to have relative growth rates of 1 : 1.51 : 2.19 suggesting that while secondary degradation is a minor factor, the model fits and that the amount of product formed is independent of concentration at those higher concentrations.

Factors that may limit the kinetic model used include,

(i) variation in quantum yield with concentration - the collisional deexcitation of molecules could be concentration dependent etc.,

(ii) secondary processes with concentration dependency,

(iii) generation of diiodofluorescein which, despite its insolubility, might distort polarographic data for the most dilute solutions,

(iv) frequency dependency is unknown and limits the estimation of ε - as does the fact that the Lambert-Beer law is only valid for single wavelengths, not over absorption bands.

Photodegradation Studies on Indigo Carmine

Introduction

Sighting studies of degradation in natural light produced conflicting results. The 100 ppm $^{w}/_{v}$ solution initially degraded faster than other food dyes except for erythrosine, but failed to degrade totally for several months. A 50 ppm $^{w}/_{v}$ solution had degraded completely leaving a yellowish solution when examined after 90 hours exposure in the 500 W light box. Another 100 ppm $^{w}/_{v}$ solution stored at room temperature in a darkened cupboard turned to a greenish-blue shade after several months.

It was decided to take measurements of the photodegradation in natural light and at a range of concentrations to try to clarify matters.

Experiment:

Aqueous solutions were prepared containing 10, 30 and 100 ppm $^{w}/_{v}$ indigo carmine respectively. These were stored in diffuse sunlight (June-July) and sampled at intervals. Controls were kept in a darkened cupboard, and measurements made by visible spectroscopy (609 nm maximum).

Results

The decay curves are presented in Figure 24.

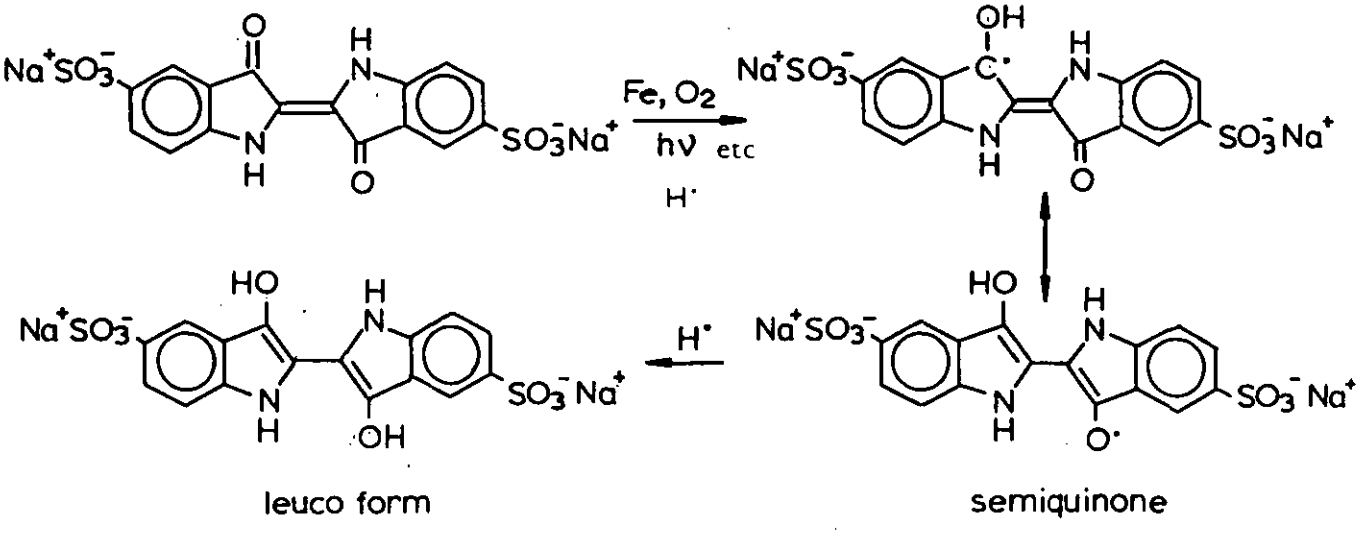
Mechanism of Indigo Carmine Photodegradation

According to Kuramoto et.al. (1958)[25], the kinetics of indigo carmine photodegradation in the presence of a range of reducing compounds, e.g. sucrose, lactose, depend upon,

(i) light intensity, and

(ii) the course of an oxidation-reduction reaction.

The sequence of their postulated reaction is:-



Photodegradation of indigo carmine in natural light

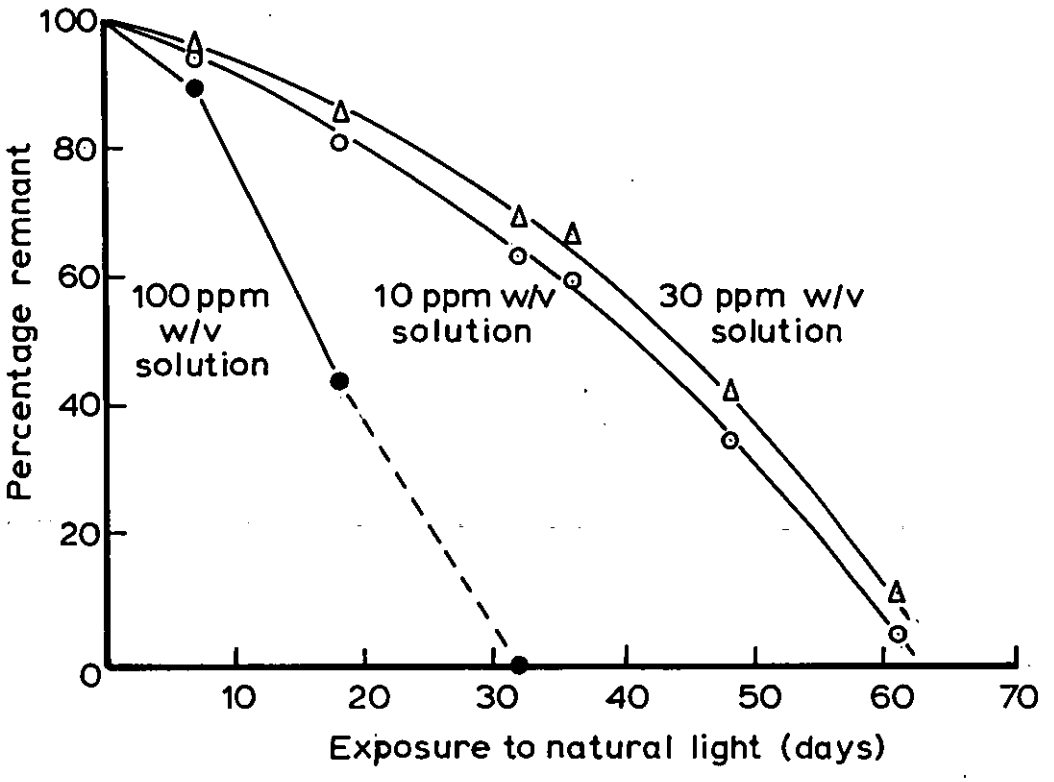


Fig. 24

Kuramoto et.al. believed that the shape of the degradation curve was explained by there being an induction period during which semiquinone was being produced, followed by a linear decay curve which they describe as "1st order". In fact their picture of the kinetics seems inconsistent with their results. The reasons are two-fold:

(i) having stated the semiquinone to be a highly unstable intermediate, they go on to consider its reduction to be the rate determining step: yet they describe an initial build up of this same species in order to explain an "induction period" prior to significant degradation, though the time scale of the experiments, with constant irradiation, is weeks rather than seconds;

(ii) despite the linearity of the decay curve over an important section of the plot, a fact that the authors remark upon (i.e. $\frac{dC}{dt}$ = constant), they go on to claim that as the rate determining step is the reduction of the semiquinone, then 1st order kinetics operate, i.e. $\frac{dC}{dt} = k[C]$. In fact such kinetics give an exponential decay, $C = C_0 \exp(-kt)$. The slope of the plot would be 'concave' where it is in fact 'convex' or linear.

However, the authors point to the following facts in previous literature to support their mechanism:

(i) an intermediate compound was believed to exist in the reduction;

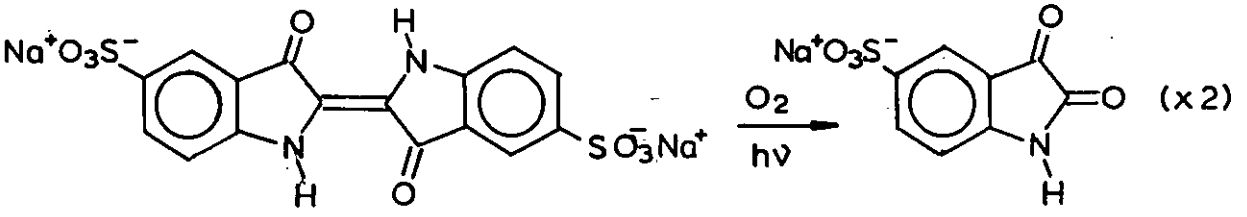
(ii) the reduction was believed to be a $2e^-$ one; and

(iii) the oxidation-reduction of anthraquinoid dyes were believed to proceed by a free-radical mechanism.

Inspection of the results presented suggests that no well

defined induction period is obvious, and that the graphs are not linear but mildly 'convex' (rate slowly increasing with time). The paper does however offer one important result: certain reducing agents do increase the rate of decay significantly, though without any obvious qualitative change in form of the decay curves.

More recent evidence for the decay of indigo carmine in aqueous solution has shown that (in the absence of reducing agents), the product obtained is:-



Kinetic Model

The unpredictability of some of the observed behaviour has led to the suspicion that catalysis by trace metal ions may influence the decay, but no proof for this has been sought. The commonly found shape of the decay curves from the present aqueous solutions, and in the work of Kuramoto et.al. using aqueous solutions containing reducing agents, strongly suggests that some product or products of the decay promote the reaction, possibly by stabilizing an intermediate or excited species which might otherwise revert to the parent compound, or by otherwise catalysing a less favoured pathway. The general type of kinetic law that this might follow may be written as below:

$$\frac{dx}{dt} = -\frac{dC}{dt} = k_1 I_0 \phi\{1 - \exp(-2.303\ \varepsilon C l)\} + k_2 x$$

where x and C are the concentrations of the degradation product and starting compound respectively ($x = C_0 - C$).

$$-\int \frac{dC}{k_1 I_0 \phi\{1-\exp(-2.303\varepsilon C l)\} + k_2(C_0-C)} = \int dt$$

(i) For $2.303\varepsilon C l \gg 5$, this tends to the limit

$$k_2 t = \log_e\{k_1 I_0 \phi + k_2(C_0-C)\} + \text{constant}$$

When $t = 0$ then $C = C_0$ and the constant $= -\log_e\{k_1 I_0 \phi\}$,

giving $k_2 t = \log_e\left\{1 + \frac{k_2(C_0-C)}{k_1 I_0 \phi}\right\}$

This formula generates degradation curves similar in shape to those obtained both in the present work and in the study reported by Kuramoto et.al., depending on the values taken by the constants k_1, k_2, I_0, ϕ and C_0 (See Figure 25).

(ii) For $2.303\varepsilon C l$ between 0.05 and 5, numerical integration is needed.

(iii) For $2.303\varepsilon C l \ll 0.05$, the integration tends to the limit,

$$t = -\int \frac{dC}{2.303 k_1 I_0 \phi \varepsilon C l + k_2(C_0-C)} = -\int \frac{dC}{k_2 C_0 + C(2.303\varepsilon l k_1 I_0 \phi - k_2)}$$

At $t = 0$, $C = C_0$ as before giving the result,

$$t = \log_e\left\{\frac{0.4343 k_2}{\varepsilon l k_1 I_0 \phi} + \frac{C}{C_0}\left(1 - \frac{0.4343 k_2}{\varepsilon l k_1 I_0 \phi}\right)\right\} \cdot \frac{1}{k_2 - 2.303\varepsilon l k_1 I_0 \phi}$$

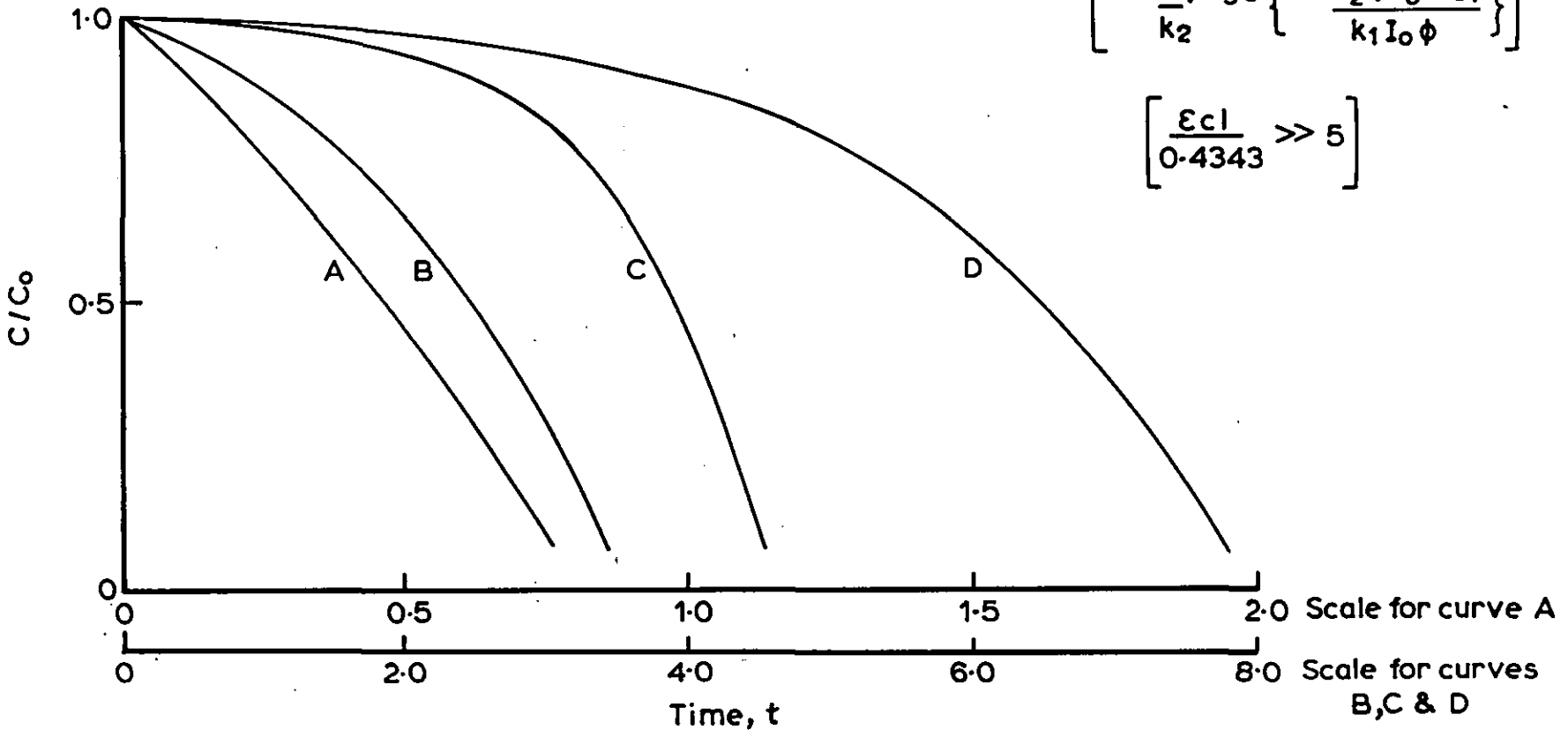
This third solution is of small importance as it describes conditions of very low light absorption which would give little decay, or where C_0 is very low, below the levels usually expected in foods.

This model does not claim any particular mechanism by which the degradation product itself accelerates the degradation, but only illustrates that if it does so, the decay curves obtained experimentally tally with curves obtained theoretically. No need arises to invoke "induction periods" to satisfy the results. Further, the simple linear dependence of $\frac{dx}{dt}$ on x chosen for this model is arbitrary and need not apply in this particular form for the general autocatalytic principle to be operating.

Theoretical decay curves for autocatalytic decay

$$\left[t = \frac{1}{k_2} . \log_e \left\{ 1 + \frac{k_2 (C_o - C)}{k_1 I_o \phi} \right\} \right]$$

$$\left[\frac{\varepsilon c l}{0{\cdot}4343} \gg 5 \right]$$



A $k_2 = 0{\cdot}5$, $C_o = 10^{-4}$, $k_1 I_o \phi = 10^{-4}$

B $k_2 = 0{\cdot}5$, $C_o = 10^{-4}$, $k_1 I_o \phi = 10^{-5}$

C $k_2 = 1{\cdot}0$, $C_o = 10^{-4}$, $k_1 I_o \phi = 10^{-6}$

D $k_2 = 0{\cdot}5$, $C_o = 10^{-4}$, $k_1 I_o \phi = 10^{-6}$

Fig. 25

Comparative Study of Food Dye Photostability

Experimental

The light box was large enough for fourteen bottles to be held simultaneously. The two colours erythrosine and indigo carmine were being dealt with separately and had already proved to be significantly less light-fast than the others, and so were omitted from the study. The other permitted food colours were all included.

Each solution comprised 48 - 52 ppm $^{w}/_{v}$ food colouring matter, with no buffer. The solutions were divided to provide controls, and the bottles containing the solutions were arranged on the shelf inside the light box or in a darkened cupboard accordingly.

The samples were withdrawn after periods of irradiation and polarographically compared with the controls.

Results

The results are presented graphically in Figures 26a - 26d.. The least light stability was shown by ponceau 4R, followed by Chocolate Brown HT. Apart from these only brown FK had very heavy loss during the degradation period of 30 days 16 hours (736 hours). The other eleven colours ranged from showing virtually no loss (sunset yellow FCF) to 60% loss (carmoisine).

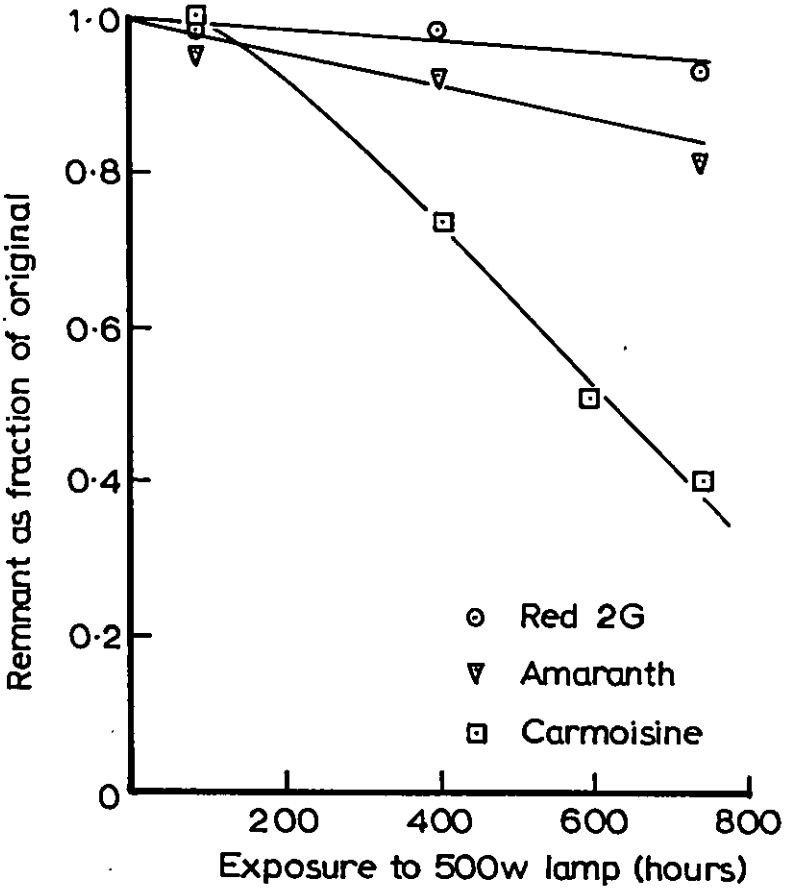
Discussion

There is no particular value in considering the shape of the decay curve for those colouring matters that suffered only slight degradation, e.g. < 15%. The reasons for this are two-fold:

(i) insufficient of the projected decay curve is obtained.

(ii) secondary features may only become apparent at higher levels of decay as is evidenced by other curves.

(a) Photostability of red colouring matters



(b) Photostability of yellow colouring matters

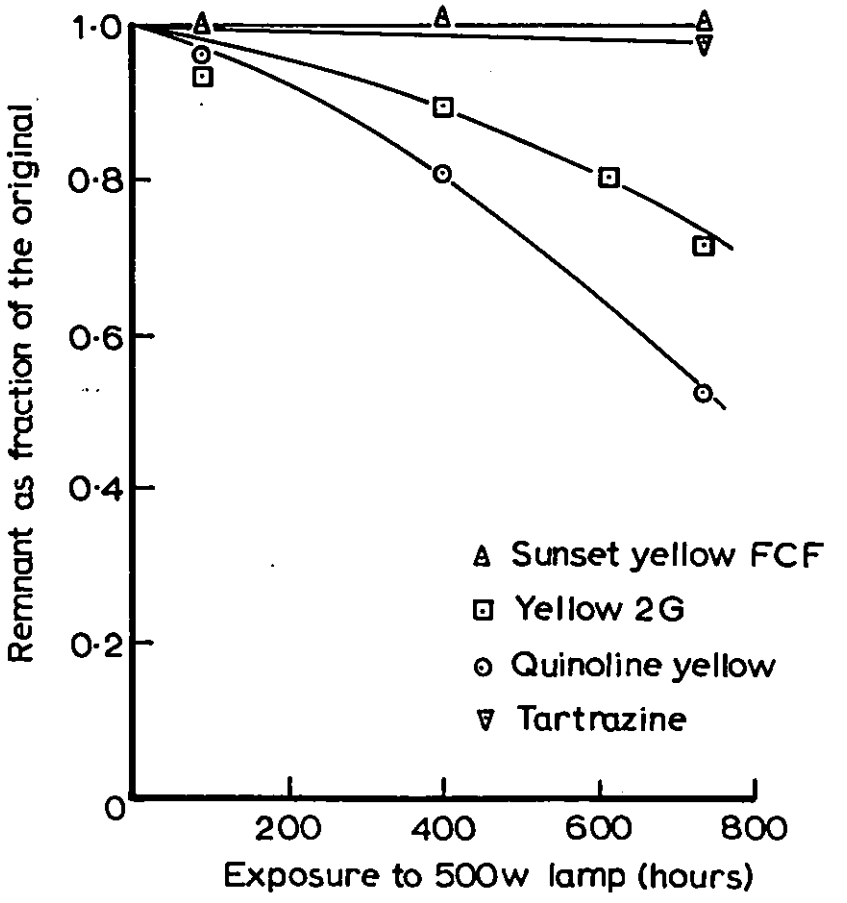
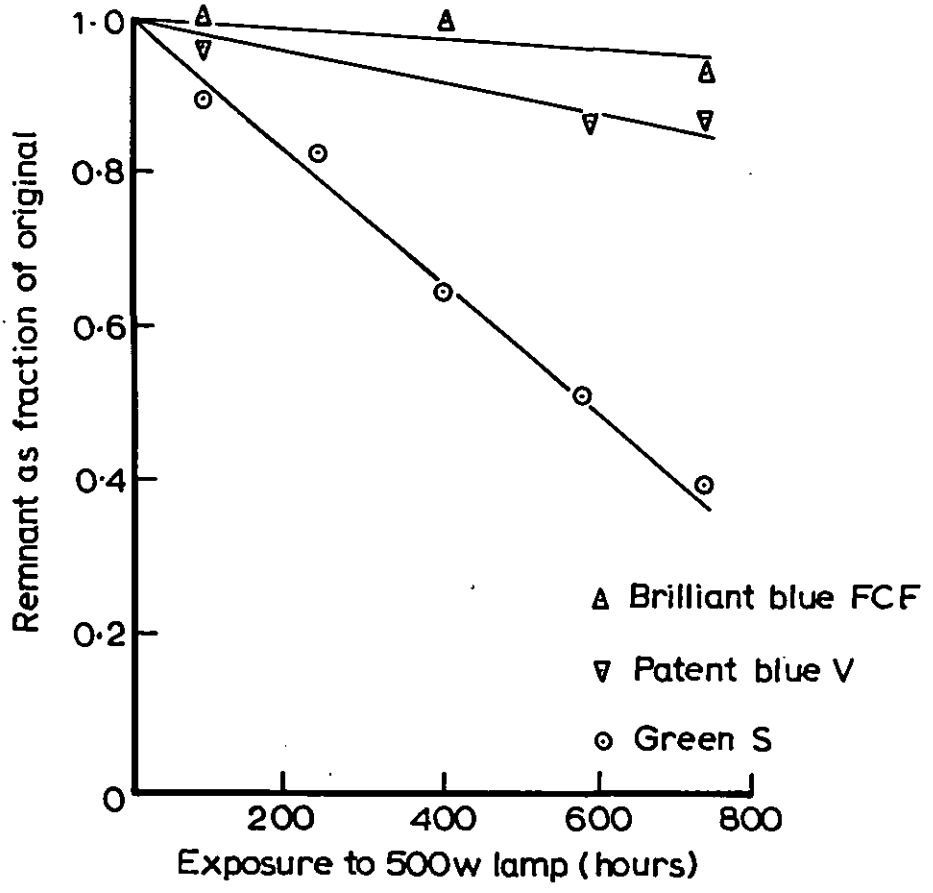


Fig. 26

(c) Photostability of blue and green / blue colouring matters



(d) Photostability of miscellaneous colouring matters

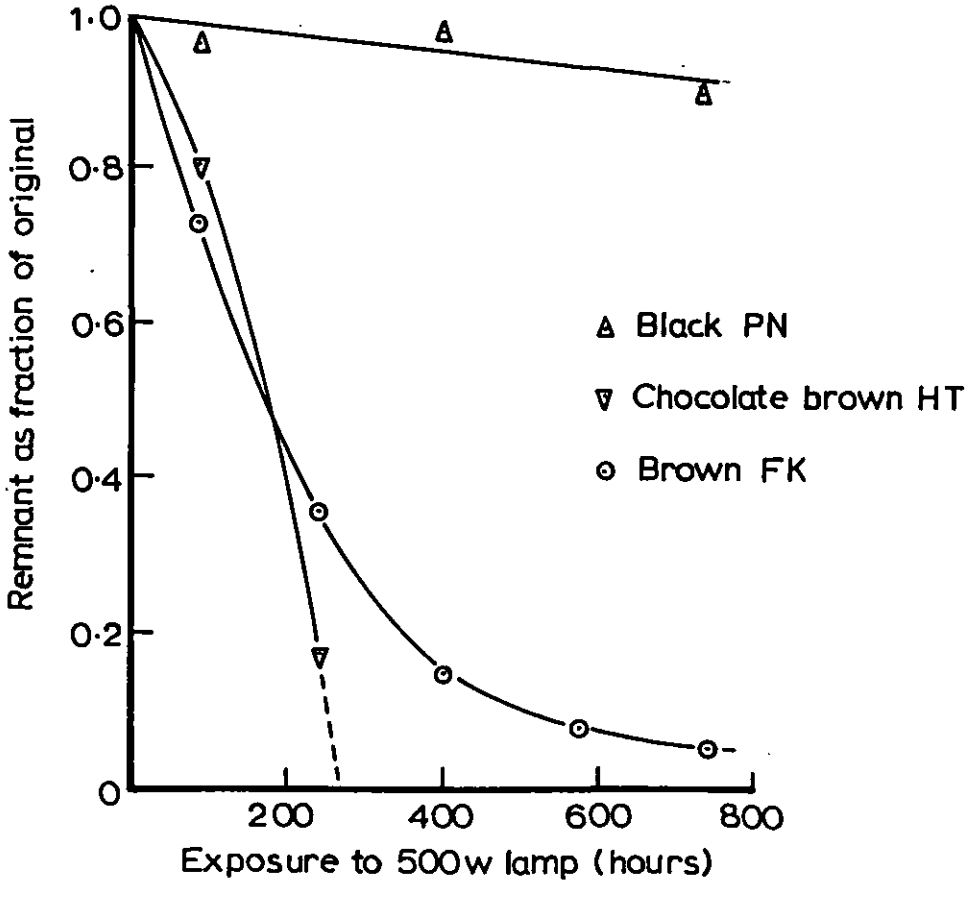


Fig. 26 (cont'd)

For these reasons it is better to state quite simply that, over the 736 hour period, brilliant blue FCF, black PN, patent blue V, red 2G, sunset yellow FCF and tartrazine all show very good light stability at 50 ppm $^{w}/v$. It is unlikely that anything short of direct sunlight over a several month period could greatly affect these dyes without the participation of other factors, e.g. the presence of chemicals.

The decay of ponceau 4R was sufficiently faster than that of other colours to make it worthwhile to undertake a more detailed, shorter term study of this separately using the light box.

For the other colours, it is possible to see a variety of kinetics operating.

Brown FK

Figure 26d showing the brown FK decay curve is redrawn as a logarithmic plot in Figure 27. The least squares best fit line corresponds to

$$-0.0195 - (0.0043 \times \text{exposure[hours]}) = \log_e (\text{remnant [fraction]})$$

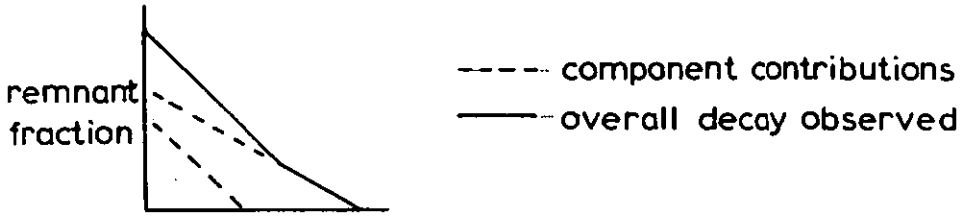
which has a correlation coefficient of -0.995. Ignoring the small zero error, the rate equation can be written as

$$C = C_0 \exp - 0.0043\ t \qquad (t \text{ in hours})$$

This is a difficult result to interpret. When the visible spectrum of brown FK is measured, a 25 ppm $^{w}/v$ solution gives an absorbance at 450 nm in a 1 cm cell of 1.24. Therefore a 50 ppm $^{w}/v$ solution in a bottle of 4.5 cm internal diameter could be estimated to give a value of $\frac{\varepsilon c l}{0.4343} > 10$. In other words, a linear decay curve might be anticipated in the simplest case.

The fact that brown FK is a mixture of six component dyes does not necessarily affect this problem. The sum of six linear decay

curves would still result in an overall linear decay curve, except that the boundary condition C > 0 might cause the overall pattern to include more than one straight line were the times for total decay of the individual dyes to differ, e.g. for two components.



Though a sequence of such steps might, from a limited number of measurements, be mistaken for exponential decay, the likelihood seems somewhat remote.

In that case, is the apparently straightforward explanation any better, namely that true 1st order kinetics are operating? There arises a mathematical query if this is so: as a single major polarographic signal is observed for the six component mixture then,

$$\text{peak current} \propto C_1 \exp - k_1 t + C_2 \exp - k_2 t \ldots + C_6 \exp - k_6 t$$

where $C_1 - C_6$ are the component concentrations at $t = 0$,
and $k_1 - k_6$ are the respective rate constants.

The difficulty is that the overall observed decay is only itself exponential for the special case that $k_1 = k_2 \ldots = k_6$, giving

$$\text{peak current} \propto \left(\sum_{n=1}^{6} C_n\right) \exp - k_1 t$$

If the rate constants do differ, one returns to the 'sequence of steps' model suggested above, albeit with the refinement that no abrupt changes in the slope occur, and no $C_n \exp - k_n t$ value ever = 0, only $\to 0$. In practice, differing k_n values could be admitted within a narrow range, and this possibility is supported by the similarities of structure of the component dyes, and indeed by the presence of a single

major polarographic peak suggesting similar reduction potentials and hence reduction rates. None of this is conclusive, nor is any basis for exponential decay particularly apparent.

Linear Decay Curves

As was shown in discussing the photodegradation of erythrosine, where the rate of decay observed is a linear function of the light absorption ($\frac{dC}{dt} = -kI_0\phi(1 - 10^{-\varepsilon Cl})$) the resulting decay curve is linear while $\frac{\varepsilon Cl}{0.4343} > 5$.

Linear decay curves were found (Figure 26) for green S and amaranth. Green S has a high extinction coefficient. The value of $\frac{\varepsilon Cl}{0.4343}$ for a 50 ppm $^{w}/_{v}$ solution was estimated from the absorbance of a 10 ppm $^{w}/_{v}$ solution (Abs. = 1.22 at 637 nm; 1 cm cell) to be 14.0 for a 1 cm pathlength, and to be very high for a 4.5 cm. internal diameter bottle. Hence the linear result is as expected. For amaranth the value of $\frac{\varepsilon Cl}{0.4343}$ was similarly estimated at 3.8 for a 1 cm path length, so that for a solution in a 4.5 cm i.d. bottle the limited degree of decay observed would be expected to take place in a linear region.

Carmoisine

The high degree of linearity of the decay curve, excepting the first 100 hours raises the possibility that there is, indeed, an induction period prior to linear decay commencing. A free radical mechanism can be delayed if free radical inhibitors are initially present. Alternatively, the data might be fitted with rather more difficulty either to simple linear decay or an autocatalytic model.

Yellow 2G, Chocolate Brown HT and Quinoline Yellow

All these compounds shown an increasing rate of degradation

with degree of degradation that suggests similarities with the indigo carmine case. Autocatalysis or any type of involvement of the decay products in promoting the decay might be involved.

Ponceau 4R

In the initial study, the 90 hour exposed solution (500 W lamp) showed only 3% loss, but the 240 hour exposed solution showed very heavy (98 - 99%) loss. As this was insufficient data to get a true picture of the decay a 120 hour study was carried out independently, the results being shown in Figure 28. A re-check of these results revealed a similarly shaped decay curve but with the decay delayed by a few hours (See Figure 28).

This indicates that a rather finely tuned induction period may occur, the controlling parameters producing an amplified effect for small variation. The main mechanism would be explicable in terms similar to those given for the indigo carmine case, with the modification that here the estimated value for $\frac{\varepsilon Cl}{0.4343}$ is rather less, i.e. 5 or below.

Conclusions

The various findings of accelerating, constant and decelerating decay rates as decay proceeds, and the involvement of the parameters concentration, extinction coefficient, path length, quantum yield and light intensity in determining both the shape and timescale of the decay curves, lead to the recognition that much of the published literature is limited in its predictive use to describing conditions, and exposure periods similar to those adopted during the trials themselves.

For instance, other factors being unchanged, a comparison of the red colouring matters ponceau 4R, carmoisine, amaranth and red 2G over a

Indication of first order decay kinetics in Brown FK photodegradation

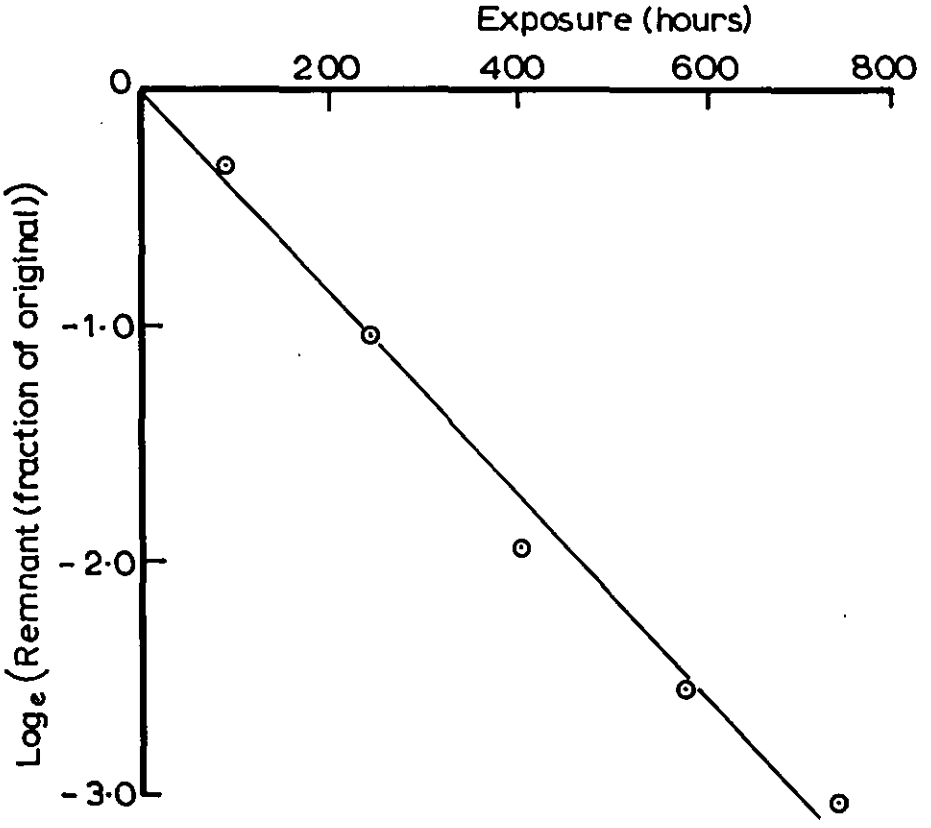


Fig. 27

Ponceau 4R photodegradation curve

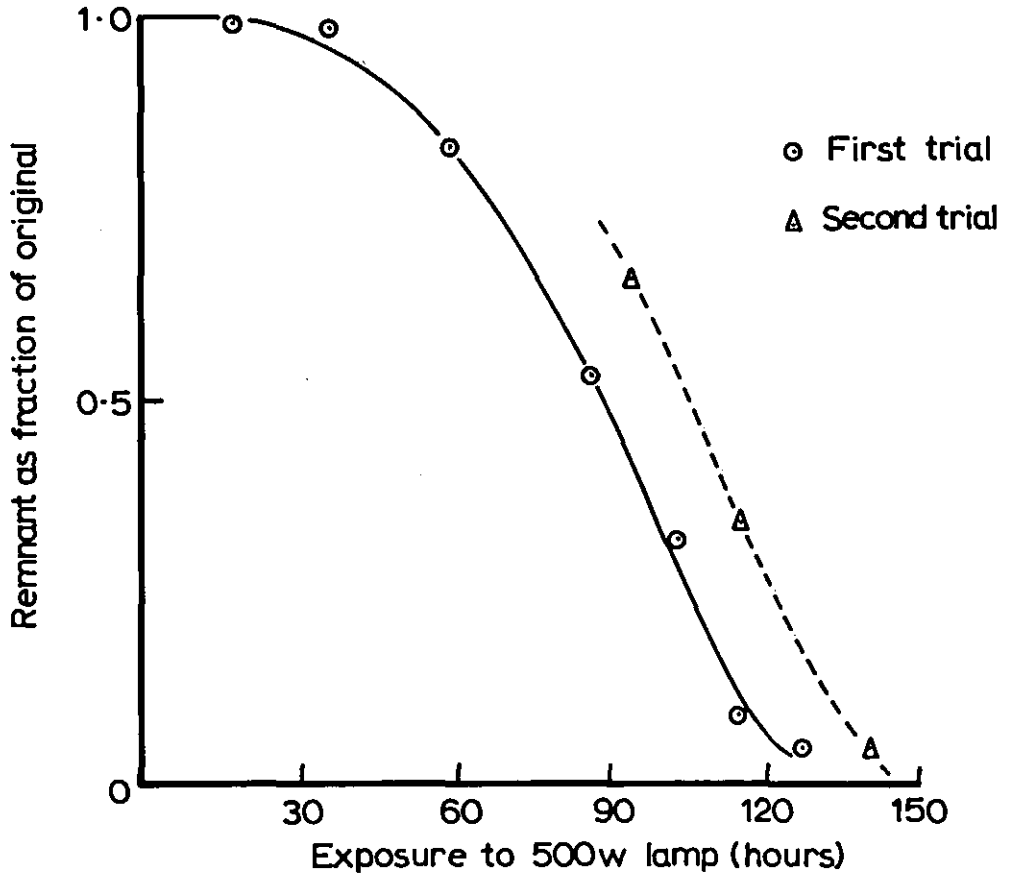


Fig. 28

100 hour trial, or of the browns, brown FK and chocolate brown HT over a 200 hour trial, would not be a good guide to the outcome of the full 700 hour experiment, or form a good basis for deciding combinations of colours for particular applications.

In general, the relative light stability of the colouring matters tested, under the conditions employed, show indigo carmine and erythrosine to have poor light stability; chocolate brown HT, brown FK and ponceau 4R to have intermediate stability; green S, carmoisine, and yellow 2G to have good stability; and the other colours to have excellent light stability.

Chapter 7: Thermal Degradation of the Food Colours

Section A: Polarographic Studies

Introduction

Aromatic azo compounds generally show good thermal stability at the azo linkage, except for syn-anti isomerisation.[140b] Aryl radicals are produced with difficulty by thermolysis unless the radical, e.g. triphenylmethyl, is able to adopt a stabilizing configuration and electronic structure.

As in the case of the light studies, initial sighting experiments were performed. For these an oven with a simple thermostatic control was employed. This oven had no window, and so was entirely dark inside.

Experimental

(i) amaranth, tartrazine and green S were prepared as 100 ppm $^{w}/_{v}$ aqueous solutions and stored in an oven at 55°C for several months, being sampled at intervals. The solutions were held in autoclavable screw-top bottles made from Duran glass (neutral glass).

(ii) glass phials were filled with 20 ml of an unbuffered, aqueous solution of 100 ppm $^{w}/_{v}$ colouring matter. The phials were sealed using a glass blowers torch, care being taken to prevent the wall thickness lessening during the sealing process by undue uneven flow of glass, which did, however, result in the micropuncture of one phial and the boiling off of the water.

The sealed phials were placed in the oven preset at 110°C (107-112°C), and left there for 15-21 days. Control samples were stored in darkened cupboards, under ambient conditions.

(iii) thicker walled phials were taken and filled with 250 ppm $^{w}/_{v}$ solutions, then heated as before but at 120°C for 21 days each.

Visual Appearance Changes

The following transformations in appearance occurred to 250 ppm $^{w}/_{v}$ solutions of the food colours after 21 days heating in sealed phials at 120°C;

amaranth - significant 'on-shade' fading.

black PN - original purple changing to pale red brown.

brilliant blue FCF - initial dark blue going to violet.

carmoisine - significant 'on-shade' fading.

chocolate brown HT - red brown to pale yellow brown.

green S - initial dark green-blue giving dark blue.

indigo carmine - dark blue going to pale green.

patent blue V - dark blue changing to lime green.

ponceau 4R - initial red changing to pale yellow.

quinoline yellow - no noticeable change.

red 2G - red going to faded pinkish red.

sunset yellow FCF - some 'on-shade' fading.

tartrazine - no noticeable change.

yellow 2G - some 'on-shade' fading.

Results

(i) None of the three colours, amaranth, tartrazine or green S, showed any degradation at this low temperature of 55°C.

(ii) and

(iii) results as per table 11.

Table 11

	100 ppm $^{w}/_{v}$: 110° study		250 ppm $^{w}/_{v}$: 120° study
Colouring Matter	% Remnant	Heating Period	% Remnant after 21 days
amaranth	7	21 days	3.0
black PN	11	19	4.0
brilliant blue FCF	89	16	15.1
brown FK	18	15	—
carmoisine	34	15	34.6
chocolate brown HT	0	19	7.1
erythrosine	85.6	15	—
green S	0	20	45.0
indigo carmine	0	20	—
patent blue V	84	15	3.1
ponceau 4R	6	16	0
quinoline yellow	52	15	60.7
red 2G	0	15	0
sunset yellow FCF	-	-	37.4
tartrazine	52	21	55.7
yellow 2G	2	16	18.5

Some of the polarograms of degraded solutions revealed new peaks. Though a number of these were quite minor, indigo carmine gave a significant new peak after thermal degradation. At pH 9.0, in the presence of tppc, the major peak at -0.42V disappears, and a strong new peak at -1.25V appears together with other small peaks (-1.57V and -0.52V etc.)

The growth and decay of these smaller peaks is shown in figure 29. Other polarograms in which new minor peaks were found included patent blue V (originally, at pH 9.0 + tppc, the major peak was -0.89 V with minor peaks at -0.65 V and -0.46 V; after thermal degradation, peaks appeared at -0.27 V and -0.80 V) and brilliant blue FCF (originally, at pH 2.0 without tppc, major polarographic peaks appeared at -0.51 V and -0.99 V with a minor peak at -0.28 V; after thermal degradation, new minor peaks appeared at -0.38 V and at -0.78 V). In the case of red 2G, a small polarographic peak appeared, after thermal degradation, at -0.83 V (pH 9.0, no tppc) partly beneath the original peak at -0.74 V.

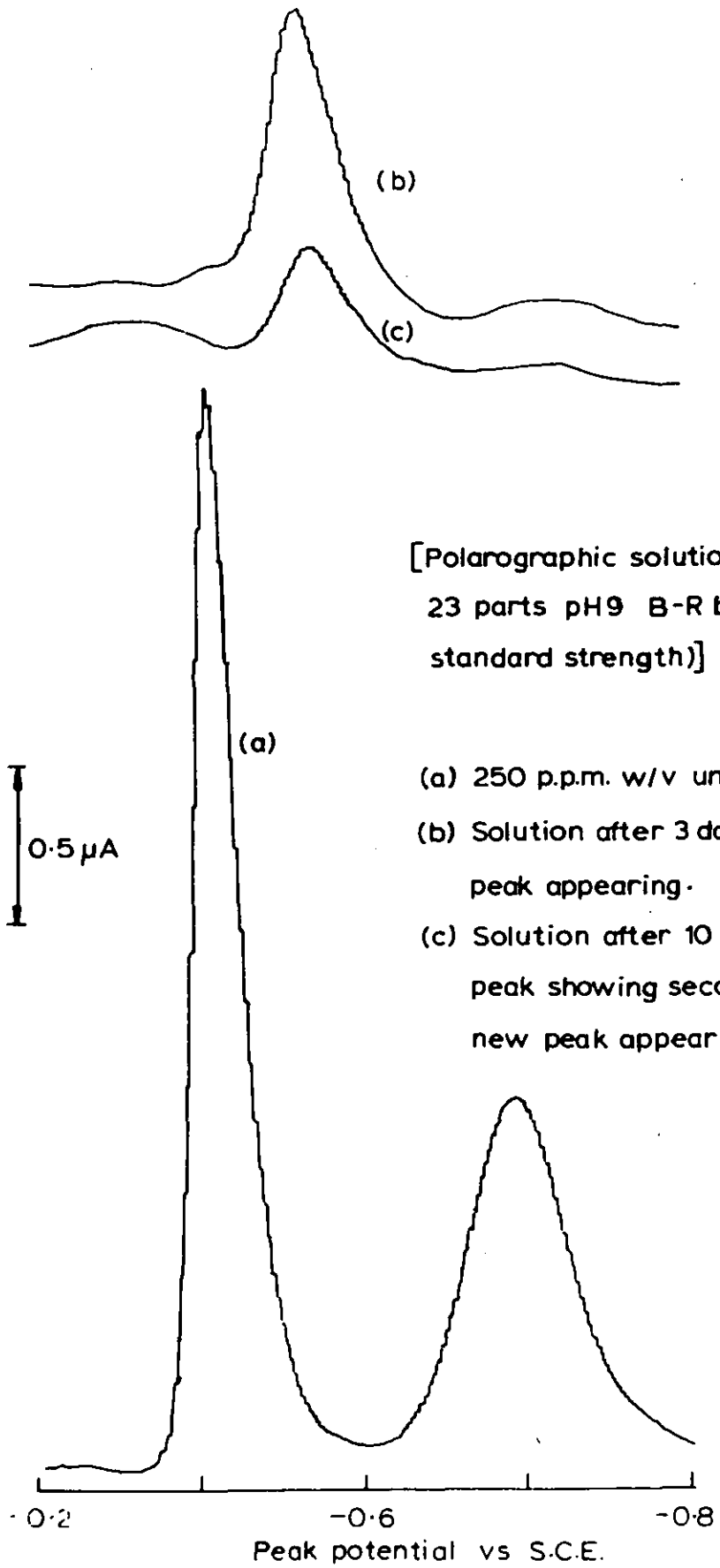
Discussion

The 110° study was conducted using a less accurate oven than the later 120° study. Also new polarographic equipment was introduced for this giving improved performance and stability. Generally, however, allowing for the effects of higher concentration and temperature, most results seem compatible from the two studies.

Some undoubted discrepancies do appear however. Brilliant blue FCF, green S and patent blue V are the major ones. Though these are all triphenylmethane dyes, the two blues show greatest loss at the higher concentration, the green S at the lower, so no trend can be ascribed on this structural basis.

Instead a further study on all the dyes was conducted in order to elucidate their degradation curves.

New polarographic peaks arising during the thermal degradation of indigo carmine at 120°C



[Polarographic solution, 2 parts sample: 23 parts pH9 B-R buffer (43·2% standard strength)]

(a) 250 p.p.m. w/v undegraded solution

(b) Solution after 3 days heating - one new peak appearing.

(c) Solution after 10 days heating - first new peak showing secondary decay; second new peak appearing.

Fig. 29

Thermal Degradation Curves

Experimental

Thick walled glass phials were used as in the previous study (iii). Each colouring matter was again made up as 250 ppm $^{w}/_{v}$ (249-257 ppm $^{w}/_{v}$) aqueous, unbuffered solutions. Control solutions were stored in the dark at room temperature as before. The samples were heated in the sealed phials in the 120^{o} oven for a month. At weekly intervals, starting 3-4 days into the trial, the phials were removed from the oven, cooled by immersion in tap water, and opened with a glass knife. After sampling, the shortened stem was resealed and the phials replaced in the oven. The samples were determined polarographically.

Table 12: Molarities of the food dye solutions

Colouring Matter	Molecular Weight	Molarity of 250 ppm $^{w}/_{v}$ solution
amaranth	604.5	4.14×10^{-4}
black PN	868.6	2.88×10^{-4}
brilliant blue FCF	792.8	3.15×10^{-4}
brown FK	mixture	
carmoisine	502.5	4.98×10^{-4}
chocolate brown HT	652	3.83×10^{-4}
erythrosine	879.9	2.84×10^{-4}
green S	576.6	4.34×10^{-4}
indigo carmine	466.4	5.36×10^{-4}
patent blue V	579.7 (for $Ca^{2+}/2$)	4.18×10^{-4}

(continued)

Table 12: Molarities of the food dye solutions (continued)

Colouring Matter	Molecular Weight	Molarity of 250 ppm $^{w}/_{v}$ solution
ponceau 4R	502	2.98×10^{-4}
quinoline yellow	mixture (c. 483 av.)	$\sim 5.2 \times 10^{-4}$
red 2G	509	4.91×10^{-4}
sunset yellow FCF	452.4	5.53×10^{-4}
tartrazine	534.4	4.68×10^{-4}
yellow 2G	551	4.54×10^{-4}

Results

The decay curves obtained are shown in figures 30a to 30d.

Discussion

Two colouring matters, indigo carmine and chocolate brown HT, decayed so quickly that single measurements only needed to be made on the timescale chosen for the experiment. Of these indigo carmine was the less stable.

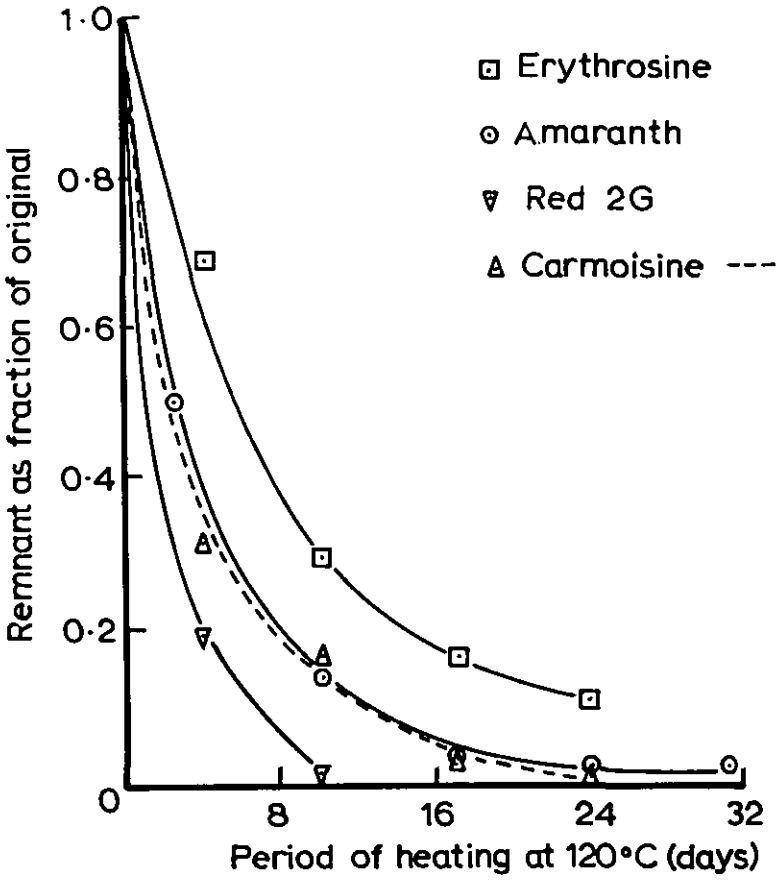
As the other decay curves all suggest first order decay as defined by the relationship

$$C = C_o \exp - kt$$

where C = remnant concentration
C_o = original concentration
t = time
k = rate constant

Comparative heat study conducted at 120°C

(a) Thermal stability of red colouring matters



(b) Thermal stability of yellow colouring matters

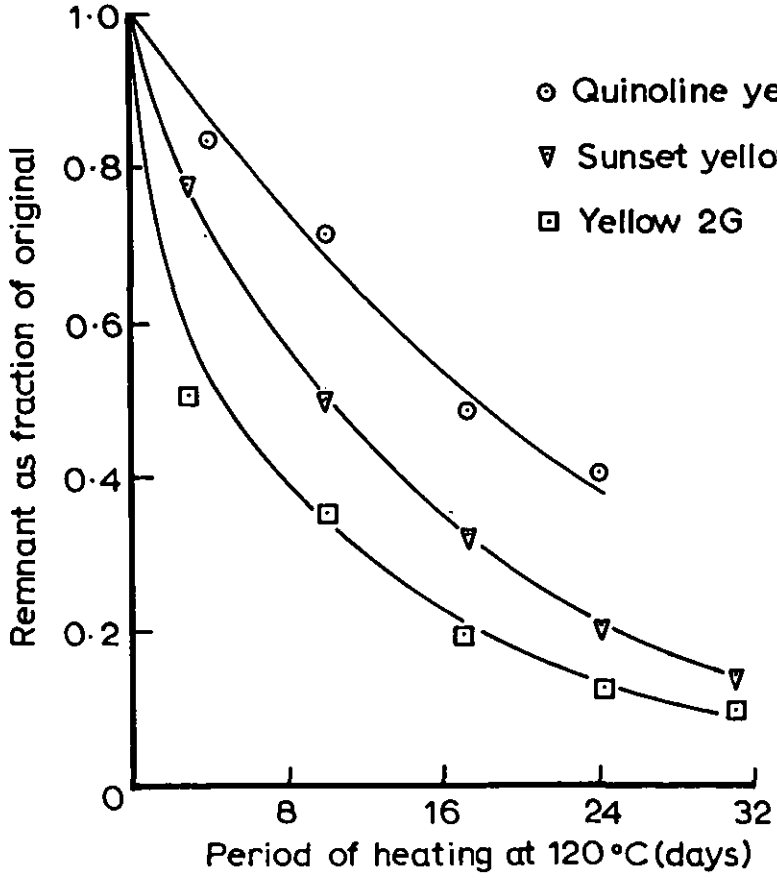
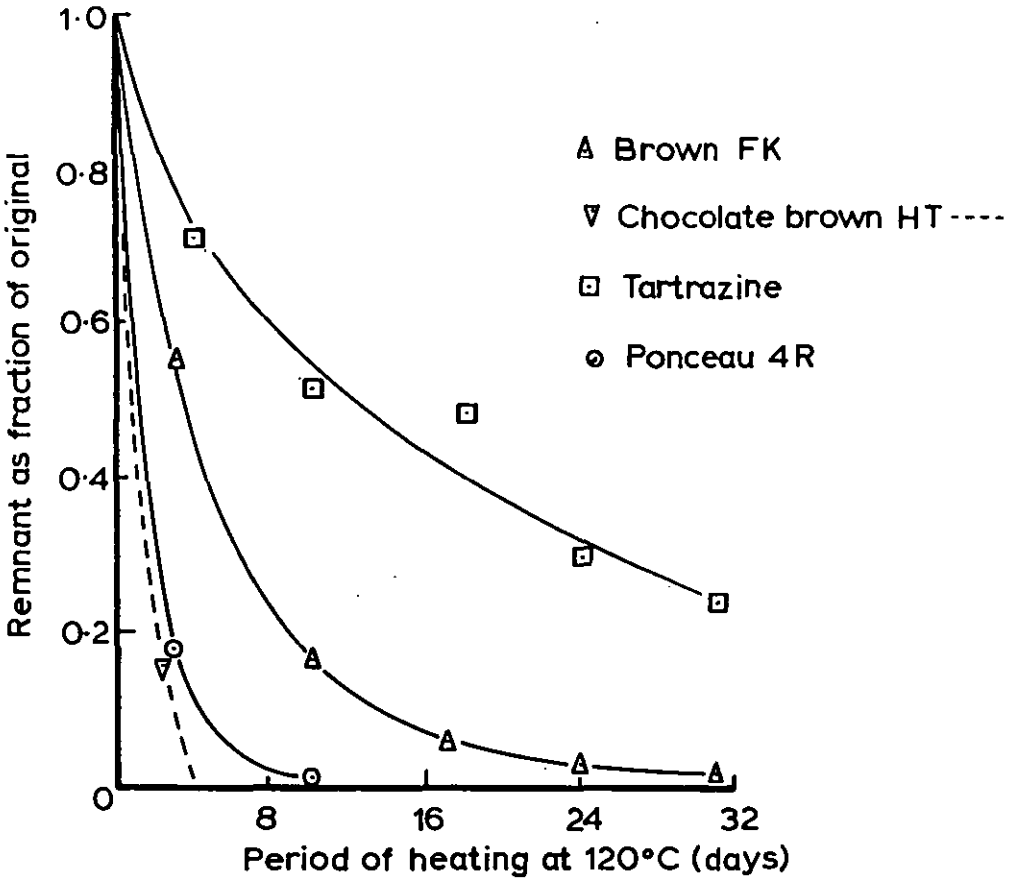


Fig. 30

Comparative heat study conducted at 120°C

(c) Thermal stability of various colouring matters



(d) Thermal stability of blue, blue-green and purple colouring matters

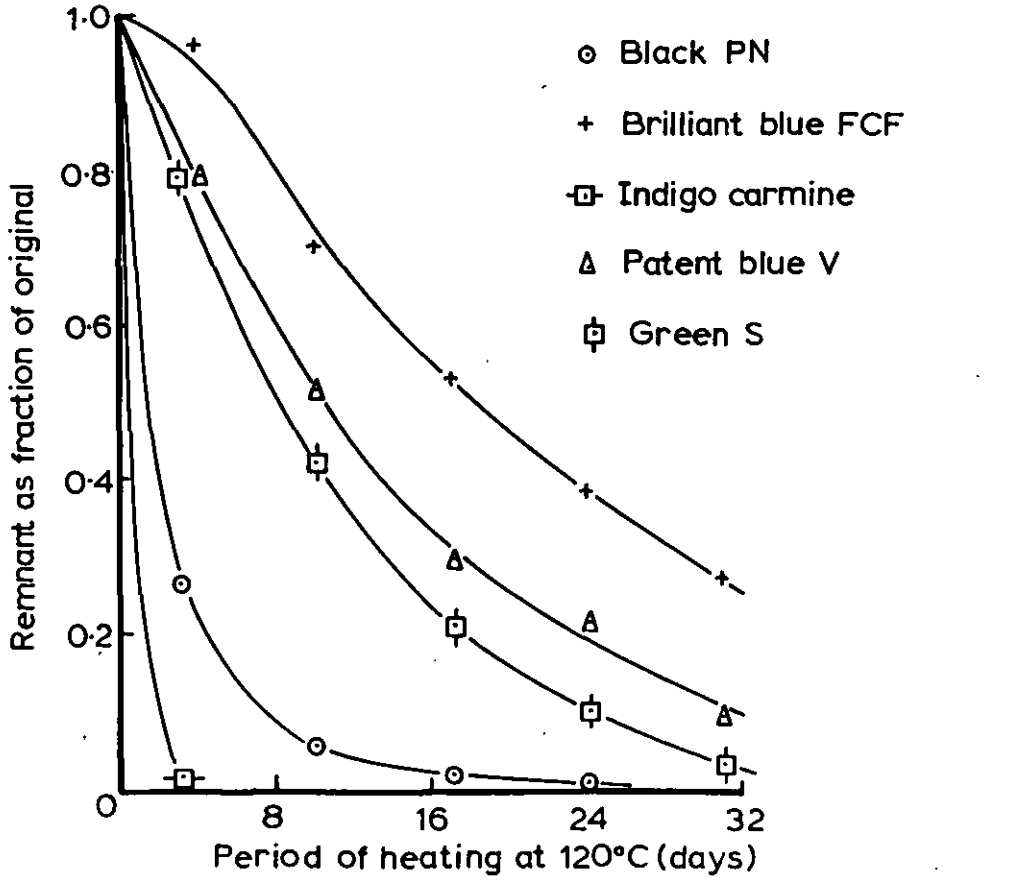


Fig. 30 (cont'd)

this was tested by plotting $\log_e (C/C_o)$ against t to see whether a linear relationship was obtainable. The plots are presented in figures 31a to 31d.

Of these, amaranth alone definitely shows non-linearity, but this is only in the range where C/C_o falls below 0.04 so that the appearance of even the most insignificant polarographic peak due to a degradation product under the original peak could have caused this distortion. The plot for $C/C_o > 0.04$ is highly linear and so first order.

The other thirteen colouring matters all give high linear correlation coefficients in the $\log_e (C/C_o)$ plots vs time. Included in the calculation of these least square best lines is, of course, the point t = 0, $(C/C_o) = 1$. The results are given in table 13.

Table 13

Colouring Matter	Rate Equation from least squares straight line	Correlation coefficient of this line
	t is in days	
black PN	$C=C_o\exp(-0.212t - 0.364)$	-0.988
brilliant blue FCF	$C=C_o\exp(-0.0427t + 0.0735)$	-0.996
brown FK	$C=C_o\exp(-0.135t - 0.218)$	-0.984
carmoisine	$C=C_o\exp(-0.198t - 0.0456)$	-0.994
erythrosine	$C=C_o\exp(-0.0962t - 0.0623)$	-0.987
green S	$C=C_o\exp(-0.108t + 0.185)$	-0.994
patent blue V	$C=C_o\exp(-0.0735 + 0.0594)$	-0.994

(continued)

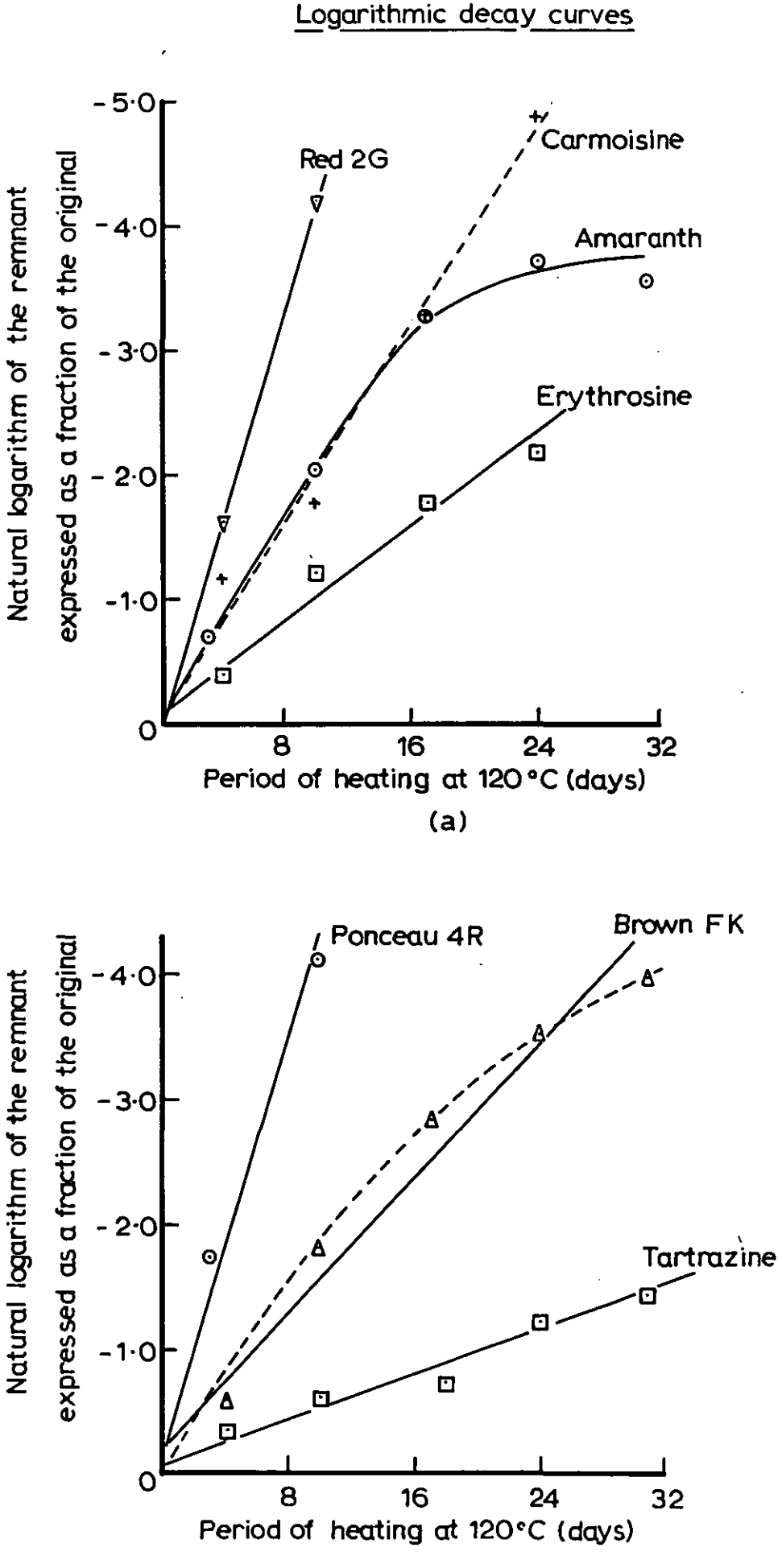
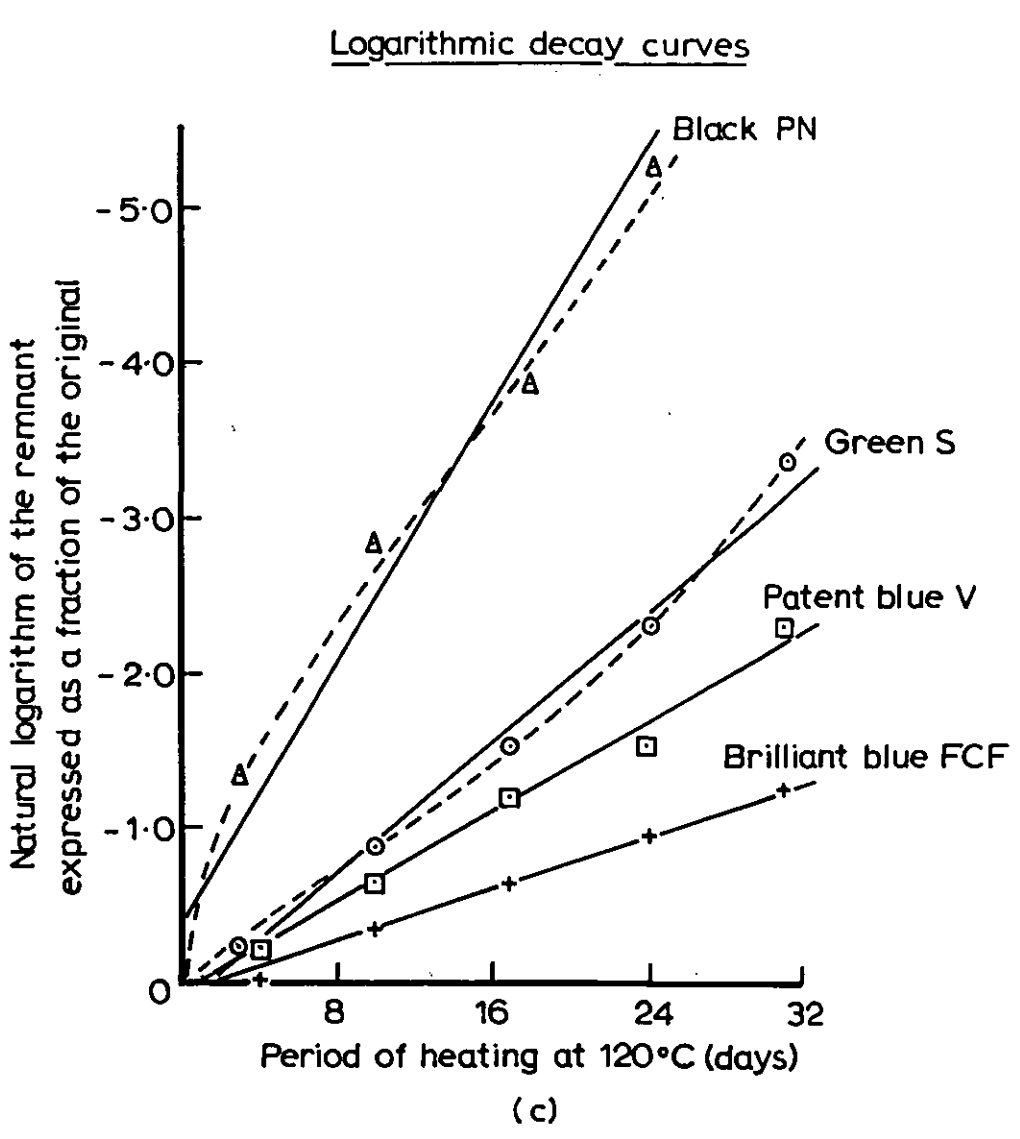

Logarithmic decay curves
Natural logarithm of the remnant expressed as a fraction of the original
-5·0
-4·0
-3·0
-2·0
-1·0
0
Red 2G
Carmoisine
Amaranth
Erythrosine
8
16
24
32
Period of heating at 120 °C (days)
(a)
Natural logarithm of the remnant expressed as a fraction of the original
-4·0
-3·0
-2·0
-1·0
0
Ponceau 4R
Brown FK
Tartrazine
8
16
24
32
Period of heating at 120°C (days)
(b)

Fig 31(a) and (b)


Logarithmic decay curves
Black PN
Green S
Patent blue V
Brilliant blue FCF
Natural logarithm of the remnant expressed as a fraction of the original
-5·0
-4·0
-3·0
-2·0
-1·0
0
8
16
24
32
Period of heating at 120°C (days)
(c)

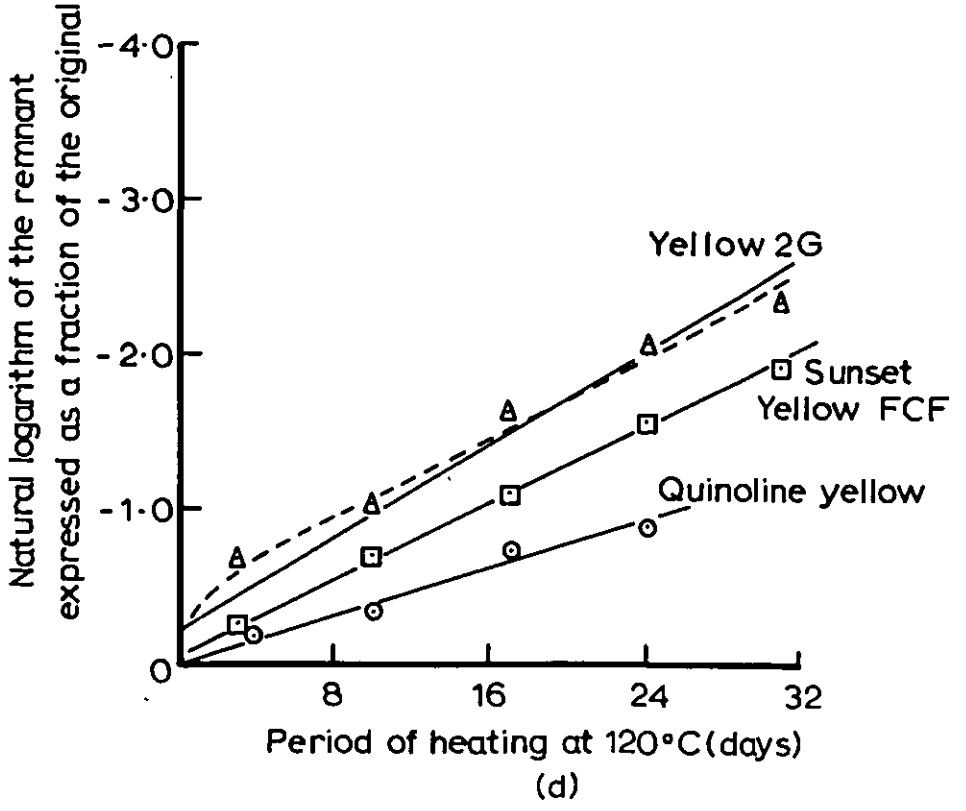

Yellow 2G
Sunset Yellow FCF
Quinoline yellow
Natural logarithm of the remnant expressed as a fraction of the original
-4·0
-3·0
-2·0
-1·0
0
8
16
24
32
Period of heating at 120°C (days)
(d)

Fig 31 (c) and (d)

Table 13 (continued)

Colouring matter	Rate Equation from least squares straight line	Correlation coefficient of this line
ponceau 4R	$C=C_o\exp(-0.408t - 0.190)$	-0.991
quinoline yellow	$C=C_o\exp(-0.0394t + 0.0026)$	-0.992
red 2G	$C=C_o\exp(-0.420t + 0.0223)$	-0.9999
sunset yellow FCF	$C=C_o\exp(-0.0625t - 0.0451)$	-0.999
tartrazine	$C=C_o\exp(-0.0457t - 0.0767)$	-0.983
yellow 2G	$C=C_o\exp(-0.0752t - 0.232)$	-0.980

Insufficient data exists, as has been said, to obtain the decay curves for some colouring matters. Nevertheless, if exponential decay is assumed, rather inaccurate rate equations may be derived:

amaranth	$C \approx C_o\exp(-0.19t)$
chocolate brown HT	$C \approx C_o\exp(-0.63t)$
indigo carmine	$C \approx C_o\exp(-1.02t)$

In all of the above, the coefficient of t is the rate constant for first order decay, k. Rather than comparing the thermal stabilities by the relative values of C/C_o at some time, t, it is better, i.e. of more general value, to compare the values of k. (units : day^{-1}).

Inspection of the decay curves, separately from consideration of the correlation coefficients given above, leaves some room for doubt as to whether higher order contributions may add to the overall kinetic

picture. What the correlation coefficients indicate is not that different order kinetics do not contribute, but rather that such contributions, if present, are minor. For several of the colouring matters, however, the point t = 0 has an unusually high error, suggesting that true exponential decay may only be adopted after a brief initial decay of another type. These particularly include black PN, brown FK, green S, ponceau 4R and yellow 2G.

Effect of pH on Thermal Degradation of Green S

Introduction

Few foods are of alkaline pH, the main examples being fish products. Also, though acid pHs are very common, these are generally in the range pH 3-7. For instance, canned rhubarb is generally at pH 3.0-3.5. Therefore, only a limited pH range is of real interest.

It was decided to gauge the effect of pH on the rate of thermal degradation of green S in oxygen-depleted solution over the pH 3-7 range. Green S was chosen as it is a triphenylmethane type dye, one of those showing marked discrepancies between the 110°, 100 ppm $^{w}/_{v}$ study and the 120°, 250 ppm $^{w}/_{v}$ one. Oxygen removal was undertaken in order to reduce the number of contributing factors involved.

Experimental

Solutions were prepared containing green S dissolved in 100% B-R buffer (adjusted to the required pH previously) as follows: 100, 40 and 16 ppm $^{w}/_{v}$ at pH 7; 100 and 40 ppm $^{w}/_{v}$ at pH 3; and 40 ppm $^{w}/_{v}$ at pH 5. Solutions were divided to provide controls, and each stored in a screw-top, autoclavable bottle of Duran glass. Solutions

were degassed with oxygen-free nitrogen on each occasion that a sample was taken, as well as prior to the initial heating period. The controls were stored in the dark at room temperature. The sample bottles were placed in an oven at 85° and sampled approximately weekly. Before sampling the bottles were weighed to ensure that the seal had been effective so that artificial concentration was avoided (average c. 0.05% per week loss). Polarographic comparison with the control was carried out.

Results

The decay curves found are presented in figure 32.

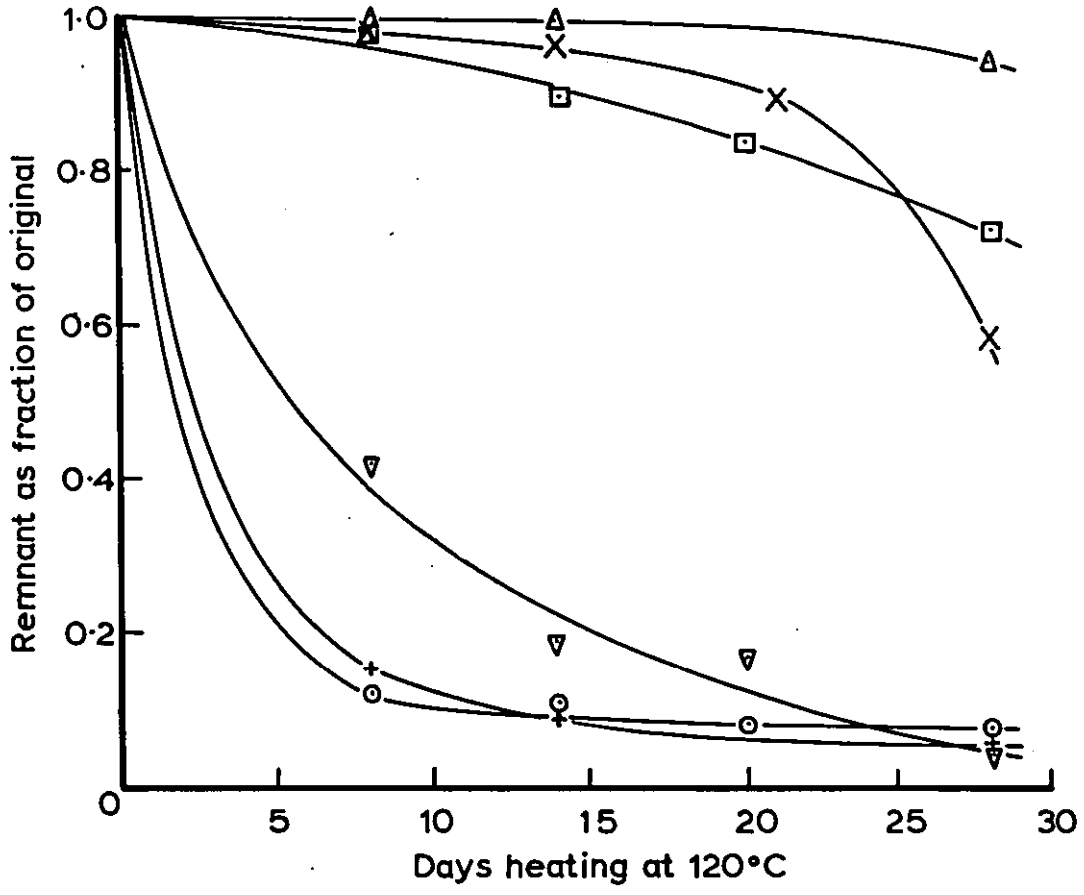
Discussion

None of the curves obtained corresponded to first order degradation, though the pH 7, 16 ppm $^{w}/_{v}$ solution could have been interpreted this way. Kinetic interpretation of the curves is limited to generalities: the pH 7 curves all show strong initial decay which after 90% loss, or thereabouts, gives a much lower decay rate for the 100 and 40 ppm $^{w}/_{v}$ cases; the pH 5 and pH 3 solutions interestingly give a very different decay pattern with very slow initial decay, the pH 5 solution being especially stable.

This suggests that base hydrolysis may play a major role in the degradation of green S under the conditions of the experiment (O_2-depletion, etc.), but that below pH 5 there may be an alternative acid hydrolysis mechanism.

The possibility was considered that the differences in behaviour at pH 3, 5 and 7 might be nothing more than a colour change with pH. In fact, the decolouration proved irreversible by change of

Effect of pH on thermal degradation of Green S



⊙ pH7 100ppm w/v

\+ pH7 40 ppm w/v

▽ pH7 16 ppm w/v

△ pH5 40ppm w/v

⊡ pH3 100ppm w/v

X pH3 40ppm w/v

Fig. 32

pH and when green S was originally made up at pH 7, its solution was identical in colour to that at pH 3 (prior to degradation), suggesting that the mechanistic difference could not be put down to a change in hydrolysis stability with the ionic structure in solution.

Experimental

Samples of green S degraded at pH 3 and at pH 7 for 35 days under the conditions already specified were subjected to chromatographic analysis for purposes of comparison. A 10 cm SAS-hypersil column was employed together with an eluent comprising 41/59/0.25/0.25 v/w/v isopropanol/water/cetrimide/acetic acid, the acid being present to overcome initial pH differences between the samples.

Results

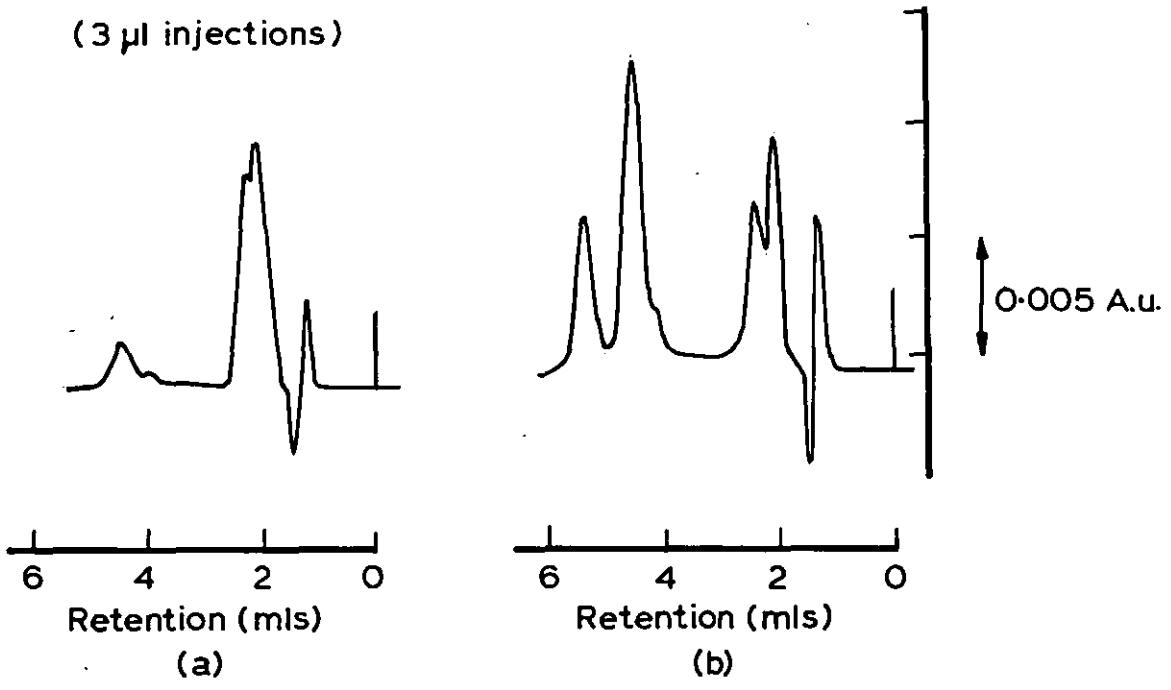
The chromatographs are reproduced in fig. 33.

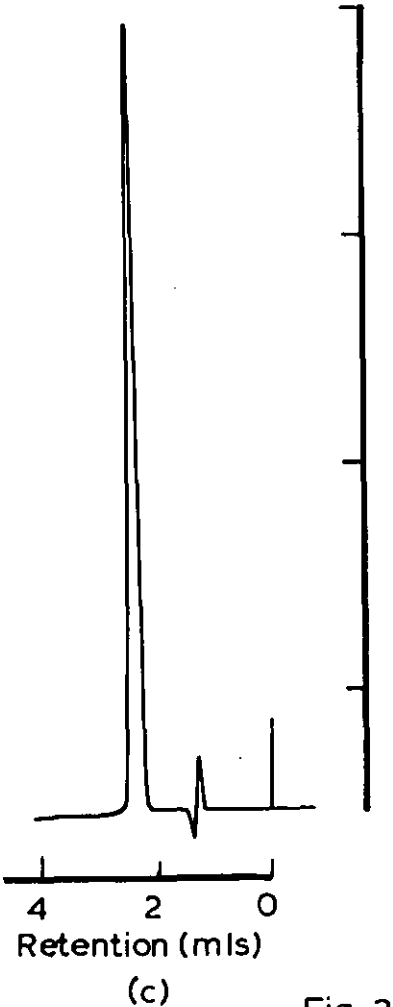
Discussion

At least one product peak (retention 5.4 mls) occurs in the pH 7 degraded sample but not in the pH 3 degraded sample, and either the major peak at retention 4.55 mls in the pH 7 degraded sample is a different compound to that giving the small peak at retention 4.45 mls in the pH 3 degraded sample, or at least it is only a major product peak in the former case. These findings indicate a probable difference in degradation mechanism at the two pH values.

HPLC comparison of pH3 and pH7 solutions of Green S after thermal degradation

(85°, 35 days, deoxygenated)





(a) Thermally degraded Green S
- 100 ppm w/v in pH3 B-R buffer

(b) Thermally degraded Green S
- 100 ppm w/v in pH7 B-R buffer

(c) Green S standard
- 200 ppm w/v aqueous soln.

Eluent: 41/59/0·28/0·28 v/v/w/v isopropanol/water/cetrimide/acetic acid

Fig. 33

Section B: Determination of Red 10B Formed from Red 2G

Introduction

In the preceding section on thermal degradation, it was noted that a small new polarographic peak was observed after thermal decay of red 2G, though this new peak was partly beneath the original red 2G peak. A search of the literature resulted in finding that the interim report of the Food Additives and Contaminants Committee on the "Review of Colouring Matter in Food Regulations" (1973)[8] stated that red 2G degrades under certain conditions, including elevated temperature, by deacetylation to the non-permitted food colour red 10B. It has not been possible to obtain more detailed information from the literature on this point.

The amino group at which deacetylation occurs in red 2G is three ring carbons removed from the azo group which is responsible, upon reduction, for the polarographic signal. This suggested the possibility that the small change in $E_{\frac{1}{2}}$ value going from red 2G to the thermal decay product, observed polarographically, could be due to just such a remote structural modification as deacetylation. So no initial conflict existed in the hypothesis that the new peak was due to red 10B forming. The first stage in trying this hypothesis was the comparison of the polarographic waves given by the degraded sample of red 2G and an authenticated red 10B sample.

Experimental

The 250 ppm $^{w}/v$ red 2G thermally degraded solution and a 100 ppm $^{w}/v$ red 10B solution were compared. Analytical solutions were made up comprising 20% $^{v}/v$ colouring matter solution, 40% 0.32 M borate

buffer (pH 7.5) and q.v. water. Differential pulse polarographic scans were made going negative from 0.0V.

Results

In both cases the main peak occurred at -0.78V with the identical half-width of 0.052V. In each case a second, minor peak occurred at -0.99_5 to -1.00V.

Discussion

These results obviously support the idea that the new peak in the thermally degraded red 2G polarogram corresponds to red 10B. However, polarography is not by itself sufficient to unambiguously identify an unknown compound, even where the possibilities are limited. Other approaches were therefore taken.

Experimental

The h.p.l.c. apparatus was set up as follows:-

eluent - 67/33/0.25 v/v/w methanol/water/cetrimide; stationary phase - SAS-Hypersil; column - 10 cm x 0.45 cm i.d., 316 steel; flow rate - 0.8 mls/min; detection at 240 nm; room temperature - 20°C; column water jacket - 40°C.

5 µl samples of thermally degraded red 2G (originally 250 ppm $^{w}/v$) and of red 10B (100 ppm $^{w}/v$) were chromatographed. Red 2G (100 ppm $^{w}/v$) was also run.

Results

During the degradation the red 2G peak (retention time 12.2 minutes) split and an incompletely resolved preceding peak appeared at 11.8 minutes. Red 10B was found to also have a retention time of 11.8 minutes.

Discussion

Taking the h.p.l.c. result into account alongside the polarographic evidence, as well as the fact that the distinctive deep pink-red of red 10B was clearly present in the degraded red 2G sample, there is adequate confirmation that the product is indeed red 10B.

Changes in pH During Red 2G Degradation

Experimental

Two phials were filled with 987 ppm $^{w}/_{v}$ red 2G aqueous solution, and a single phial was filled with distilled water. The pH values of these liquids were measured by means of a digital pH meter which had been calibrated using pH 4 and pH 9.2 buffers for slope and zero error. One of the red 2G solutions was stored in the dark at room temperature. The other sealed phials were heated in an oven at 130°C for 172 hours. The phials were then opened and the pH measurements repeated.

Results

	Initial pH	pH after 172 hours
Distilled water (130° heating)	6.4	7.2
Red 2G, 987 ppm aq. (130°C)	7.4	3.6
Red 2G, 987 ppm aq. (room temperature)	7.4	7.1

Discussion

The pH change of the heated red 2G solution is very marked and confirms the production of acid during the thermal degradation. It can be shown that if activities = concentrations then $H^+ = \{([CH_3COO^-]+[CH_3COOH]) \times K_a\}^{\frac{1}{2}}$ where K_a is the acid dissociation constant = 1.75×10^{-5} for acetic. If the red 2G deacetylation proceeds to completion, then the original red 2G molarity will equal the final acetic acid molarity. Now, 987 ppm $^w/_v$ for a molecular weight of 509 gives a molarity of about 0.002 (0.00194).

$$[H^+]^2 = 0.002 \times 1.75 \times 10^{-5} = 3.5 \times 10^{-8}$$

$$[H^+] = 1.9 \times 10^{-4} \text{ and pH} = 3.7.$$

This is strikingly close to the observed value, though it should be considered as subject to various approximations including

(i) total degradation assumed - in fact a few percent remained,

(ii) other components have been ignored - initially the commercial red 2G was slightly alkaline, w.r.t. water,

(iii) activities may differ from unity, (etc.).

Approach to a Quantitative Method

In order to follow the decay of red 2G and the build up of red 10B, a quantitative means was required that could estimate these species simultaneously and without mutual interference. The h.p.l.c. method did not seem an attractive choice. Resolution was poor with the eluent composition (and under the other conditions) stated above, and the prospect of changing to a far more polar mixture giving better separation but large increases in retention time, was not a welcome one. Further, any use of buffers could be expected to affect the ion-pair mechanism and complicate the separation.

As an alternative, an attempt was made to modify the polarographic method.

Experimental

Red 2G, red 10B and their mixture were each made up in 40% B-R buffer at an analytical concentration of 50 ppm $^{w}/v$ (in the mixture, 50 ppm $^{w}/v$ each). Differential pulse polarograms were obtained at values over the range pH 2-10 by adjustment of the pH with 3M NaOH (aqueous).

Results

Measurement of the individual red 2G and red 10B polarograms gave the following:-

Colour	pH	d.p.p. peak position	peak height	peak half width
red 10B	2.2	-0.24V	2.87 μA	0.17_5V
	4.3	-0.60V	2.95	0.14_5V
	6.7	-0.73V	7.11	0.06 V
	10.0	-0.85V	5.06	0.09 V
red 2G	2.2	-0.20V	5.81 μA	0.17 V
	4.0	-0.38V	8.05	0.12 V
	5.8	-0.55V	10.6	0.11 V
	7.4	-0.66V	12.7	0.10 V
	9.9	-0.80V	10.7	0.10 V

Other features observed included:

(i) a second peak occurred on both the red 2G and red 10B polarograms whose position (potential), but not size/shape, was largely pH independent, and approximately the same on both polarograms, viz. -0.98V (pH 2.1) to -0.92V (pH 9.6);

(ii) a shoulder on the red 2G peak at, or below, pH 4 was most pronounced at low pH (pH 2.1);

(iii) the polarograms of the colour mixture showed no resolution over pH values 2-4 and at very high values, e.g., pH 10.9.

Discussion

Two factors were shown to be overriding in determining resolution:-

(a) separation of peak maxima increases with decreasing pH values e.g. at pH 9.6 separation between maxima is 0.06V, while at pH 6.8 it is 0.12V;

(b) peak half-widths generally increase with decreasing pH values.

These contrary trends resulted in a pH value of c. pH 7 giving optimum resolution: unfortunately even this proved insufficient.

Previous work in this laboratory had shown that in favourable cases, e.g. a mixture of tartrazine and sunset yellow FCF, the addition of special surfactants caused differential shifts between the peak potentials of closely positioned polarographic peaks, so increasing their resolution. This was based on the original idea of Pietrzyk and Rogers,[78] and has been discussed more fully already in the introduction. It was

therefore thought worthwhile to undertake a trial to find out whether any useful differential shift could be obtained in the red 2G-red 10B analytical system.

Experimental

Red 2G, red 10B and their mixture were made up in the same manner as before except that tetraphenylphosphonium chloride was included in the analytical solution at a concentration of 1000 ppm $^{w}/v$, other concentrations being unchanged. Differential pulse polarograms were again obtained over the pH range 2-10.

Results

Variations in peak position, half-width and height are given below for red 10B and red 2G when run independently.

Colour	pH	d.p.p. peak position	peak height	peak half width
red 2G	2.2	-0.28V	3.00μA	0.11 V
	4.7	-0.48V	2.73	0.07V
	6.7	-0.58V	2.73	0.06_5V
	8.3	-0.67V	2.40	0.08V
	10.1	-0.77V/-0.87V	Wave split (0.79μA)	-
red 10B	2.1	-0.53V	3.27μA	0.10V
	4.4	-0.64V	2.19	0.09_5V
	6.5	-0.76V	2.58	0.09V
	9.0	-0.84	3.46	0.09V

Other features noted included:-

(i) minor peaks occur in the red 10B polarogram, viz., -0.13V and -0.28V at pH 2.1; -0.39V and -0.07V at pH 4.4; -0.39V at pH 6.5; and -0.36V at pH 9,

(ii) minor peaks also occur in the red 2G polarogram, but these are less prominant than in the red 10B case. A small peak appears at -0.43V both at pH 8.3 and at pH 10.1.

Results for the red 2G/red 10B mixture were:-

pH	R2G peak	R10B peak	Separation
4.3	-0.48V	-0.66V	0.18V
6.6	-0.59V	-0.75V	0.16V
8.0	-0.65V	-0.81V	0.16V
9.7	-0.75V	-0.90V	0.15V

Discussion

From the results of the separate red 2G and red 10B runs, it is clear that inclusion of tetraphenylphosphonium chloride decreases peak half-widths and much decreases variation of half-width with pH. Fig. 34 illustrates the effect of the tppc on the differential pulse polarogram of a red 2G/red 10B mixture.

While the red 2G/red 10B mixture was found to give excellent resolution of the red 10B peak at pH 2.1, unfortunately a small polarographic minimum preceded this peak and the red 2G peak was merged

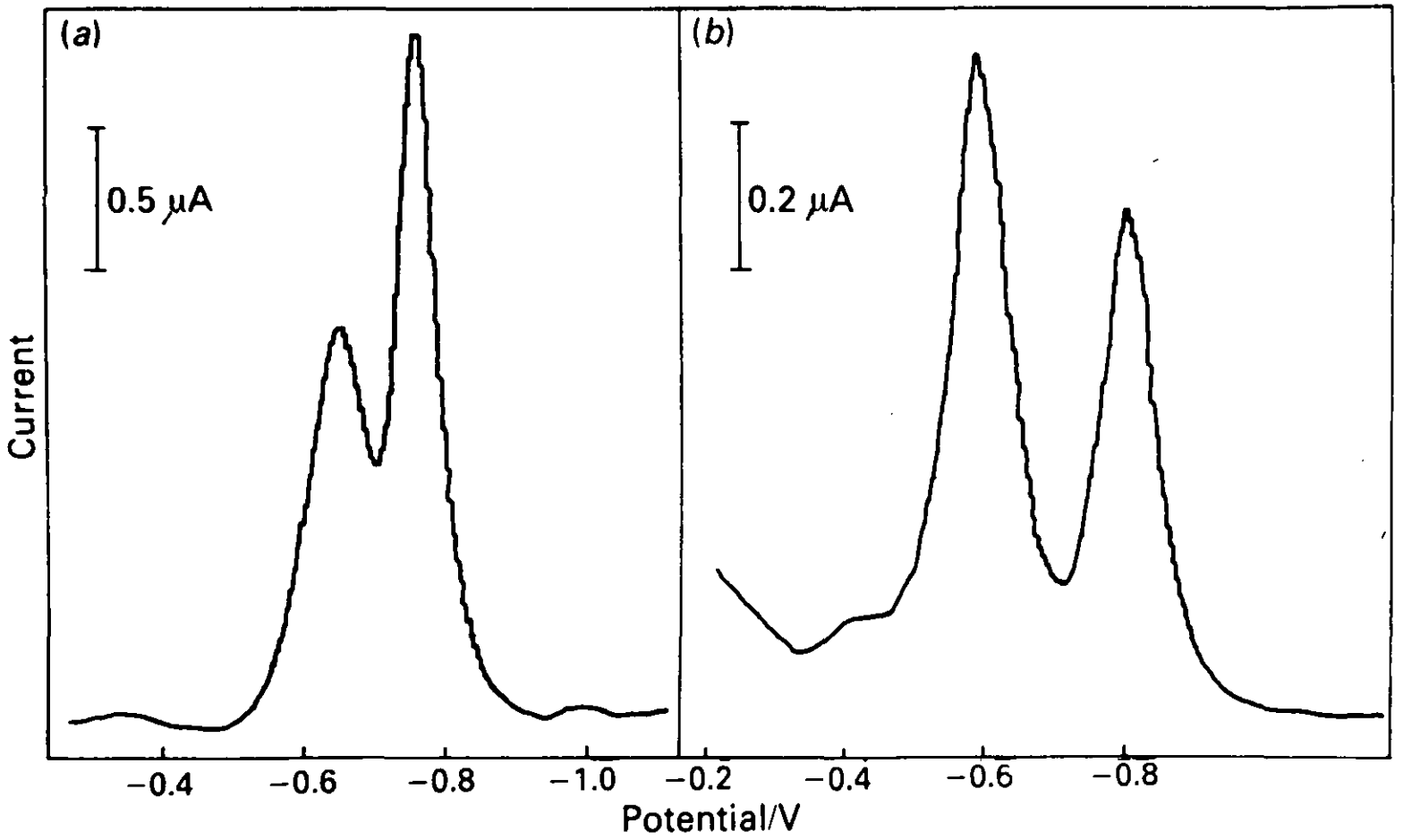


Fig. 34 Effect of tetraphenylphosphonium chloride (TPPC) on the differential pulse polarograms of a mixture of Red 2G and Red 10B in pH 7 Britton - Robinson buffer. TPPC concentration: (*a*) 0 and (*b*) 500 p.p.m.

with the minor peaks, listed above. The interfering minor peak was partly removed at pH 4.3 and well separated at higher pH.

Consideration of peak half-widths, shapes and separation led to the adoption of pH 6-7 as providing overall optimum resolution. Use of tetraphenylphosphonium chloride gives:

(i) increased peak separation (30+ % at pH 7),

(ii) decreased peak half-widths (except for red 10B at pH 6.6).

Though the separation obtained with tetraphenylphosphonium chloride seemed adequate, it was felt that tests with the arsonium analogue were appropriate, as previous work in this laboratory had shown that different results were often obtained using these two surfactants.

Experimental

The previous experiment was repeated substituting tetraphenylarsonium chloride for the phosphonium surfactant.

Results

Essentially comparable results were obtained. For instance, at pH 9.0 reasonable polarograms were obtained having the following features:

Colour	Peak Position	Peak Half-width	Peak Height Ratio
Red 2G	-0.69V	0.09_5	$\frac{\text{Red 2G pk.ht}}{\text{Red 10B pk.ht.}} = 2.0$
Red 10B	-0.86V	0.09_5	

So peak separation at pH 9.0 was 0·17.

Discussion

Taking peak separation and half-widths into account, no real advantage is achieved by the substitution of tpac for tppc. Further, the general polarographic form obtained is less good owing to a sloping baseline, and, finally, tpac is rather more toxic than tppc, though tppc is by no means non-toxic.

Looking at all the results to this point in the derivation of a suitable analytical procedure, one slight drawback to the use of tppc is that some fall-off in signal occurred due to peak suppression. This was not critical: for red 2G at pH 6.5-6.8, the fall-off is only 17%, while for red 10B at this pH range, it is larger at 59% but still quite acceptable. Generally red 10B showed far more pH dependency as regards peak height than did red 2G except at high pH values (>9).

An initial calibration curve was constructed at pH 7 in the presence of tppc for red 2G (fixed concentration) and red 10B (varying). The red 10B curve was found to be approximately linear, but it was found that the red 2G peak declined with increasing red 10B concentration. This will be discussed more fully later.

It was necessary to ascertain whether the peak suppression occurred in the absence of tppc. Therefore a calibration curve was constructed accordingly.

Experimental

Solutions were prepared containing 40% pH 7.0 B-R buffer, 40 ppm $^{w}/v$ red 2G and 0-40 ppm $^{w}/v$ (8 ppm $^{w}/v$ increments) red 10B. No tppc was added. Differential pulse polarograms were obtained of these six solutions.

Results

Concentration of red 10B	Red 2G peak height	Red 10B peak height
0 ppm $^{w}/v$	13.6 µA	1.57 µA
8	12.9	6.93
16	12.8	11.3
24	12.2	15.7
32	12.6	20.5
40	12.6	24.2

Discussion

The suppression of the red 2G peak with increasing red 10B concentration clearly occurs in the absence of tppc, and so is not a disadvantage induced by the surfactant. It does, however, constitute an interference to the accuracy of the method. This will be dealt with later.

First, however, the question arose as to whether the tppc concentration was a critical factor requiring very exact measurement.

Experiment

The effects of tppc concentration were investigated at pH 2.0 for a 1:1 red 2G/red 10B mixture over a range of tppc concentrations 200-800 ppm $^{w}/v$ in the analytical solution and at pH 7 for tppc concentrations 200-600 ppm $^{w}/v$. As before, 40 ppm $^{w}/v$ each colour and 40% B-R buffer were used.

Results

pH	Concentration (ppm $^{w}/v$) tppc	200	400	500	600	700	800
2.1	red 2G peak height (μA)	6.3	6.9	7.0	7.6	7.5	7.3
2.1	red 10B peak height	1.6	2.1	2.1	2.4	2.4	2.4
7.0	red 2G peak height	5.1	5.3	5.4	5.4	-	-
7.0	red 10B peak height	2.1_3	2.2_4	2.3_2	2.2_4	-	-

At pH 2.1: red 2G peak position constant at -0.19V

red 10B peak position constant at -0.24V

At pH 7.0: red 2G peak position: -0.59_5 → -0.60 (almost constant)

peak half width: 0.075V (200 ppm tppc) → 0.08 (600 ppm tppc)

red 10B peak position: -0.775→-0.785 (almost constant)

peak half width: 0.09V (fairly constant over range).

Discussion

At pH 2.1 a tppc concentration of 700 ppm $^{w}/v$ would minimize concentration dependency: at pH 7.0 a figure of 500 ppm $^{w}/v$ would do the same.

It was noted earlier that the red 2G peak height was not independent of red 10B concentration and showed suppression. Two trials were conducted as below, one at 0-25 ppm $^{w}/v$ each colour and one at 0-4 ppm $^{w}/v$ each colour for comparison.

Experimental

Analytical solutions were prepared comprising 40% B-R buffer (pH 7.0), 25 or 4 ppm $^{w}/v$ one colouring matter, and 0-25 or 0-4 ppm $^{w}/v$ the other colour. The solutions also contained 500 ppm $^{w}/v$ tppc. Calibration curves were obtained and the consistency in height of the polarographic peak from the fixed concentration colour was monitored.

Results

Concentration of red 2G (ppm $^{w}/v$)	0	5	10	15	20	25
Red 2G peak height (μA)	0.08	1.5	3.1_5	4.8_0	6.6_1	8.8_9
Red 10B (25 ppm $^{w}/v$) peak height (μA)	9.09	10.7	11.5	12.0	12.8	12.4

Concentration of red 10B (ppm $^{w}/v$)	0	5	10	15	20	25
Red 10B peak height (μA)	0.24	2.3_6	4.3_7	7.8_0	9.5_1	12.3
Red 2G (25 ppm $^{w}/v$) peak height (μA)	9.5_3	10.4	9.5_3	9.7_6	8.4_6	7.8_0

Concentration of red 2G (ppm $^{w}/v$)	0	0.8	1.6	2.4	3.2	4.0
Red 2G peak height (μA)	0.114	0.299	0.457	0.705	0.835	1.04
Red 10B (4.0 ppm $^{w}/v$) peak height (μA)	0.760	0.787	0.829	0.858	0.799	0.811

Concentration of red 10B (ppm $^{w}/v$)	0	0.8	1.6	2.4	3.2	4.0
Red 10B peak height (μA)	0.008	0.154	0.260	0.402	0.516	0.697
Red 2G (4.0 ppm $^{w}/v$) peak height (μA)	0.874	0.906	0.882	0.921	0.874	0.913

In the above, red 2G peak appeared at -0.59 V with half-width 0.095-1.0 V; red 10B appeared at -0.79V → -0.80V with half-width 0.090 → 0.095V.

Discussion

One inconsistency occurs in these results: the 25 ppm $^{w}/v$ red 2G/25 ppm $^{w}/v$ red 10B measurements of 8.89 and 7.80 μA are unusually discrepant for polarography: the average 8.35 μA fits the linearity better in both calibration curves.

For the more concentrated, 25 ppm $^{w}/v$ solutions the linearity of the calibration curves are acceptable. Red 2G: peak height (μA) = -0.19 + 0.35 x red 2G $\underline{\text{conc}}^{n}$ (ppm): correlation coefficient = 0.997. Red 10B: peak height (μA) = 0.05 + 2.04 x red 10B $\underline{\text{conc}}^{n}$ (ppm): correlation coefficient = 0.997 . However, the consistency in the peak height is not acceptable: the red 2G peak is suppressed by increasing red 10B concentration, while the red 10B peak is enhanced by increasing red 2G concentration. As these effects are large they render the method inaccurate at the 25 ppm $^{w}/v$ level.

For the 4 ppm $^{w}/v$ solutions the problem largely disappears. The calibration curves remain linear:

(i) peak height red 2G (μA) = 0.112 + 0.232 x red 2G conc^{n} (ppm)

correlation coefficient = 0.998

(ii) peak height red 10B (μA) = 0.006 + 0.167 x red 10B conc^{n} (ppm)

correlation coefficient = 0.998

so if anything, these are better than at 25 ppm $^{w}/v$. Also, there is no longer any trend showing peak suppression or enhancement. For constant 4 ppm $^{w}/v$, red 2G peak height varies around 0.895 μA with standard deviation estimated as 0.02 μA. For red 10B the same measurements give 0.807 $\pm$ 0.034 μA.

To summarize, inclusion of 500-700 ppm $^{w}/v$ tetraphenylphosphonium chloride increases the resolution between the differential pulse polarographic peaks of red 10B and red 2G allowing linear calibration graphs to be constructed for both species at pH 6-7. Polarographic interaction between the two species, causing suppression of the red 2G peak and enhancement of the red 10B peak may be effectively eliminated by operating with analytical concentrations not much larger than 4 ppm $^{w}/v$, which is within the accessible range of a modern polarograph.

Thermal Degradation of Red 2G

Experimental

An aqueous solution containing 5000 ppm $^{w}/v$ red 2G was placed in an autoclavable screw-top bottle (Duran glass). This was heated at c. 85°C in a dark oven for 149 days. The solution was

examined visually and polarographically. The latter entailed dilution to give an analytical concentration of 250 ppm $^{w}/_{v}$, which was compared with a standard, undegraded red 2G solution of this same concentration (pH 9.0: no tppc).

Results

The solution had taken on the characteristic pink-red shade of red 10B. A maximum of 2% of the original red 2G remained. A new polarographic peak appeared at -0.85V (cf red 2G peak -0.77V) corresponding to red 10B. The peak heights of the standard red 2G and of the red 10B produced by degradation were 7.60 µA and 7.88 µA respectively, a ratio of 1:1.04.

Discussion

While this was an experiment conducted previous to the adoption of the tppc method, it is reported here because it led to the realisation that there must be alternative reaction pathways open to red 2G in its decay. The lower concentration (e.g. 250 ppm $^{w}/_{v}$) trials had shown that, as the red 2G degraded, red 10B was formed but that its peak height never approached the original size of the red 2G peak. Therefore it was apparent that one of two possibilities must be true:

(i) red 2G has the choice of reaction pathway

  (a) red 2G ⟶ red 10B

  (b) red 2G ⟶ products not giving polarographic peaks in the usual polarographic range; or

(ii) red 2G has only one significant reaction pathway, viz. deacetylation to give red 10B, and that the secondary reaction i.e. red 10B ⟶ products not giving polarographic peaks, could be greatly impaired at high concentrations.

In fact, elements of both possibilities might contribute. In particular all the azo dyes tested showed loss of the azo group (revealed by lack of polarographic signal) as a thermal decay mode, so that (i) above seemed likely, while the remarkable stability of the red 10B peak formed in the high concentration solution may well indicate that it is stabilized by some factor, either the concentration itself or, more plausibly, by the amount of acetic acid produced by the deacetylation and its effect upon the pH.

More detailed investigation of the concentration dependency (incorporating the tppc method) was regarded as an important step in forming a quantitative picture supporting these speculations.

Two types of trial were proposed: firstly, a comparison of percentage decay for red 2G solutions of differing concentration; and secondly, the monitoring of the decay pattern by hplc over a period.

Experimental

Solutions were prepared containing 1000, 500, 200 and 100 ppm $^{w}/v$ red 2G respectively. The solutions were sealed in glass phials and placed in a dark oven at 130^{o}C for 41.5 hours, then analysed by the polarographic method involving tppc previously described. The experiment was repeated using solutions of red 10B, but allowing two days for the decay instead of 41.5 hours.

Results

Original concentration of red 2G	1000	500	200	100 (ppm $^{w}/v$)
Percentage Remnant	28.8	30.9	43.2	37.6
Original concentration of red 10B	1000	500	200	100 (ppm $^{w}/v$)
Percentage Remnant	84.0	74.3	74.8	72.0

Discussion

The minimum decay rate at 200 ppm $^{w}/v$ red 2G is surprising, but not implausible. There is concentration dependence for both red 2G and red 10B decay, but it is slight in the latter case over the 500-100 ppm $^{w}/v$ range. It is noteworthy that for red 2G the maximum decay rate (percentage) is at the highest concentration, while the opposite applies in the case of the red 10B. This could simply reflect differences between deacetylation and azo-cleavage processes. This work has been published.[144]

Section C: Checks on the Interpretation and Reproducibility of Results

Effects of pH Changes During Degradation

The fall in pH detected following the thermal degradation of red 2G highlighted the possibilities for error in the interpretation of results. In particular, it must be ascertained whether a change in shade of colour during a degradation reaction heralds the formation of a new coloured compound, or whether it is simply an effect of pH modification upon the original colouring matter.

Polarographic measurements guard against this problem by the use of buffer in the analytical solution. Further, it is possible to construct plots of pH vs. $E_{\frac{1}{2}}$ value which show high linearity over several units of pH enabling fine corrections to be made.

Nonetheless, there is no clear guarantee that polarography will show the presence of new species formed during decay. It was felt to be a simple and obvious check to compare the pH colour range of the original compounds with that of their differently coloured products. A single example of this is given below, though the method was used in other cases also.

The example chosen is that of patent blue V. After thermal decay the solution had turned green.

Experimental

Two solutions were taken: the first was 250 ppm $^{w}/_{v}$ patent blue V freshly prepared (dark blue); the second the same solution after three weeks heating at 120° (green - no bluish tinge). The pH of each solution was measured.

Each solution was treated with measured amounts of 0.1 M hydrochloric acid and the amount of acid required to effect colour changes

was noted. A sample was also treated with 5M hydrochloric acid and observed. Alkali was added to find out whether the colour changes observed could be reversed.

Results

For a 4 ml aliquot of 250 ppm $^{w}/_{v}$ patent blue V solution (pH 6.55) 0.35 mls 0.1 M HCl were needed to produce a turquoise colour, and a total of 0.60 mls in order to give a green colour (still bluish tinge). These correspond to values of pH 3.1 and pH 2.9 respectively. No further change occurred on addition of a further 0.9 mls 0.1 M HCl (pH 2.7 measured, pH 2.6 calculated). Addition of 5 M hydrochloric acid to a fresh sample gave a yellow/pale lime green colour. These changes were reversed by alkali. Addition of 0.05 M NaOH to fresh patent blue V failed to change its colour. Addition of 4 M NaOH resulted in a yellow colour being formed.

The thermally degraded solution showed an initial pH of 6.9. Addition of a few drops of 0.01 M hydrochloric acid appeared to clarify or lighten the solution a little, but though no clear transition point was seen, a slow transformation from green to yellow/pale lime green (pH 2.5) to yellow took place (pH 2.2). One drop of concentrated acid added to a fresh aliquot of the solution produced a vivid orange colouration. Again these changes proved reversible. An excess of alkali turned the solution purple.

Discussion

The two green colourations, that of the standard and that of the degraded solution were clearly different when compared. The especially vivid colourations at the pH extremes provided clear indication of the differences between starting material and decay product. (This obviously did not necessarily show complete absence of starting material in the

degraded solution).

The advantages of this type of study are,

(i) speed and simplicity,

(ii) utilisation of the self-indicating properties of the dyes,

(iii) accurate pH measurement coupled with colour comparisons could ultimately prove useful as a structural check against standards, e.g. in confirmation of an hplc study.

Reproducibility of Thermal Degradation Conditions

Introduction

During tests on the photostability of food colouring matter solutions already described it was found that ponceau 4R and indigo carmine could behave unpredictably. For instance, whereas the shape of the ponceau 4R kinetic decay curve remained essentially the same for repeat experiments, the period after the commencement of the experiment and prior to the onset of heavy decay (perhaps an "induction period"?) did vary (cf. Figure 28).

The question therefore arose as to whether individual thermal degradation trials could be assumed to be typical of the general behaviour of similar solutions. The fact that all the thermal decay curves proved approximately exponential in form, rather than sigmoidal as for ponceau 4R, gave some basis to hope that the problem of irreproducibility would be less likely to arise. A trial was conducted on amaranth to confirm this.

Experimental

An aqueous solution of 254 ppm $^{w}/_{v}$ amaranth was placed into three separate phials. The sealed phials were heated at 120° for 34 days,

then opened and a sample of each (5 µl) chromatographed. The chromatograms were compared.

[Eluent, 77/33/0.25 (v/v/w) methanol/water/cetrimide; detection at 254 nm].

Results

The following peaks appeared in the three chromatograms:

Retention (mls)	1.38	1.75	1.95	2.3	2.5	2.9	3.15	3.33	3.55
Peak Height (AU)									
Phial 1	Solvent	.011	.013	.014	.092	.004	.005	.005	.004
Phial 2	Peak	.012	.012	.010	.094	.005	.004	.004	.004
Phial 3		.012	.014	.014	.094	.005	.005	.005	.004
Average (AU)		.012	.013	.013	.093	.005	.005	.005	.004
Standard Deviation (AU)		.0004	.001	.002	.001	.0004	.0004	.0004	0

These figures compare to that of the standard amaranth solution (254 ppm $^{w}/v$; non-degraded) which showed a retention of 4.08 mls and a peak height of 0.288 AU.

Discussion

The similarity of the three degradation solution chromatograms is so striking that there is no further doubt, at least for amaranth, as to the predictability of the degradation. To some extent this may reflect the greater facility with which consistent conditions can be provided for thermal degradation as opposed to photodegradation.

In general no case was discovered at any time where the thermal decay trials gave extraordinary deviations from expected results for any of the food colours under test.

CHAPTER 8

Chromatographic and Ancillary Techniques

SECTION A : USES OF UV-VISIBLE SPECTROSCOPY

The UV-visible spectra of the 16 food colours were obtained for two purposes:-

(i) for reference purposes in selecting detection wavelengths in hplc; and

(ii) for comparison with degraded solution spectra.

As regards (i), all 16 colours showed sufficient absorptivity at 254 nm to be detected. However, brilliant blue FCF, green S and patent blue V are all fairly weakly absorbing in this region (these are all triphenylmethane type dyes). Use of 235 - 240 nm significantly improves the absorption for green S and patent blue V, but brilliant blue FCF remains of low extinction coefficient until the wavelength is reduced below 230 nm, and this is unacceptable for an eluent comprising methanol/water/cetrimide whose UV cut-off is in this region.

Comparison of standard and degraded solutions by UV-visible spectroscopy was carried out for a number of solutions. The uses and limitations of this approach may be well illustrated by reference to Figure 35 where UV-visible spectra are given for ponceau 4R (a) for a standard, undegraded solution, (b) for a heavily thermally degraded solution, and (c) for a heavily photodegraded solution.

Firstly, this figure shows that ponceau 4R (in common with the majority of the food colours tested) degrades to give a colourless solution under the influence of heat or of light. The visible absorption peak may therefore be used very simply to follow the decay of the dye - this method was used for indigo carmine in producing the data for Figure 24. No new peaks appear beneath the declining peak

U.V.-visible spectra of Ponceau 4R solution showing the effects of degradation

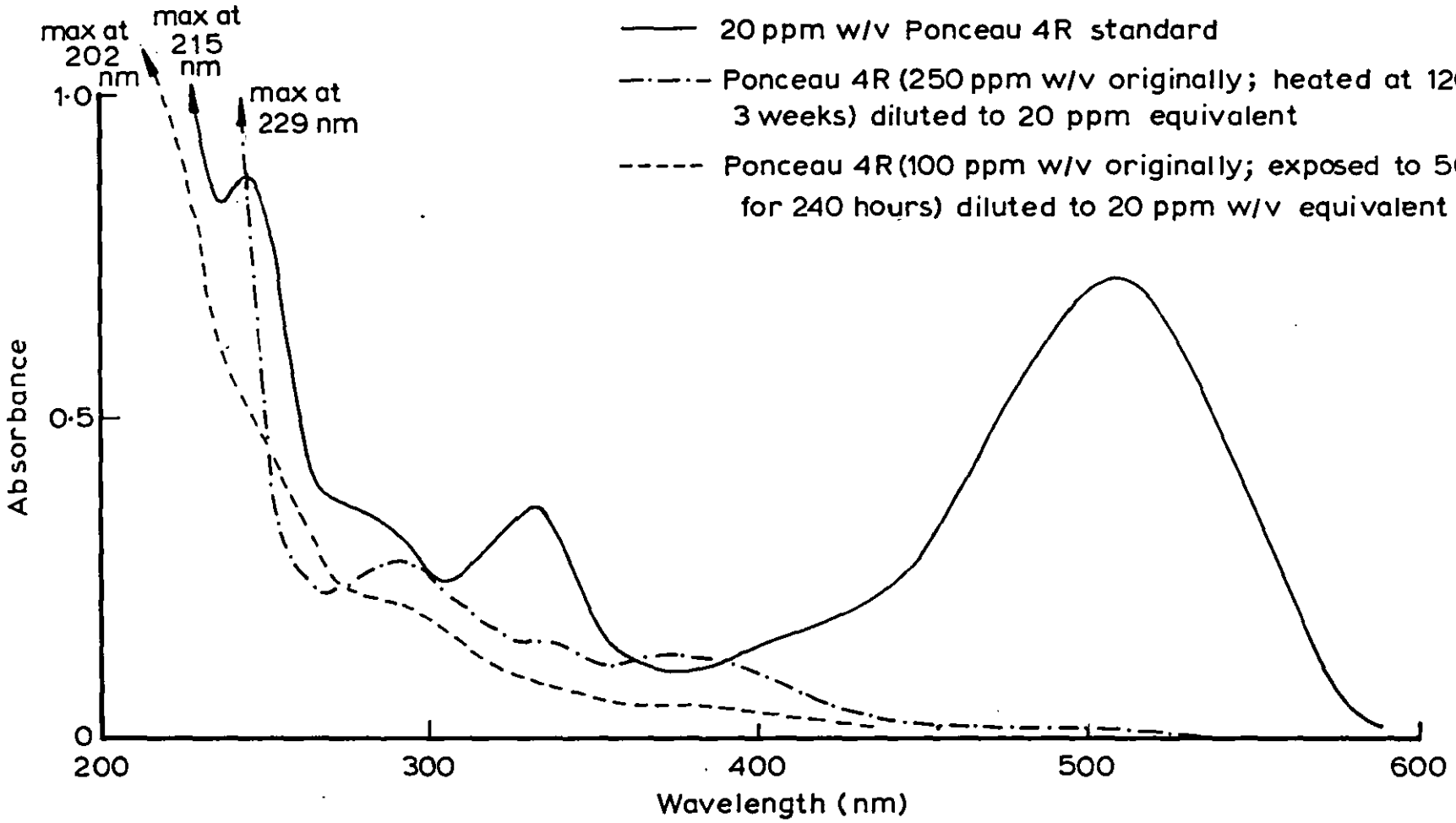


Fig. 35

to complicate the measurement.

Secondly, the UV spectra show at a glance that the products of photodegradation differ (certainly in a major degree) from those of thermaldegradation. For instance, the far UV maxima of the photo-degraded and thermally degraded solutions are 202 and 229 nm, respectively, compared to 215 nm for the undegraded ponceau 4R.

The limitations of the method are also apparent. It has already been mentioned that all aromatic compounds absorb in the far UV (below 270 nm). This fact, an advantage in hplc where it permits a wide range of compounds to be detected at a single wavelength, is a disadvantage in simple spectroscopy where specificity is rather low. Computers or microprocessors can help to investigate the cluttered 190 - 280 nm region of the UV spectrum, either by direct use to produce high derivative spectra[145] and hence enhanced resolution of poorly defined peaks, or by use of published computer programs for determining the number of species present in a solution when a series of solutions are available - such as samples taken at various stages of a degradation, for instance.[146] Neither of these methods offers the potential benefits of hplc as an aid to increased specificity when dealing with complex mixtures containing many minor constitutents. For this reason, all efforts were directed towards hplc analysis, with UV-visible spectroscopy only serving an ancillary role as outlined originally.

Simple UV-visible spectroscopy might have a role to play, however, in solving a perennial problem of hplc - knowing whether all the solution components have finally eluted. The possible check on this by summating the spectra of the eluted peaks (derived, for instance, as in figures 39 – 41), and comparing this with the spectrum of the sample prior to chromatographic separation, might show the absence of

important components. This would be a project in itself and would benefit greatly from electronic peak-integrator equipment. The major problems to be tackled might prove to be,

(i) individual compounds might suffer loss during chromatography; and

(ii) the bandwidth of the hplc detector is much wider (~10 nm) than the usual bandwidth of standard spectrometers (~1 - 2 nm), and the resulting distortion would need to be compensated for when undertaking the comparison.

SECTION B : MODIFICATIONS TO THE CHROMATOGRAPHIC PROCEDURE

Several of the chromatograms of degraded solutions showed incomplete resolution of complex separation patterns. As theory states that reduced flow rates should benefit separation (except where this is extended to very slow flow indeed) a sequence of runs at differing flow rates was undertaken to estimate thevalue best suited to present requirements.

Experimental

An aqueous solution containing 255 ppm $^{w}/_{v}$ patent blue V was heated in a sealed glass phial at 130° for 31 days. The solution was then chromatographed at flow rates of 1.2, 1.0, 0.8, 0.6 and 0.4 mls/min, other conditions including: detection at 235 nm; 5 µl injection; 0.04 AUFS; eluent 75/25/0.25 methanol/water/cetrimide (v/v/w).

Results

All the significant peaks were seen in all the chromatograms. The main benefits in slower flow rate were found in small peaks seen as broadening or barely discernible shoulders on larger peaks becoming better defined at slower flow rates. The response time of the equipment may have contributed to this effect in part. The major degradation product peak (r.t. 3.53 mls.) appeared less symmetric at slower flow rates possibly indicating an unresolved shoulder.

Discussion

The improvement obtained by reducing the eluent flow rate seems quite limited. Generally0.8 mls/min seemed a worthwhile compromise giving a visible improvement in resolution w.r.t. 1.2 mls/min, but remaining acceptable in analysis time required. Other methods were therefore sought to improve resolution and little variation in eluent

flow rate was practised.

Step Elution Chromatography

Introduction

The effort towards complete chromatographic separation of thermal degradation mixtures met with a recurrent difficulty. Often the prominent peaks appeared early and were incompletely resolved, such that the adoption of a substantially more polar eluent would have been recommended but for the presence of small, late-eluting peaks, whose retention times would have been so increased by this measure that the total analysis time would have become quite unacceptably long.

The usual remedy is to use gradient elution, but the type of single piston pump available was not suited to this, and there are doubts as to whether gradient elution can be effective in ion-pair chromatography because of the generally recognised need for prolonged equilibriation. One example of gradient elution ion-pair chromatography was, however, listed in Table 7 and used in the separation of food colouring matters.[126] The chromatograms reproduced in this paper are of acceptable quality and so an investigation was conducted to assess the method. Step-elution was employed because of the type of apparatus available.

Experimental

The thermally degraded solution of carmoisine described in the previous section was again taken as the test solution. The initial eluent comprised 60/40/0.25 (v/v/w) methanol/water/cetrimide. This was used for initial column equilibration and was run for the first 18.2 mls of the elution. The eluent was then switched to 75/25/0.25 (v/v/w) for the remainder of the elution.

Results

A rise of over 0.04 AU in the baseline occurred from retention 29 mls to 35 mls, which then sloped down again to give an overall, constant rise of about 0.024 AU. The most prominent of a number of peaks appeared at 35.1 and 46.7 mls respectively.

Discussion

This trial while providing adequately Gaussian peaks (low degree of tailing) showed up the flaws of the method. Though an improved result in this case could undoubtedly have been obtained by using initial and secondary eluents of closer composition (e.g. 67/33/0.25 and 77/23/0.25) the real problem of analysis time is clearly not significantly shortened. This is because of the reequilibriation period necessary prior to each run in order to obtain reproducible results. Moreover the hump effect in the baseline following change of eluent makes estimation of the baseline underneath the peaks inexact. It is believed that the hump may be due to leaching of cetrimide out of the stationary phase after changing to a less polar eluent, while the overall change in baseline is due to the difference in UV absorbance between the two compositions of eluent. This in turn shows that the chromatographic process is operating during a period of inequilibrium, and this augurs poorly for good reproducibility and renders column parameters such as relative retention quite meaningless.

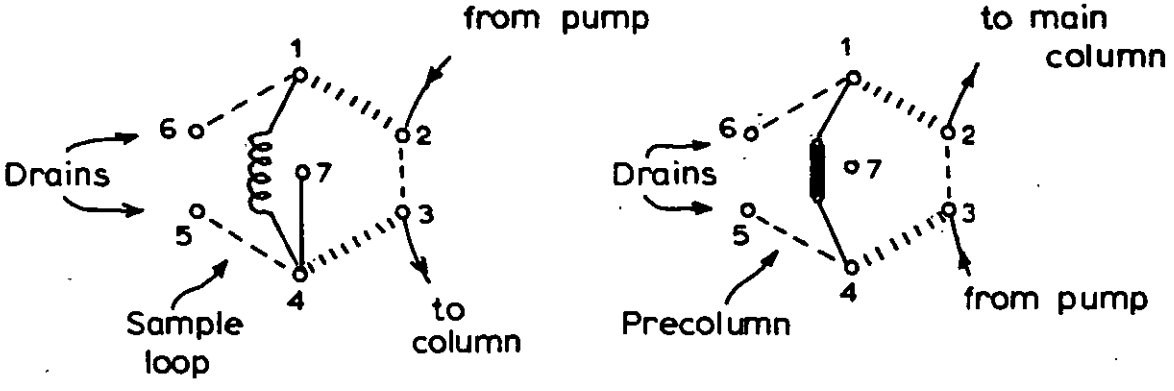
This method was thereforenot pursued further. More concentrated mixtures might prove easier to work with in this context, but there would remain difficulties as have been outlined.

Column-switching

The technique of column-switching (or "cutting") was investigated as a method which offered clear advantages in ion-pair work for resolving early eluting peaks without extending analysis time

unacceptably in the presence of high-retention peaks. As a practical method this has developed further recently[147,148]. The basic concept is that the sample is runthrough a short (e.g. 4 cm) pre-column: after elution of the group of low-retention peaks onto the main analytical column, the eluent flow is by-passed around the pre-column and directly through the main column, leaving high-retention peaks retained on the pre-column. Back-flushing or switched, forward-flushing to waste can eliminate these retained peaks. Sufficiently, polar eluents may then be employed throughout the operation to ensure good resolution of the early peaks without the unacceptable retention times, due to late peaks that would otherwise result.

When applied to the available apparatus, the method seemed adaptable in principle. The figure shows the usual configuration of the rheodyne valve as set for normal hplc, and the configuration adopted for column switching.



(syringe injection into port 7)

Usual Configuration Column-Switch Mode

Alternative connections at the switching positions ---- and ''''''''

The problem of constructing a high-efficiency pre-column was the major concern. A workshop-modified column was assembled, but no adequate means was found to high-pressure pack this. Moreover, the column in its present form has given rise to high back pressures, possibly due to the narrow gauge tubing used in its connections. At the present time Shandon-Southern do not produce a suitable equipment package including pre-column and packing connectors/packing column to pursue the method. It is included here however, because it seems to offer the solution to the remaining hplc difficulties in this work, and to present no substantial disadvantages at all, other than the need to practise 'trial and error' methods in the evaluation of the optimum switching time after injection.

Construction of a Column Heating System

Introduction

One of the means of increasing the separating power in reversed phase chromatography, which many workers in the field have increasingly espoused over the past couple of years, is the use of elevated temperatures (e.g. 60°C) during the separation.[82a] Generally the hplc column and eluent inlet system are both enclosed in an oven such that preheated eluent enters the system. Indeed, this latter point has been stressed by some research groups.[93]

Whereas it may be true that optimal results are to be achieved in this manner, no suitable oven was available for the present work. The practical questions to be faced were,

(i) whether a means of heating the column be devised,

(ii) whether the apparatus constructed would produce a significant increase in resolution relative to there being no such apparatus available.

A third, and very important consideration was also involved: it had been apparent that (in the absence of such apparatus) a chromatogram run on one day produced significantly different retention times to an analogous chromatogram run the following day. It was suspected that ambient temperature variations were the cause of these discrepancies. It seemed that, if any progress was to be made, a method was urgently required to provide consistent temperature conditions, whether or not they produced greater resolution, so that sets of results could be compared.

Design of a Column Heating Jacket

It was found that a section of $1\frac{1}{4}$ inch diameter bicycle inner tube could be fitted over the column head and base so exactly that only a single restraining clip was required at the base to render the assembly watertight (Figure 16). This left a space for water between the tubing wall and the outer wall of the column proper. To enable water flow to take place through this jacket, two eyelets were punched in the tubing, one at the top and one at the bottom of the jacket space. Tyre valves, with their internal mechanisms removed were then fitted through the eyelets. These were of the type that may be sealed by constriction under screw pressure. The lower hole acted as the inlet. Plastic tubes were led from both inlet and outlet to a thermostatted water bath, the inlet tube being attached to a water pump incorporated into the bath. Flow through the jacket was regulated by adjustment of screw pressure on the inlet tube leading from the pump, automatic compensation being made by the pump by transfer of torque to the bath stirring mechanism. The bath was filled with distilled water as it was found that tap water produced some scale deposits which were drawn through the pump, constraining the flow as they built up. No other

maintenance problems arose over many months of operation and daily checks with a mercury thermometer showed the thermostat to be highly reliable. The steel column did however eventually suffer surface rust. It is likely that occasional treatment of the column with a polymer wax sealant (as used on cars) could inhibit this rusting.

Testing the Apparatus

The apparatus was set up as in Figure 16. The water pump was started and half an hour allowed for complete equilibration at each new temperature, during which time eluent was also circulated. A 5 µl injection of a solution of 255 ppm $^{w}/_{v}$ patent blue V was then made and the resulting chromatogram measured. The range 20° - 54°C was investigated.

Results

Heating Jacket Temperature (°C)	20	30	40	46	54
Retention Time (mls)	3.90	3.20	2.78	2.44	2.18
Solvent Retention (mls)	1.40	1.36	1.33	1.31	1.29
$\frac{\text{Peak Width at } {}^{1}/_{10} \text{ Height}}{\text{Peak Width at } \frac{1}{2} \text{ Height}}$	2.15	2.10	2.00	2.13	2.23
Efficiency of Column (theoretical plates)	1160	1420	1480	1470	1560
Peak Height (Absorbance Units)	2.16×10^{-2}	2.87×10^{-2}	3.47×10^{-2}	4.08×10^{-2}	4.46×10^{-2}

Discussion

There was a significant improvement in column efficiency when the heating jacket was used, much of this taking place during the rise from 20°C to 40°C. Peak shape as measured by the peak width ratio ($W_{\frac{1}{2}}$: $W_{\frac{1}{10}}$) does not show consistent improvement with increasing temperature but rather seems marginally better around 30 - 46°C than outside this range. Overall 40°C seemed a good choice of jacket temperature. It was feared that long term use of 54°C might result in some deterioration or distension of the tubing. [At a temperature of 60°C, some workers noted declining column efficiencies[94]].

SECTION C : CHROMATOGRAPHIC BEHAVIOUR OF STANDARD COMPOUNDS. COMPARISON OF ION-PAIRING WITH NON-ION-PAIRING SUBSTANCES

Selection of compounds for comparison: one compound involved in ion-pairing and one compound clearly not so involved were required which were of comparable retention times and which both contained UV chromophores. 5 µl injections were made of 250 ppm $^{w}/_{v}$ solutions of the following compounds in water or methanol: green S , patent blue V (examples of ion-pairing species), n-butylbenzene, dioctylphthalate, dimethylphthalate, di-n-butylphthalate, anthracene, sym-diphenylethane and 5,8-dimethyltetralin. Conditions included use of an eluent comprising 65/35/0.25 (v/v/w) methanol/water/cetrimide, and ambient temperature (c. 20°C)

Results

Compound	Retention (mls)	Compound	Retention (mls)
green S	2.52	dioctylphthalate	5.80
patent blue V	4.04	dimethylphthalate	1.64
n-butylbenzene	2.76	di-n-butylphthalate	2.88
anthracene	2.48	sym-diphenylethane	2.80
		5,8-dimethyltetralin	3.32

Discussion

Of the available compounds, dioctylphthalate had too high a retention time. That of 5,8-dimethyltetralin was next greatest and this was chosen as being intermediate between the retention times of green S and patent blue V.

Two types of comparison were made:

(i) green S with 5,8-dimethyltetralin : retention time variation with

temperature;

(ii) green S, patent blue V and 5,8-dimethyltetralin: retention time variation with constituency of the eluent.

Experimental

(i) effect of temperature: the heating jacket was set at the required temperature and eluent circulated through the hplc column for 20 - 30 minutes prior to any injection to allow equilibriation. The retention times of green S and of 5,8-dimethyltetralin were found at a range of temperatures (22.5°C to 62°C) using an eluent comprising 70/30/0.25 (v/v/w) methanol/water/cetrimide.

Results

The results are shown in graphical form in Figure 36.

Discussion

The important feature of these results is the change in relative retention of green S, the ion-pairing eluate, and of 5,8-dimethyltetralin, the non-ion-pairing eluate ranging from 1 : 0.85 at 22.5°C to 1 : 1.22 at 54°C. This cross-over effect means that resolution increases above and below a small temperature range around 31° where there is peak overlap. Whether or not this is typical behaviour would require the experiment being repeated with a wide range of compounds. By itself, however, this finding highlights the difficulties in developing good chromatographic procedures for mixed compounds of different type.

Experimental

(ii) effect of eluent composition: the heating jacket was set at 25°C throughout. The column was equilibriated with a sequence of methanol/water/cetrimide eluents of composition ranging from 75/25/0.25 (v/v/w)

to 65/35/0.25 (v/v/w). For each eluent chromatograms were obtained of green S, patent blue V and 5,8-dimethyltetralin.

Results

The variation in retention of the three compounds with changing eluent composition is shown graphically in Figure 37.

Discussion

All three compounds show an exponential relationship between retention and the percentage of water in the eluent. The logarithmic linear regression lines show this:

green S : $\log_e$ retention (mls) = -1.29 + 0.0790 x percent water

linear correlation coeff., r = 0.987

patent blue V : $\log_e$ retention (mls) = -2.22 + 0.129 x percent water

r = -.993

5,8-dimethyltetralin : $\log_e$ retention (mls) = -0.590 + 0.0612 x percent water

r = 0.996.

Two features to be seen are,

(i) that despite the close structural relationship between green S and patent blue V, the slopes of their respective regression lines (0.129 and 0.0612) differ greatly;

(ii) that the linear regression line of 5,8-dimethyltetralin is of lesser slope than that of the ion-pairing compounds - but not much less in fact than that of one of these (green S).

Given the difficulty of demonstrating any theoretical correlation between structure and retention, it was necessary to compare simpler molecules. Five possible decay products of the food colours were therefore selected and their retention behaviour observed with

Variation in retention with temperature for Green S (ion-pairing) and for 5,8-dimethyltetralin (not ion-pairing)

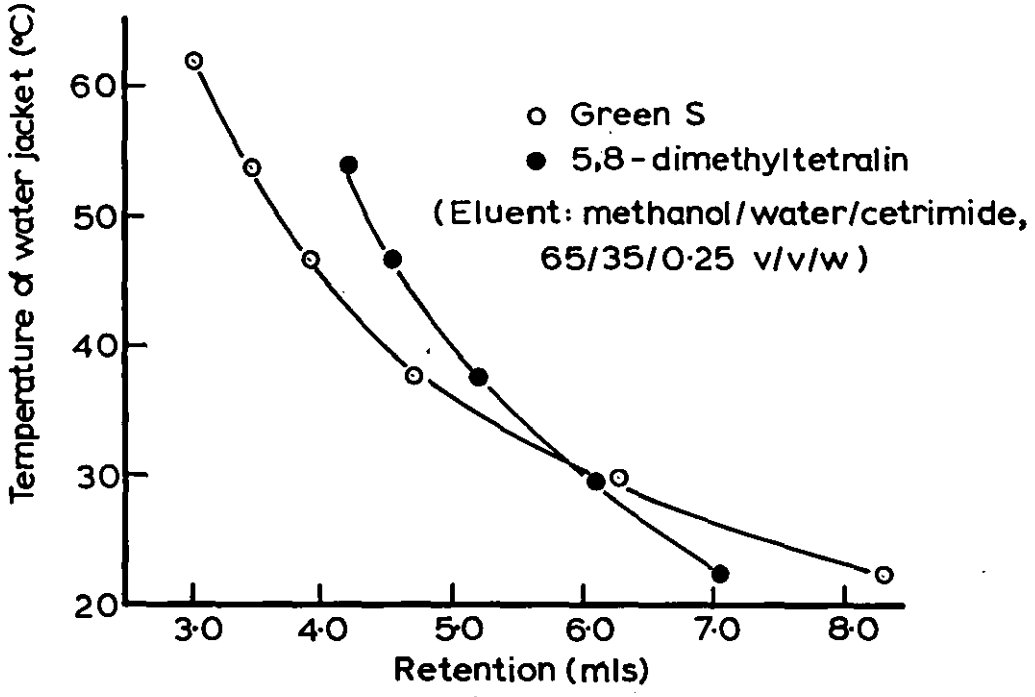


Fig. 36

Variation in retention of Green S, 5,8-dimethyltetralin and Patent Blue V with composition of the eluent

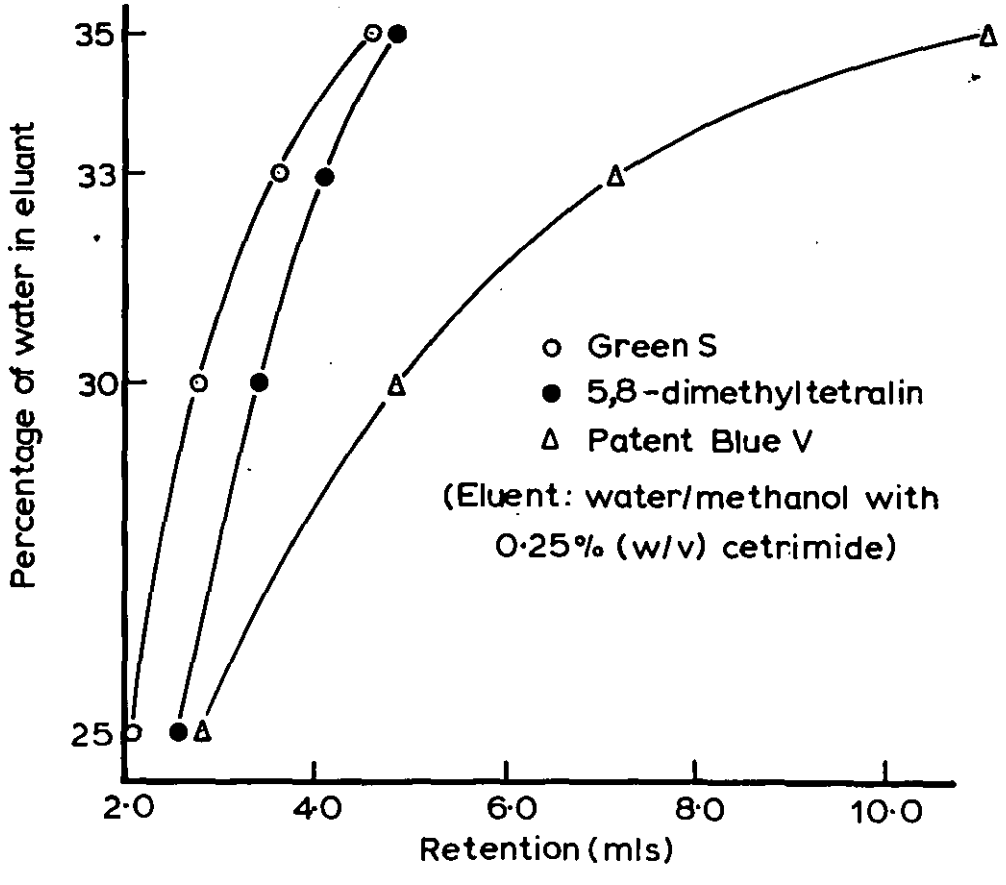


Fig. 37

changing eluent composition.

Experimental

Aqueous solutions were prepared containing ca. 100 ppm $^{w}/v$ sodium naphthionate, sodium naphthol-4-sulphonate, sulphanilic acid, phenol-4-sulphonic acid or aniline hydrochloride. Each was chromatographed on an SAS-hypersil column thermostatted at 39.5°C (eluent temperature 14.5°C before reaching the column) at a range of methanol/water eluent compositions, all containing 0.25% $^{w}/v$ cetrimide.

Results

These are shown graphically in Figure 38. The retention of phenol-4-sulphonic acid was virtually identical to that of sulphanilic acid when the methanol percentage in the eluent was in the range 66.7 to 75%.

Discussion

Clearly, substitution of the aromatic amino group by a hydroxy group leads to increased retention. However, the effect is difficult to observe for smaller molecules and this leads to problems with resolution when complex degradation mixtures are involved with high retention components present which limit the use of highly polar eluents.

A means other than simple chromatographic resolution would therefore, in practice, be needed to differentiate (e.g.) sulphanilic acid, phenol-4-sulphonic acid and their mixtures one from another and in the presence of other chromatographically resolvable solution components.

Variation in retention of possible decay products with composition of the eluent

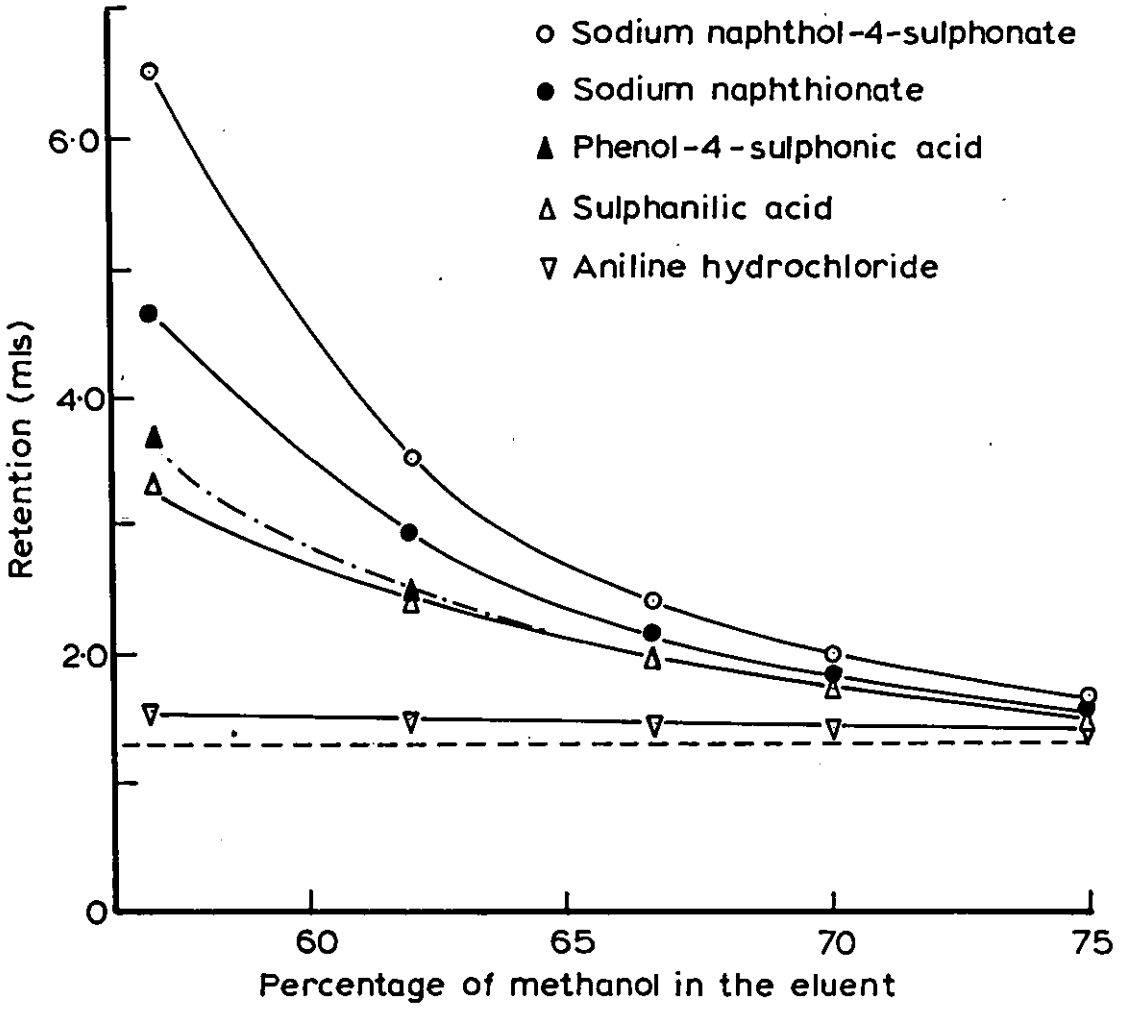


Fig. 38

Experimental

UV-spectra were obtained for ca. 12.5 ppm $^{w}/_{v}$ aqueous solutions of phenol-4-sulphonic acid, sulphanilic acid, sodium naphthol-4-sulphonate and sodium naphthionate.

Results

Measurement of the ratio of absorbance at 248 nm to absorbance at 230 nm gave the following:

Phenol-4-sulphonic acid	0.088
Sulphanilic acid	2.75
Sodium naphthol-4-sulphonate	0.047
Sodium naphthionate	0.69

Discussion

With the resolution available from a standard laboratory UV-spectrophotometer (bandwidth $\sim$ 1 - 2 nm) there is no difficulty in quantifying mixtures of phenol-4-sulphonic acid and sulphanilic acid, or of naphthol-4-sulphonic acid and naphthionic acid by choosing measurement at 248 and 230 nm. For an hplc detector, however, with its 8 - 10 nm bandwidth, it is dubious whether the latter pair could be well distinguished. The former pair is however the one that is difficult to resolve chromatographically, and a double run with the detector centred at each of 230 nm and 248 nm in turn could be used to quantify mixtures of sulphanilic and phenol-4-sulphonic acid, or to identify which component was present.

A trial was carried out to ascertain the result of the method. 255 nm was substituted for 248 nm to avoid the rapid peak rise of phenol-4-sulphonic acid below 248 nm (wide bandwidth detector).

Experimental

The hplc peak height ratios of phenol-4-sulphonic acid and of sulphanilic acid were measured (on separate chromatograms) at 230 and 255 nm. Because no analytical grade of phenol-4-sulphonic acid was avilable, no attempt at measuring detector response per unit concentration was made. Chromatographic conditions were as previously described with 60% methanol in the eluent.

Results

Ratio of peak height at 255 nm to that of 230 nm:

Phenol-4-sulphonic acid : 0.046

Sulphanilic acid : 1.93

Discussion

Two causes for the difference in ratios obtained by hplc detection and by straightforward spectrophotometry may be,

(a) the levelling effect of a wider bandwidth,

(b) the presence of impurities in the phenol-4-sulphonic acid sample which contribute to the UV-spectrum, but not when separated chromatographically before detection.

CHAPTER 9

Hplc Investigation of Thermally Degraded Food Colour Solutions

SECTION A: ANALYSES CONDUCTED PRIOR TO THE INTRODUCTION OF A COLUMN THERMOSTAT

Note Regarding Hplc Analyses Conducted without Thermostatting

The initial absence of a column thermostat was somewhat compensated by use of amaranth as a reference material to which the retention times of other chromatographic peaks could be related. Later (after adoption of the thermostat) it was realised that this procedure had been of limited benefit, as different compounds showed markedly differing temperature dependency in their retention times.

The earlier, non-thermostatted chromatography work was, however, still of value in showing the number of degradation products formed, their spectra (by repeated chromatography at various detection wavelengths) and their approximate relative retentions.

Investigation of Thermally Degraded Solutions by Hplc

Introduction

There are characteristic difficulties associated with the optimisation of separation when chromatographing a mixture of unknown compounds. For instance, whereas compounds belonging to homologous series may directly reflect a polarity change in the eluent (leading to increased retention times) by showing increased separation and resolution, it is not always true that the same effect will prevail where dissimilar compounds are involved. Although early in the present work the assumption was made that, in reversed phase ion-pair

chromatography, the dominance of the sulphonate moiety permitted all aromatic compounds containing this group to be treated as in some sense homologous, eventually it was realised that this was not being born out in practice. Chromatograms obtained with eluents of even moderately different polarities in some cases bore little recognisable similarity: some peaks overlapped with the more polar eluents which did not do so with less polar eluents (and vice versa); some peaks even overlapped at intermediate values of polarity, one peak overtaking another across the polarity range.

A second difficulty resides in the broad range of retention times observed. At low retention in several chromatograms there are found to be a number of peaks clustered together. Adoption of major polarity changes in the eluent in order to increase retention and so resolution - even where this benefit does follow - is thwarted by later eluting peaks having their retention times increased out of proportion to the analytical period acceptable.

The analysis of the thermal degradation product mixture of carmoisine was undertaken while having regard to these difficulties, and analytical methods were tried in order to improve the data obtained.

Experimental

A 255 ppm $^{w}/_{v}$ carmoisine aqueous solution was sealed in a glass phial and heated in an oven at 120^{o}C for 31 days, as described in an earlier section. The solution was then sampled and investigated chromatographically.

Hplc conditions:-

Detection at a range of wavelengths from 235 nm to 355 nm.
Eluent composition 77/23/0.25 (v/v/w) methanol/water/cetrimide.
Flow rate 1 ml/min. [No thermostatting].

Results

A series of chromatograms at different wavelengths of detection was obtained. An example is shown at 254 nm in figure 39. The chromatograms have been measured and are reproduced in abstracted form in figure 40 showing the positions and heights of the principal peaks. Comparison of the 254 nm chromatogram presented in the ordinary and abstracted forms shows the degree of simplification and hence the limits of use of such a treatment.

Four peaks have been selected as being prominant and these have their UV spectra constructed as shown in figure 41.

One feature that shows up well on the different chromatograms is the slow drift to longer retention times that occurred during the analysis period. This amounted to perhaps 5% overall.

Discussion

Those chromatographic peaks which reveal maxima in their UV spectra within the accessible wavelength range provide some clues to their possible identities in this way. However, UV information is not as useful in this respect as e.g. IR spectral data may be.

Chromatogram of carmoisine solution (255 p.p.m. w/v) after 31 days heating at 130°C

5µl injection

Detection wavelength: 254 nm

Eluent: 77/23/0·25 v/v/w methanol/water/cetrimide

W, X, Y, Z : peaks labelled as in figures 40 and 41

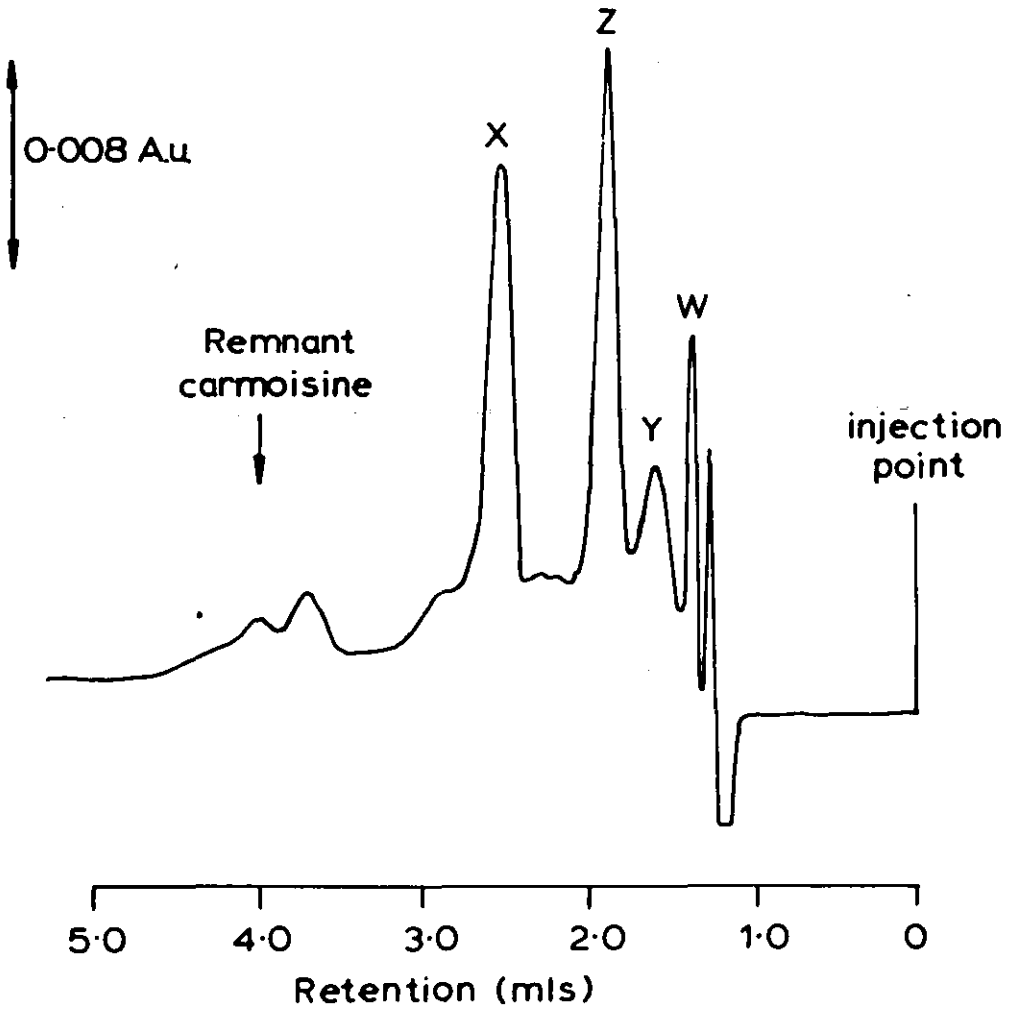


Fig. 39

Peak heights at different wavelengths in chromatograms of carmoisine solution (255 p.p.m. w/v) after 31 days heating at 120°C

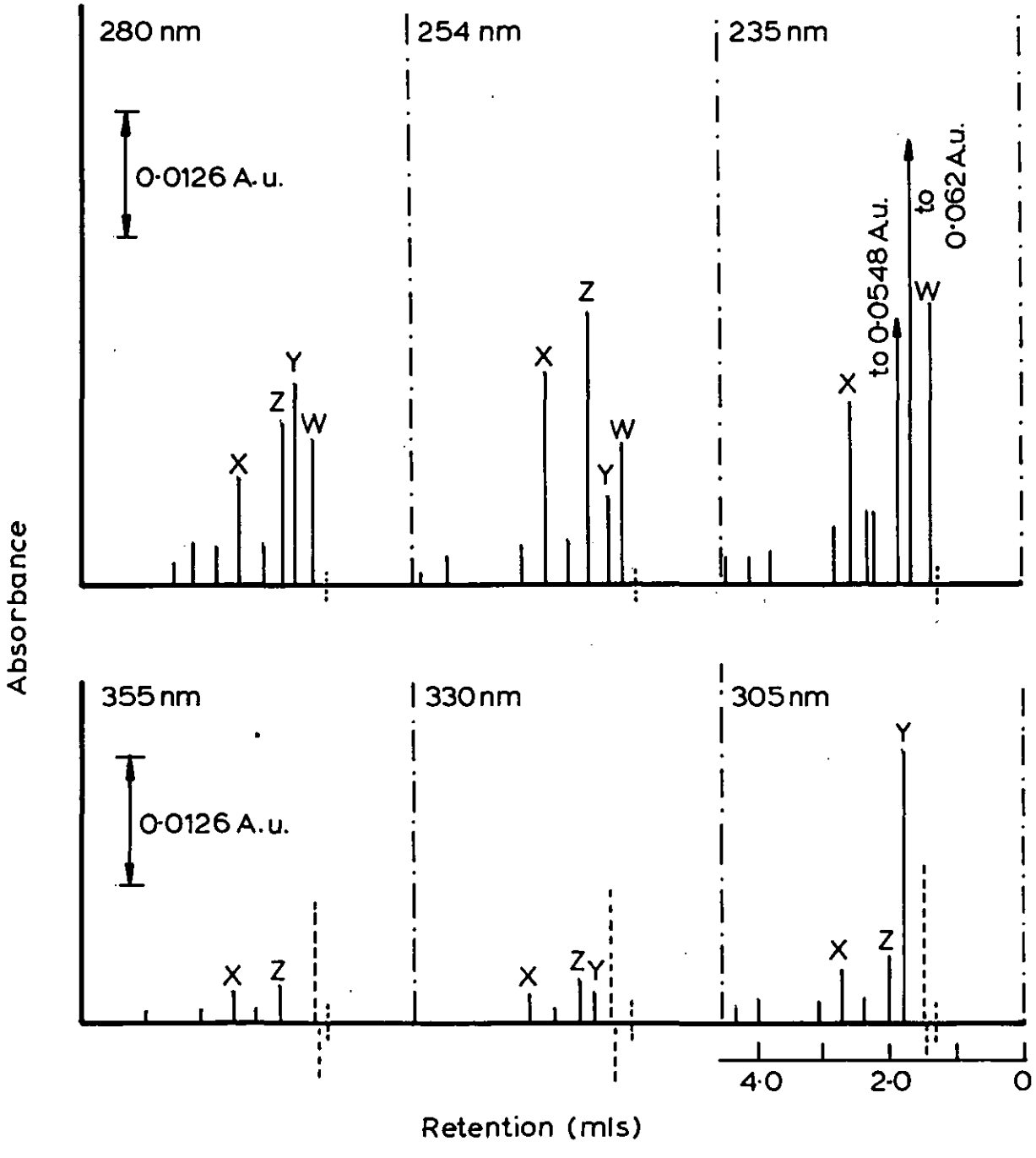


Fig.40

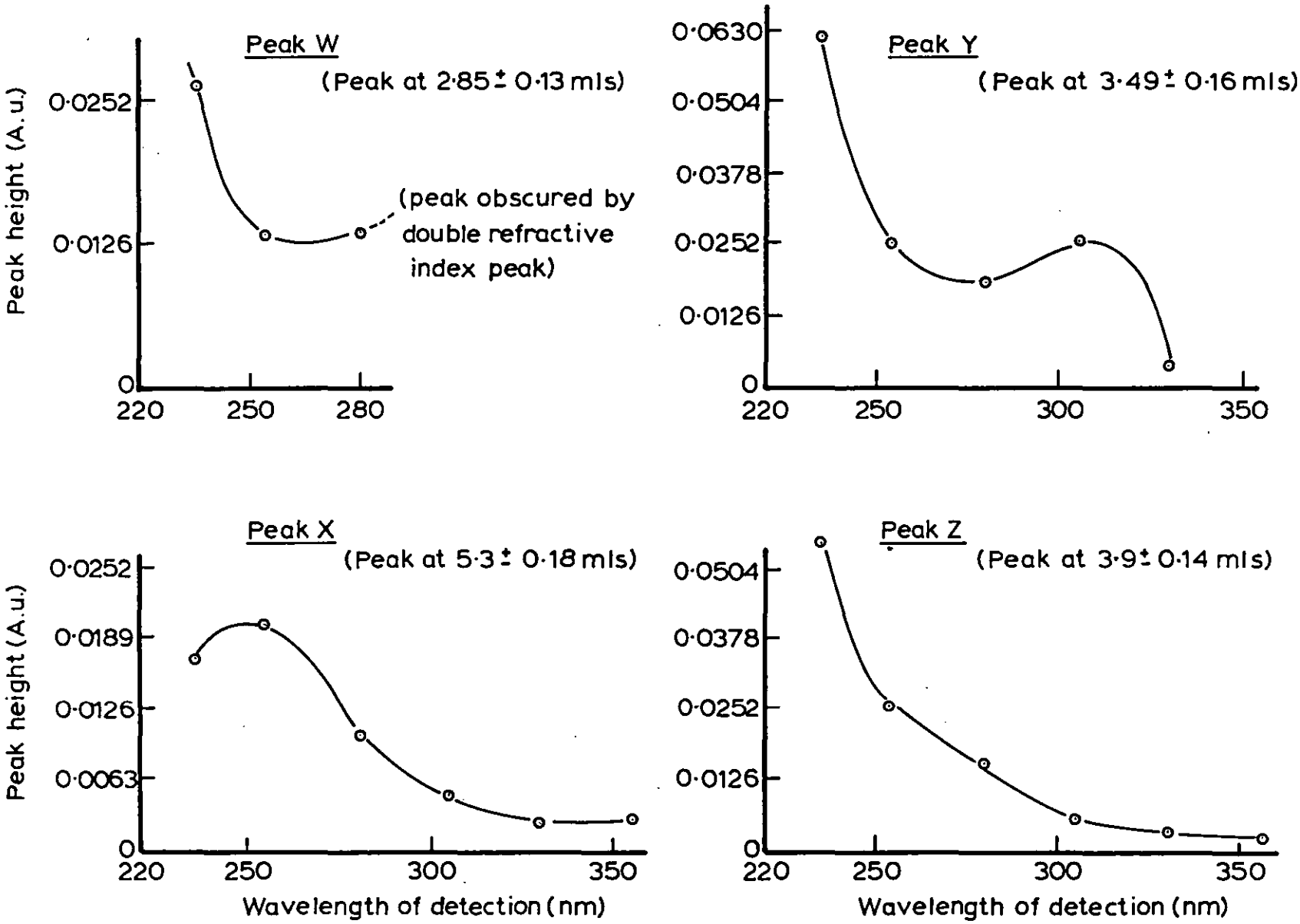

Peak W
(Peak at 2·85 ± 0·13 mls)
(peak obscured by double refractive index peak)
Peak height (A.u.)
0·0252
0·0126
0
220
250
280
Peak Y
(Peak at 3·49 ± 0·16 mls)
0·0630
0·0504
0·0378
0·0252
0·0126
0
220
250
300
350
Peak X
(Peak at 5·3 ± 0·18 mls)
Peak height (A.u.)
0·0252
0·0189
0·0126
0·0063
0
220
250
300
350
Wavelength of detection (nm)
Peak Z
(Peak at 3·9 ± 0·14 mls)
0·0504
0·0378
0·0252
0·0126
0
220
250
300
350
Wavelength of detection (nm)

Fig. 41

Also there seem to be other good purposes to which such information may be profitably applied:-

(i) choice of wavelengths of detection such that incompletely resolved peaks may be measured with least mutual interference: this may entail measuring the chromatograms at a number of different wavelengths of detection;

(ii) a check to ensure that peaks appearing at restricted ranges of wavelength are not missed by merely looking at chromatograms obtained at a single wavelength;

(iii) at least in theory (this was not tried in practice) one could correlate the peaks obtained on chromatograms obtained at different wavelengths for a given eluent composition with those obtained with another eluent by the spectra of the respective peaks, even where the chromatographic pattern had altered beyond recognition; and lastly

(iv) checking a chromatograph peak for its dependency on wavelength could help to demonstrate whether that peak was identical with a standard, or whether the peak was another compound of similar retention, or an unresolved peak that ideally might have its components quantified at different wavelengths.

The drift in retention times observed was not understood initially. The possibility of surface modification of the stationary phase by gradual build up of slow-moving impurities from the sample was at first suspected. This was discounted because of the minute amounts of such impurities. Later it was recognised that the most probable cause was fluctuation in ambient temperature during the course of a day's work. (The construction of a hplc column thermostat, which followed on this finding, has already been described.)

Other thermally degraded food dye solutions were also investigated in the manner described for carmoisine, and these are described below.

Chromatograms of Other Thermally Degraded Food Dye Solutions

The following figures (42-46) are produced in an analogous manner to that used for carmoisine. Brilliant blue FCF, patent blue V, quinoline yellow, sunset yellow FCF, and yellow 2G are given: these are all chromatograms obtained before thermostatting was developed. The eluent employed in each case was 75/25/0.25 v/v/w methanol/water/cetrimide. A standard flow-rate of 1.0 ml/min of eluent was applied. Injection volume was 5 µl.

Discussion

The hplc detector employed was not suited to recording the full visible spectrum as it was fitted with a deuterium lamp only (maximum around 385 nm useable). Therefore it was not ideal for investigating coloured degradation products.

But in tandem with this should be considered the fact that most aromatic components without ring conjugated chromophoric groups show falling absorptivities above 280 nm. Therefore compounds showing rising absorptivities toward the 380+ nm limit are definitely absorbing significantly in the visible while other compounds showing maxima between e.g. 290-350 nm present interesting cases in so far as sulphonic acid/salt groups are electron-withdrawing and may therefore suggest category (d) in the list provided below.[137a]

Peak heights at different wavelengths in chromatograms of Brilliant Blue FCF (256 p.p.m. w/v) after 31 days heating at 120 °C

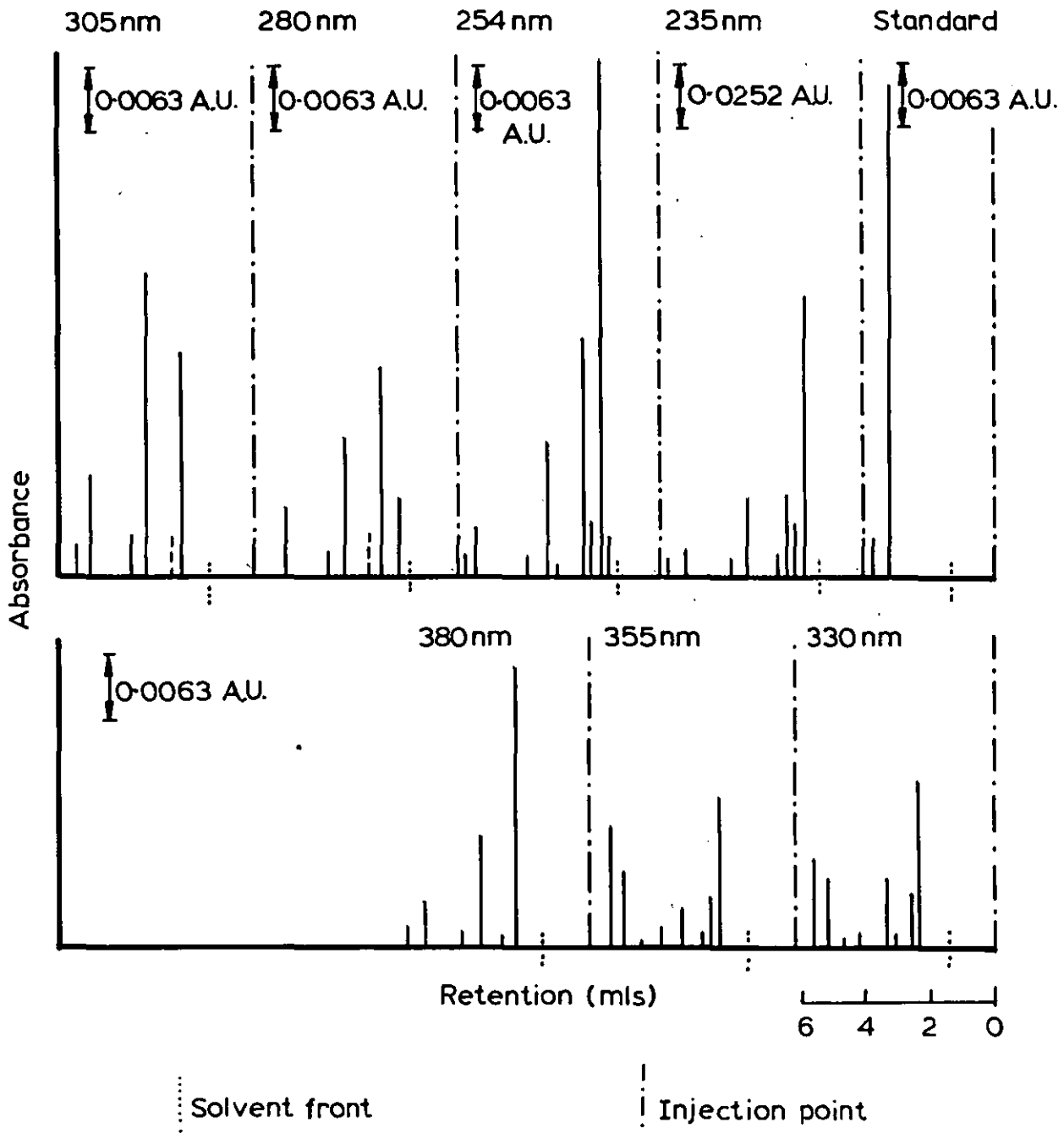
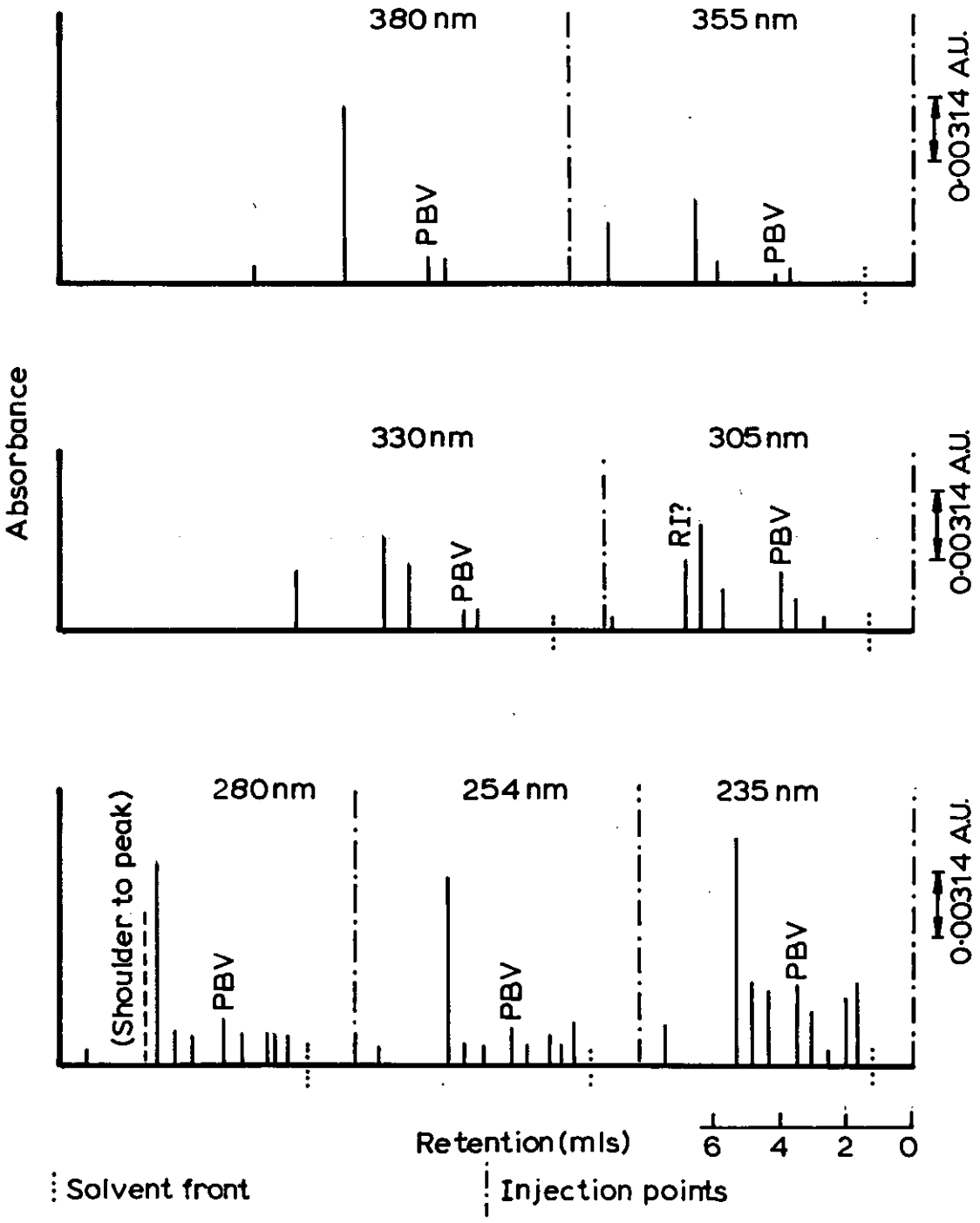


Fig. 42

Peak heights at different wavelengths in chromatograms of Patent Blue V (255 ppm w/v) after 31 days heating at 120°C



RI? = Differential peak

Fig. 43

Peak heights at different wavelengths in chromatograms of Quinoline Yellow (255 p.p.m. w/v) after 31 days heating at 120°C

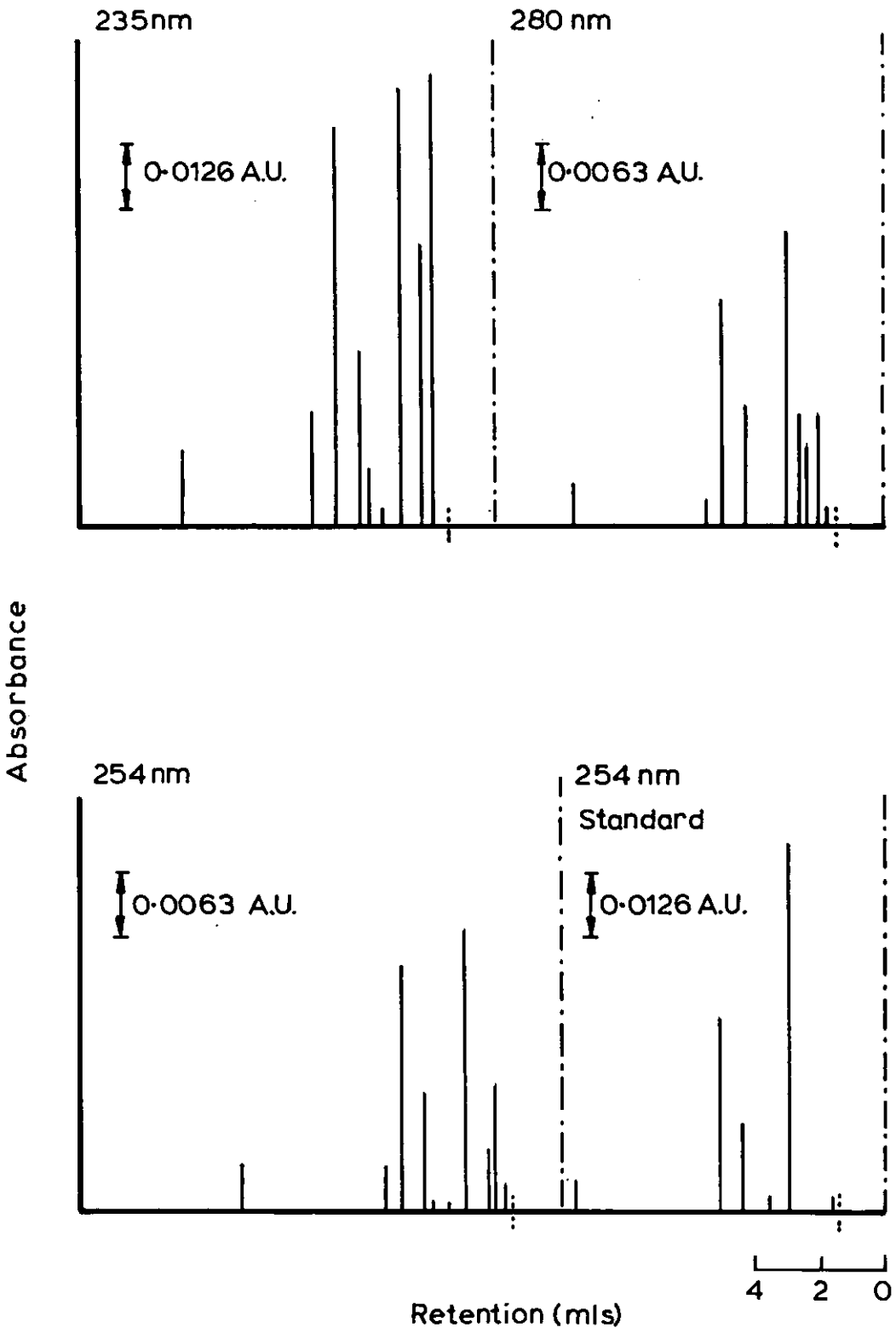


Fig. 44 (a)

Peak heights at different wavelengths in chromatograms of Quinoline Yellow (255 p.p.m. w/v) after 31 days heating at 120°C

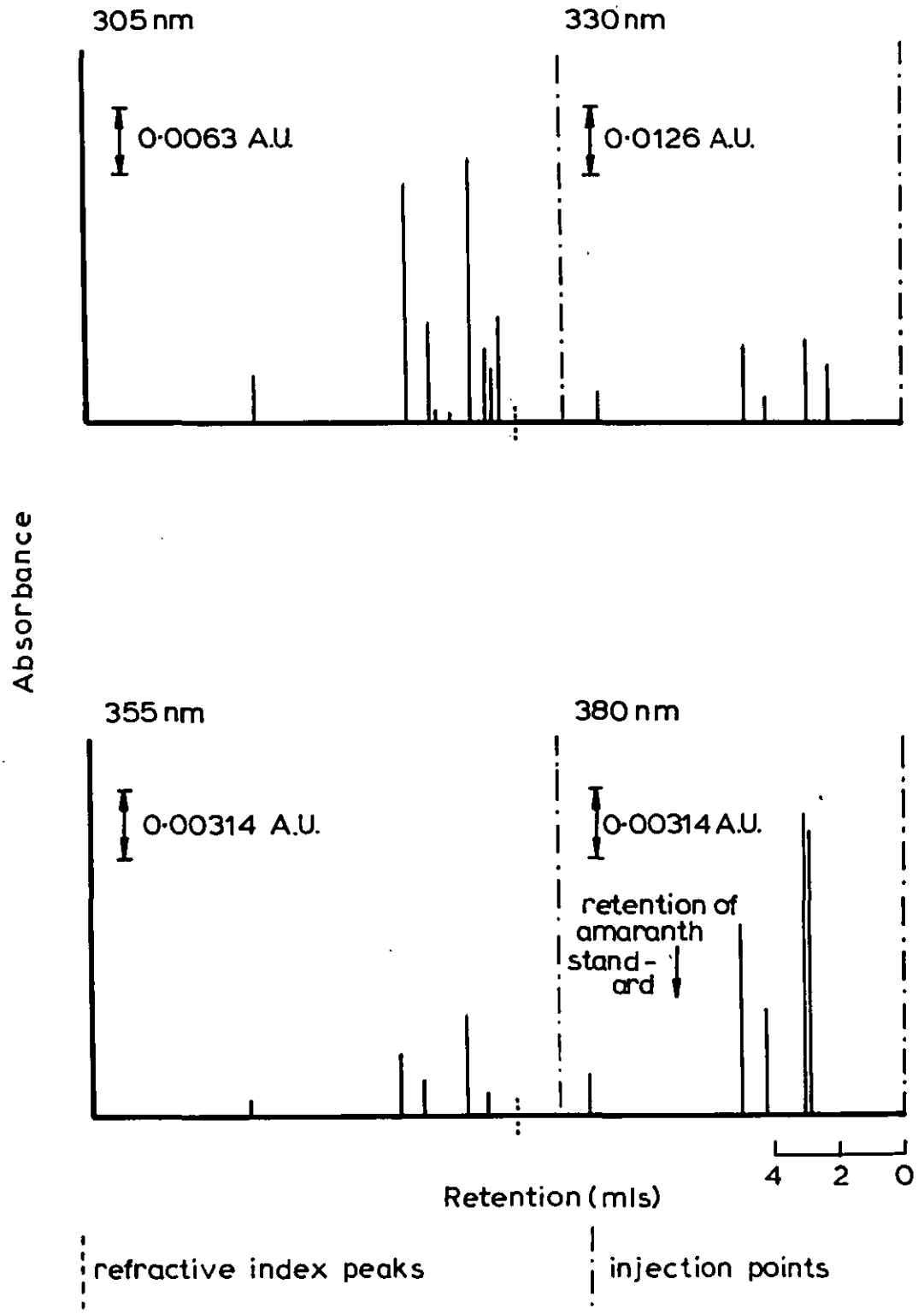
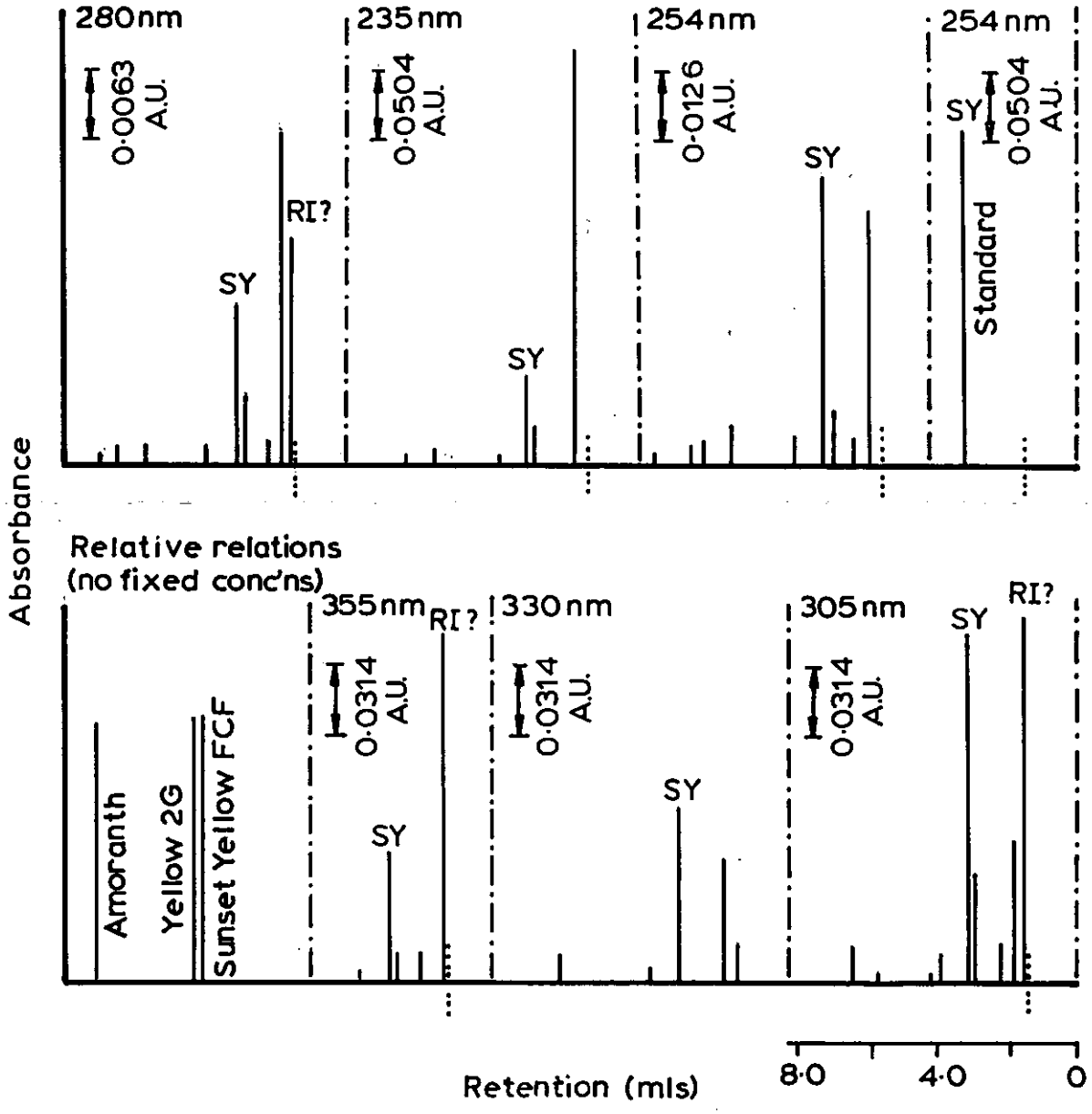


Fig. 44(b)

Peak heights at different wavelengths in chromatograms of Sunset Yellow FCF (254 ppm w/v) after 31 days heating at 120°C.



⋮ Solvent front

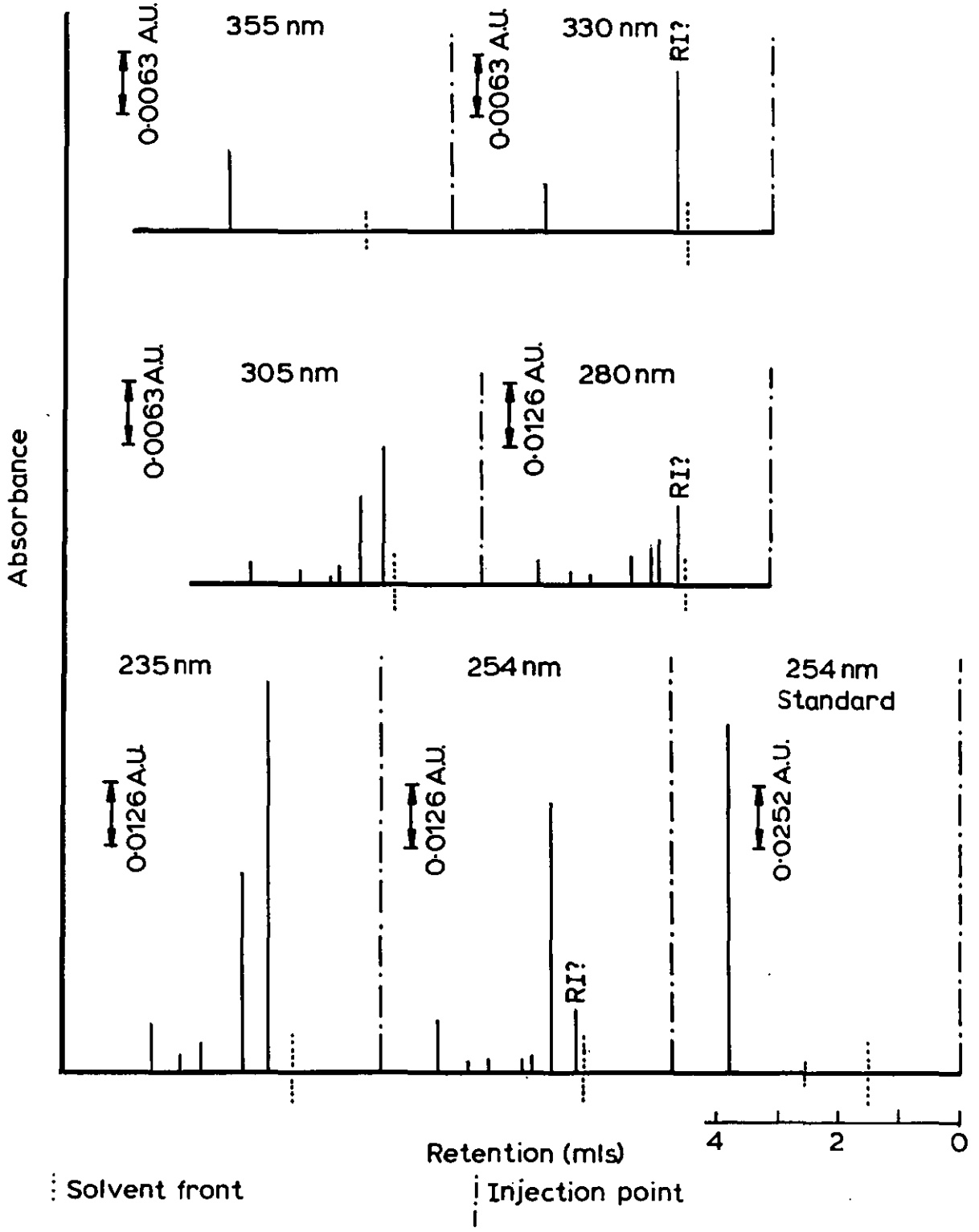
RI? = Differential peak

| Injection points

SY = Sunset Yellow

Fig. 45

Peak heights at different wavelengths in chromatograms of Yellow 2G (255 ppm w/v) after 31 days heating at 120°C



RI? Differential peak

Fig. 46

In an inert medium benzene shows absorption bands at 193-204 nm (ε = 5000) and at 230-270 nm (ε = 250). Other aromatics produce modified spectra as follows:

(a) polynuclear systems (e.g. naphthalene derivatives) - bands displaced to longer wavelengths and increased in intensity;

(b) electron-donating or conjugative substituents - the 230-270 nm band is shifted to longer wavelength;

(c) electron-withdrawing substituents - little change occurs;

(d) para di-substituted aromatics where one substituent is electron-donating and the other electron-withdrawing - shift in the absorption to longer wavelength;

(e) para di-substituted (but not complementary as in (d)), ortho or meta substituted aromatics - little change to that expected on the basis of summing the individual substituent effects as in (b) and (c).

Comparison of spectra between acidic and neutral media for aminoaromatics, or between neutral and alkaline media for phenolic compounds, can confirm the identification of individual compounds where standards are available. Generally UV-visible spectroscopy is limited as a tool for identifying complete unknowns.

SECTION B: HPLC WITH THERMOSTATTING: THERMALLY DEGRADED SOLUTIONS

1) Green S

An aqueous solution of 257 ppm $^{w}/_{v}$ green S was prepared. This was sealed in a glass vial and subjected to 31 days heating at 120^{o}C. The solution was monitored polarographically during this period (results

already given in figure 30). At the end of the trial the solution was stored ca. 6 months prior to hplc analysis. Conditions were varied to give the best compromise between speed and efficiency, and finally fixed at:

Eluent: 63/37/0.25 v/v/w methanol/water/cetrimide.

Detection wavelength: 240 nm.

Flow rate: 0.8 mls/min

Thermostat set at 39.5°C: room temperature 17.5°C.

Results

The chromatogram obtained is given in figure 47. The chromatogram has been labelled in common with the following table which gives the most important parameters from the chromatogram.

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative peak area*
1	2.11	4.25×10^{-3}	2.7%
2	2.45	8.03×10^{-3}	5.9%
3	2.88	9.53×10^{-3}	8.2%
4	3.22	3.31×10^{-3}	3.2%
5	3.41	1.12×10^{-2}	11%
6	3.89	2.36×10^{-3}	2.7%
7	4.42	2.36×10^{-3}	3.1%
8	6.14	1.26×10^{-3}	2.3%
9	7.39	1.42×10^{-3}	3.1%

continued

Chromatogram of Green S solution (257 ppm w/v) after 31 days heating at 120°C

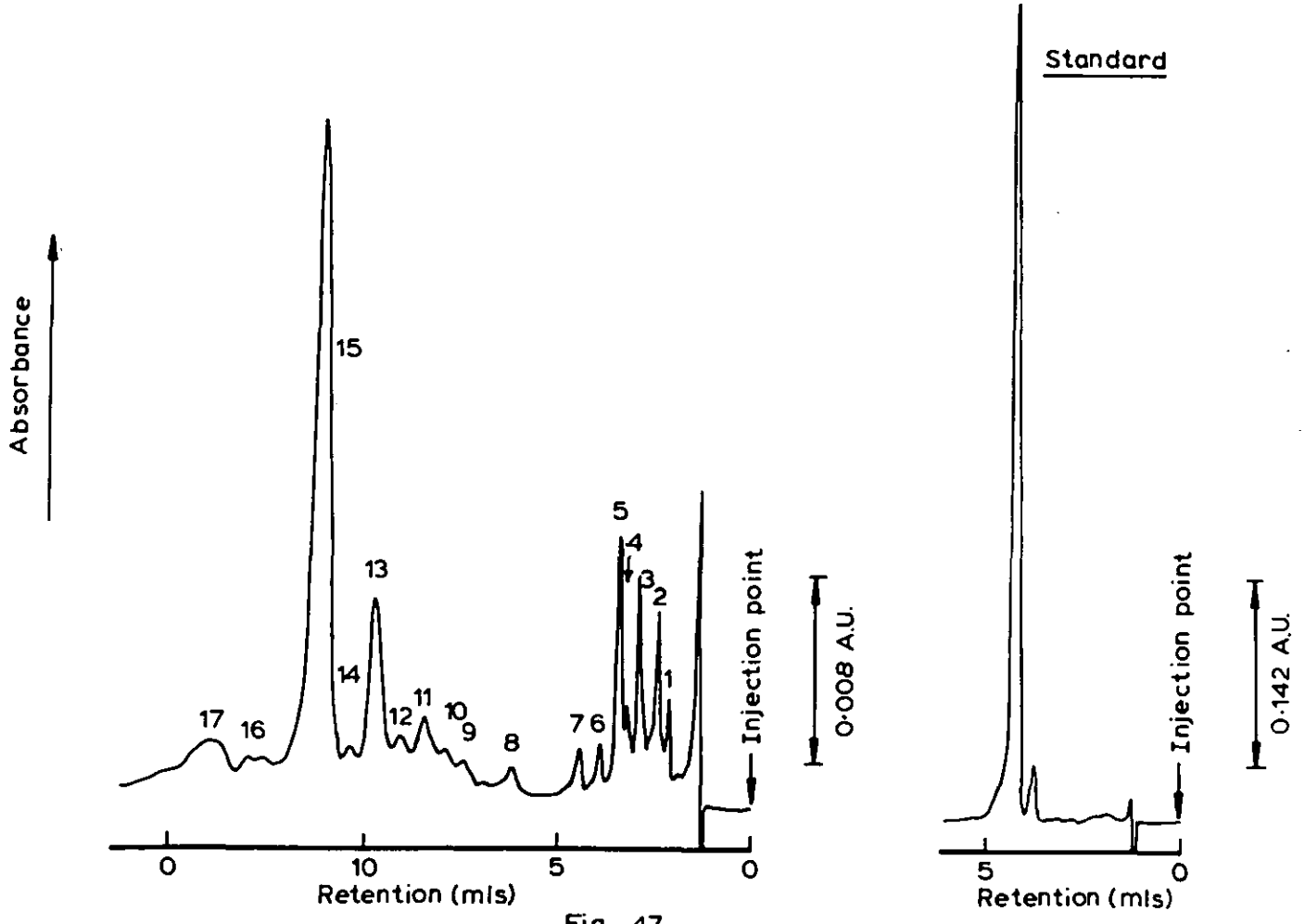


Fig. 47

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area*
10	7.92	1.97×10^{-3}	4.6%
11	8.40	3.46×10^{-3}	1.2%
12	9.02	2.52×10^{-3}	6.8%
13	9.70	8.50×10^{-3}	25%
14	10.37	2.05×10^{-3}	6.3%
15	11.14	2.88×10^{-2}	95%

*Peak areas have been estimated relative to the original peak area of a 5 µl injection of 257 ppm $^{w}/_{v}$ green S standard according to the approximate formula (for peak N)

$$\text{Relative peak area (N)} = \frac{\text{Peak height (N)} \times \text{retention (N)} \times 100}{\text{Peak height (green S)} \times \text{retention (green S)}}$$

This formula is derived simply from the equations linking the basic chromatographic parameters and assumes peak shapes to be the same, e.g. all Gaussian.

Peaks shown in figure 47 labelled 16 and 17 were too poorly defined to include in the table above.

Discussion

No certain assignment for a remnant green S peak can be made in the chromatogram. Peak 7 seems most likely, having a retention only 2% greater than the green S standard and a peak area 3% that of the standard - very close to the value for remnant green S obtained polarographically. Also, the retention times of samples with two

orders of magnitude concentration difference are likely to be affected by loading effects on the column shortening the retention slightly of the heavier sample.

A striking feature of this chromatogram is that there are so many degradation products. Without a knowledge of the extinction coefficients at 240 nm of the various compounds, it is not possible to specify which peaks represent the major products. It is, however, possible in the present special case to indicate that peak 15 is indeed a significant decay product by the following reasoning. Measurement of a UV spectrum of green S showed that a 10 ppm $^{w}/_{v}$ solution had an absorbance of 0.947 at 240 nm (the wavelength of detection used in the hplc study) in a 1 cm cell. The extinction coefficient at 240 nm may be found from the Beer-Lambert Law (see Chapter 6).

$$\text{Molarity} = \frac{10 \times 10^{-6}}{\text{m.wt.}} = \frac{10^{-2}}{576.64} = 1.734 \times 10^{-5}\text{M}$$

$$\text{Extinction coefficient at 240 nm} = \frac{\text{Absorbance}}{\text{Molarity x path length}}$$

$$\varepsilon_{240} = \frac{0.947}{1.734 \times 10^{-5} \times 1} = 54{,}600 \text{ l mol}^{-1} \text{ cm}^{-1}$$

Now, it has been shown that ordinary, simple molecular species can not have extinction coefficients in excess of about 10^5 l mol^{-1} cm^{-1}.[138a] Therefore, the lower limit for the molar ratio of

green S (before decay) : decay product (peak 15)

must be that ascribed by supposing the decay product to have this 'maximum' extinction coefficient, viz.,

$$1 : \text{relative peak area} \times \frac{54,600}{10^5}$$

or

$$1 : 0.95 \times 0.546, \text{ i.e. } 1 : 0.52.$$

If not more than a single molecule of the decay product can be produced per molecule of green S then the extinction coefficient corresponding to a 1 : 1 ratio would be 54,600 x 0.95 = 51,900 l mol^{-1} cm^{-1}. All these ratios assume 100% loss of green S . The presence of so many other decay peaks must make a 1 : 1 ratio unlikely, and a high ε_{240} value for the decay product observed in peak 15 is indicated. The product could be a derivative of green S that retains the skeletal triphenylmethane structure. If so the increased retention time relative to that of green S could be consistent with the preservation of the sulphonic acid groups responsible for the ion-pairing, but with the loss of the quaternary ammonium function thereby decreasing the affinity of the molecule for the polar mobile phase. Further evidence would obviously be required to test this possibility.

2) Sunset Yellow FCF

A 254 ppm $^{w}/v$ sunset yellow FCF solution was thermally degraded in the same manner as for green S (see figure 30 for decay curve). The degraded sample was subjected to hplc analysis, first without thermostatting of the column (results in figure 45), and then (after 6 months storage) at 240 nm under the same conditions used for green S above.

Results

The chromatogram obtained is given in figure 48. The chromatogram has been labelled in common with the following table which gives the major chromatographic parameters.

Chromatogram of Sunset Yellow FCF solution (25.4 ppm w/v) after 31 days heating at 120°C

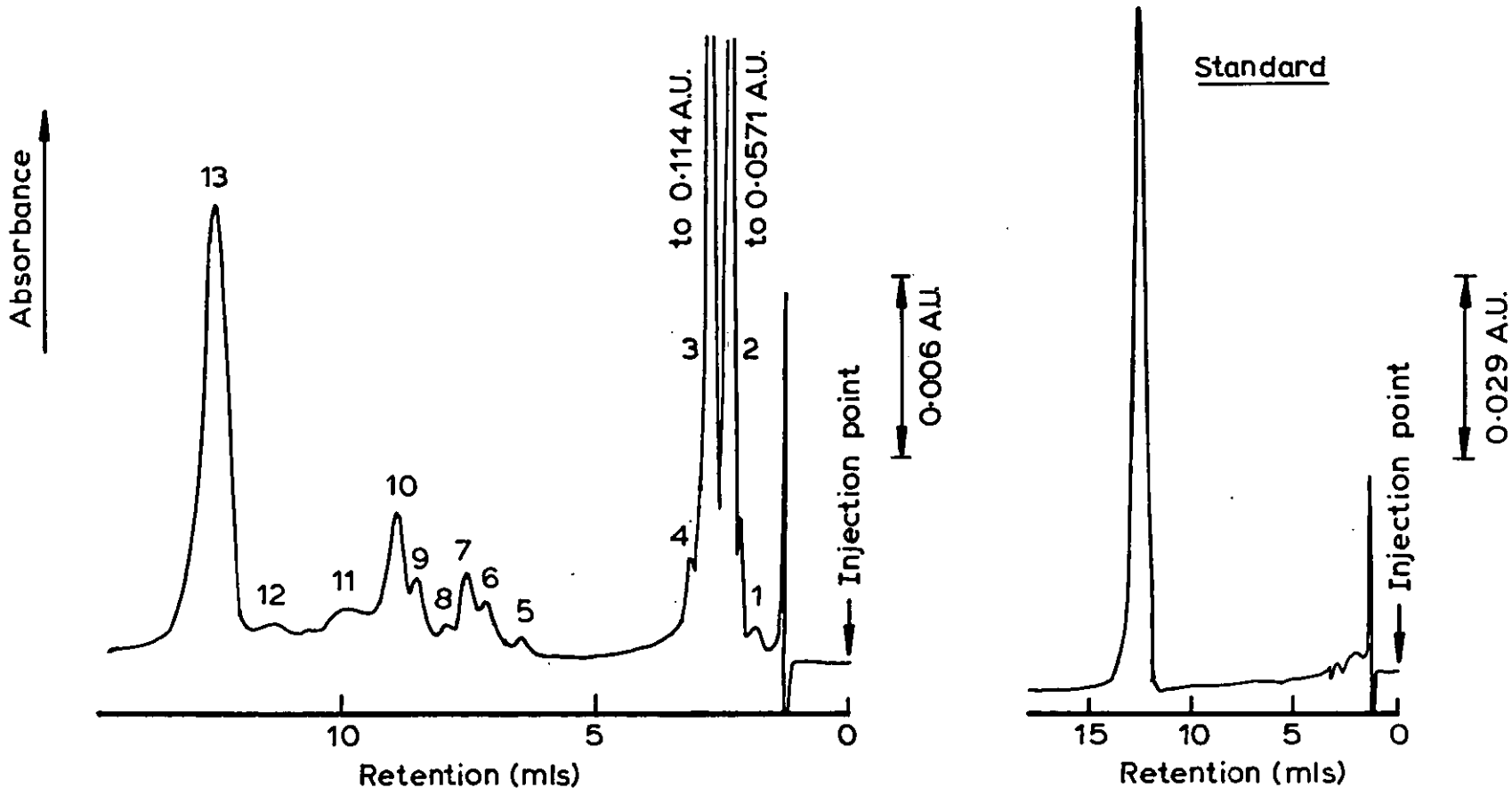


Fig. 48

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area*
1	2.16	3.15×10^{-3}	0.5%
2	2.40	5.72×10^{-2}	10%
3	2.78	1.13×10^{-1}	23%
4	3.12	1.10×10^{-3}	0.25%
5	6.53	6.30×10^{-4}	0.3%
6	7.20	1.73×10^{-3}	0.91%
7	7.54	2.83×10^{-3}	1.6%
8	7.97	1.10×10^{-3}	0.64%
9	8.54	2.52×10^{-3}	1.6%
10	8.93	4.72×10^{-3}	3.1%
11	9.89	1.42×10^{-3}	1.0%
12	11.3	9.4×10^{-4}	0.78%
13	12.5	1.37×10^{-2}	12.6%

* Calculated relative to sunset yellow FCF undegraded 257 ppm $^{w}/_{v}$ standard (cf green S above for details).

Discussion

As with green S, sunset yellow FCF produces numerous thermal decay products when heat treated over prolonged periods. However, subsequent storage under ambient conditions does not seem to further degrade the colouring matter: the final polarographic measurement showed 14.3% remnant. Hplc here shows that storage has only reduced this to 12.6%, less than a 2% fall.

Comparison of figure 48 with figure 43, shows that major peaks 2 and 3 were not resolved sufficiently to appear as separate peaks using the less polar, low retention eluent mixture (75% methanol rather than 63%). The higher retention minor peaks seen in Figure 43 do not appear in figure 48 - when small peaks elute at very high retention they can be missed because of their low peak height and flattened shape.

3) Quinoline Yellow

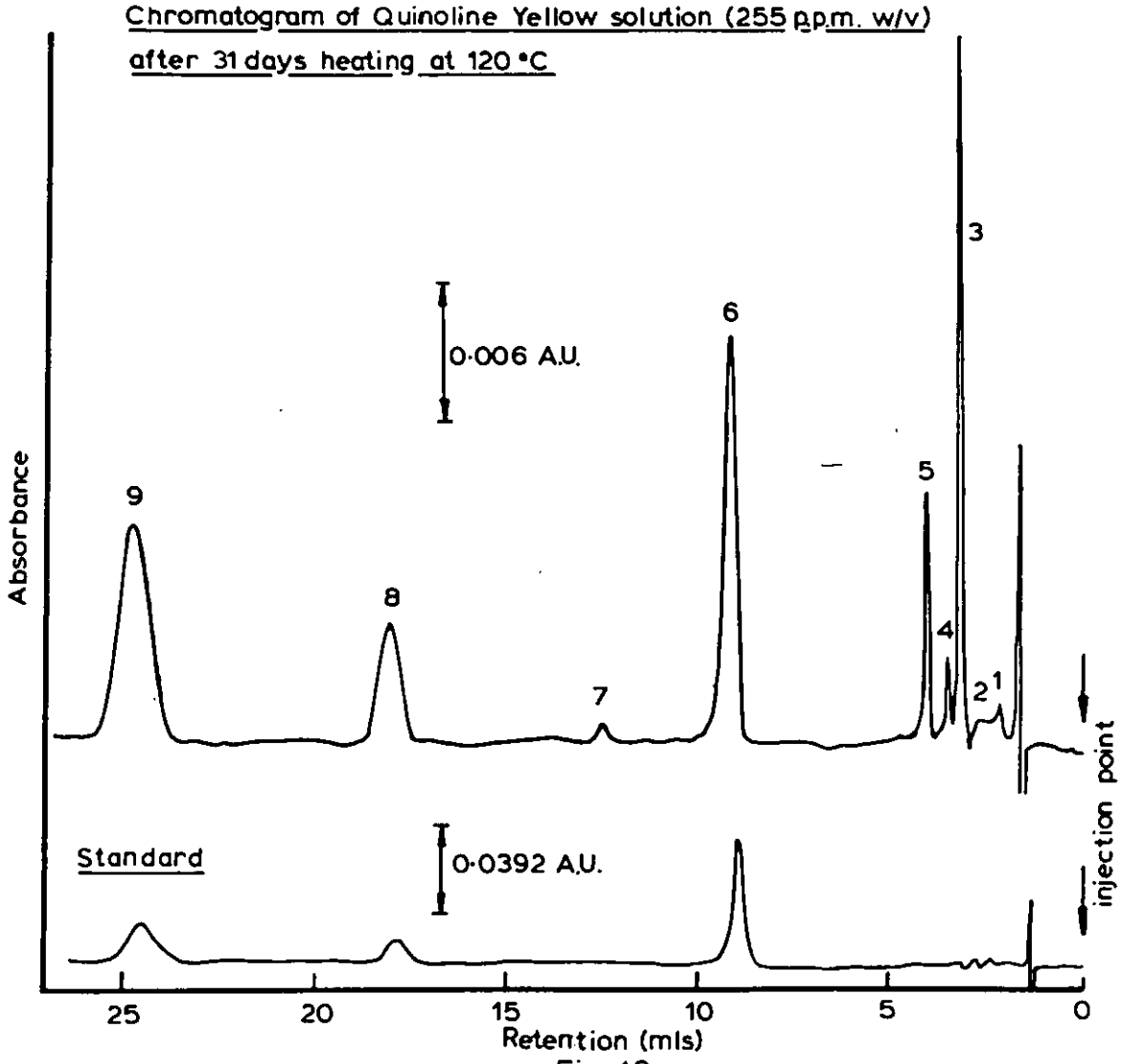
A 255 ppm $^{w}/_{v}$ quinoline yellow solution was thermally degraded as for green S above (see fig. 30 for decay curve). Fig. 44 shows early hplc results as already described. After 6 months storage in the dark, the quinoline yellow FCF sample was chromatographed under the conditions listed above for green S.

Results

The chromatogram obtained is given in fig. 49. The peaks are labelled in common with the table below.

Peak No	Retention (mls)	Peak Height (A.U.)	Estimated Relative Peak Area*
1	1.77	2.1×10^{-3}	0.8%
2	2.30	1.2×10^{-3}	0.57%
3	2.43	3.16×10^{-2}	16%
4	3.15	4.1×10^{-3}	2.7%
5	3.68	1.15×10^{-2}	8.8%

(continued)


Chromatogram of Quinoline Yellow solution (255 p.p.m. w/v)
after 31 days heating at 120 °C
0·006 A.U.
Absorbance
9
8
7
6
5
4
3
2
1
injection point
Standard
0·0392 A.U.
25
20
15
10
5
0
Retention (mls)
Fig. 49

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area*
6	8.91	1.82×10^{-2}	34%
7	12.2	9.6×10^{-4}	2.4%
8	17.8	5.4×10^{-3}	20%
9	24.5	9.7×10^{-3}	49%

* Calculated relative to the first of the three major quinoline yellow FCF peaks (peak 6) for an undegraded 255 ppm $^{w}/v$ solution (cf green S above for details).

Discussion

Measurement of the quinoline yellow FCF standard chromatogram given in figure 49 shows that the three component peaks (peaks 6, 8 and 9) are in the height ratio prior to degradation of 1 : 0.14 : 0.30. In the degraded sample the new ratio is 1 : 0.30 : 0.53 showing the ratio of thermal stabilities to be 1 : 2.1 : 1.8 (this is assuming that thermal isomerisation between the components is not operating).

This throws some uncertainty over the polarographic study reported in Chapter 7. The polarogram of quinoline yellow run in pH 1.9 B-R buffer shows a major peak at -0.65V (vs S.C.E.) and a minor peak at -1.02V (peak height ratio 2.85 : 1). After 24 days of thermal degradation at 120^{o}C this peak height ratio had been narrowed to 2.41 : 1, but the proximity of the -1.02V peak to the solvent cut-off

made this discrepancy unremarkable given the inherent error in measurements close to such cut-offs. The major polarographic peak was used as an overall measure of the amount of the quinoline yellow components remaining for the construction of the decay curve (figure 30).

Clearly it would be preferable to conduct a thermal decay study using the present hplc method to analyse the degraded solution, and to produce 3 decay curves based on chromatographic peaks 6, 8 and 9 respectively.

The degraded solution gives a fairly simple chromatogram given that there are 3 components in the undegraded dye mixture.

4) Amaranth

A 252 ppm $^{w}/v$ amaranth solution was thermally degraded and analysed as described for green S above (see figure 30). Hplc conditions were as for green S also.

Results

The chromatogram obtained is given in fig. 50 (peaks labelled as in the table below). In comparison, an undegraded 252 ppm $^{w}/v$ amaranth solution gave a peak of height 0.0243 AU at a retention of 56.4 mls.

Chromatogram of Amaranth solution (252 p.p.m. w/v) after 31 days heating at 120°C

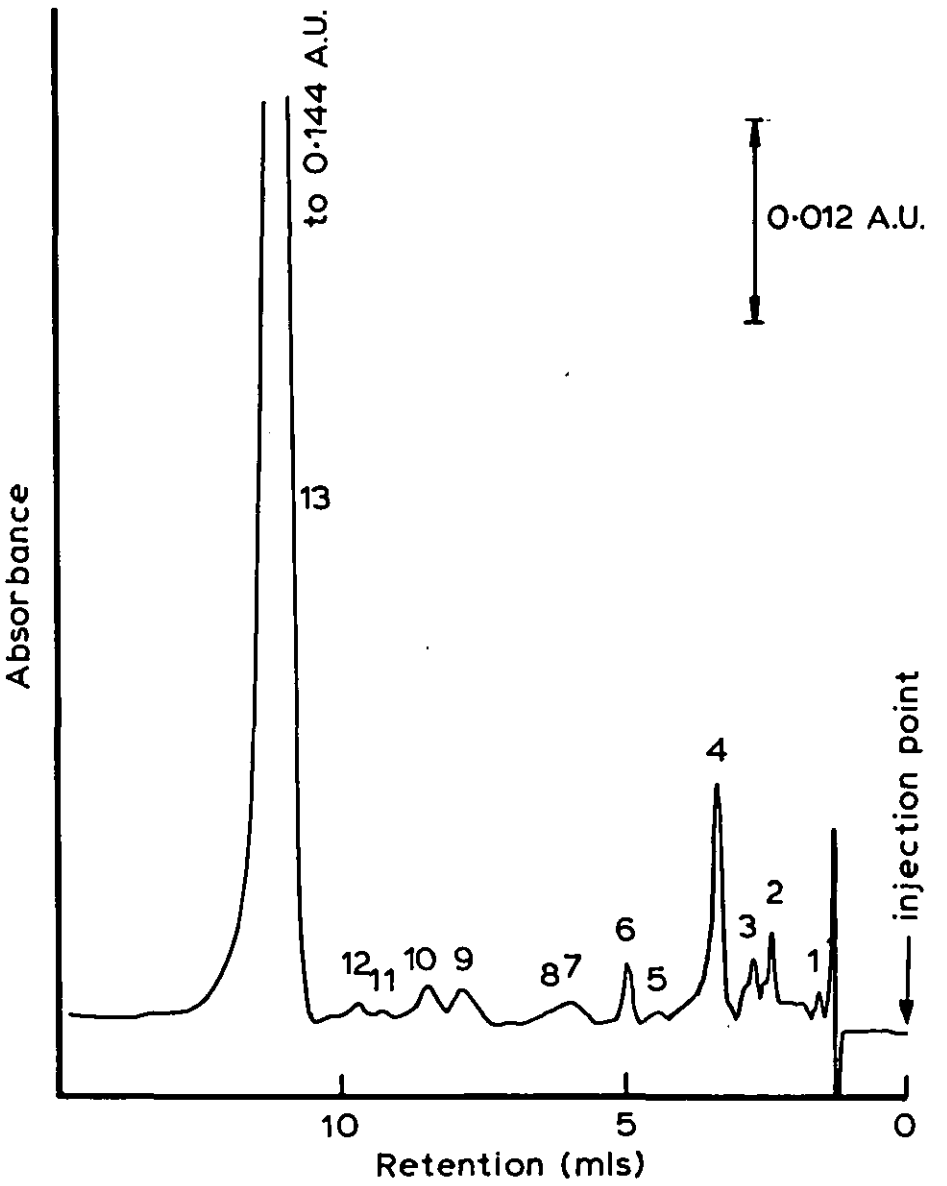


Fig. 50

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area*
1 (ts)	1.54	2.20×10^{-3}	0.25%
2 (ls,ts)	2.40	5.98×10^{-3}	1.0%
3 (ts)	2.74	4.09×10^{-3}	0.82%
4 (ls,ts)	3.36	1.42×10^{-2}	3.5%
5	4.46	6.30×10^{-4}	0.21%
6	4.99	3.78×10^{-3}	1.4%
7 (u)	5.86	1.26×10^{-3}	- (u)
8 (u)	6.14	1.10×10^{-3}	- (u)
9	7.87	2.05×10^{-3}	1.2%
10	8.50	2.20×10^{-3}	1.4%
11	9.31	6.30×10^{-4}	0.43%
12	9.74	1.10×10^{-3}	0.78%
13	11.18	1.44×10^{-1}	117%

ts = trailing shoulder on peak

ls = leading shoulder on peak

u = unresolved from adjacent peak of similar height

$$* = \frac{\text{retention of peak (mls)} \times \text{peak height (AU)} \times 100\%}{56.4 \times 0.0243}$$

(see green S above for details)

Discussion

From the polarographic study an amaranth final concentration of about 2-3% was indicated (see Chapter 7). No remnant amaranth peak was visible in the chromatogram (the relevant high retention section is not shown for reasons of space in figure 50). The lack of peak is not surprising; either further decay took place during subsequent storage or, equally possibly, so small a peak would not be seen at such high retention.

The chromatogram is incompletely resolved but shows a very complex array of minor products together with the single major decay product peak (peak 13).

5) Tartrazine

A 252 ppm $^{w}/v$ tartrazine solution was thermally degraded and analysed as described for green S above (see figure 30). Hplc conditions were also as for green S.

Results

The early peaks are reproduced in figure 51. It is not possible to reproduce the whole chromatogram effectively on a single scale, as will be discussed below. Labelling of peaks is as in the table. In comparison, an undegraded 252 ppm $^{w}/v$ solution had a peak height of 0.0201 AU, at a retention of 46.0 mls. These are the figures used in the estimation of the relative peak areas (see green S above for calculation details).

Initial section of chromatogram of tartrazine solution (252 ppm w/v) after 31 days heating at 120°C

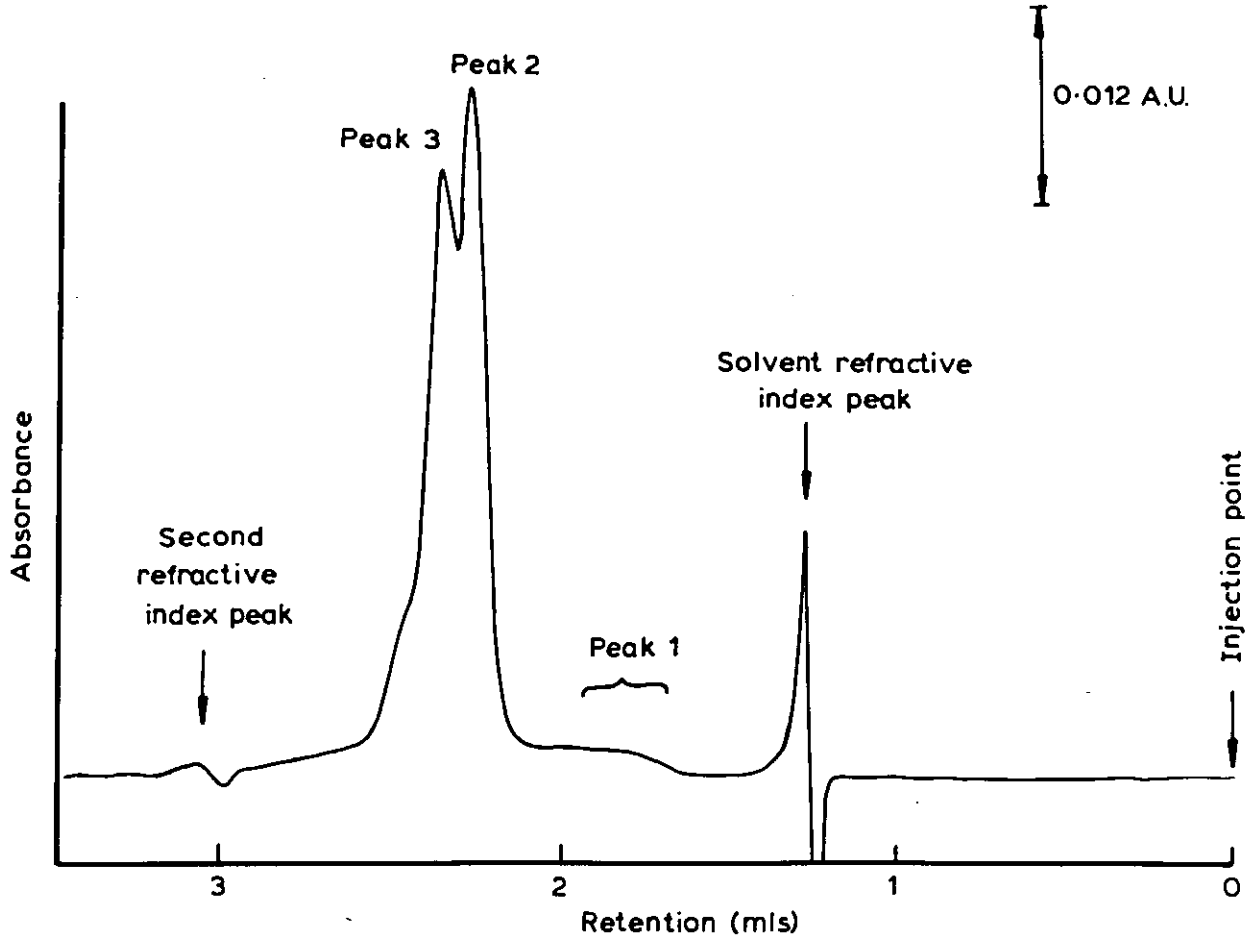


Fig. 51

Peak No	Retention (mls)	Peak height (AU)	Estimated Relative Peak Area
1 (hump)	—		
2 (u)	2.28	3.95×10^{-2}	ca. 10% (u)
3 (u, ts)	2.36	3.47×10^{-2}	ca. 9% (u)
4	7.09	3.8×10^{-3}	2.9%
5	7.50	1.3×10^{-3}	1.1%
6 (ls)	9.62	1.0×10^{-3}	1.0%
7	12.6	4.7×10^{-4}	0.64%
8 (parent)	46.0	4.1×10^{-3}	20.4%

u = incompletely resolved (see figure 51).

ts = trailing shoulder on the peak.

ls = leading shoulder on the peak.

Discussion

Peak 1, termed a 'hump' in the table, is clearly associated with the sample matrix. The reverse refractive index peak shows the end of the 'plug' of high-water content liquid as it travels through the column/detector carrying with it any non-ion pairing, water soluble material. This effect can be seen (less clearly) on other chromatograms reproduced here.

The very great range of retention times in this chromatogram coupled with the difficulty of resolving peaks 2 and 3 has prevented the useful reproduction of the whole chromatogram. This example speaks

very eloquently for the introduction of a column cut technique as discussed in Chapter 8. Only then could adequate separating power be obtained without leaving the tartrazine parent peak non-eluted on the column.

The figure of 20.4% tartrazine remaining compares with that of 23.7% found by polarography (figure 30), showing that the 6 months storage between the two studies does not have a pronounced effect on stability.

6) Indigo Carmine

A 257 ppm $^{w}/v$ indigo carmine solution was thermally degraded and analysed as described for green S above, except that the heat treatment was discontinued after 10 days rather than 31 days because all trace of indigo carmine had been lost (see figure 30). Hplc analysis was by the method used for green S.

Results

The chromatogram obtained is given in figure 52. The retention of the 257 ppm $^{w}/v$ indigo carmine shown (1st peak) is 7.58 mls and the peak height is 0.0928 AU. The decay product peak areas are estimated relative to this peak (cf. green S).

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1	1.87	7.97×10^{-4}	0.21%
2	2.35	2.08×10^{-1}	69.5%
3	2.88	1.02×10^{-2}	4.2%

Chromatogram of Indigo Carmine solution (257 p.p.m. w/v) after 10 days heating at 120 °C

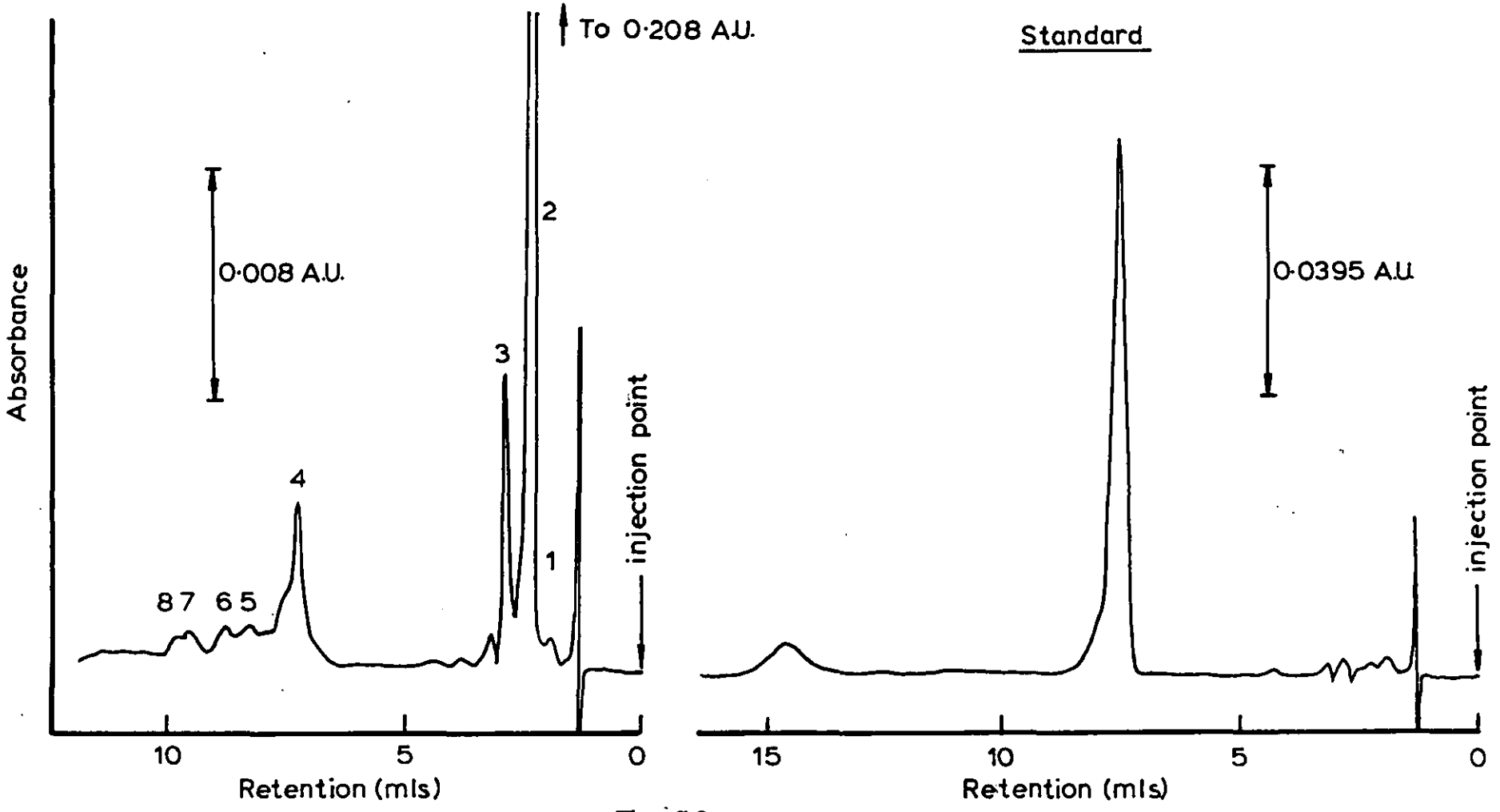


Fig. 52

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
4 (ts)	7.25	5.74×10^{-3}	5.9%
5 (u)	8.26	1.35×10^{-3}	- (u)
6	8.78	1.35×10^{-3}	1.7%
7 (u)	9.50	1.12×10^{-3}	- (u)
8 (u)	9.84	9.56×10^{-4}	- (u)

ts = tailing shoulder on the peak.

u = incompletely resolved peak.

Discussion

Hplc shows little parent peak remaining.

7) Brilliant Blue FCF

A 256 ppm $^{w}/_{v}$ brilliant blue FCF solution was thermally degraded as for green S above (see figure 30 for decay curve). Fig. 42 shows early hplc results already described in this Chapter. Hplc analysis with thermostatting was conducted as described for green S etc. above.

Results

The chromatogram obtained is shown in Fig. 53 together with a 256 ppm $^{w}/_{v}$ brilliant blue FCF undegraded standard. [Labelling as in the table]. The standard, showing two partly resolved peaks,

Chromatogram of Brilliant Blue FCF solution (256 ppm w/v) after 31 days heating at 120°C

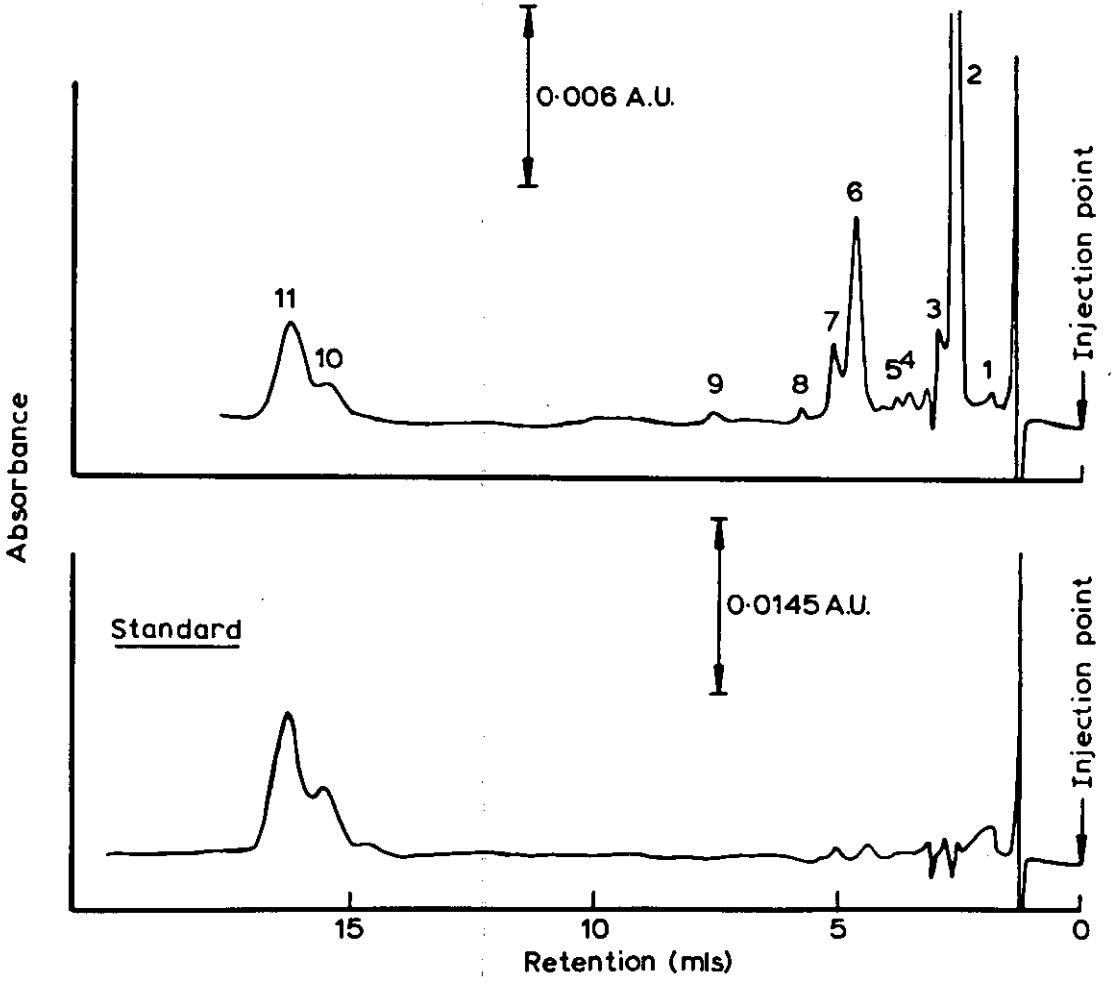


Fig. 53

has its latter peak at retention 16.2 mls, and a peak height of 0.0115 AU. This peak is used in the estimation of the relative peak areas [Cf. green S above].

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1	1.82	9.45×10^{-4}	0.9%
2	2.59	6.76×10^{-2}	94.0%
3	2.93	1.73×10^{-3} (s)	2.7%
4	3.55	9.45×10^{-4}	1.8%
5	3.84	8.66×10^{-4}	1.8%
6	4.61	6.93×10^{-3}	17.1%
7	5.09	2.76×10^{-3}	7.5%
8	5.81	4.72×10^{-4}	1.5%
9	7.58	3.94×10^{-4}	1.6%
10 (u) p	15.5	1.34×10^{-3}	ca. 11% (u)
11 (u) p	16.2	3.31×10^{-3}	ca. 29% (u)

u = incompletely resolved.

s = measured by skimming peak 2.

p = parent peak.

Discussion

The fall off in peak 11 to 28.8% is very close to the value obtained after 31 days heating by polarographic means (28.1% - see figure 30). This indicates that 6 months storage has had no further degradative effect.

The ratio of peak heights for parent peaks 10 and 11 is 1 : 2.3 for the undegraded standard compared to 1 : 2.6 for the degraded sample. This is in contrast to the differential fall-off in component peaks found for quinoline yellow (discussed above), and indicates that the components giving rise to peaks 10 and 11 must have very similar thermal stabilities.

8) Yellow 2G

A 255 ppm $^{w}/_{v}$ yellow 2G solution was thermally degraded as described for green S above (see fig. 30 for decay curve). Fig. 46 shows variation in chromatographic peak height with wavelength as already described earlier in this Chapter. Hplc was later performed with thermostatting as described above for green S.

Results

The decay product and a yellow 2G standard have their chromatograms shown in fig. 54. The standard is of peak height 0.0178 AU and retention 19.5 mls, and is used to assess the relative peak area of the decay product peaks (see green S above). Labelling of peaks is as in the table.

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1 (u)(p)	1.82	1.34×10^{-3}	- (u)
2 (ls)	2.26	3.54×10^{-2}	23.0%
3	2.69	3.07×10^{-2}	23.8%

(continued)

Chromatogram of Yellow 2G solution (255 p.p.m. w/v)

after 31 days heating at 120 °C

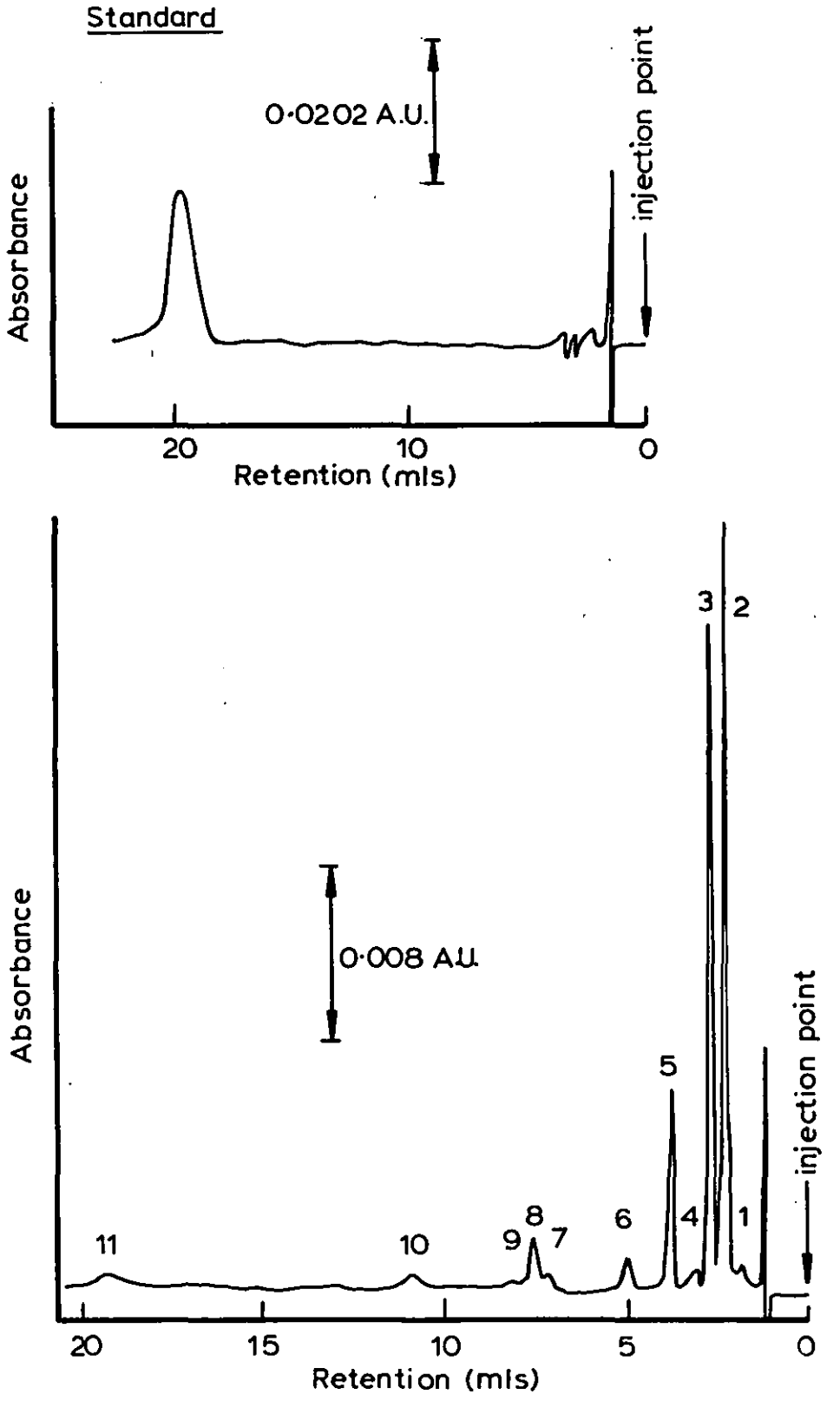


Fig. 54

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
4	2.98	1.02×10^{-3}	0.88%
5	3.74	9.29×10^{-3}	10.0%
6	4.94	1.42×10^{-3}	2.0%
7	7.15	5.51×10^{-4}	1.1%
8	7.54	2.28×10^{-3}	5.0%
9	8.06	3.15×10^{-4}	0.73%
10	10.8	6.30×10^{-4}	2.0%
11 (p)	19.2	6.30×10^{-4}	3.5%

u = incompletely resolved.

p = present in standard chromatogram

ls = leading shoulder on peak.

Discussion

A full degradation profile of yellow 2G is given later in this Chapter. Fig. 54 is included here as part of the set of chromatograms performed at 40°C with a 63/37/0.25 v/v/w methanol/water/cetrimide eluent.

The value of 3.5% remnant found here (after 6 months storage following heat treatment) compares with the 9.8% remnant found immediately after the 31 days heating at 120°C indicating some further loss has occurred during storage.

9) Black PN

A 249 ppm $^{w}/_{v}$ black PN solution was thermally degraded at 120°C for 31 days (see fig. 30) after which there was no trace of the original colouring matter. Hplc analysis (40°C) was carried out using the 63/37/0.25 v/v/w methanol/water/cetrimide eluent employed throughout this series of chromatograms, but in order to find the retention time of, and dectector response to black PN itself, a separate run was made at 70/30/0.25 v/v/w methanol/water/cetrimide, as black PN can scarcely be made to elute at any more polar an eluent composition.

Results

The retention of black PN at 70/30/0.25 v/v/w methanol/ water/cetrimide (40°C) was found to be 44.85 mls. Peak height for the 249 ppm $^{w}/_{v}$ standard was 0.0214 AU. These values are used for the estimation of the peak areas of the decay product peaks in the table below. At the same eluent composition, peak 3 showed a retention of 5.48 mls. Figure 55 shows the decay product chromatogram obtained with 63/37/0.25 v/v/w methanol/water/ cetrimide eluent (for improved resolution). It is labelled in common with the table.

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1	2.45	1.64×10^{-2}	4.2%
2	2.88	1.43×10^{-3}	0.43%
3	3.46	2.29×10^{-2}	8.3%

(continued)

Chromatogram of Black PN solution (255 p.p.m. w/v) after 31 days heating at 120°C

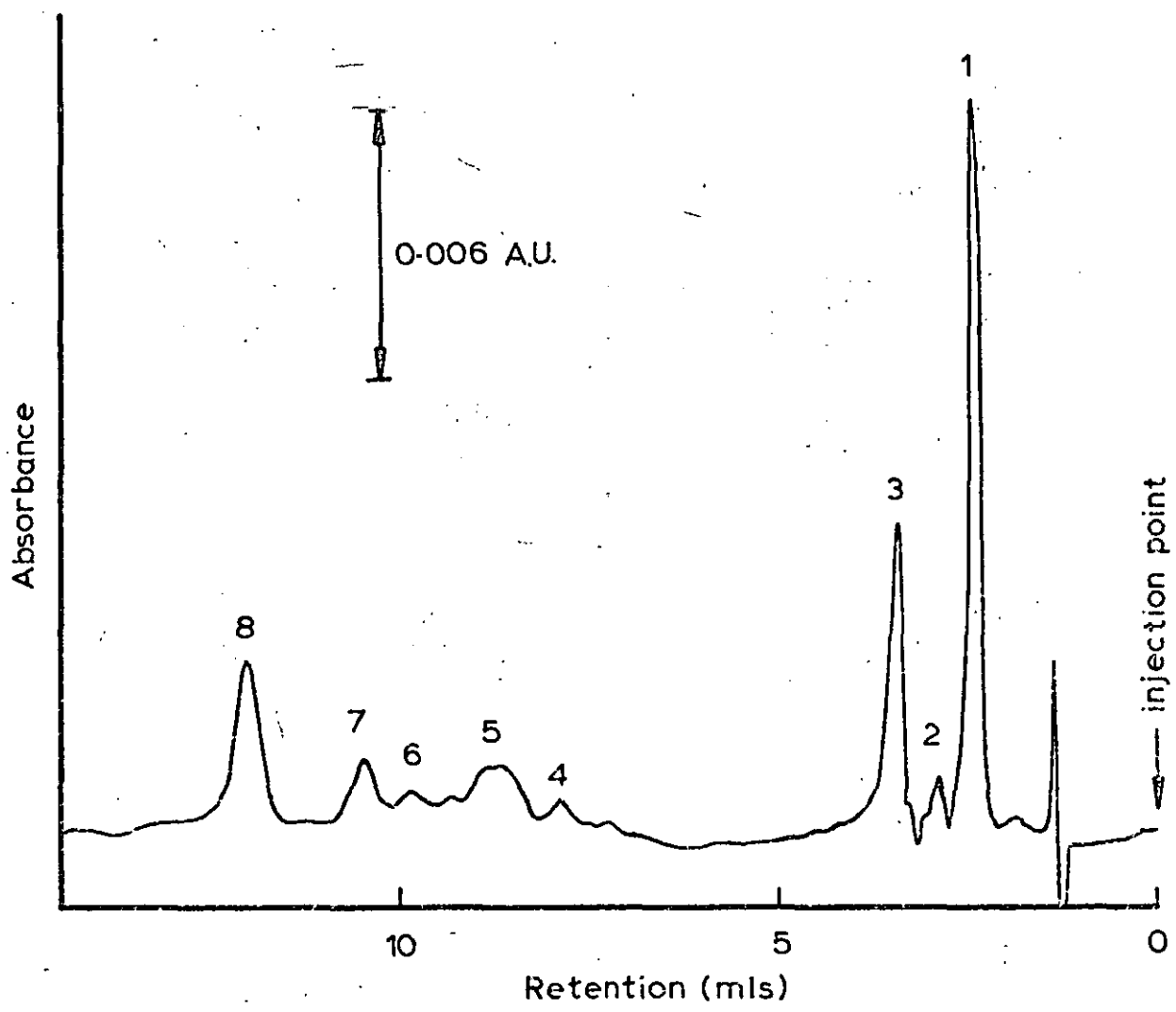


Fig. 55

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
4	7.87	9.56×10^{-4}	0.78%
5 (u)	8.74	1.75×10^{-3}	- (u)
6	9.84	1.04×10^{-3}	1.1%
7	10.5	1.83×10^{-3}	2.0%
8	12.0	3.90×10^{-3}	4.9%

u = unresolved

Discussion

The use of the constant, retention x peak height, is unaffected by the use of different eluents for standard and product peaks as long as the peak shapes remain similar. No remnant black PN was found.

10) Ponceau 4R

A 250 ppm $^{w}/_{v}$ ponceau 4R solution was thermally degraded at 120°C for 10 days (see fig. 30) after which there was less than 2% remnant colouring matter. Because of the high retention time of ponceau 4R in 63/37/0.25 v/v/w methanol/water/cetrimide, the same approach was taken as for black PN, the degradation product being separated with this eluent mixture (40°C), and the standard being run with an eluent containing 70% methanol instead.

Results

The retention of the standard was found to be 37.2 mls, while the peak height was 0.0417 AU. Decay product peak areas were estimated relative to these values (cf black PN above). Fig. 56 shows the chromatogram obtained (using 63% methanol in the eluent) of the decay products. The early peaks are very minor and have not been labelled.

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1 (u)	9.55	1.43×10^{-3}	- (u)
2	10.4	1.12×10^{-3}	0.75%
3	14.8	6.61×10^{-3}	6.3%
4	15.6	5.42×10^{-3}	5.5%

u = incompletely resolved.

Discussion

No remnant ponceau 4R peak was seen. The chromatogram was investigated further as discussed in Chapter 10.

11) Patent Blue V

A 254 ppm $^{w}/v$ patent blue V solution was thermally degraded at 120^{o}C for 30 days (fig. 30). Fig. 43 shows the variation in chromatographic peak height with wavelength as already described in this Chapter. Hplc was performed on the solution after 6 months storage as described for green S above.

Chromatogram of Ponceau 4R solution (250 p.p.m. w/v) after 10 days heating at 120°C

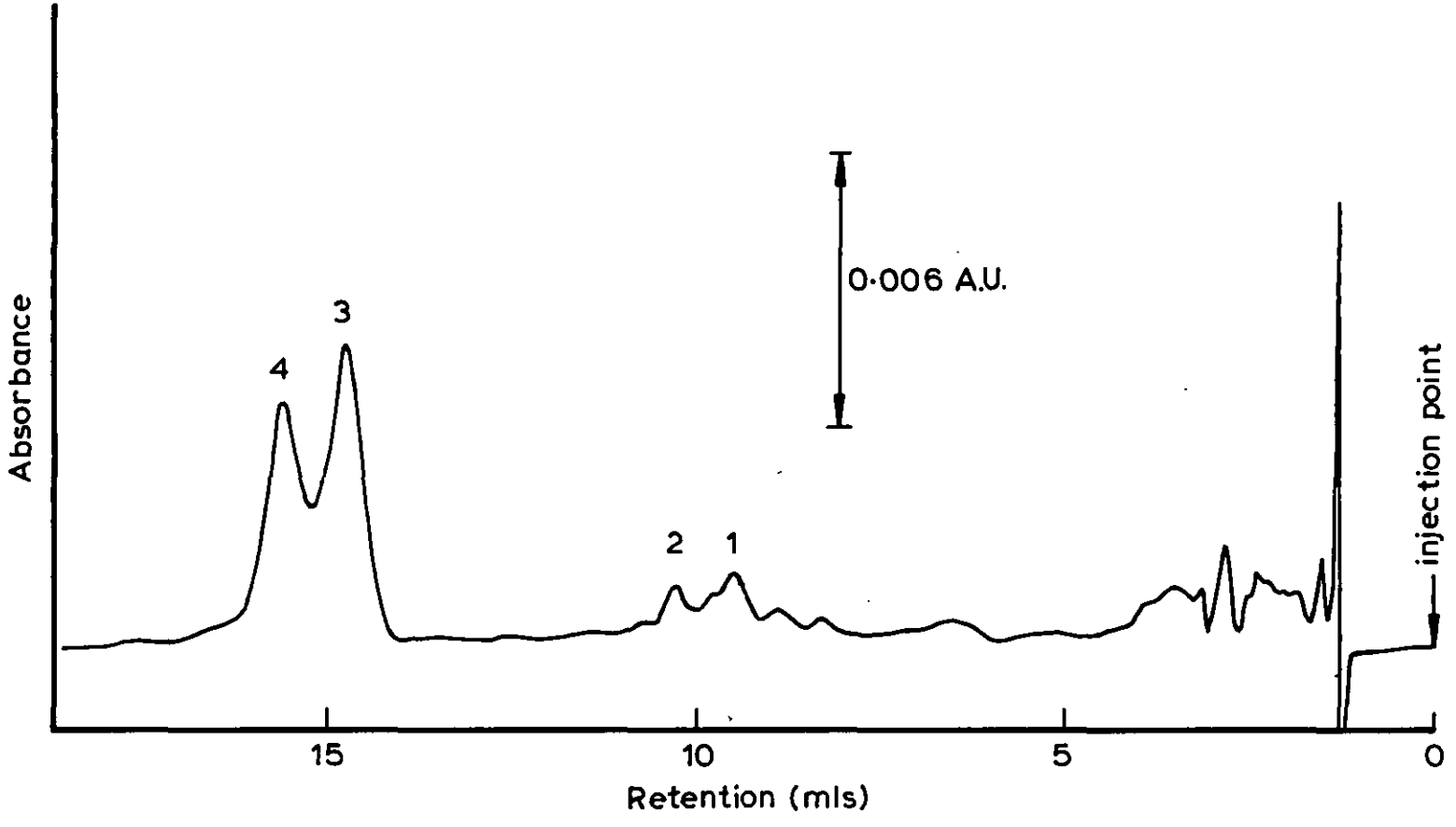


Fig. 56

Results

The decay products and a patent blue V standard have their chromatograms shown in fig. 57. The standard shows a retention of 15.2 mls and a peak height of 0.0289 AU. The other peak areas in the table are estimated relative to these values. Some very minor early peaks are not listed in the table.

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1	1.68	4.62×10^{-3}	1.8%
2 (ls)	7.25	7.97×10^{-4}	1.3%
3 (p)	9.79	2.79×10^{-3}	6.2%
4	15.4	1.43×10^{-3}	5.0%
5	21.7	3.11×10^{-3}	15.4%

ls = leading shoulder on peak.

p = parent peak.

Discussion

Patent blue V is somewhat atypical in that the major decay product peaks on the chromatogram appear at higher retention than the parent peak. This may suggest that peaks 4 and 5 represent compounds which retain the triphenylmethane structure in their molecules, but which have have undergone substitution of certain functional groups. [The diethylamino group is a good leaving group and might, for instance, have been displaced by hydroxyl]. Cleavage of the triphenylmethane

Chromatogram of Patent Blue V solution (254 p.p.m. w/v) after 31 days heating at 120 °C

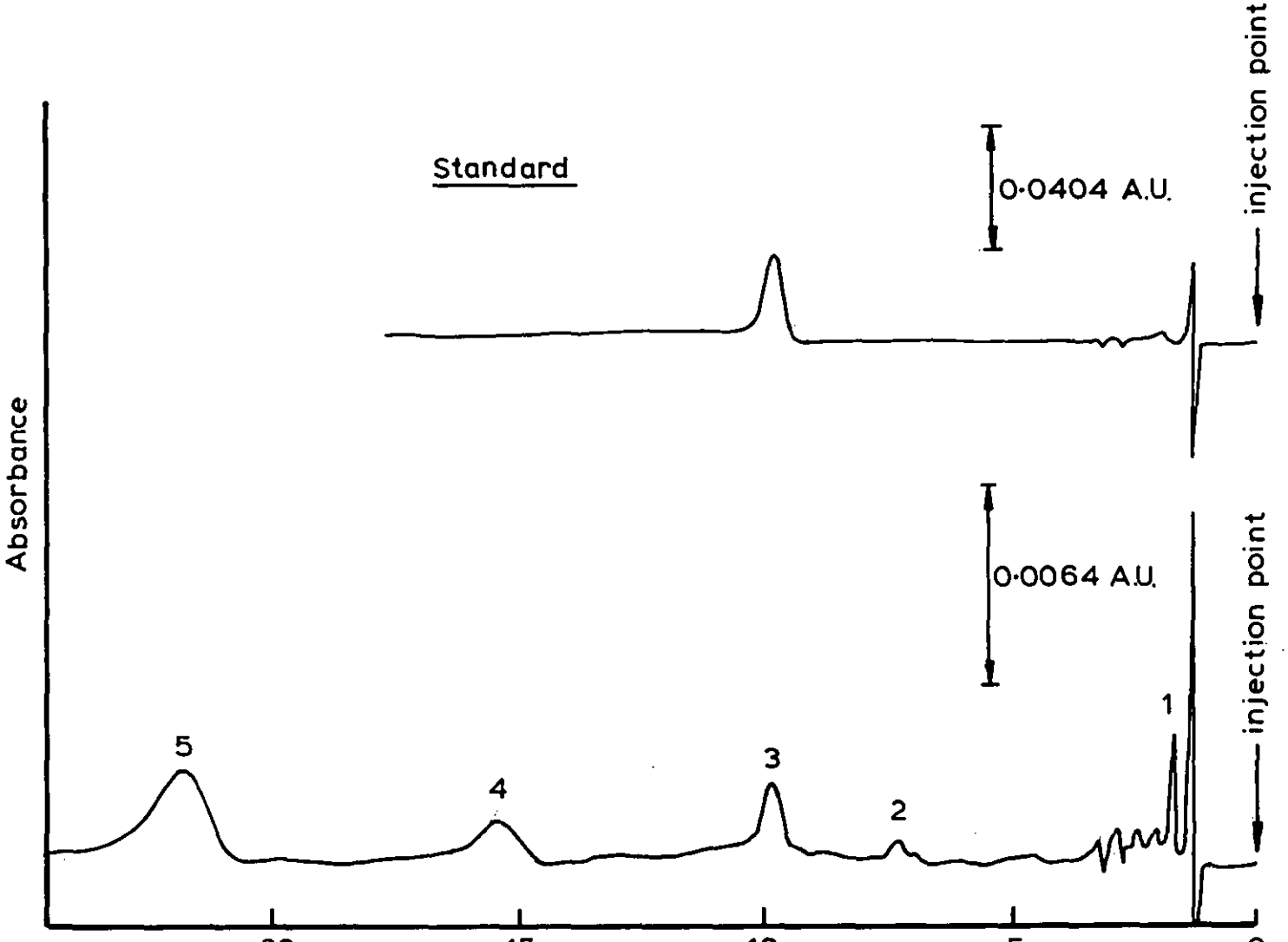


Fig. 57

structure to give polar benzene derivatives would be shown by the lower retention of these species. Compare the retention behaviour of phenol-4-sulphonic acid, aniline etc, discussed in Chapter 8.

After the 31 days degradation, polarographic analysis (Chapter 7) showed a retention of 9.8% patent blue V. Six months storage has reduced this, therefore, by a further small amount to 6.2% (peak 3).

12) Carmoisine

A 255 ppm $^{W}/_{v}$ carmoisine solution was thermally degraded as described for green S above (see fig. 30 for decay curve). Hplc analysis (without column thermostatting) has been described in some detail earlier in this chapter (see figs. 39, 40 and 41). Hplc was also conducted with thermostatting using 63/37/0.25 v/v/w methanol/water/cetrimide as described already for green S.

Results

The chromatogram obtained is given in fig. 58. A 255 ppm $^{W}/_{v}$ carmoisine standard gave a peak height of 0.0164 AU at a retention of 49.0 mls - much higher than the retention of any of the observed products. These values are used to assess the relative peak areas of the product peaks (see green S for details).

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1 (ts)	1.49	2.83×10^{-3}	0.5%
2	1.87	6.30×10^{-4}	0.2%

(continued)

Chromatogram of Carmoisine solution (255 p.p.m. w/v) after 31 days heating at 120°C

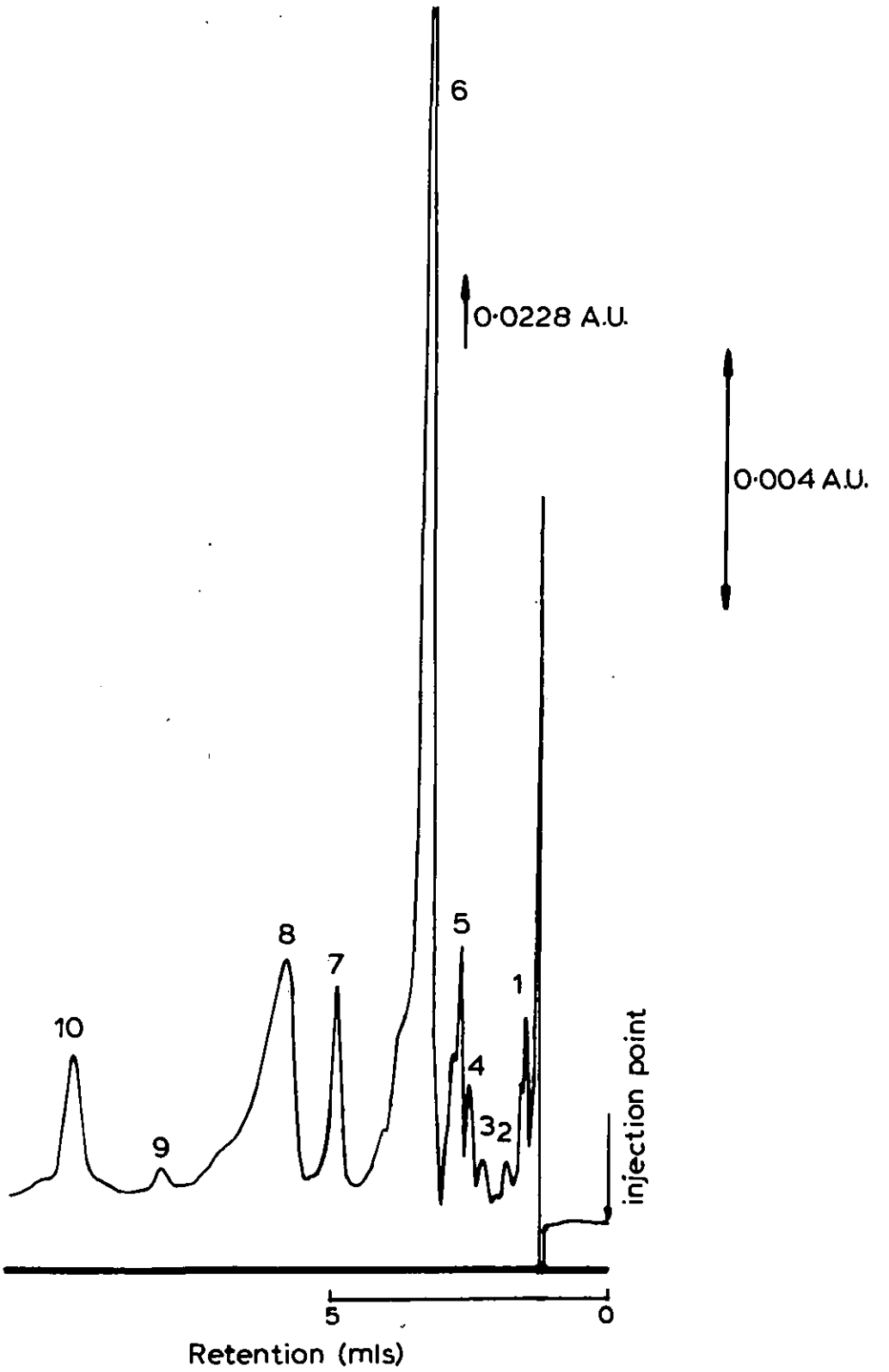


Fig. 58

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
3	2.30	7.09×10^{-4}	0.2%
4	2.54	1.85×10^{-3}	0.6%
5 (ts)	2.69	3.86×10^{-3}	1.3%
6 (2ts)	3.36	2.28×10^{-2}	9.5%
7	4.90	3.31×10^{-3}	2.0%
8 (z)	5.86	3.66×10^{-3}	- (z)
9	8.06	5.12×10^{-4}	0.5%
10	9.74	2.20×10^{-3}	2.7%

ts = trailing shoulder

z = peak non-Gaussian

Discussion

Though it would be possible to analyse this completely degraded sample by using a more polar eluent to increase the separating power, this would be difficult to do were the sample only partially degraded because of the very high retention time that the parent peak would then show. Once again it might be easiest to use a column cut technique, as discussed in Chapter 8, were a degradation profile of carmoisine to be attempted.

Peak 8 could be simply an unresolved peak. However, it has the characteristic appearance of a single peak where the chromatographic mechanism is functioning imperfectly. One reason for this happening

in ion-pair chromatography is that a second ionic equilibrium competes with the ion-pair equilibrium which is responsible for efficient separation.

13) Red 2G

A 255 ppm $^{w}/_{v}$ red 2G solution was thermally degraded as described for green S above (see fig. 30 for decay curve). Hplc was conducted under the conditions given for green S.

Results

The chromatogram obtained had a number of rather small peaks, mainly incompletely resolved. It is given in fig. 59. The 255 ppm $^{w}/_{v}$ red 2G standard is shown: peak height is 0.0417 AU, and retention is 27.6 mls. These values are used to assess the relative peak areas of the other peaks - most values being very approximate owing to the limitations of the chromatogram obtained in this case.

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
1 (u)	2.24	3.07×10^{-3}	ca 0.6% (u)
2 (u)	2.38	1.65×10^{-3}	- (u)
3 (u)	ca. 2.8	1.14×10^{-3}	- (u)
4 (ts)	3.04	2.83×10^{-3}	0.8%
5	3.62	5.51×10^{-4}	0.2%
6	7.04	3.94×10^{-4}	0.2%

(continued)

Chromatogram of Red 2G solution (255 p.p.m. w/v) after 31 days heating at 120 °C

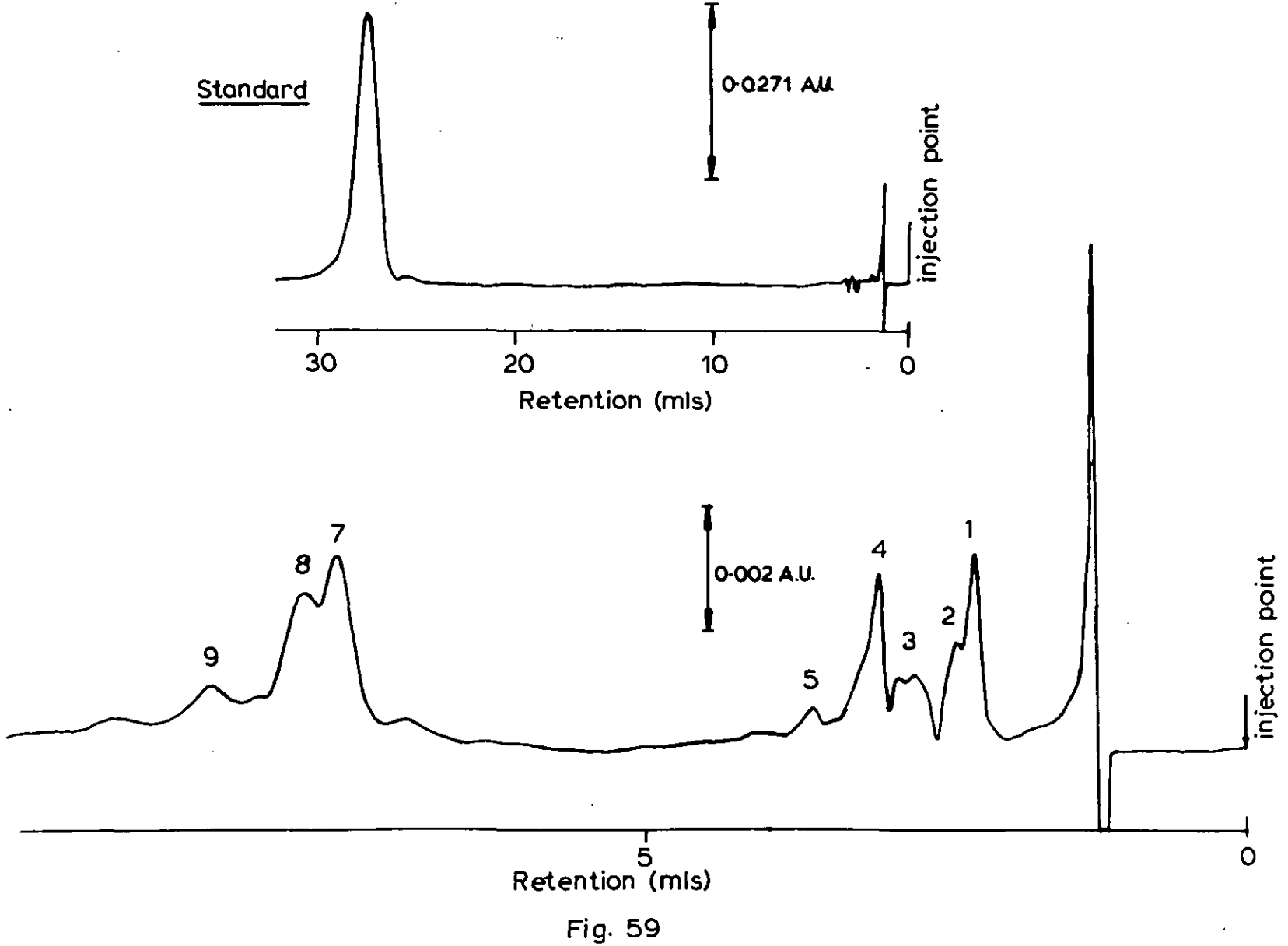


Fig. 59

Peak No	Retention (mls)	Peak Height (AU)	Estimated Relative Peak Area
7 (u)	7.64	2.99×10^{-3}	ca. 2.0% (u)
8 (u)	7.90	2.36×10^{-3}	ca. 1.6% (u)
9	8.68	8.66×10^{-4}	0.7%

u = incompletely resolved peak

ts = tailing shoulder

Discussion

The inadequacy of the chromatogram obtain in this case - other than for serial comparison with the other chromatograms obtained under the same conditions - seems due to the very complete degree of degradation undergone. Neither red 2G nor its primary decay product red 10B remain in detectable amounts.

Erythrosine, Chocolate Brown HT and Brown FK

Both reference to previous reports in the literature (see Table 7) and attempts at chromatography under the standard conditions used above showed that three food colours were ill-suited to this method. Erythrosine is not a sulphonate but a carboxylate salt. Its retention increased far more rapidly than that of the other dyes (which all contain sulphonate groups) as the percentage of methanol in the eluent was decreased, and the percentage of water increased. Both brown FK and chocolate brown HT are not eluted under conditions even approaching those employed here and were excluded from this section of the study.

SECTION C: DEGRADATION PROFILE OF YELLOW 2G

Introduction

Hplc is a natural choice of technique for the construction of degradation profiles as both starting material and products may be quantified from a single chromatogram.

Selection of Chromatographic Conditions

A solution of 255 ppm $^{w}/_{v}$ yellow 2G was sealed in a glass phial and heated at 120°C in an oven for 21 days. The partly degraded solution was chromatographed initially under the following conditions:-

Column: 10 cm column packed with SAS-hypersil.

Detection: 254 nm.

Sensitivity: 0.16 or 0.32 AUFS.

Flow rate: 1.0 ml/min

Injection volume: 5 µl.

Eluent: 75/25/0.25 v/v/w methanol/water/cetrimide.

Room temperature: 22.5°C

Column jacket temperature: 25°C

Variation was made in the column jacket temperature and in the eluent composition in order to improve the resolution obtained. Final conditions were fixed at:-

Column jacket temperature: 40°C, and

Eluent: 67/33/0.25 v/v/w methanol/water/cetrimide.

In addition, the detection wavelength was lowered to 240 nm in order to make the peaks at retention times of 2.0 and 2.24 mls of more equal size, facilitating their independent, accurate measurement. Fig. 60 shows an example of the chromatogram obtained under these conditions.

Experimental

An aqueous solution of 255 ppm $^{w}/_{v}$ yellow 2G was sealed in a glass phial and placed into a 130°C oven. The phial was sampled at intervals and the contents chromatographed by the method selected above. The sampling was continued over a 500 hour (3 week) period.

Results

The decay curve of the yellow 2G, and the growth curves of the major product peaks are shown in Fig. 61. The peak labelling of Fig. 61 is common with that of Fig. 60. Peak D was only observed on the first day of the experiment and had disappeared after 46 hours. A duplicate experiment confirmed its early existence.

Discussion

Reference to the thermal decay curve obtained for yellow 2G in Section A of Chapter 7 shows that the curve obtained could be considered as approximately exponential with a logarithmic linear regression line of correlation -0.980 and giving

$$C = C_o \exp\{-0.0752t - 0.232\}$$

where t is in units of days. The fact that the zero error was rather large - at $t = 0$, $C = 0.79\ C_o$ - left doubts as to the correctness of

Chromatogram of partially thermally degraded Yellow 2G

(255 p.p.m. w/v; 162 hours at 130°C)

H.p.l.c. conditions as given in text

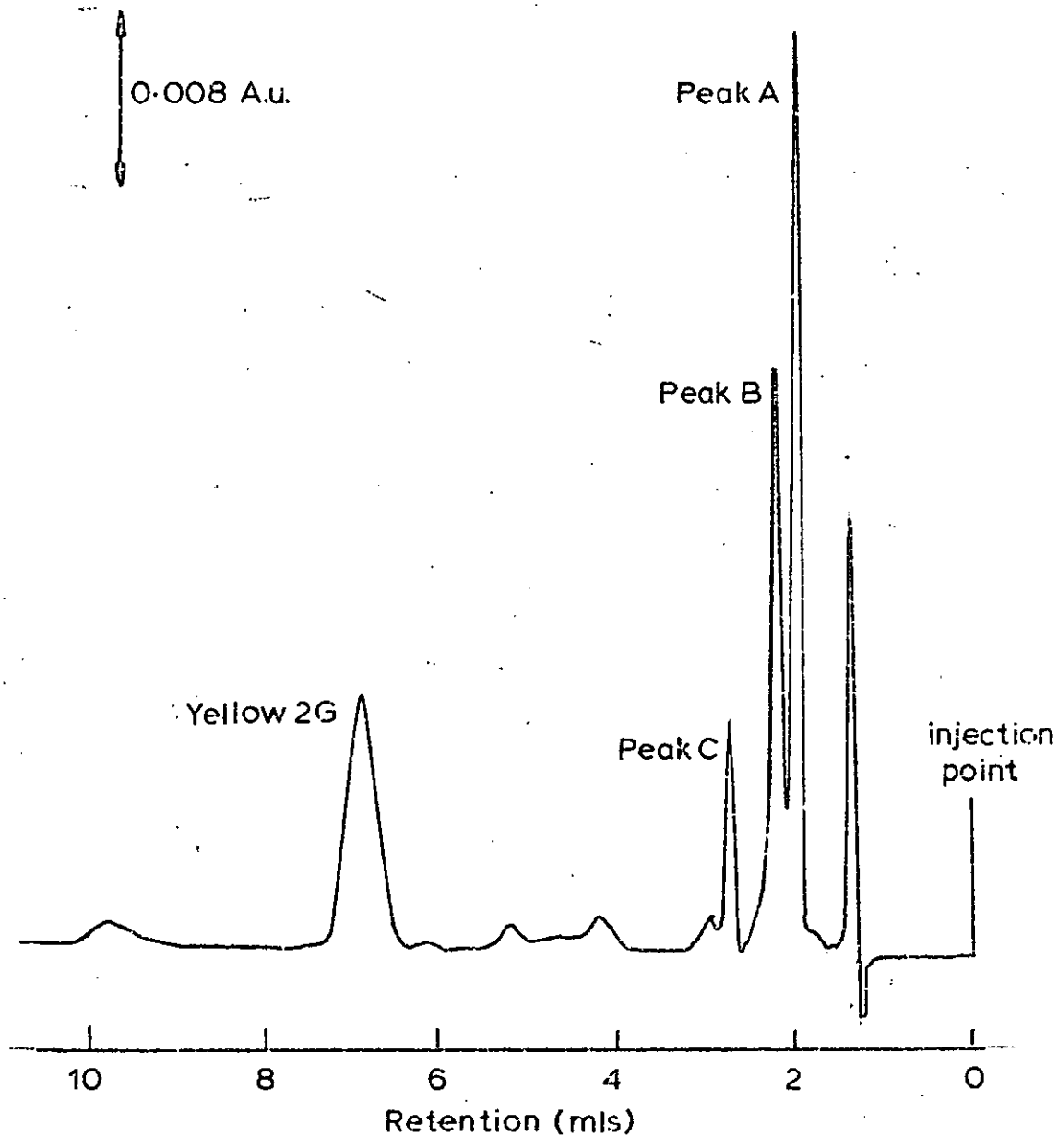


Fig. 60

Thermal degradation profile of Yellow 2G

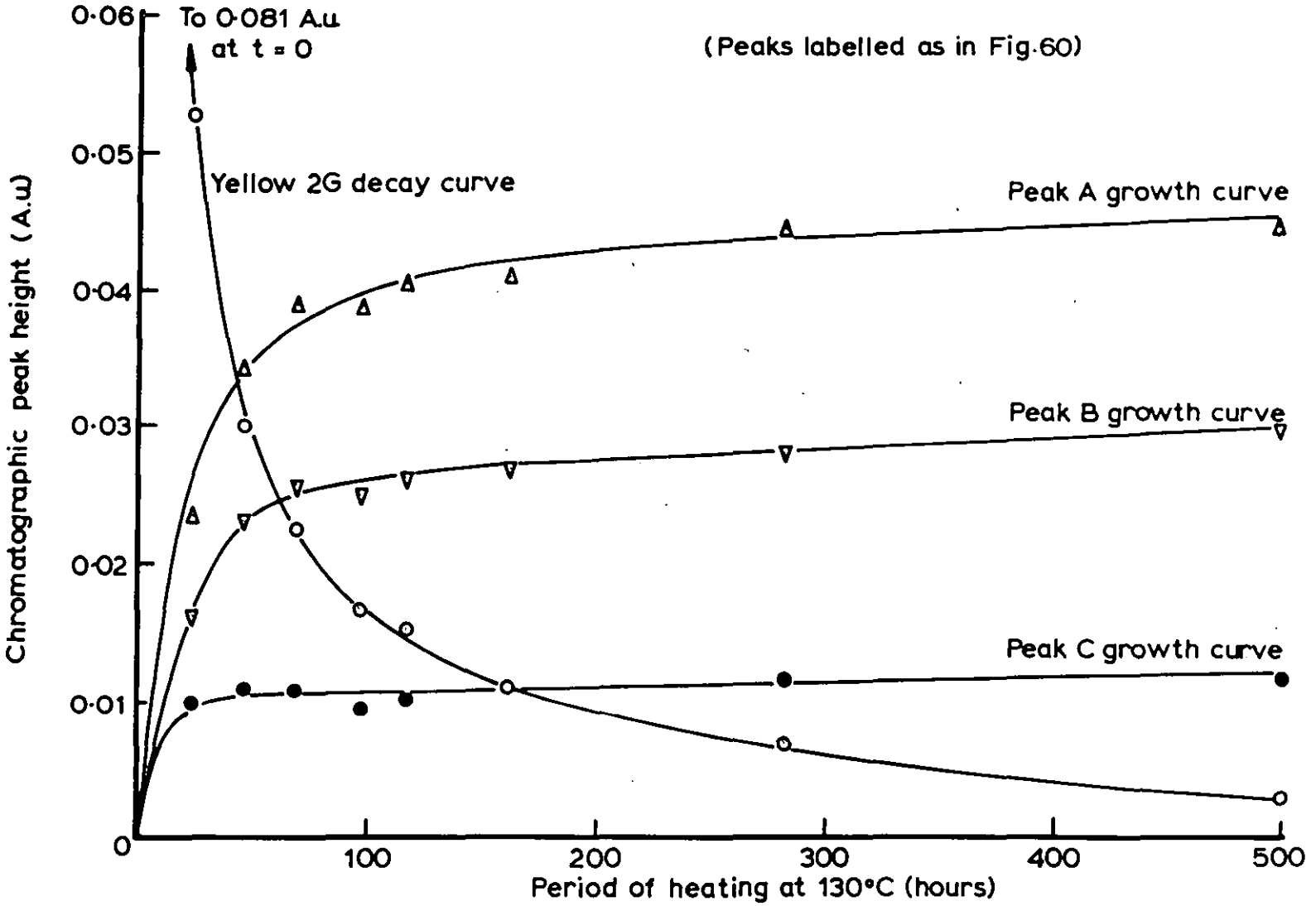


Fig. 61

the simple exponential law in this case. The new study at 130°C, not 120°C as before, and with measurement by hplc rather than polarography, gave the opportunity to consider this again.

Using the data given in Fig. 61, a logarithmic regression line was obtained for yellow 2G at 130°C. This corresponded to:-

$$C = C_o \exp\left\{-0.00611\ t\ -0.652\right\}$$

where t is again in units of days. Here the zero error is so large - at $t = 0$, $C = 0.52\ C_o$ - that the correlation coefficient of −0.937 cannot disguise the non-exponential nature of the decay. Indeed, the attempt to fit an exponential curve to the line results in the coefficient of t at 130°C actually looking smaller than that at 120°C because of this large zero correction.

Inspection of the growth of curves of peaks A, B and C in Fig. 61 shows that whereas peaks A and B take around 100 hours to reach the region of slow growth, peak C must rise very early indeed - the first measurement after 23 hours already has the peak in the "plateau" region. Inspection of the first chromatogram (23 hour sample) revealed a peak at retention 2.1 mls of peak height 0.016 AU that was present in both the duplicate samples but which had disappeared by the time the 46 hour samples were run - probably by this point it was obscured by the other peaks from which it was only partially resolved. These factors point strongly to the decay of yellow 2G being a complex process, either a multistage decay of which peaks A, B and C represent final products, or a complex decay with several decay pathways.

The following table shows the ratio, height of peak A : height of peak B, at differing times during their respective growths. The ratio is fairly consistent throughout at 1.521 ± 0.047 which is equivalent to a coefficient of variation of just 3%.

Time (Hours)	23	46	69	97	117	162	283	499
Ratio	1.43	1.49	1.51	1.55	1.54	1.53	1.59	1.53

This contrasts with the result obtained by comparing the ratio, decay in peak height of the yellow 2G peak : growth in peak height of peak A, over successive decay periods early in the trial. For instance, the original peak height of the undegraded yellow 2G at time, t = 0, was 0.0808 AU and at t = 23 hours was 0.0527 AU, a decay of 0.0808 -0.0527 = 0.0281 AU. The growth of peak A over the same period was 0.0233 -0 = 0.0233 AU. The ratio is 0.0281/0.0233 = 1.21. From t = 23 to t = 46 hours the changes in peak height give a ratio of 0.0228/0.011 = 2.62. From t = 46 to t = 69 hours, the ratio given is 0.0074/0.0045 = 1.64. These differences provide an effective means of demonstrating that peaks A and B do not rise at rates which are in simple linear proportion to the decay of the yellow 2G.

To summarize, the kinetics of decay of yellow 2G are not simple, 1st order, nor do they correspond in any direct and simple manner with the growth of the product peaks. Peaks A and B do grow in close proportion to one another, but not to peak C. The presence of one early major peak which subsequently decayed rapidly and the continuing presence of a number of minor peaks, as visible in Fig. 60, further testify to the complexity of the decay.

CHAPTER 10

CONCENTRATION AND QUALITATIVE ANALYSIS OF THE DECAY PRODUCTS

Concentration of the Decay Products

Many of the decay product solutions are dilute and complex mixtures. This goes back to the early findings that in both heat and (especially) light degradation, decay can be heavily inhibited at concentrations higher than 100 or 250 ppm $^{w}/v$. This means that separation of significant quantities (e.g. 50-100 mg) of individual products has proved difficult. The following experiments addressed this problem.

Extraction Systems

Experimental

Extraction systems were set up to concentrate aqueous tartrazine solutions. These systems comprised cetrimide and an organic solvent in addition to these solutions. The effect of ether, ethyl acetate, t-butanol, n-butanol, amyl alcohol, chloroform and methylethyl-ketone as organic phase and of $Al(NO_3)_3.9H_2O$ as salt additive were tried.

Results

Both phases contained tartrazine where t-butanol, chloroform, n-butanol and methylethylketone were used. However, an intensely yellow "oil" formed between the two phases in the case of chloroform. No extraction occurred into the organic phase for ether or ethyl acetate. For amyl alcohol virtually complete extraction into the alcohol layer occurred. Addition of $Al(NO_3)_3.9H_2O$ to this only partially reversed the process, both layers becoming yellow.

Discussion

Use of amyl alcohol/cetrimide to concentrate the system for subsequent chromatographic separation (e.g. where a mixture of sulphonates comprised the degradation product) was an attractive proposition. Manifold concentration seemed feasible. However, chromatography of the product showed that the amyl alcohol solvent was a very slow band moving down the SAS-hypersil column, extending analysis times unacceptably. The method could be well employed were column-switching techniques to be adopted to exclude the amyl alcohol from the main column. Disruption of the ion-pair (tartrazine-cetrimide) by aluminium nitrate to effect back extraction proved too incomplete to be worthwhile. Strong complex-breaking agents such as perchloric acid were precluded because of their equally disruptive effect on subsequent chromatography.

Simpler means of product concentration were considered. In the same way that the use of ion-pair hplc in the present study relies on the likely presence of sulphonate moieties in the putative breakdown products, so too the involatility conferred by their presence was taken as a basis for solution concentration.

Rotary Evaporation

Experimental

A portion of the solution remaining from the thermal degradation of green S - discussed in Chapters 7 and 9 - was taken and 5.024 g were weighed out. The sample was transferred to a 100 ml round bottomed flask and heated under reduced pressure on a rotary evaporator until several-fold concentration had occurred.

Chromatograms were obtained of both the non-concentrated and the concentrated solutions. Hplc conditions included:

Eluent: 62/38/0.25 v/v/w methanol/water/cetrimide.

Detection wavelength: 240 nm.

Flow rate: 0.8 mls/min.

Thermostat set at 39.5°C.

Results

The weight of the final solution was found to be 0.9204 g. The chromatograms obtained are shown as photographs (not redrawn or traced) in figure 62.

Peak No*	Initial Peak Height (AU)	Final Peak Height (AU)
2	0.00550	0.0287
3	0.00590	0.0306
5	0.00749	0.0414
13	0.00502	0.0268
15	0.0163	0.0912

* As in figure 47.

Discussion

The concentration factor is 5.024/0.9204 = 5.46. The expected final peak height for peak 2 is therefore 5.46 x 0.00550 = 0.0300 AU. The

Chromatograms of thermally degraded Green S sample before and after concentration (x 5·46) by rotary evaporation

[Peak numbering as per Fig. 47]

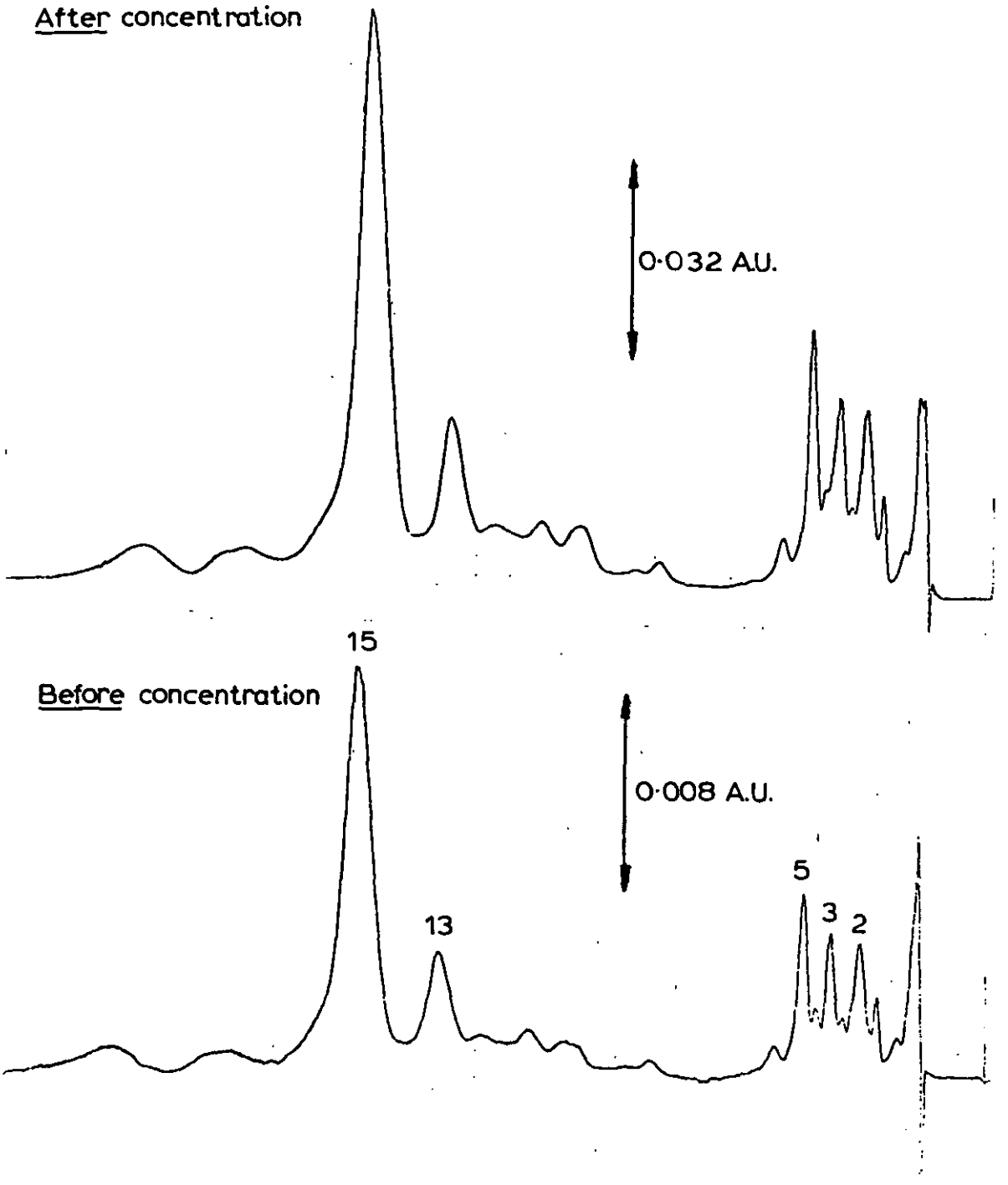


Fig. 62

value found was 0.0287 AU, which is 0.0287/0.0300 x 100 = 95.7% of the expected value. Performing the same calculation for the other peaks shows values of 95.0%, 101.3%, 97.7% and 102.7% for peaks 3, 5, 13 and 15 respectively.

Perhaps it is surprising that a solution that has previously been heated at 120°C for a month in a sealed phial should show sensitivity to boiling under reduced pressure, even to the extent that this does. It is unlikely that any component of the chromatogram is particularly volatile - most, perhaps all, are sulphonate salts. Aerosol loss is, of course, quite possible. Inspection of figure 62 shows that the concentration does modify the early peaks somewhat, but that the most important effect is a general improvement in baseline noise (because the 0.16 A.U.F.S. scale can be used rather than the 0.04 A.U.F.S. scale).

Overall, the changes in peak height are sufficiently small to encourage further attempts at straightforward concentration, monitored by this comparative hplc method, or perhaps by related technique such as freeze drying. Though this method (sometimes referred to as lyophilisation) is mostly used by biochemical analysts for aqueous solutions of e.g. enzymes etc., its introduction in the present field might remove the uncertainties surrounding simple rotary evaporation.

Qualitative Analysis of the Degradation Products

The greatest difficulty in this study has undoubtedly been the identification of degradation products. The approaches that have been taken are best understood in the light of these difficulties.

The first and foremost problem is the availability of standards for the putative degradation products. These include the amine cleavage products of azo compounds, and possibly the hydroxyl analogues (NH_2 displaced by OH). [Another worker in this laboratory, Mr. A.M. Summan, has investigated the production of ammonia during the light degradation of the food colours].[149] Although this displacement is not a reaction that is presented in elementary texts, a distinction should be made between reactions suitable for high yield syntheses, operating over one or two hours perhaps, and between possible reactions occurring at high temperature over a period of weeks in moderate yield. Enquiries made to the Chemical industry (E.g. ICI) have failed to locate a source of these compounds: it seems that though they must all have been synthesised at one time, stocks have not been maintained. The absence of suitable standards has prevented several means of identification from being fully pursued, notably the comparison of retention times, the comparison of UV-visible spectra, and the comparison of retention dependence on variable factors, e.g. eluent composition. Therefore the presence of the ion-pairing group (generally sulphonate, though carboxylate cannot be excluded) was already indicated as being present by the nature of the chromatography itself in the peaks showing retention times substantially exceeding that of the solvent front.

Qualitative analysis conducted by means of, or in tandem with gas chromatography has been a well-known technique for a number of years.[150] The analogous topic in hplc has shown far less rapid growth, partly because of the undoubted success of thin layer chromatography in this regard. A resume of methods for hplc is given by Snyder and Kirkland.[83i]

The approach adopted in the present work comprised performing functional group-selective reactions on the degradation products and then detecting these by the displacement of peaks in the chromatogram, or by use of a fluorescence detector where the reaction had created suitable fluorescent derivatives.

Fluorescent Labelling

The commonest reason that analytes are fluorescence-labelled is to decrease their detection limit. The low concentrations involved in the present study, while not being below the levels accessible to UV detection, were a supporting factor in selecting this as the first approach as derivatisation may involve some dilution of the sample.

There is no very good means to fluorescence label phenols selectively. Most of the reagents which are able to do this also work for aminoaromatics, or at least are non-general in action[151] and therefore inappropriate for identifying unknowns.

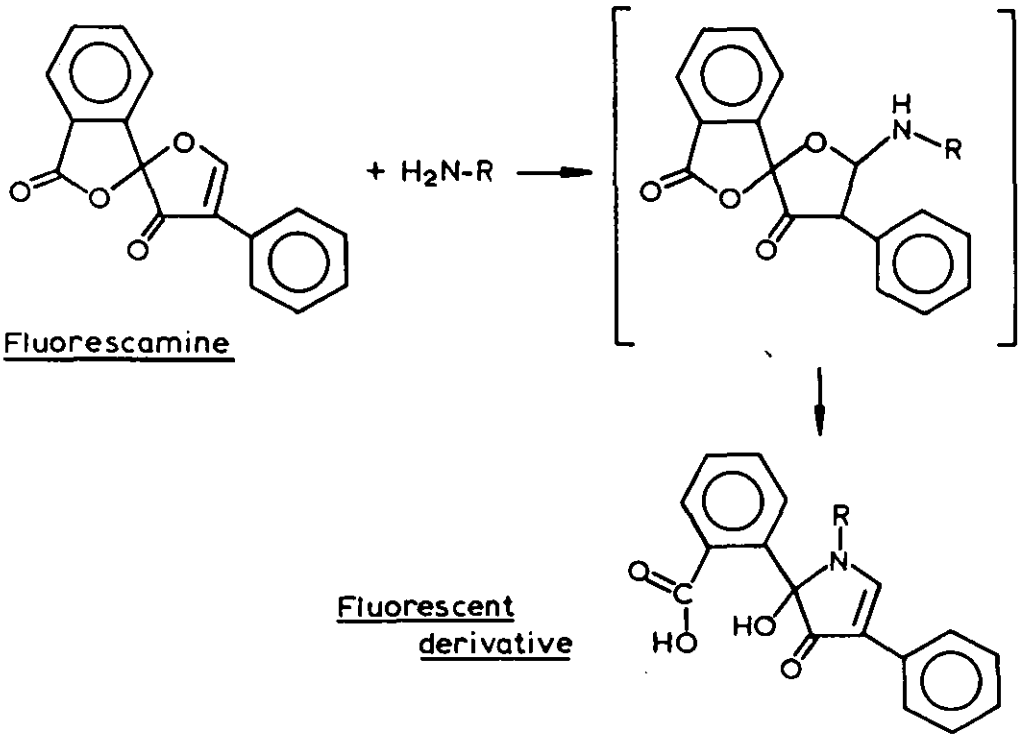
Aliphatic amino groups have received very considerable attention[152] unlike both phenols and aromatic amines, because of their biological significance. Most current textbooks and review articles list far more reagents that have been introduced for this category of compound than for all other categories combined.[153]

The earliest reagent for aliphatic amines was 5-dimethylaminonaphthalene-1-sulphonylchloride, the so-called "dansyl chloride". An entire family of related compounds, preferred for their stronger fluorescence or for the enhanced hydrolytic stability of the derivative formed, have followed in its wake. These compounds and another reagent, 4-chloro-7-nitrobenzo[C]-1,2,5-oxadiazole, share the characteristic that they give fluorescent products not only with amines, but also with at least some phenolic OH groups. This renders them unsuitable for present purposes where the two types of group require to be distinguished.

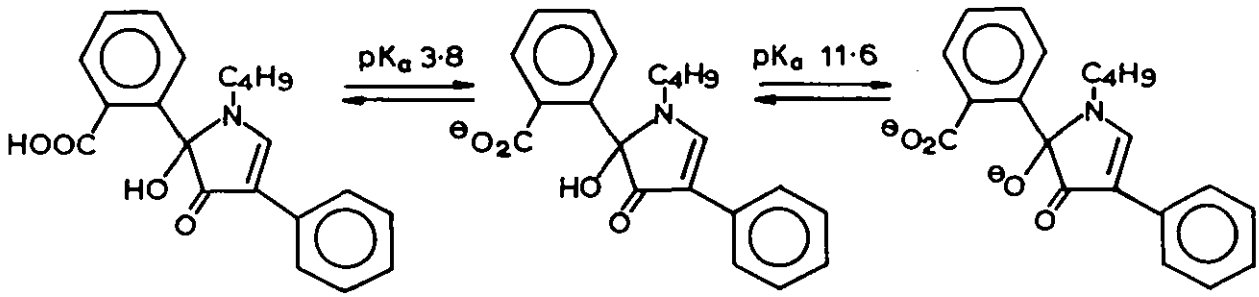
Other types of reagent that have found use for fluorescence-labelling of aliphatic amines include isocyanates, isothiocyanates, pyridoxal (derivatives), o-phthaldialdehyde and various aliphatic aldehydes. The aldehydic reagents have tended to be used for post-chromatographic derivatisation.

There was one reagent, however, that seemed to offer unique benefits in the present work, viz., 4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione, more generally known as fluorescamine (or fluram).[154] Fluorescamine is itself non-fluorescent and will hydrolyse in aqueous media to give non-fluorescent products. It solutions in e.g. acetone or acetonitrile (carefully dried) are stable for perhaps, three months. It undergoes nucleophilic attack by primary and secondary amines, alcohols, water, etc., as shown in figure 63, but its essential feature is that only the products formed with primary amines are fluorescent.[153] This degree of specificity was its great attraction in the present study.

Use of fluorescamine in the fluorescence-labelling of primary amines



(a) Reaction of primary amines with fluorescamine

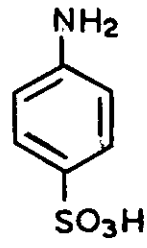


(b) pH-dependence of derivative fluorescence and stability

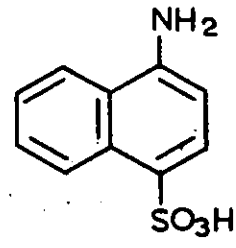
Fig. 63

The other main attraction of fluorescamine is that it is an established pre-column technique, the derivatives formed being separable by reverse-phase hplc.[155]

All the references given refer to fluorescamine as a reagent for aliphatic primary amines. It was decided to conduct the derivatisation on aromatic amines to ascertain its adaptability in this regard. Sulphanilic and naphthionic acids were chosen as the simplest sulphonated examples of this category, and because they are possible degradation products from some of the food colours.



Sulphanilic acid



Naphthionic acid

Experimental

A stock solution was prepared containing 34 mg fluorescamine in 10 ml hplc-grade acetonitrile. This concentration was equivalent to 1.2×10^{-5} moles/ml. The water content of the acetonitrile, quoted as <0.05% in the unopened solvent bottle, was controlled by storing the solution over type 4A molecular sieves.

Borate buffer ($^{M}/3$) was prepared at pH 8. Aqueous solutions of sulphanilic and naphthionic acid were prepared (250 ppm $^{w}/v$) and diluted to 12.5 ppm $^{w}/v$. Care was taken that the water used in these

solutions and in the buffer was distilled and stored in glass apparatus (to avoid the fluorescence interference which may occur when water is stored in plastic containers).

A test tube was wrapped in aluminium foil to exclude light leaving the mouth of the tube open. 1 ml sulphanilic (or naphthionic) acid solution and 1.5 ml buffer were pipetted into the tube which was then placed onto a vortex mixer. Next 0.5 mls fluorescamine solution were added while the mixer was operating at a medium-high speed to ensure "instant" and thorough mixing. The vibration was continued for two minutes. The solution was transferred to a 1 cm fluorimeter cell and an emission spectrum was obtained over the 400-650 nm range with an excitation wavelength of 395 nm.

Results

The sulphanilic acid solution treated with fluorescamine gave rise to intense fluorescence at 505 nm (peak half-width was 78 nm). In the case of naphthionic acid no peak was found, only an ill-defined shoulder on the decay curve from the scatter line using highest sensitivity at 500-510 nm.

Discussion

These results were observed over the pH 7.5-9.5 range with no change in the essential result that the sulphanilic acid reaction took place and the naphthionic acid reaction did not. The improbable chance that the sulphanilic acid peak was a Raman peak was checked as a matter of course by changing the excitation line to 400 nm - no shift in emission maximum resulted. The possibility that the naphthionic acid reaction had produced an even more intensely absorbing/fluorescing

species than had the sulphanilic acid reaction, and that the non-observation of fluorescence was due to an inner filter effect required to be tested.

Experimental

The naphthionic acid derivatisation with fluorescamine was again attempted as before. Both the UV-visible absorption spectrum and the fluorescence emission spectrum (395 nm excitation) were obtained.

Results

The fluoresence spectrum was unchanged. The UV-visible absorption spectrum showed no maximum above the 316 nm peak indicative of unreacted naphthionic acid. [Cf. sulphanilic acid after fluorescamine derivatisation with 397 nm absorption maximum].

Discussion

The non-appearance of the naphthionic acid-fluorescamine derivative fluorescence is a real effect caused by non-reaction rather than an inner filter effect.

An explanation for the differential reactivity of the two amino-sulphonic acids was sought in the literature. In an early paper on the evaluation of fluorescamine as a colorimetric reagent for amines, several pertinent points were discussed.[156] Variations in reactivity with pH and reagent concentration were mentioned but these do not seem to apply in the current case for naphthionic acid. [A range of pH, 7.5-9.5 has failed to give reaction, and attempts using 10-fold the fluorescamine concentration stated above has also proved unsuccessful]. However, this paper also states that very weakly basic amines (e.g.

p-nitroaniline) may require non-aqueous solvents and a tertiary amine catalyst to cause reaction. If this is the case for naphthionic acid then this reaction could prove difficult to apply as the original matrix would be water. The rate of fluorescamine hydrolysis might always exceed that of amine derivatisation.

For this reason the otherwise advantageous use of the selective reagent fluorescamine was prevented. The method was however so quick and reliable that it could be applied as an analytical method for the rapid estimation of sulphanilic acid in the presence of naphthionic acid and/or hydroxyaromatic sulphonic acids. A colorimetric method with measurement at 397 nm could be used, or the fluorimetric method for more dilute solutions.

Also, fluorescamine might be used to separate the phenol-4-sulphonic acid peak from the sulphanilic acid peak in chromatograms containing both. This problem has already been dicussed in Chapter 8 where an alternative approach was taken (i.e. twin wavelength detection). The use of fluorescamine might be preferred where the two compounds were present to greatly differing extents.

Diazotisation

Initially diazotisation was considered less advantageous than fluorescence-labelling as an amine-specific reaction. However, one difficulty seemed perfectly avoidable. The dilution of the sample solutions and the fact that phenols couple in alkaline solution rather than at pH 0-2 meant that the presence of both aromatic amines and phenols among the degradation products would not result in the coupling of one product (a phenol) with the diazotised amine. Only the aromatic

amine chromatographic peak would be abstracted/displaced, maintaining recognisable specificity.

Previous work in this laboratory had evolved the following procedure.

Experimental

5 ml c 10^{-4}M amine solution was adjusted to c. pH 7. 2 ml. 0.1% $^{w}/v$ sodium nitrite and 2.5 ml 20% $^{w}/v$ sodium bromide aqueous solutions were added. The mixture was transferred to a flask containing 3 ml concentrated sulphuric acid to which a few crystals of ice had been added, and the whole flask was cooled in an ice bath. This transfer was completed over a c. 5 minute period, more ice being added as required to the solution. After 3 minutes, 2 ml 0.5% $^{w}/v$ sulphamic acid was added in order to destroy any surplus nitrous acid. A further 5 minutes was allowed for reaction. The solution was then introduced into a 50 ml volumetric flask containing 4 mls of the coupling agent, viz., N-naphthylethylenediamine dihydrochloride, and the solution made up to volume with water. The purple colour was gradually generated: an hour was allowed for complete reaction.

Discussion

The role of the bromide was to promote the diazotisation. The method had two serious drawbacks. The already low quantities of decay products were further diluted without any concomitant gain in sensitivity in the window of the spectrum that was open to the hplc detector. Also, when the mixture was chromatographed without modification the whole chromatogram was useless, comprising a heavy absorption peak with heavy tailing at the solvent front. Furthermore, the final pH

was too low to permit standard chromatography (pH was below 1.0) because of column deterioration below about pH 2.0 (generally true of reverse-phase packing materials). It was further suspected that the acidity and high ionic strength of the solution was disrupting the ion-pairing mechanism despite the small injection volume because sulphonate-containing species would continue within the "solvent plug" as it travelled down the column, never forming a stable complex with the pairing ions present.

Various attempts were made to overcome these difficulties, but none was successful. Slurrying the product solution with barium hydroxide followed by centrifuging and decanting the supernatant was tried, but it seemed likely that adsorption of polar species onto the precipitated barium hydroxide and sulphate would disrupt the analysis, even though this method had the advantage of avoiding further sample dilution and of reducing the acidity and ionic strength of the sample solution greatly.

Finally diazotisation was abandoned. Whereas labelling with fluorescamine had been prevented because of problems with the reaction, diazotisation was prevented because of the subsequent chromatographic difficulties it presented, and because of the sample dilution involved.

Use of Retention Times in Qualitative Analysis

1) Direct Comparison with Standards

Despite the general unavailability of suitable standards, four very useful ones were available. These were sulphanilic acid and phenol-4-sulphonic acid (which are possible decay products of black PN,

brown FK, sunset yellow FCF, tartrazine and yellow 2G) and sodium naphthionate and naphthol-4-sulphonate (which might be produced from amaranth, carmoisine, chocolate brown HT or ponceau 4R). The relevant chromatographic behaviour of these standards was set out in Chapter 8 (see fig. 38) together with that of aniline (a possible product from red 2G). As a résumé, the retentions of these standards using 63/37/0.25 v/v/w methanol/water/cetrimide eluent at 39.5°C column jacket temperature (i.e. the same hplc conditions as for the series of chromatograms in Chapter 9, fig. Nos. 47-59) are given in the following table:-

Standard	Retention (mls)
Aniline	1.49
Sulphanilic acid	2.32
Phenol-4-sulphonic acid	2.38
Sodium naphthionate	2.71
Sodium naphthol-4-sulphonate	3.24

The following tentative assignments have been made (see Chapter 9 for the tables of decay product retention times):-

Peak	Chromatogram	Assignment	Retention Difference*
2	tartrazine	sulphanilic acid	1.7%
3	tartrazine	phenol-4-sulphonic	0.8%
2	yellow 2G	sulphanilic	2.6%
2	sunset yellow FCF	phenol-4-sulphonic	-0.8%
1	black PN	phenol-4-sulphonic	-2.9%
3	amaranth	naphthionate, Na	-1.1%
5	carmoisine	naphthionate, Na	0.7%
4	amaranth	naphthol-4-sulphonate	-3.7%
6	carmoisine	naphthol-4-sulphonate	-3.7%

$$*\text{retention difference} = \frac{\text{retention [standard]} - \text{retention [product]} \times 100\%}{\text{retention [standard]}}$$

Confirmation of these assignments would require other techniques as discussed in this or preceding chapters.

2) Variation of Retention with Eluent Composition

Fig. 64 shows the same degraded sample of ponceau 4R previously shown in fig. 56, run instead at 67/33/0.25 and 70/30/0.25 v/v/w methanol/water/cetrimide respectively. Peaks 3 and 4 are seen to superimpose at 67% methanol and to switch over their elution order completely from 70% to 63% methanol. Such very great sensitivity to the eluent composition has been noticed in only one other case, that of erythrosine. Here it was assumed that the cause was the presence

Peak cross-over effect in chromatograms of thermally degraded Ponceau 4R run at different eluent compositions

(A) eluent 67% methanol: peaks 3 and 4 overlap

(B) eluent 70% methanol: peak 4 precedes peak 3

(C) see Fig. 56 run at 63.% methanol for peak 3 preceding peak 4

[eluent 0 25% w/v cetrimide; q.v water in each case]

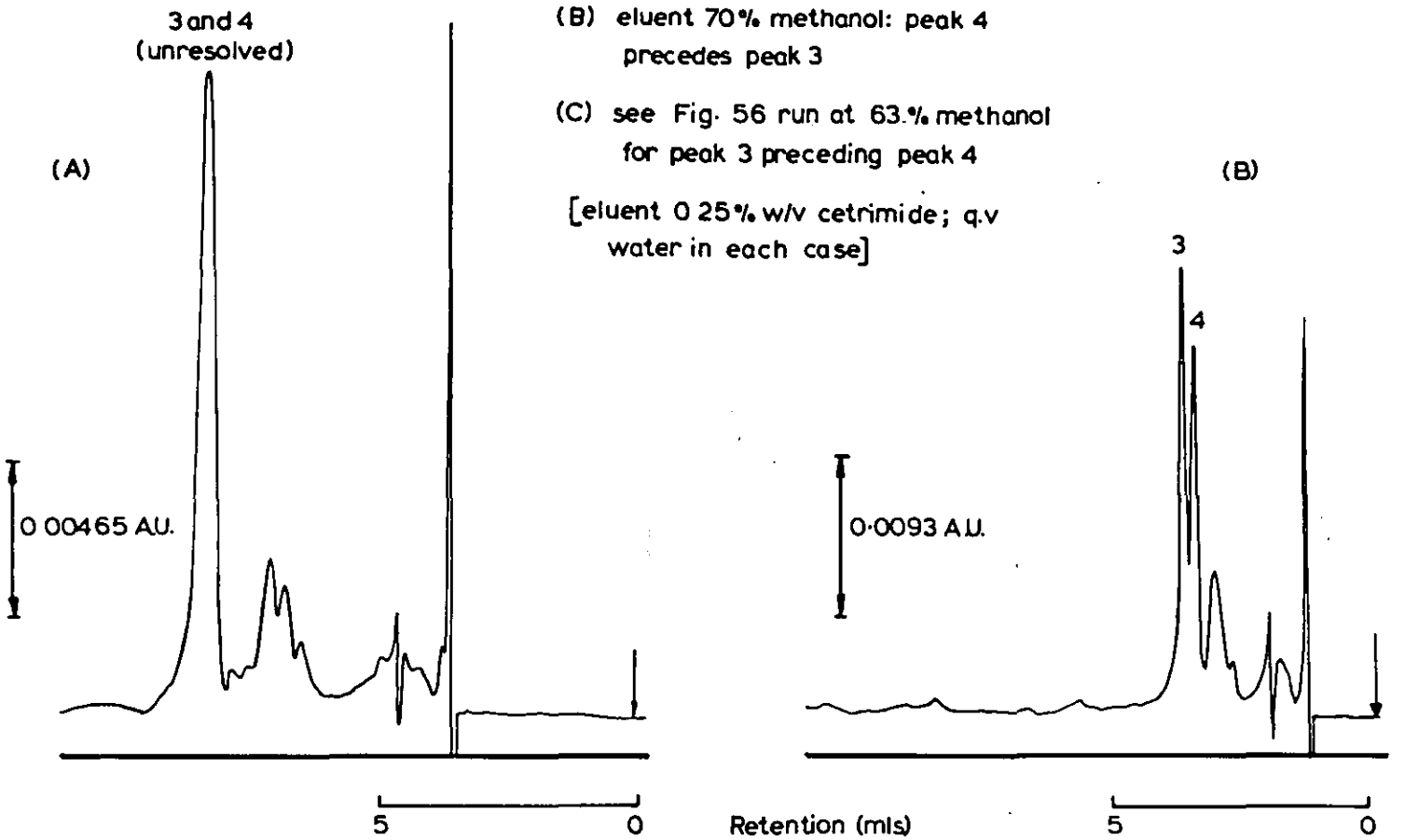


Fig. 64

of the carboxylate group (no sulphonate group is present in erythrosine making it unique among the UK-permitted synthetic food colouring matters). There is no likelihood of any carboxylate group appearing in a ponceau 4R decay product unless oxidative ring opening has occurred. The study begun in Chapter 8 investigating the effect of temperature or eluent composition on the retention behaviour of standard compounds might profitably be extended with the object of identifying certain functional groups in this way. This method would then be similar in scope to the search for new polarographic peaks (see Chapter 7) which gives the important clue to molecular structure that an electroactive decay product has been formed, but by itself is not suitable for complete identification.

GENERAL CONCLUSIONS

The stability of the 16 UK-permitted synthetic food colouring matters has been investigated under conditions of exaggerated heat or light. For the latter study an apparatus was devised to provide strong, even illumination by white light (tungsten filament lamp with mercury trigger) while maintaining the trial solutions at close to ambient temperature.

Decay curves were obtained using differential pulse polarography for both heat and light degraded solutions. Most of the thermal decay curves were approximately 1st order decay, though in some cases it was apparent that more complex kinetics were involved in modifying this simple picture. The light degradation curves, on the other hand, showed a substantial variety of shapes depending on the specific colouring matter. For both heat and light decay curves it was found that the commonly met practice of comparing stabilities on the basis of the ratio of the decay losses at a single time was inadequate, especially for some of the light degraded solutions.

Comparison of photostability taking account of the whole decay curve showed that amaranth, black PN, brilliant blue FCF, patent blue V, red 2G, sunset yellow FCF and tartrazine had excellent stability, while that of carmoisine, green S, quinoline yellow, and yellow 2G was still very good. Brown FK showed rapid initial fall-off, but the decay rate slowed, leaving the solution coloured pale brown even at high exposure times. Of the remaining colouring matters, the shape of the decay curves and overall decay periods of chocolate brown HT and of ponceau 4R suggest that under normal lighting, over intermediate storage periods, there might well be little decay. The two colouring matters showing minimal photostability were indigo carmine and erythrosine. Both these cases have previously been reported in the literature.

As regards thermal stability, brilliant blue FCF, quinoline yellow and tartrazine stood above the rest. Fairly good stability was shown by erythrosine - in contrast to its photostability - and by green S, patent blue V, sunset yellow FCF and yellow 2G. Intermediate stability was shown by amaranth, black PN, chocolate brown HT, carmoisine and brown FK. Chocolate brown HT and ponceau 4R degraded rapidly, but the least thermally stable colour by far was indigo carmine.

Few assignments of stability seem related simply to the primary structure of the dyes. The indigoid structure is obviously not stable to heat or light, while the xanthene structure of erythrosine behaves differently to heat and light, but these are unique representatives of their dye types among the UK-permitted food colouring matters and posssibly atypical of these types. Among the three triphenylmethane dyes, all show adequate heat and light stability, but among the ten azo colours a wide range of stabilities was apparent. It may be the case that chocolate brown HT and black PN show lower heat stability because they have two azo groups per molecule.

The same factor could help explain the shape of the photo-decay curve of brown FK, which is a mixture of mono-, dis- and trisazo molecules of otherwise similar structure, which might vary in stability according to the number of azo groups present.

During the polarographic analysis of some solutions new polarographic peaks appeared as the thermal degradations progressed. Among these were indigo carmine (cf. Fig. 29) and red 2G. The new peak in the latter case was identified by comparison with a standard as being red 10B (the deacetylation product of red 2G). Confirmation of the identification was obtained by hplc.

The determination of red 2G in the presence of red 10B was initially difficult. Red 2G and red 10B proved to have highly similar retention times (hplc) and $E_{\frac{1}{2}}$ values (polarography) in the systems employed. A method was developed using differential pulse polarography in which tetraphenylphosphonium chloride was added to the polarographic solution. This surfactant produced a differential shift in the $E_{\frac{1}{2}}$ values of the respective colouring matters and so increased the resolution of their polarographic peaks.

The thermally degraded solutions were subjected to hplc analysis. In the absence of any large body of recently published work on the chromatography of the decay products from the food colours, a literature search was made of the hplc analysis of the food dyes themselves and their subsidiaries. Among the methods used, reversed phase ion-pair chromatography stood out as offering greatest flexibility in use. Trials showed the superiority of a methanol/water/cetrimide eluent on an SAS-hypersil column over an isopropanol/water/acetic acid/cetrimide eluent on the same column.

Difficulties were initially encountered with varying retention times. These difficulties were largely eliminated by the development of a water jacket to thermostat the hplc column. Other benefits which accrued from this included a substantial improvement in chromatographic resolution and an increased flexibility in the chromatographic system.

The thermally degraded food colour solutions were separated by hplc. The complexity of the degradation patterns was greater than had been anticipated by reference to the previous literature (see Chapter 2 and Appendix A). A series of 13 chromatograms was run under the same chromatographic conditions to compare the thermal degradation solutions of all the food colours except for erythrosine, chocolate brown HT and brown FK which were excluded as being unsuited to the chromatographic

conditions. Among the 13 chromatograms obtained, only those of ponceau 4R and of patent blue V were at all simple, each of these having about 4 product peaks. All the others showed 8 or morepeaks with green S actually having at least 14 decay product peaks! Referring back to the polarographically-obtained decay curves, it is reasonable to make the statement that, for the comparatively large molecules that these dyes are, the general stability is high and no particular weak point in most of the structures seems to exist. The decay pathways taken therefore comprise a number of more or less low yield and low probability reactions, which require the extreme conditions (temperature + time) utilised in this study in order to occur to any significant extent. The alternative possibility, namely that the complex nature of the breakdown is due to early primary decay by a single major decay pathway (a 'weak link' in the molecule) followed by subsequent secondary, tertiary etc. decay also exists: however, the hplc study of the thermal degradation of yellow 2G revealed the greatest complexity in the chromatogram early, not late, in the study, before loss of the parent molecule had even exceeded 50% [this result proved reproducible].

Comparison of retention times indicated that phenol-4-sulphonic acid, sulphanilic acid, sodium naphthionate and sodium naphthol-4-sulphonate were possible thermal decay products from a range of azo-type colouring matters (see Chapter 10). Obviously, those azo-colouring matters which fade or decolourize (as opposed to changing colour) must be losing the azo linkage and the known instability of the hydrazine analogues makes cleavage the expected outcome.

Retention behaviour was also seen as a possible indicator of product structure, e.g. presence of carboxylate or sulphonate group, retention of the triphenylmethane structure after decay, etc. In particular, investigation of a ponceau 4R thermal degradation

solution showed one product peak to have unusual retention sensitivity to the eluent composition. This may indicate the presence of an ionizable group, and if this turned out to be carboxylate it would show that ring opening had taken place (naphthalene ring opening to give a carboxy-alkylbenzene derivative).

The UV-spectra of decay products were found to be more easily plotted by repeat chromatography at different detection wavelengths than by peak elution and collection followed by the running of spectra in a conventional spectrometer. The former method avoided problems of judging the collection limits of the eluting peak and in practice required far fewer chromatographic runs as many peaks could be measured from the same set of chromatograms. [No stopped-flow UV-scanning detector was available]. From this a technique was found for estimating the amounts of phenol-4-sulphonic acid and of sulphanilic acid in admixture, when unresolved chromatographically, by measurement of their common peak at two detection wavelengths.

Diazotisation and reaction with fluorescamine were investigated as possible amine-selective reactions to identify aromatic amines in the presence of phenols, and so to distinguish the two possible types of decay product. Diazotisation was found to leave a solution that had too many ionic species present (especially acidic species) for subsequent analysis by ion-pair hplc. The ion-pair mechanism was disrupted and the unresolved mixtures of sulphonic acid group-containing decay products were swept through the column at the solvent front. Reaction with fluorescamine was found to be simple and rapid, and to provide high sensitivity in the analysis of sulphanilic acid in aqueous solution (standard fluorescence spectrometer). The method failed to work for naphthionic acid and indeed would provide an especially fast method for the sensitive detection of sulphanilic acid in the presence of

naphthionic acid. The non-generality of the reagent prevented its use in the qualitative analysis of the complex product mixtures by hplc.

APPENDIX A

Stability Data for Individual Food Colours

Some of the most comprehensive data in the following paragraphs is taken from Helou[36] to avoid repetition his formulas, etc. are given separately here. (These do not apply to other references).

Formulations:-

(a) aqueous solution: 1 mg colour; buffer*; q.v. to 100 ml distilled water.

(b) simple syrup: 1 mg colour; buffer*; 85 g sucrose; 0.1 g methyl-p-hydroxybenzoate; q.v. to 100 ml distilled water.

(c) modified syrup: 1 mg colour; buffer*; 45g sucrose; 5 g propylene glycol; 0.1 g methyl-p-hydroxybenzoate; q.v. to 100 ml distilled water.

(d) simple elixir: 1 mg colour; buffer*; 0.2 mls spirit of flowers of 'laranja' (sic); 23 ml alcohol; 38 ml simple syrup; 0.1 g methyl-p-hydroxybenzoate; 0.05 g vanillin; q.v. to 100 ml with distilled water.

* Buffer: for pH 2, 3.15 ml 0.1M citric acid added;
for pH 4, 2.7 ml 0.1M citric acid and 1.7 ml 0.2M disodium phosphate added;
for pH 6, 0.3 ml 0.1M citric acid and 0.4 ml 0.2M disodium phosphate added;
for pH 7, 2.5 ml 0.2M disodium phosphate and 1.47 0.2M sodium hydroxide added;
for pH 8, 2.5 ml 0.2M disodium phosphate and 2.34 ml 0.2M sodium hydroxide added.

[Each pH is ±0.05 units].

Conditions:-

Storage for 7 to 11.5 months,

(a) refrigerated,

(b) at ambient temperature in diffuse light,

(c) at ambient temperature in the dark.

Subsequent analysis being by visual inspection and by spectophotometry.

Each colouring matter will now be discussed individually.

Amaranth

Helou[36] studied the light and storage stability (formulations above). Stability is average in aqueous solution (pH 2-8) for storage under refrigeration or at ambient temperature, in the dark or exposed to diffuse light. In simple and modified syrups, stability to light is poor (pH 2-8), as is dark storage at pH 7-8. Storage stability in simple elixir is good except when exposed to light at low pH where it is poor (pH 2) or average (pH 4).

Peacock[21] describes the light-fastness of aqueous amaranth solution as moderate, while Spiess[141] describes it as adequate. Goodhart[20] tested the light stability of an amaranth lake (0.027-0.081% amaranth) which proved to be moderate to good. Cool, white fluorescent light, 1000 ft.-candles (10 days) was used in the latter study.

Wojcik[46] states that amaranth is unaffected by irradiation with a bactericidal lamp (254-260 nm) for 336 hours when in the pure form or in dragee mass, but that aqueous solutions decolourize, depending on the amaranth concentration and the exposure time.

Jouffrey[23] describes the light fastness of amaranth in aqueous solution as average, and that stability towards oxidants is limited while there is little stability towards reducing agents (e.g. ascorbic acid) and that fading occurs in gelatin preparations. However, amaranth shows compatibility with glucose and sucrose in solution.

Peacock[21] gave the stability of amaranth as: good towards 10% and glacial acetic acid; good towards 10 and 30% hydrochloric acid; good towards 10% sodium hydroxide solution, but only fair towards a 30% solution; moderate towards 5% ferrous sulphate solution; good towards 5% alum solution; fair towards oxidants; and very poor towards reducing agents. Hence Jouffrey and Peacock concur on the last two cases, while Peacock is somewhat more generous than Helou in assessing acid stability.

Banerjee et.al.[49] found that amaranth was fairly stable toward glucose and lactose, but far less stable towards ascorbic acid - in agreement with Jouffrey. They also found amaranth to be unstable in the presence of sodium sulphite, but to be destroyed only slowly in the presence of sodium nitrite or bisulphite. Loss of colour within one day revealed the poor stability of amaranth (40 µg) towards 0.5% citric or tartaric acid solution (10 ml) containing iron or tin metal, and little better stability (loss of colour within 2 days) towards 5% potassium nitrate solution containing either of these metals. Benerjee et. al. referred back to work by Lolua et. al.[28] who discussed the primary reduction of amaranth to the hydrazo analogue by nascent hydrogen from acid-metal couples (citric, malic, tartaric acids; iron and copper metals). This hydrazo analogue may be further reduced, cleaving the molecule to form amines, or may be re-oxidised by dissoved oxygen to amaranth.

More recently Drozdova et. al.[33] have studied the stabilities of acid solutions (2.5% acetic, citric, tartaric, malic and 0.1N hydrochloric) of amaranth in contact with aluminium A-5, steel-3 and steel-20. For steel-20/ 0.1N hydrochloric acid, two degradation products were identified, viz., 1-amino-2-naphthol-3,6-disulphonic acid and 1-naphthylamine-4-sulphonic acid (a known carcinogen(sic)).

Driver and Francis[32] have investigated the storage stability of amaranth when incorporated into pectin and gelatin gels, flushed with nitrogen, sealed, then stored at -28°, 4.4°, 21° and 38°C. (Gelatin gel: 2.8% gelatin; 13.9% sucrose; 83.3% water. Pectin gel; 0.2% citric acid; 0.3% pectin; 48.2% sucrose; 51.2% water. Amaranth incorporated at 3 ppm $^{w}/_{v}$ level). At the three lower temperatures, the storage stability of amaranth in the pectin gels proved very good, and even at 38° good stability lasted for 6 days. In gelatin gels, amaranth showed good stability at -28° of 4.4°, and for 6 days at 21°, but at 38° marked, erratic bleaching occurred. Various stabilizers were tested for the gelatin gel, viz., pectin, citric acid and an ascorbic/citric acid mixture. None of these proved totally effective at 38°, though improvements at some temperatures were noted. The stabilizing action was tentatively ascribed to the metal chelating effect of citric acid and pectin.

Luck[29] carried out a number of compatibility tests with oxidizing and reducing agents. He found that sterilizing at 120° for half an hour produced no determinable degradation (pH 7 phosphate buffer solution, $^{M}/150$; 0.01% amaranth). Addition of 0.1% $^{w}/v$ sodium sulphite, followed by sterilizing at 120° for 30 minutes completely degraded the amaranth producing 3 unidentified uncoloured products (found by tlc). In the same solution, stored for 3 days at 20° instead of being sterilized, partial degradation produced two uncoloured products. When ascorbic acid (50 mg/ 100 ml) was added to the amaranth buffer solution, pH 7, neither boiling (10 minutes) nor standing at room temperature (3 days) showed any effect. Heating a solution containing 0.06%$^{w}/_{v}$ hydrogen peroxide at 120° for 30 minutes produced only one, uncoloured product (tlc), while 3 days standing at 20° gave no change. Luck repeated these tests at pH 3, substituting citric

acid buffer, $^{M}/150$, for the phosphate. Heating at 120° for 30 minutes produced no change. Addition of 0.01% sodium sulphite gave partial degradation with 4 products, one of which was coloured brownish yellow. Standing at 20° for 3 days yielded three non-coloured products. Addition of ascorbic acid (0.05% $^{w}/_{v}$) gave one product after 10 minutes boiling, or a single product after 3 days stored at room temperature. Addition of H_2O_2 (0.06% $^{w}/_{v}$), followed by heating at 120° gave 2 products, while standing the solution at room temperature for 3 days yielded no change. On a comparative basis, Luck concluded that amaranth was among the more stable colouring matters which his study included.

The thermal stability of amaranth in aqueous solution is also referred to by Jouffrey[23] who reports it as being good, and by Nursten and Williams.[18] The latter heated amaranth, 0.005% buffered at pH 7 or pH 3 ($^{M}/150$ phosphate buffer) with and without ascorbic acid (1 g/l) for 20 minutes each at 250°F (121°C). The stability proved excellent in the absence of ascorbic acid (99% retained at pH 7; 97% at pH 3) and still very good with ascorbic acid present (96% retained at pH 7; 91.7% at pH 3). On a comparative basis amaranth rated as among the most stable colours.

The Trace Materials (Colours) Committee of the British Food Manufacturing Industries Research Association[42] investigated the degradation of food colours during processing and subsequent storage. Their results are limited as they did not report fading as it was said to be almost universal, nor did they investigate colourless breakdown products. The amaranth sample under test initially contained 2.2% Fast Red E. Processing to produce boiled sweets, madeira cake or fresh sausages gave no change. However, when toffees were prepared, a new red colour was found, while the Fast Red E partly faded. With fish paste, a yellow-brown colour was formed.

Knowles et. al.[24] report that, after mixing with the other ingredients of pork luncheon meat (50 ppm amaranth) and processing of the meat, more amaranth was lost in the absence of nitrite than with sodium nitrite present (100 ppm). A yellow subsidiary (not identified) was formed in each case. The protective action was tentatively ascribed to preferential reactivity of nitrite towards sulphur-containing compounds liberated during processing.

Black PN

Jouffrey[23] describes black PN as showing good stability towards natural light in aqueous solution, while Spiess[141] merely gives it as adequate.

Black PN is perhaps the least stable of the synthetic colouring matters permitted in the U.K. towards reducing agents. Koether[17] remarks on the pronounced susceptibility of black PN to ascorbic acid. Nursten and Williams[18] heated black PN (0.005% buffered at pH 7 or 3 with $^{M}/_{150}$ phosphate buffer) first with and then without 1 g/l ascorbic acid present (250°F; 20 mins). Without the ascorbic acid there was complete retention at pH 7 and very good retention at pH 3 (but with a shift from 577 to 573 nm in the absorption maximum at pH 3). In the presence of ascorbic acid, there was by contrast poor retention at both pH's, especially at pH 7.

Jouffrey[23] confirms the good heat stability of black PN in aqueous solution, and remarks on a change of colour to violet-red in the presence of ascorbic acid (but also a change in hue to violet-blue in the presence of citric acid).

Luck[29] found that black PN was the only colour from the range

he tested which, when buffered at pH 7, gave rise to decay products after boiling for only 30 minutes. He found five such products. However, sterilizing at 120° for 30 minutes (pH 7) yielded only a single degradation product, while boiling for 10 minutes in the presence of ascorbic acid (50 mg/100 ml) gave eight products, one of which was red. Storage for 3 days at 20°, instead of boiling, gave two products. At pH 3, boiling for 30 minutes gave 3 products, as did sterilizing at 120° for 30 minutes. Boiling with ascorbic acid (as per at pH 7) gave 5 products, one of which was yellow, while standing for 3 days gave 3 products, one being orange.

Eisenbrand and Lang[55,56] refer to the air-oxidation products of the products of reductive cleavage of black PN, viz., 1-acetylamino-7-amino-8-naphthol-4,6-disulphonic acid and 1,4-diamino-naphthalenesulphonic acid as having an inhibitory effect upon the action of lactic acid bacteria. Also, they refer to the reductive cleavage of black PN (fading to a yellow solution) by ascorbic acid at pH 7, and describe the pronounced autocatalytic fading of the colour (accelerated from hours to minutes) through the activity of the cleavage product, 1,4 -diamino-6-naphthylenesulphonic acid. Naphthionic acid has an analogous, but comparatively minor effect.

Ruiz and Laroche[35] identified an orange dye formed in various confectionery items which were coloured with black PN (their extraction procedure gave rise to a second orange dye). They determined that the transformation, which occurred during processing of the confectionery, came about because of a combination of heat and the presence of reducing sugars (e.g. glucose, invert sugar). However, Jouffrey[23] reports that black PN is compatible with sucrose, a non-reducing sugar, but fades in gelatin preparations.

Luck[29] found that in pH 7 buffer containing 100 mg/100 ml sodium

sulphite, black PN (0.01%) upon sterilization (120°, 30 mins) gave 5 degradation products (tlc), while storage (3 days at 20°) gave 2. With regard to oxidizing medium (60 mg/100ml hydrogen peroxide) Luck reports than sterilizing gave one product at pH 7, but 3 at pH 3, while storage gave no change at either pH.

The Trace Materials (Colours) Committee of the British Food Manufacturing Industries Research Association[42] have considered changes due to processing and subsequent storage. The sample of black PN used was found to contain two red, one orange and one grey-blue subsidiary. Processing to give toffees or fresh sausages resulted in no change in the black PN used. However, in boiled sweets and fish past, changes in colour to yellow and orange respectively occurred, while in fondant, an orange and a yellow formed and one subsidiary was lost.

Wurziger[47] investigated the degradation of black PN in fish roe. Here a change from black to brown occurred, mainly as a result of overlong or inappropriate storage. Indeed, the degradation proved to be an indicator of the freshness of the produce!

Brilliant Blue FCF

In the range of formulations and conditions studied by Helou[36], poor stability was noted only (i) in simple syrup at pH 2 in diffuse light, (ii) in modified syrup at pH 2-7 in diffuse light, and (iii) in simple elixir at pH 2 in diffuse light. It may be noted that at pH 2 in aqueous solution stability was good towards diffuse light, while in the absence of light, all formulations showed average or good stability (pH 2-8) whether or not refrigerated.

According to Cooper[64], the relative stability of certain food colours varies according to whether exposure is to normal levels of lighting or to exaggerated levels. However, brilliant blue FCF showed little difference (5th instead of 6th most stable from a group of 10 colours compared), but this possibility should be realised in any photodegradation study.

In a test employing the sophisticated MacAdam method of assessing colour difference, Goodhart et. al.[20] found brilliant blue FCF lake (0.01-0.03% colour) showed poor stability to cool, white fluorescent light. Here, the mode of formulation appears to decrease stability.

Brownley and Lachman[61] tested brilliant blue FCF stored (i) at room temperature, (ii) at 60° and (iii) under exaggerated lighting for 90 days when present at 0.15% concentrations in the following:- (a) aqueous solution, (b) 0.15M phosphate buffer solution (pH 6.6), (c) 5% lactose solution, (d) 5% spray-dried lactose solution, (e) 5% D-glucose solution, (f) 5% D-galactose solution. For (c), (d), (e) and (f), i.e. the sugar solutions, heat at 60° for 90 days, the absorption maximum shifted from 307 to 280 nm indicating the formation of an unidentified degradation product. All other cases showed less decay, the most being a fall in absorbance from 0.117 to 0.105 at 307 nm for the D-galactose solution over 90 days of exaggerated illumination.

When buffered in tablet form,[40] brilliant blue FCF showed good stability at 25° at pH 3-7, but less stability at higher temperatures. 19% degradation occurred at pH 5 during 60 days storage at 80° compared to 13, 11 and 12% loss respectively for unbuffered tablets and tablets buffered at pH 3 and pH 7.

Peacock,[21] using a scale of fastness from 1 (very poor) to 7 (excellent) found brilliant blue FCF to rate: 3 for fastness to light; 5 for fastness to 10% glacial acetic acid; 5 to glacial acetic acid; 4 and 2 respectively to 10 and 30% hydrochloric acid; 4 to 10% sodium hydroxide; 4 to 5% ferrous sulphate solution; 2 to oxidising agents; and only 1 to reducing agents. Although experimental detail is lacking, the comparison made by Peacock is useful for the number of colours compared (all the F, D & C, D & C and Ext. D & C colours) and for the comprehensive list of tests performed.

Brown FK

Nursten and Williams[18] report that brown FK is only slightly degraded (ca. 3%) by heating at 121°C, pH 7.0 for 20 minutes. At pH 3.0 a little more is lost. However, the presence of ascorbic acid during the heat treatment increased the loss at pH 7.0 (ca. 11%) and particularly at pH 3.0 (ca. 56%). In these studies the brown FK was 0.005% concentration and the ascorbic acid was 1 mg/ml.

Carmoisine

Jouffrey[23] describes the lightfastness of carmoisine in aqueous solution as average. Spiess[141] describes it as being quite good.

Jouffrey[23] also considers carmoisine (i) to have good heat stability, (ii) to show very slight decolouration in the presence of 0.3 g/l ascorbic acid, but to be stable towards oxidants and reductants, and (iii) to have good heat stability towards 10% sucrose solution, but (iv) to fade in gelatin preparations.

Koether[17] singles out carmoisine (and black PN) as giving especially easy and complete reduction with ascorbic acid.

Nursten and Williams[18] showed that carmoisine, when buffered ($^{M}/150$ phosphate) at pH 3 and at pH 7, did not fade when heated for 20 minutes at 250°F (121°C). However, when the experiments were repeated, this time with 1 g/l ascorbic acid present, the 516 nm absorption maximum was reduced to 65.3% at pH 7 and to 83.4% at pH 3. This does not seem to justify Koether's opinion[17] regarding the reaction with ascorbic acid, and carmoisine certainly compared well with black PN in the Nursten trial.

Banerjee et al.[49] found that carmoisine was highly unstable towards ascorbic acid, bur fairly stable towards glucose and lactose. They also found carmoisine to be unstable towards sodium sulphite, though less so than indigo carmine and amaranth. Stability was fairly good toward sodium bisulphite, but rather mediocre towards sodium nitrite compared to most colours. Loss of colour within one day showed the poor stability of carmoisine (40 µg) towards 0.5% citric or tartaric acid solution (10 ml) containing iron or tin metal, and little better stability (loss of colour within 3 and 2 days respectively) towards 5% potassium nitrate solution.

Eisenbrand and Lohrscheid[54] note that, when air-oxygen is introduced into a fermentation sediment containing the cleavage product of carmoisine, 1-hydroxy-2-amino-4-naphthalenesulphonic acid, strong fermentation inhibitors are formed. This fails to occur under nitrogen or e.g. with naphthionic acid. Later, Eich and Eisenbrand[52] purified this oxidation product (a 'red substance') and analysed it as being $(C_{20}H_{16}N_2SO_6)_X$.

Knowles et al.[24] mixed carmoisine (50 ppm in the form of a 0.05% aqueous solution) with the other ingredients of a pork luncheon meat,

then processed the meat. One batch incorporated 100 ppm sodium nitrite, while the other had none present. The latter showed cleavage of the azo group as evidenced by the loss of the 508 nm absorption band. However, the nitrite-containing batch retained the band though it was much reduced, while a new pink subsidiary appeared that did not appear in the absence of nitrite (paper chromatography).

The effect of baking when carmoisine is incorporated (100 ppm) into a cake mix was evaluated by Mukherjee et. al.[30] The percentage recovery of colour fell from 99.8% before baking to 93% after, and subsequently to 90% after 11 months of storage - thereby showing good stability overall.

A more wide ranging assessment of changes undergone during processing and subsequent storage was carried out by the British Food Manufacturing Research Association (Trace Materials (Colours) Committee).[42] They did not refer to partial fading (all colours faded to some degree) or to colourless breakdown products. The carmoisine sample used contained 0.4% of a red subsidiary. No change was noted upon processing to, or storage of toffees, madeira cake or fresh sausages. In the case of boiled sweets, red and purple colours were formed, while, for fish paste, yellow and orange colours were formed on processing, while additional yellow, orange, pink and brown colours had formed after 14 months storage (paper chromatography).

Chocolate Brown HT

Nursten and Williams[18] heated chocolate brown HT (0.005%) in buffered aqueous solution ($^{M}/150$ phosphate) at 250°F (121°C) for 20 minutes. The temperature was determined from the 15 lb excess pressure (initial heating period was 6 minutes). At pH 7 the absorbance at 464 nm dropped to 80.9%

during the heating, and at pH 3 to 89.5%. When the tests were repeated with 1g/l ascorbic acid present, absorbance for the pH 7 solution fell to 81.2% with a slight shift in absorption maximum from 464 to 460 nm, while at pH 3 the fall was to 51.2% with the same shift occurring. Hence ascorbic acid has little effect at pH 7, but a significant effect at pH 3.

Erythrosine

As long ago as 1904, Jodlbaur and Tappeiner[142] reported that an aqueous solution of erythrosine developed acidity when exposed to light. Evenson,[57] in 1939, showed the lack of stability of erythrosine towards sunlight. He demonstrated that organically-bound iodine is split off during exposure and suggested that this iodine reacts with the small excess of sodium carbonate that is present in the commercial colour (via the formation of hydroiodic acid and iodic acid). Carbon dioxide is formed, acidity is increased and the bulk of the erythrosine, which is insoluble at low pH, precipitates. Furthermore, Evenson showed that erythrosine sensitises other colours, e.g. indigo carmine, to destruction by light. Brilliant blue FCF and tartrazine were among the few water-soluble colours not to show this sensitisation.

Jouffrey[23] concurs that lightfastness is poor, while Peacock assesses it as fair, Spiess[141] assesses it as very weak and Lachman et. al.[19] rate erythrosine as the least stable of ten colours tested using fluorescent light.

Denoel,[31] in a review article, notes that erythrosine aluminium lake is weak in light-fastness, but this varies according to the excipients employed. Twice as strong a colour change occurs upon exposure with calcium sulphate as excipient as with calcium hydrogen phosphate or lactose. Goodhart et. al.[20] also rated an erythrosine lake (0.019-0.057% erythrosine concentration) as being of poor relative light stability .

Helou[36] has assessed the light-fastness of erythrosine in various formulations (described at the start of this appendix). Dark storage stability (pH 4-8) under refrigeration is good for aqueous solution, for simple and modified syrups, and for simple elixir. Dark storage stability at ambient temperature is good for aqueous solution, for simple and modified syrups, and for simple elixir. Dark storage stability at ambient temperature is good for aqueous solution (pH 4-8), modified syrup (pH 6-8) and simple elixir (pH 4-8), and is average for simple syrup (pH 4-8) and modified syrup (pH 4). At ambient temperature, under diffuse lighting, stability is poor for aqueous solution (pH 4-8), simple syrup (pH 4-8), modified syrup (pH 4, pH 7-8) and simple elixir (pH 4), and is average for modified syrup (pH 6) and simple elixir (pH 6), while it is good for simple elixir (pH 7-8). This increased stability toward light in partially alcoholic solution was noted also by Evenson[57] in his paper in 1939.

Dickinson and Raven[62] found that where cherries coloured with erythrosine are stored in unlaquered cans, the combination of organic acid (cherries are usually canned at pH 4, at which pH erythrosine is almost insoluble, and so precipitates onto the fruit) and metallic iron and/or tin results in the electrochemical reduction of the erythrosine, removing iodine, to yield fluorescein. Tin and/or iron or stannous chloride without organic acid gave no fluorescein. Likewise, citric acid alone gave none. The loss of iodine results in an off-flavour being imparted to the fruit. Lacquered cans do not provide the conditions for the reduction - no fluorescein has been found in rhubarb tinned in lacquered cans even after 4 years storage. Nevertheless Raven[7] points out that the erythrosine still degrades - obviously to a product other than fluorescein.

Jouffrey[23] describes the heat stability of erythrosine as good. This is confirmed by Nursten and Williams[18], who found that heating erythrosine in $^{M}/_{150}$ phosphate buffer (pH 7) at 250°F (121°C) for 20 minutes gave only

a 3.3% fall in absorbance of the 528 nm maximum. Repeating this with 1 g/l ascorbic acid has no effect (or at least no negative effect) on the stability of erythrosine, which is presumably because this colour is of the xanthene type, not the azo type.

Luck[29] found that sterilizing at 120°C for 30 minutes did not modify the erythrosine (0.01%, pH 7 - an especially pure sample) nor did the presence of sodium sulphite (1 g/l), ascorbic acid (0.5 g/l - boiled for 10 minutes not sterilized) or hydrogen peroxide (0.6 g/l) produce any degradation products (tlc) upon sterilizing for 30 minutes at 120° or upon storage for 3 days at room temperature. Few other colours proved so stable.

However, Dickinson and Raven[62] point out that erythrosine does lose iodine when boiled in water, but that as fluorescein is not detected, diiodofluorescein seems the likely product. Even when erythrosine is stored dry for long periods, a sublimate of iodine can be found on the tops of some containers.

Also, Jouffrey[23] describes erythrosine as incompatible with ascorbic acid. He describes its stability towards oxidants as small, and towards reducing agents as very small. Erythrosine is compatible with gelatin , with 10% sucrose solution and with saturated bicarbonate solution, but incompatible with 0.3 g/l citric acid (though compare Dickinson and Raven, fourth paragraph preceding), and with 10% glucose solution.

Banerjee et. al.[49] found that erythrosine (4 ppm) was poor in storage stability towards 5% ascorbic acid solution (colour lost within 2 days) but showed fair stability towards 5% ascorbic acid solution (colour lost within 2 days), but showed fair stability towards 10% glucose and 10% lactose solutions. Towards 1% sodium sulphite solution the stability was fairly poor (became fluorescent within 3 days), towards 5% sodium nitrite solution storage

for 30 days gave loss of colour, but towards 0.5% sodium bicarbonate solution stability was a little better (20% remnant after 30 days). Loss of colour within 3 or 4 days respectively showed relatively good stability toward 0.5% citric acid solution containing tin or iron. Similar good stability was shown in 0.5% tartaric acid solution (lost colour in 4 days with tin; 5 days with iron). Exceptional stability was shown towards 5% potassium nitrate solution (12 days for colour loss with tin; 13 days with iron).

Peacock[21] gives the following stability ratings: very good towards 10% soldium hydroxide solution; good in 30% sodium hydroxide at 98°C (turns blue but at 25° precipitates); fair towards oxidizing agents; very poor towards reducing agents.

Knowles et.al.[24] incorporated erythrosine (as a 0.05% aqueous solution) into a pork luncheon meat at a level of 50 ppm. One batch also comprised 100 ppm sodium nitrite, while the other had no nitrite present. Erythrosine, in the sample without nitrite, seemed to be unaffected by processing (very heat stable). However, in the presence of nitrite, a new pinky/orange subsidiary was formed in about equivalent amounts (spectrophotometric analysis) to the remnant erythrosine.

The effects of processing and subsequent storage on erythrosine in foods were investigated by the Trace Materials (Colours) Committee set up by the British Food Manufacturing Industries Research Association.[42] They did not report colourless breakdown products or partial fading (because this happened to all the colours). The erythrosine used had at least 6 subsidiaries present. Processing in toffees gave no changes. For boiled sweets, fondants and fish paste, though no change occurred during processing, some subsidiaries were lost after seven months in the first two cases, and a brown colour resulted after an equivalent period in the last case. A

yellowish colour was formed in fresh sausages, and red and orange colours in madeira cake (the original subsidiaries were possibly intensified in this case).

Finally, Mukherjee et.al. looked at the changes occurring with baking and subsequent storage. Here, a pronounced loss of colour took place during baking - percentage recovery fell from 99.8% before baking to 46% after baking, falling further to 32% after 5 months' storage, but maintaining this level thereafter for at least 6 more months.

Green S

Green S is commonly used in colouring canned vegetables (peas). Raven[7] reports that the colour is stable over long periods under these conditions.

Nursten and Williams[18] heated green S (0.005%) in aqueous solution at pH 7 and pH 3 ($^{M}/150$ phosphate buffer) at 121°C for 20 minutes. The absorbance at the 635 nm absorption maximum fell by 14% at pH 7, but by only 2.3% at pH 3 during the processing. This suggests that green S is not especially heat stable at neutral pH. When the tests were repeated with 1g/l ascorbic acid present, the 635 nm absorbance fell by only 2.7% at pH 7 and by 4.5% at pH 3, thereby actually showing greater stability at pH 7 with ascorbic acid than without it. Heating at pH 7 increased the absorption at low wavelengths, e.g. from 0.510 to 0.785 at 380 nm.

The Trace Materials (Colours) Committee set up by the British Food Manufacturing Industries Research Association[42] investigated the effect of processing and subsequent storage on green S incorporated into various foodstuffs. The original green S sample contained two green subsidiaries. No changes took place with boiled sweets, fresh sausages, fondants, toffees or fish paste, but a yellow colour was produced in madeira cake.

Indigo Carmine

Jouffrey[23] reports that the stability of indigo carmine to natural light is poor, both in aqueous solution and on cellulosic support. Peacock[21] found the lightfastness to be very poor, as did Spiess[141]. When incorporated in tablet form, however, indigo carmine proved fairly stable according to Cooper.[64] Also, Goodhart et.al.[20] showed the stability to be good when in lake form (0.013 - 0.039% indigo carmine).

Evenson has shown[57] that the photostability of indigo carmine may be greatly decreased in the presence of erythrosine, so the stability of colour mixtures may sometimes not be predictable from a knowledge of the component colour stabilities.

Helou[36] tested indigo carmine in the formulations described at the start of this appendix. Refrigerated storage stability was poor in aqueous solution (pH 2), in simple syrup (pH 2-8), in modified syrup (pH 2, pH 7-8) and in simple elixir (pH 2); it was average in aqueous solution (pH 4-8), in modified syrup (pH 4) and in simple elixir (pH 4-8); and it was good in modified syrup (pH 6). At ambient temperature, the dark storage stability proved to be poor in aqueous solution (pH 2) in simple syrups (pH 2-8), in modified syrup (pH 2-4, pH 7-8) and in simple elixir (pH 2, pH 7); it proved average in aqueous solution (pH 4-8), and in simple elixir (pH 4-6, pH 8); and it proved good in modified syrup (pH 6). In the presence of diffuse light at ambient temperature, the stability proved poor in all cases (aqueous solution, simple and modified syrup and simple elixir - all from pH 2-8), except that it was average in simple elixir at pH 8. These findings clearly illustrate the general susceptibility of indigo carmine to light and to more acid conditions in the various formulations.

Brownley and Lachman [61] studied indigo carmine in the presence of D-glucose, lactose and galactose when exposed to light. The sugars

proved to have a pronounced deleterious effect on the stability of this colour, the monosaccharides showing more effect than did the non-hydrolysed disaccharide. There were erratic changes during testing which prevented kinetic interpretation.

Jones et.al.[59] found that the main photodegradation product was isatinsulphonic acid (2,3-dioxoindolinesulphonic acid) and that faded solutions gave off a bluish fluorescence characteristic of the sulphonated anthranilic acids. Therefore, a two stage process seems to occur, initial oxidation to the isatinsulphonic acid followed by conversion to sulphonated anthranilic acid.

According to Crosby et.al.[110] photodegradation in the presence of oxygen gives rise to 2,3-dioxo-5-indolinesulphonic acid, sodium salt, as indicated by infrared spectroscopy.

Jouffrey[23] states that indigo carmine is unstable towards oxidizing and reducing agents; shows little compatibility with citric acid or with 10% sucrose solution; is incompatible with ascorbic acid, 10% glucose solution and saturated bicarbonate solution; and fades in gelatin preparations. However, Zuckerman[143] notes that the structure of indigo carmine makes it the most stable of all F, D & C certified colours towards reductants.

Peacock[21] describes fastness towards oxidizing agents as poor; towards reducing agents as moderate; towards 10% and glacial acetic acid as very good; toward 10% and 30% hydrochloric acid as good and moderate respectively; towards 10% sodium hydroxide solution as moderate, but towards 30% as very poor (yellows in hue); and towards 5% ferrous sulphate solution as moderate. The stability towards acids is somewhat contrary to Helou's findings[36] reported above.

Banerjee et.al.[49] showed indigo carmine to be moderately stable towards ascorbic acid at ambient temperature (4 ppm colour took 4 days to be destroyed in 5% ascorbic acid solution). Towards 10% lactose stability was similar, and in 10% glucose solution all the colour was destroyed within 16 days. Stability was very low towards 1% sodium sulphite and 0.5% sodium bisulphite solutions, complete loss of colour within one day taking place. Loss of colour in 0.5% citric acid or 0.5% tartaric acid solution containing tin within 3 and 2 days respectively shows moderate stability - greater than that of the azo colours tested. Replacement of tin with iron gave the same result. In 5% potassium nitrate solution, loss of colour took place within 2 days each for solutions containing tin or iron, showing comparability with various azo colours, e.g. amaranth.

Luck[29] investigated the heat stability and chemical compatibilities at pH 3 and pH 7 of indigo carmine (0.01% in $^{M}/_{150}$ phosphate buffer). At pH 7: sterilizing (120° for 30 minutes) gave no transformation products; addition of 1 mg/ml sodium sulphite followed by sterilizing completely changed the solution giving seven degradation products (tlc); storage for 3 days (instead of sterilizing) gave six products and a completely changed solution. With 0.5 mg/ml ascorbic acid, boiling for 10 minutes gave two products, while storage similarly gave two products. In a solution containing 0.6 mg/ml hydrogen peroxide, storage gave one product , while sterilizing changed the solution giving three products. At pH 3: sterilizing in buffer alone gave no new products. With 1 mg/ml sodium sulphite, storage completely changed the indigo carmine giving rise to two products, while sterilizing also completely altered the solutio and gave 3 products. With 0.5 mg/ml ascorbic acid, four products arose upon boiling for 10 minutes, while two products were formed after storage. For the solutions containing 0.6 mg/ml hydrogen peroxide, storage produced no

new species, though sterilizing completely altered the solution giving three detected products.

Nursten and Williams[18] simulated an extreme food processing method by heating indigo carmine in aqueous solution ($^{M}/150$ phosphate buffer) at pH 3 and pH 7 at 121°C for 20 minutes. The 610 nm absorbance decreased by 10.5% at pH 7 and by 5.5% at pH 3. When the process was repeated with the inclusion of 1 g/l ascorbic acid, the absorbance decreased by 5.4% at pH 7 and by 6.4% at pH 3, showing good tolerance of the ascorbic acid.

Changes taking place in indigo carmine during proessing and subsequent storage of foodstuffs in which it was incorporated were studied by the Trace Materials (Colours) Committee of the British Food Manufacturing Industries Research Association.[42] The original sample of indigo carmine had four blue subsidiaries. No chemical transformations were detected in the case of toffees, fish paste or fresh sausages, except almost complete fading occurred in the latter two cases. A faint yellow produced in fondants was lost after 7 months' storage.

Similarly, Mukherjee et.al.[30] observed the changes taking place during baking and subsequent storage. A large amount of colour was lost by baking, the percentage recovery falling from 99.8% to 63%. On subsequent storage, the colour completely decomposed.

Crosby et.al.[50 110] studied the effects of heat and alkali upon indigo carmine, both in air and under nitrogen. Using 25 mg/l indigo carmine solutions, recoveries were as follows (for comparison recoveries for repeated test under nitrogen are given in parentheses):-
90% (96%) after 30 minutes at 100°; 87% (90%) after 60 minutes; 65% (86%) after

standing in 10% $^{v}/_{v}$ aqueous ammonia at room temperature for 30 minutes, or 55% (82%) after 60 minutes; 14% (80%) after heating in 10% $^{v}/_{v}$ aqueous ammonia solution at 100° in a water-bath; and 12% (76%) after 60 minutes.

Patent Blue V

Spiess[141] gives the lightfastness of patent blue V as being good, and that it is comparable to that of tartrazine or sunset yellow FCF.

Jouffrey[23] supplies more widespread data. Like Spiess, he gives lightfastness in aqueous solution as being good, but weak when on a cellulosic support. Jouffrey also gives the stability towards heat as good, and that patent blue is compatible with 10% glucose solution, gelatine and 10% sucrose solution. He states patent blue V to be incompatible with citric acid, becoming greenish yellow, and that it changes to a yellow-green in ascorbic acid solution. Generally it yellows in the presence of reducing agents, and is changed by the presence of oxidants.

According to Raven[7], early experiments indicated that patent blue V used to colour canned peas faded upon storage. Later experiments indicated that this was not likely to be the case, and that increased absorption of the colour occurred during storage resulting in loss of colour in the brine.

Ponceau 4R

Lightfastness is aqueous solution reported to be good by Jouffrey[23], and to be very good by Spiess[141]. Using a bactericidal lamp (254-260 nm), Wojcik et.al.[46] showed that ponceau 4R was unaffected after 336 hours irradiation when in the form of the pure substance or in dragée mass, but that decolourisation took place in aqueous

solution.

Van Beek et.al.[45] applied flash-photolysis and rapid-flow techniques to the photoreduction of a range of azo dyes including ponceau 4R in the presence of hydrogen donors, e.g. D,L-mandelic acid. They showed that a multistep process was taking place, including an initial stage forming hydrazyl radicals, followed by disproportionation to give a molecule of original dye and a molecule of hydrazo compound. The latter was highly unstable when irradiated in water, though stable enough to be observable in alcohol, giving rise ultimately to amine cleavage products.

Jouffrey[23] described the chemical compatibilities as follows: weak stability towards oxidants; unstable towards reducing agents; little compatibility with ascorbic acid (decolouration occurs); good compatibility with 10% glucose or sucrose solutions; fading in gelatin preparations. Jouffrey also described the heat stability as good.

Luck[29] investigated the effect of heat with and without the presence of oxidizing or reducing agents. He found that sterilizing 0.01% ponceau 4R (120°, 30 minutes) formed no identifiable products (pH 7, $^{M}/_{150}$ phosphate buffer). When the sterilization was repeated with 1 mg/ml sodium sulphite present, five degradation products (one coloured yellow) were detected. During storage (3 days at 20°) a similar solution containing sodium sulphite was also completely changed, but only two products (one yellow) were detected. In the presence of 0.5 g/l ascorbic acid neither boiling for 10 minutes, nor storage gave changes. With 0.6 g/l hydrogen peroxide however, sterilizing gave two products (one yellow), while storage gave no changes.

Luck repeated these trials at pH 3. Boiling for 30 minutes, gave two products, while sterilizing gave a single product. Sterilizing

or storage with sodium sulphite present completely altered the solution giving two products (one yellow). With ascorbic acid, four products were formed with either boiling for 10 minutes or with storage. In the presence of hydrogen peroxide, sterilizing gave two products while storage gave none.

Nursten and Williams[18] heated ponceau 4R (0.005%) buffered at pH 3 or pH 7 ($^{M}/150$ phosphate buffer) for 20 minutes at 121°C in two trials, one in the presence and one in the absence of 1 g/l ascorbic acid. Without ascorbic acid at pH 7 a new peak appeared at 390 nm (which conflicts with Luck's findings). Otherwise the 508 nm absorbance fell as follows: pH 7, no ascorbic - 54% loss; pH 7, ascorbic - no loss; pH 3, no ascorbic - 4.6% loss; pH 3, ascorbic - 8.2% loss.

Banerjee et.al.[49] showed that ponceau 4R (4 ppm) was fairly unstable towards 5% ascorbic acid (colour loss within 3 days) but quite good towards 10% glucose and lactose solutions. Fastness towards sodium bisulphite and sodium nitrite proved to be not very good (loss of colour with sodium nitrite in 4 days, change to yellow within 3 days with sodium bisulphite), and very poor towards sodium sulphite (yellows within 0.2 days). Loss of colour within 2 days each showed average stability towards 0.5% citric or tartaric acids containing tin or iron, but comparatively low stability towards 5% potassium nitrate solution containing tin (loss within 2 days) or iron (3 days).

Knowles et.al.[24] incorporated 50 ppm ponceau 4R (as a 0.05% aqueous solution) into a pork luncheon meat mix. In one batch 100 ppm sodium nitrite was included. Without nitrite, processing destroyed the 505 nm absorption maximum, producing a weak absorption at 419 nm (yellow-brown). In the presence of nitrite the 505 nm absorption was lost with no new absorption band in the visible being formed.

The First Report of the Trace Materials (Colours) Committee set up by the British Food Manufacturing Industries Research Association[42] outlined changes occurring in ponceau 4R when processed into various foodstuffs. The original colour used was found to contain 0.9% orange-red and yellow subsidiaries. Partial fading was not recorded as it almost always occurred to some extent. No other change occurred in processing to produce boiled sweets, madeira cake or fresh sausages. With toffees, an increase in the subsidiaries was observed. With fish paste, the greatest changes were apparent: the yellow subsidiary greatly increased and a pink was formed; after 14 months storage the yellow had become as intense as the main colour.

By comparison, Dickinson and Raven[62] have noted a yellow colour present in strawberry marshmallows containing ponceau 4R as the colouring agent.

Raven[7] noted that in canned rhubarb (lacquered cans; usual pH is 3.0-3.5) coloured with mixtures of erythrosine and ponceau 4R (the latter colours the syrup), ponceau 4R was still detectable after several months' storage, but not after 2 years. Raven speculated that electrochemical reduction was occurring to give the amino cleavage products, but he produced no evidence, remarking, however, that naphthionic acid (one of the expected amine products) had been identified in fish paste coloured with ponceau 4R. The non-detection of the other amine formed by cleavage, viz., 8-amino-7-hydroxy-1,3-naphthionic acid, disodium salt, was conjectured to be due to instability resulting in the formation of a quinone type substance as the final product. Initially up to 90% of the colour added to the fish paste was lost upon heat treatment being carried out, but if more colour was then added and the processing repeated, little if any further loss occurred. One possibility seemed to be

that volatile sulphur compounds were liberated on the first heating only, and that these were responsible for the colour loss. (A similar explanation was offered by Knowles et. al. [24] for the protective action of sodium nitrite by preferential reaction with those sulphur compounds - though this mechanism does not seem to work well for ponceau 4R). An alternative explanation is that non-volatile reducing agents in raw fish-paste might be responsible.

Sulphur-bearing compound (e.g. hydrogen sulphide) have been known to reduce ponceau 4R to a yellow colour. Meat and potato pie coloured with ponceau 4R turned green owing to loss of the ponceau 4R, and a brown soup powder which was boiled with cabbage and water behaved likewise.[7]

Quinoline Yellow

Jouffrey[23] found that fastness to natural light was good in aqueous solution, and average on a cellulosic support. Spiess[141] also gave the lightfastness as good, whereas Peacock[21] described it as only moderate. Goodhart[20] found that quinoline yellow incorporated into pharmaceutical tablets (at 0.30% concentration) had relatively good light stability.

Chemical compatibilities have been investigated by several workers. Peacock[21] has given the following fastness ratings: towards 10% and glacial acetic acid, good; towards 10% and 30% hydrochloric acid, good; towards 10% sodium hydroxide solution, moderate (turns redder in hue); towards 30% sodium hydroxide solution, fair (turns redder and precipitates); towards 5% alum solution, moderate; towards oxidants poor; towards reducing agents, good.

Jouffrey[23] found that in oxidising media, slight modification

of the colour occurred; in reducing media, quinoline yellow was stable. There was compatibility with citric acid, with 10% glucose and sucrose solutions and with ascorbic acid solutions of concentration less or equal to 2 g/l. He also noted reddening above pH 9.

Luck[29] investigated quinoline yellow (0.01% solution) for heat stability (sterilizing, i.e. heating at 120° for 30 minutes) and for storage stability (3 days at 20°). The solutions were buffered at pH 7 or at pH 3 with $^{M}/_{150}$ phosphate buffer. Solutions were also tested that contained 1 g/l sodium sulphite, 0.5 g/l ascorbic acid or 0.6 g/l hydrogen peroxide. At pH 7: sterilizing the simple buffered solution brought about no changes; sterilizing or storage of the sulphite-containing solution likewise formed no products; boiling for 10 minutes produced no changes in the ascorbic acid-containing solution, while storage gave one product; sterilizing the hydrogen peroxide solution gave two products, storage gave none. At pH 3: sterilizing the simple buffer solution gave no products; sterilizing or storage of the sodium sulphite solution likewise formed no products; boiling the ascorbic acid solution for 10 minutes gave four products, while storage gave three products; sterilizing the hydrogen peroxide solution completely altered the original colour and gave three products, while storage produced no changes.

Red 2G

Porter[14] found that red 2G showed good stability when exposed in an Atlas 'Fade-Ometer' apparatus (carbon arc source). An approximately linear rate of colour loss occurred over a 200 hour period, reducing a solution of ca. 1.15×10^{-3} moles/litre concentration to 72% remnant.

Van Beek et.al.[45] have investigated the rate and mechanism of the photoreduction of red 2G and other colours in the presence of hydrogen donors, e.g., D,L-mandelic acid, using flash-photolysis and rapid flow techniques. They believed that the observed reactions were readily explicable in terms of the scheme,

$$X_1\text{-}N{=}N\text{-}X_2 + H^{\cdot} \xrightarrow{k_1} X_1\text{-}\overset{H}{N}\text{-}\dot{N}\text{-}X_2$$

then,

$$2\ X_1\text{-}\overset{H}{N}\text{-}\dot{N}\text{-}X_2 \xrightarrow{k_2} X_1\text{-}\overset{H}{N}\text{-}\overset{H}{N}\text{-}X_2 + X_1\text{-}N{=}N\text{-}X_2.$$

The hydrazo compounds can then also disproportionate to give amines and azo compounds. The $H^{\cdot}$ radical is produced by photodissociation of the D,L-mandelic acid. For red 2G, k_1 was measured as being 2×10^9, and k_2 as 1×10^9 (order of magnitude accuracy only - reproducibility 50% or less).

Nursten and Williams[18] looked at the heat stability of red 2G, and the effect of ascorbic acid on heat stability. Using pH 3 and pH 7 buffered ($^M/_{150}$ phosphate) aqueous solutions containing 0.005% red 2G (and 1 g/l ascorbic acid where used), they heated each solution over a 6 minute period to bring the excess pressure to 15lb. (equivalent to 121°C) and maintained this for 20 minutes. At pH 7 with no ascorbic acid, no absorbance decrease occurred in the 532/4 nm maximum, but a 1.75% loss took place at 506 nm. Repeated with ascorbic acid present, the 506 nm fall was 5.25%. Similarly, at pH 3 with no ascorbic acid, no loss took place at 532/4 nm, while at 506 nm the fall was 1.7%. With ascorbic acid present, this drop grew to 9%. Overall this showed both good heat stability and compatibility with ascorbic acid.

Knowles et.al.[24] introduced 50 ppm red 2G (as a 0.05% aqueous solution) into a pork luncheon meat. In one test, 100 ppm sodium nitrite was also incorporated. In the absence of nitrite, most of the red 2G was

destroyed, and a yellow-brown subsidiary was produced. In the presence of nitrite, a pink-red colour was formed, together with a trace of another subsidiary.

A more widespread study of changes occurring during processing has been carried out by the Trace Materials (Colours) Committee set up by the British Food Manufacturing Industries Research Association.[42] They limited their report to the formation of new colours during processing and subsequent storage. The original red 2G used was found to contain 0.2% of a pink subsidiary. No changes occurred for red 2G in boiled sweets, madeira cake, fresh sausages, or toffees during processing, though, in the case of toffees, subsequent storage for 9 months produced a trace of pink. For red 2G in fish paste, processing destroyed almost all the colour, forming several yellows. After 13 months storage a mauve and traces of pink also resulted.

Sunset Yellow FCF

Peacock[21] reported that sunset yellow FCF had fair light stability, while Spiess[141] rated it as good. Jouffrey[23] also considered that fastness to natural light was good in aqueous solution, but weak when on a cellulosic support. When incorporated (0.06 - 0.10%) as a lake into pharmaceutical tablets, fastness to fluorescent light proved poor according to Goodhart et.al.[20]

Helou[36] obtained comprehensive data for light and storage stability at room temperature, and for storage stability while refrigerated. The formulations are set out at the start of this appendix. Refrigerated storage stability was good in aqueous solution (pH 2-8), in simple syrup (pH 2, pH 6-8), and in modified syrup (pH 2-8), and was average in simple syrup (pH 4) and in simple elixir (pH 2-8). Dark storage stability at ambient temperature was good in aqueous solution (pH 2-8), in simple syrup (pH 2, pH 6-8) and in modified syrup (pH 2-7); and was

average in simple syrup (pH 4), in modified syrup (pH 8) and in simple elixir (pH 2-8). Lightfastness at ambient temperature was good in aqueous solution (pH 2-7); it was average in aqueous solution (pH 8), in simple syrup (pH 2-4), in modified syrup (pH 6) and in simple elixir (pH 2-8); and it was poor in simple syrup (pH 6-8), and in modified syrup (pH 2-4, pH 7-8).

Nursten and Williams [18] tested the heat stability of sunset yellow FCF in aqueous solution, and also the influence of ascorbic acid (1 g/l) on this stability. Solutions containing 0.005% of the colour were heated (6 minutes) to an excess pressure of 15 psi over atmospheric pressure, which was equivalent to a temperature of 121°C, then maintained at this temperature for 20 minutes. No loss in absorbance occurred at 482 nm for the pH 7 solutions, either with or without ascorbic acid present, while at pH 3, the solution with no ascorbic acid showed no loss, but that with ascorbic acid present showed 6.8% loss. This indicates that sunset yellow FCF is especially heat stable.

Luck [29] studied the heat and storage stabilities and chemical compatibilities of sunset yellow FCF (0.01%) in aqueous solution at pH 3 and pH 7 ($^{M}/_{150}$ phosphate buffer). He identified products formed by tlc. At pH 7: sterilization (i.e. heating at 120° for 30 minutes) gave no transformation products in simple buffered solution; in the presence of 1 g/l sodium sulphite however, sterilization gave 3 products, one of which was coloured yellow, while storage (i.e. 3 days at 20°) gave 2 products, one of which was yellow; in the presence of 0.5 g/l ascorbic acid, boiling for 10 minutes formed one product, while storage formed 2 products; in the presence of 0.6 g/l hydrogen peroxide, sterilization gave two products, while storage gave none. At pH 3: sterilization of the simple buffered solution gave no degradation products; sterilization of the sodium sulphite-containing solution however, completely altered

the sunset yellow FCF, forming 3 products, one being yellow, while storage also completely changed the solution forming one yellow product; boiling the ascorbic acid-containing solution for 10 minutes resulted in 4 new substances, whereas storage (3 days at room temperature) gave one new substance; finally, sterilization of the hydrogen peroxide-containing solution completely transformed the colour, giving 3 products, whereas storage did not give any product.

Jouffrey[23] described sunset yellow FCF as having the following stability or compatibility ratings: towards oxidants, stability very slight; towards reducing agents, unstable; compatible with citric acid, with 10% glucose of 10% sucrose solution, and with saturated bicarbonate solution; fades in the presence of gelatine; and colour changed by strong concentrations of ascorbic acid.

Peacock[21] also lists various chemical compatibilities. Fastness to 10% or glacial acetic acid is good; fastness to 10% or 30% hydrochloric acid is good; fastness towards 10% sodium hydroxide solution is good, but in 30% sodium hydroxide solution stability is only fair, and the hue becomes darker and reddens; fastness to 5% ferrous sulphate solution and to 5% alum solution is moderate; stability towards oxidants is fair, but towards reducing agents is very poor.

Banerjee et.al.[49] compared the chemical compatibilities of a range of colours by noting the number of days taken to destroy all the colour (40 µg) or the remnant after a month in 10 ml. aqueous solution was fair (3 days for colour loss); toward 10% glucose or lactose solutions was good (27 and 31 µg remnant after 32 days respectively); towards 1% sodium sulphite solution was poor (0.2 days); towards 0.5% sodium bisulphite was moderate (5 µg remnant after 30 days); towards 5% sodium nitrite was moderate (30 days); towards 0.5% citric or tartaric acid solutions containing iron or tin, poor (1 day in each case);

towards 5% potassium nitrate solution containing tin or iron, poor (2 days in each case). The low stabiliity towards chemical or electrochemical reduction is obvious (cf. Peacock and Jouffrey above).

Drozdova et.al.[33] studied the effect on 10^{-3} mole/l sunset yellow FCF of exposure to solutions containing 2.5% citric, malic or tartaric acids, together with steel-3 or aluminium. After 24 hours, the degradation products were found to include sulphanilic acid (showing reductive cleavage). In addition, the known carcinogen (sic) 1-amino-2-hydroxy-6-naphthalenesulphonic acid was found on the metal surfaces.

Eisenbrand and Lang[56] showed that this same reductive cleavage product of suset yellow FCF, 1-amino-2-hydroxy-6-naphthalenesulphonic acid, undergoes air oxidation giving product(s) characterised by their inhibitory effect on the action of lactic acid-fermenting bacteria.

Raven[7] noted that the use of sunset yellow FCF in canned butter beans (to mask a greying which may afflict the beans on canning) had a useful life of around 6 months only, and that the storage instability of this colour was well known throughout the food industry. No theory or mechanism regarding the loss was given.

Changes taking place on processing or during subsequent storage were investigated in the First Report of the Trace Materials (Colours) Committee set up by the British Food Manufacturing Industries Research Association.[42] The committee only reported new coloured products formed as detected using paper chromatography - not partial loss of colour or non-coloured products. The original sunset yellow FCF sample was found to contain three subsidiaries, 0.42%, 0.32% and 0.42%. No changes were noticed on processing in boiled sweets, toffees, fish paste, madeira

cake, or fresh sausages. This seems, in the light of the above limitations to the technique not necessarily to indicate any particular stability of this colour.

Another study on similar lines was conducted by Mukherjee et.al.[30] They incorporated the colour into a cake mix, and measured colour loss during baking and subsequent storage. A 6% loss on baking, and a subsequent further loss to 9% total loss after 11 months storage, showed good stability.

Tartrazine

Peacock[21], Spiess[141] and Jouffrey[23] concur that the photostability of tartrazine in aqueous solution is good. Goodhart[20] found that the photostability of tartrazine incorporated (0.028 - 0.084%) into a lake and used in a tablet formulation was good when exposed to fluorescent white light.

Helou[36] used the formulations set out at the start of this appendix in order to test the light and storage stability of tartrazine under a range of conditions. Stability was good when refrigerated (pH 2-8) in aqueous solution, simple syrup or modified syrup, and was average when in simple elixir. Dark storage at ambient temperature resulted in the following stability ratings: good stability in aqueous solution, simple syrup and modified syrup (each pH 2-8); and average stability in simple elixir (pH 2-8). Photostability at ambient temperature when exposed to diffuse light was good in aqueous solution (pH 4-8), in simple syrup (pH 7) and in simple elixir (pH 8); it was average in aqueous solution (pH 2), simple syrup (pH 2, pH 6) and in simple elixir (pH 4-7); and it was poor in simple syrup (pH 4, pH 8), modified surup (pH 2-8) and in simple elixir (pH 2).

Luck[29] investigated the heat stability of tartrazine in aqueous

solution, and the effects of oxidizing and reducing agents. He subjected 0.01% tartrazine in $^{M}/_{150}$ phosphate buffer (pH 7 and pH 3) to sterilization (heating at 120° for 30 minutes) but found no products formed (tlc). At pH 7: addition of 1 g/l sodium sulphite gave two products upon sterilization, and one product on storage (3 days at 20°); addition of ascorbic acid (0.5 g/l) gave no new products upon boiling (10 minutes) or storage; addition of hydrogen peroxide formed no new products on storage or sterilization. At pH 3: sterilization of the simple solution gave no changes; sterilization or storage of sodium sulphite-containing solutions likewise gave no new products; however, boiling of the ascorbic acid-containing solution for 10 minutes gave 3 products (one coloured orange), these products corresponding to dye components already present, while storage gave a single, orange coloured product; sterilization of the hydrogen peroxide-containing solution gave 3 products, one being yellow, while storage gave no changes.

Nursten and Williams[18] likewise looked at heat stability and the effect of ascorbic acid. They buffered 0.005% tartrazine at pH 7 or pH 3 ($^{M}/_{150}$ phosphate) and heated over a 6 minute period to create an excess pressure of 15 psi over atmospheric (equivalent to 121°C) then held this for 20 minutes. The cycle was repeated at pH 3 and at pH 7 with and without 1 g/l ascorbic acid present. At pH 7 (no ascorbic) a loss in absorbance at 428 nm of 3.6% was recorded, while no loss occurred with ascorbic acid present! At pH 3 no loss occurred with or without ascorbic acid present, showing tartrazine to be among the most stable of the colours tested.

Jouffrey[23] noted that tartrazine was compatible with citric acid, 10% glucose, 10% sucrose and saturated bicarbonate solutions and with gelatine, but incompatible with ascorbic acid; and that decolouration occurred with powerful reducing agents, while with oxidants, stability was

doubtful.

Peacock[21] also studied chemical compatibilities. He found that fastness to 10% and glacial acetic acid was good; to 10% and 30% hydrochloric acid was good; to 10% sodium hydroxide was good, but to a 30% solution was only fair, with the tartrazine reddening in hue. In 5% ferrous sulphate, the hue darkened. In 5% alum solution, tartrazine showed moderate stability. Fastness towards oxidants was fair, but towards reducing agents was very poor.

Banerjee et.al.[49] investigated the effect of various preservatives on the stabilities of a range of food colours. Using 40 µg of tartrazine in 10 ml of solution, they quoted for particular solutions (i) the number of days required for total loss to occur, or (ii) the remnant (µg) of tartrazine out of the initial 40 µg after 30 or 32 days. Stability towards 5% ascorbic acid (4 days) was the best among the azo-colours tested. Stability towards 10% glucose (15 µg remnant at 32 days) and towards 10% lactose (20 µg remnant at 32 days) was not quite as good as other azo-colours, e.g. amaranth. Stability was excellent (no loss over one month) towards 0.5% sodium bisulphite and towards 5% sodium nitrite solutions, while stability towards sodium sulphite (3 days) was fairly average for an azo-colour. Fastness in 0.5% ctiric acid containing tin or iron was moderate (2 days), in 0.5% tartaric acid containing tin or iron was poor (1 day), and in 5% potassium nitrate solution containing these metals was comparatively poor (2 days).

Raven[7] reported that tartrazine, used as a component in a mixture to colour canned fresh peas, completely degraded so that, after 13 months storage at ambient temperature, none was detectable. This led to his trying to find a substitute colouring matter.

Also, for canned peas, a rare set of circumstances was described by Hall and Raven,[63] under which yellow 2G and (less readily) tartrazine could turn red. The reaction was believed to be a property of the azo-pyrazolone system which is common to both colours. Bacterial reduction was instrumental in this reaction (for details see the section below on yellow 2G).

Changes taking place during processing or subsequent storage were considered by the Trace Materials (Colours) Committee set up by the British Food Manufacturing Industries Research Association.[42] The original tartrazine was found to contain no subsidiaries. No new colours resulted when processed in boiled sweets, toffees, fish paste, madeira cake or fresh sausages. However, in the case of fish paste alone, subsequent storage did result in traces of mauve and brown after 15 months.

Yellow 2G

The photoreduction of various azo dyes in the presence of hydrogen donors, e.g. D,L-madelic acid or acetone, was studied by van Beek.[45] Light dissociates a hydrogen radical from the donor molecule. The ensuing reaction may be represented thus:

(i) $H^{\cdot} + X_1\text{-N=N-}X_2 \xrightarrow{k_1} X_1\text{-}\overset{H}{N}\text{-}\overset{\cdot}{N}\text{-}X_2$ (k is the rate constant).

(ii) $2\ X_1\text{-}\overset{H}{N}\text{-}\overset{\cdot}{N}\text{-}X_2 \xrightarrow{k_2} X_1\text{-}\overset{H\ H}{N}\text{-}N\text{-}X_2 + X_1\text{-N=N-}X_2$

(iii) $2\ X_1\text{-}\overset{H\ H}{N}\text{-}N\text{-}X_2 \longrightarrow X_1\text{-N=N-}X_2 + X_1\text{-NH}_2 + H_2N\text{-}X_2$

For yellow 2G k_1 is ca. 3×10^8, while k_2 is ca. 1×10^8. Van Beek employed flash photolysis and rapid flow techniques in deriving these results.

Nursten and Williams [18] studied the stability of yellow 2G to heat and the effect of ascorbic acid upon this stability. Yellow 2G

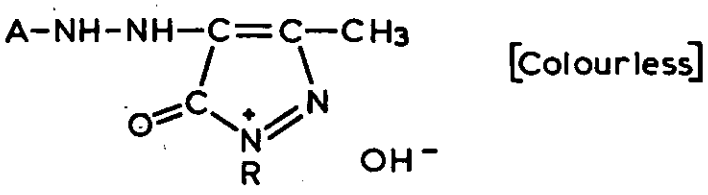
(0.005%) was buffered at pH 3 and pH 7 ($^{M}/150$ phosphate buffer); 1 g/l ascorbic acid was used. The solutions were heated for 6 minutes creating an excess 15 psi over atmospheric pressure (equivalent to 121°C) then held at this for 20 minutes. At pH 7 (no ascorbic) the absorbance maximum at 404 nm shifted to 398 nm, and a 9.8% fall in absorbance was noted at the peak maximum. When this was repeated with ascorbic acid present, no shift occurred in the peak position and no loss of colour took place. At pH 3, both with and without ascorbic acid, no loss or shift occurred. Overall, therefore, yellow 2G showed very good heat stability and ascorbic acid compatibility.

Changes in yellow 2G accompanying processing and storage of foodstuffs were looked at by the Trace Materials (Colours) Committee set up by the British Food Manufacturing Industries Research Association.[42] Partial fading and colourless products were not reported. The original yellow 2G sample was found to contain two yellow subsidiaries. No changes occurred on processing in boiled sweets or fresh sausages. Traces of new colour were found in fondants, and a trace of red was found in madeira cake, while in fish paste, the original subsidiaries were lost.

Finally, yellow 2G used in canned peas was found by Hall and Raven[63] to undergo decolouration, followed by reddening upon exposure to air. Highly specific conditions were required, viz., (a) yellow 2G must be present in the brine, (b) infection with a specific microorganism (tentatively identified as B.michaelisii) must occur, (c) storage must be under suitable conditions to allow incubation, (d) the can contents must then be exposed to air, and (e) the can contents must be ca. pH 6.0. The rarity of these circumstances arising does not detract from the value of this paper, especially as the decolourisation was shown as capable of being brought about by other reducing agents (zinc dust). The mechanism was suggested to be as follows:

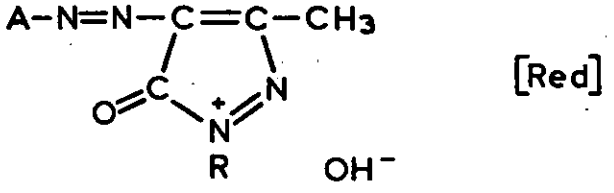
Bacterial action

Quaternary base formation and H-addition



[Colourless]

Atmospheric oxidation



[Red]

[A is p-$SO_3Na \cdot C_6H_4$-]

APPENDIX B: THE PHYSICAL MEANING OF STANDARD DEVIATION IN THE GAUSSIAN DISTRIBUTION AND ITS RELATION TO PEAK MEASUREMENT

Figure 12 shows the optimal shape for a chromatographic peak, which follows the line of the Gaussian distribution curve. The values of p(x) and x may be given in any units, but those of x are most conveniently given in units of the standard deviation, σ, of the distribution, as this makes greatest use of the symmetry of the curve. The physical significance of σ is easily demonstrated:

$$p(x) = \exp\left\{-\frac{x^2}{2\sigma^2}\right\} \qquad \text{(Gaussian distribution)}$$

$$p'(x) = -\frac{x}{\sigma^2} \exp\left\{-\frac{x^2}{2\sigma^2}\right\}$$

At the points of inflection, $p''(x) = 0$

$$p''(x) = \left\{-\frac{1}{\sigma^2} + \frac{x^2}{\sigma^4}\right\} \exp\left\{-\frac{x^2}{2\sigma^2}\right\} = 0$$

or, $\frac{1}{\sigma^2} = \frac{x^2}{\sigma^4}$ and so $x = \pm\sigma$

Therefore, it is seen that the standard deviation, $\pm\sigma$, is the x-coordinate of the inflection points on the curve.

The tangents to the curve at the points of inflection are now found by substitution of $x = \pm\sigma$ in $p'(x)$:

$$p'(x) = \pm\frac{1}{\sigma} \exp\left\{-\frac{1}{2}\right\}$$

Let the equation to the tangents be

$$y = \pm\, p'(x).\, x + c$$

Now, at $x = \pm\sigma$, $y = p(x) = \exp\left\{-\frac{\sigma^2}{2\sigma^2}\right\} = \exp\{-0.5\}$

From this the constant, C, may be evaluated:

$$C = \exp\{-0.5\} - \left(\mp\frac{1}{\sigma} \exp\{-0.5\}\right).(\pm\sigma)$$

$$C = 2 \exp\{-0.5\}$$

To find where the tangents to the curve at the inflection points cut the

x-axis we substitute y = 0.

$$x = \frac{-C}{\pm M} = \mp \frac{2 \exp\{-0.5\}}{1/\sigma \cdot \exp\{-0.5\}}$$

and so $x = \mp 2\sigma$.

From Figure 12 it may be confirmed that the tangents to the curve at the inflection points, $x = \pm\sigma$, cut the x-axis 4σ apart at $x = \mp 2\sigma$. It is for this reason that in assessing chromatographic peaks, tangents are dropped to the baseline and peak width taken as the difference in retention time (or volume) between these points.

APPENDIX C: FOOD COLOURING MATTER INDEX NUMBERS

C.I. - Colour Index: classification of dyes by number — the British Society of Dyers and Colourists, 3rd Edition (1971).

E.E.C. - European Economic Community: additive number.

F, D & C - Production certified by the U.S. Food and Drug Administration (F, D & A) for use in Foods, Drugs and Cosmetics. D & C is for Drugs and Cosmetics only.

Common U.K. Name	C.I. No.	E.E.C. No.	F, D & C or D & C
Amaranth	16185	E123	F, D & C red 2
Black PN	28440	E151	-
Brilliant Blue FCF	42090	Provisional	F, D & C blue 1
Brown FK	-	Provisional	-
Carmoisine	14720	E122	-
Chocolate Brown HT	20285	Provisional	-
Erythrosine	45430	E127	F, D & C red 3
Green S	44090	E142	-
Indigo Carmine	73015	E132	F, D & C blue 2
Patent Blue V	42051	E131	-
Ponceau 4R	16255	E124	-
Quinoline Yellow	47005	E104	D & C yellow 10
Red 2G	18050	Provisional	-
Sunset Yellow FCF	15985	E110	F, D & C yellow 6
Tartrazine	19140	E102	F, D & C yellow 5
Yellow 2G	18965	Provisional	-

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