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The Determination of Added Azo Dye in Soft Drinks via its Reduction Products

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ABSTRACT

The stability of amaranth (E123), an azo dye widely used in foods, was investigated in soft drinks. The rate of degradation of dye was monitored by spectrophotometry, and degradation products were separated and identified by HPLC. The formation of specific amines was clearly demonstrated and the levels of those amines produced in model systems and soft drinks were determined. These values were used to estimate the amounts of dye from which the amines had been derived. The method was applied to commercial soft drinks to estimate levels of amaranth initially added.

INTRODUCTION

Most methods for the analysis of synthetic colourants in foods have concentrated on the identification of the dyes present, simply to establish whether the material used is permitted or not. The permitted lists vary from country to country; for example, amaranth is permitted in the UK but not in the USA. However, in general, there are no restrictions on the quantities of these dyes that may be used and so there has been little demand for quantitative methods, apart from quality control procedures during production. For qualitative analysis thin-layer chromatography (TLC) is often suitable, providing adequate differentiation within the relatively limited range of dyes likely to be encountered. The technique is also simple and can be used simultaneously for many samples.

There is now a growing movement worldwide to restrict the use of synthetic colourants further, both in terms of the number permitted and, perhaps more importantly, the amounts used. Such legislation will clearly demand quantitative analytical methods for the range of permitted dyes in foods. Furthermore, it would be highly desirable to be able to estimate the initial concentration of colourant added from the residual dye concentration and the amount of specific dye degradation products. Identification and quantification of these degradation products may also be important in discussions of potential toxicity of dyes, as these products may react further with other food components to form undesirable compounds. The stability of azo dyes in food systems depends on a number of parameters, the most important of which is related to the presence of reducing species which result in the cleavage of the azo double bond, ultimately forming primary amines (Nursten & Williams, 1969; Fogg & Summan, 1983). The present EEC regulations limit the amounts of aromatic amines allowed in foods to 0·01% (Wadds, 1984), and the degradation of azo dyes could produce a significant contribution to these levels.

A major source of synthetic dyes in the diet is from soft drinks. These are consumed in large quantities by children, who are one of the most likely groups to be adversely affected. Ascorbic acid (vitamin C) is often added to these drinks as a nutritional supplement and/or as an antioxidant, and this provides a reducing environment in which azo dyes are likely to be degraded, leading to fading.

In the present study the fate of amaranth (E123), an azo dye widely used in soft drinks, was studied under a range of conditions similar to those encountered in such drinks. The dye was also reduced under established conditions to provide authentic degradation products that could be used to identify the same compounds in the model systems and subsequently in soft drinks. The levels of specific degradation products were determined and these values used to estimate the amount of dye from which they had been derived, from correlations established for model systems. Finally, this method was used to estimate the levels of amaranth initially added to commercial soft drinks.

EXPERIMENTAL

Apparatus and reagents

Liquid chromatography:
(a) ACS gradient system (Applied Chromatography Systems Ltd, Luton, Great Britain), model 750/03, with a decilinear programmer (model 750/36).
(b) ACS model 750 pump.

(c) Gradient system (Anachem Ltd, Luton, Great Britain), consisting of two Gilson International pumps (model 302), a manometric dynamic mixer (model 811) and a gradient control with a 702 manager (Apple Europlus e).
Other equipment used included a Rheodyne injection valve (model No. 7125, Rheodyne Inc., Cotati, USA), fitted with a 20 μl loop.
The detectors used were:
(a) Pye Unicam model LC3 variable wavelength detector.
(b) Spectroflow 757 (Kratos Analytical Instruments, Ramsey, Jersey, USA).
(c) Refractive Index detector (ACS, Luton, Great Britain).
Chart recorder: Bryans model BS6000.
Integrator: Hewlett Packard model 3390A.
Spectrophotometers:
(a) Perkin-Elmer Lambda 5 UV/VIS with model Pp-1 Plotter printer.
(b) Perkin-Elmer model 552 with chart recorder model 561.
(c) Infrared Pye Unicam SP200.
Liquid chromatography column:
Stainless steel, 12·5 cm × 4·6 cm inside diameter, packed with 5 μm Spherisorb ODS-2.
For sugars, stainless steel, 25 cm × 4·6 mm inside diameter, packed with 5 μm Spherisorb NH_2.
Mobile phases:
(a) Solvent A: 0·006M tetra-*n*-butylammonium hydroxide (pH 7).
(b) Solvent B: methanol (Rathburn).
For sugars: acetonitrile (Rathburn)/water (75:25, v/v).
Operating conditions:

Chart speed, 1 cm/min.
Eluant flow rate, 1 ml/min.
Detection Pye LC3 0·32 AUFS
Spectroflow 737 0·50 AUFS

Gradient profiles:
(a) Gilson 702, 30–45% B in 10 min, hold at 45% B for 10 min, return to 30% B in next 10 min before next injection. Stop integrating time 40 min and total time 50 min.
(b) ACS 750/36, segment 1: 30–40% B in 8 min, segment 2: 40–45% B in 12 min, segment 3: 45–50% B in 10 min, segment 4: hold at 50% B for 5 min, segment 5: 50–30% B in 5 min. Stop integrating time 40 min.
For sugars:

Flow rate, 2 ml/min.
Chart speed, 1 cm/min.
Detection, refractive index.

Citrate buffer (pH 3) prepared from 21 g/litre citric acid and 8·4 g/litre NaOH, adjusted with HCl.

The chemicals were supplied by Fisons, BDH and Sigma. Naphthionic acid and R-salt were donated by the Ministry of Agriculture, Fisheries and Food (MAFF) and amaranth by Horace Cory.

The commercial samples were purchased from local supermarkets and the fresh samples were kindly donated by producers.

Methods

Soft drink model solutions containing amaranth were made in citrate buffer, preserved with sodium benzoate and analysed for dye degradation in the presence of ascorbic acid during storage. The effects of other components, such as sugar, were investigated and also the effect of removing oxygen by flushing with nitrogen. The rate of dye degradation was measured spectrophotometrically. HPLC was used to study the degradation products of both amaranth and ascorbic acid. Preliminary separation of amaranth and its degradation products was carried out using polyamide column chromatography (Boley *et al.*, 1980) and cellulose column chromatography (Link, 1961*a*), followed by HPLC analysis of the resulting fractions. UV/visible spectra of the resulting fractions were also recorded.

The degradation products were identified by comparison of retention times and spectra with reference compounds. Quantification of the degradation products was carried out by referring to linear calibration plots of the reference compounds. The reference compounds used were naphthionic acid, 1-amino-2-naphthol-3,6-disulphonic acid (amino-R-salt), 2-naphthol-3,6-disulphonic acid (R-salt) and 1,2-naphthoquinone-3,6-disulphonic acid. The first two were donated by MAFF and the last two were prepared in the laboratory (see below). R-salt was purified by the method of Link (1961*b*).

The purity of all the products was checked by HPLC, UV/visible spectrophotometry and infrared examination of KBr disks. All methods for recovery of amaranth and its degradation products were evaluated and demonstrated good precision; for example, for recovery of naphthionic acid at concentration levels of 10–50 ppm, CV values were 12–1·2%, decreasing with increasing concentration, as expected. All the results shown in Tables 1 to 5 are averages of triplicate analyses.

Preparation of sodium 1-amino-2-naphthol-3,6-disulphonate

The amino-R-salt which was used as a reference compound was prepared by the method of Green (1949). Amaranth (10 g) was dissolved in boiling water (200 ml) and decolorised by adding sodium dithionite (3 g). The decolorised solution was quickly filtered while hot and naphthionic acid

precipitated by adding concentrated HCl. The filtrate was treated with saturated sodium chloride solution and amino-R-salt separated on standing. The product was purified by recrystallising from water.

Preparation of 1,2-naphthoquinone-3,6-disulphonic acid
The naphthoquinone was prepared by the method of Singh (1970). The yellow crystals were identified by infrared and UV/visible spectrophotometry.

Analysis of commercial samples
Samples of soft drinks containing amaranth, purchased from local supermarkets or obtained from factories, were analysed for total amaranth content and levels of naphthionic acid. The samples were stored on a bench shelf in indirect sunlight and illuminated with fluorescent lighting (0·6 m from double 6 ft 75–85 w/36 white tube) and were analysed for dye degradation at intervals of one month. The levels of ascorbic acid and sodium benzoate were also monitored. Calibration curves (peak area) were used to calculate the quantity of amines, dye, sodium benzoate and ascorbic acid present in the sample. The controls, which contained dye and buffer or dye, sugar and buffer, were also treated in the same way as the samples.

RESULTS AND DISCUSSION

The effects of ascorbic acid, sugars and exclusion of oxygen on the stability of amaranth in model systems are shown in Tables 1 and 2. Ascorbic acid is known to be able to degrade azo dyes by reduction (Nursten & Williams,

TABLE 1
Effect of Sugars on Stability of Amaranth in the Presence of Ascorbic Acid

Storage period (days)	*Per cent total dye recovered*			
	No sucrose		*Sucrose added*[a]	
	C	*S*	*C*	*S*
0	100	98	97	97
30	99	93	97	65
60	99	89	97	41
90	99	82	96	28
120	98	76	96	17

C—control (dye (100 ppm) + buffer).
S—sample (100 ppm dye + 250 ppm ascorbic acid).
[a] Sucrose added, 50 g/litre.

TABLE 2
Effect of Exclusion of Oxygen on Stability of Amaranth in the Presence of Ascorbic Acid and Sugars

Storage period (days)	*Per cent total dye recovered*					
	No sucrose			*Sucrose added*[a]		
	C	*S1*	*S2*	*C*	*S1*	*S2*
0	100·00	98	99	98	98	99
4	99	95	98	98	90	92
6	6	94	97	98	86	77
14	99	91	93	98	84	57
22	99	90	89	85	82	42

C—control (dye (50 ppm) + buffer).
S1—50 ppm dye with 200 ppm ascorbic acid added.
S2—50 ppm dye with 200 ppm ascorbic acid added and flushed with nitrogen after each reading.
[a] Sucrose added, 50 g/litre.

1969; Fogg & Summan, 1983) and it would appear that the effects observed in Tables 1 and 2 can be rationalised in terms of the amount of ascorbic acid available to degrade the dye. Thus, sugars have been reported as having a protective effect on ascorbic acid in solution, as a result of their ability to chelate metal ions which can otherwise catalyse ascorbic acid oxidation (Birch & Pepper, 1983). In other words, sugars protect ascorbic acid which is then able to reduce the dye. The catalytic effect of trace metal

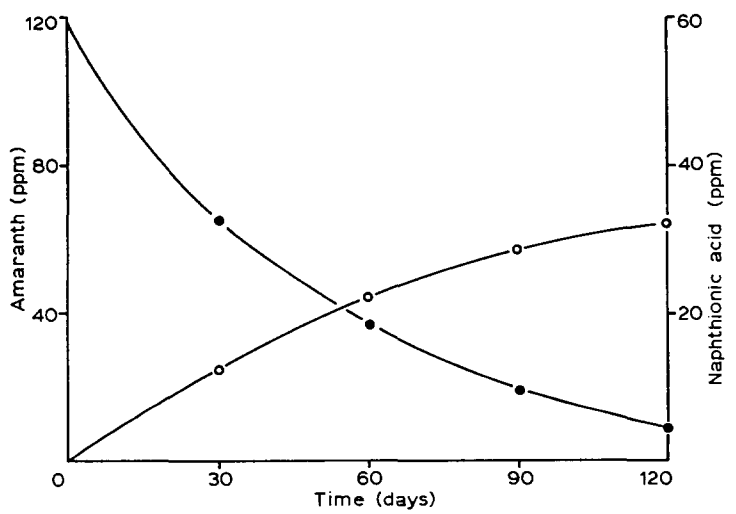


Fig. 1. Comparison of data of degradation of amaranth (●) with formation of naphthionic acid (○) in model solutions.

ions, and the subsequent protective effect of sugars on ascorbic acid, has been confirmed in other model solutions containing added metal ions (data not presented here). Ascorbic acid is also readily oxidised by atmospheric oxygen (Birch & Parker, 1974) and, in this instance, particularly by dissolved oxygen. Thus, in those samples that have been purged by nitrogen (Table 2) there will be a higher residual level of ascorbic acid and hence a greater reduction of azo dye. The higher ascorbic acid levels have been confirmed by analysis.

Amaranth is reduced to naphthionic acid and amino-R-salt by cleavage of the azo bond as shown below.

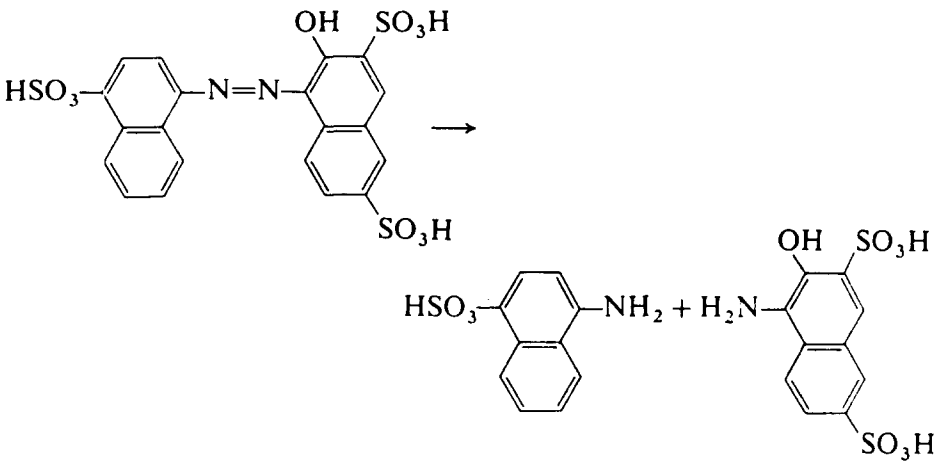
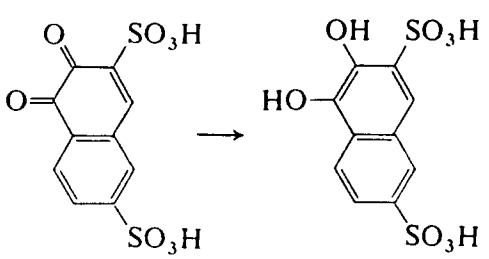


Figure 1 shows that the production of naphthionic acid increases as the concentration of amaranth decreases in model solutions. From Fig. 2 it can be seen that, in the presence of ascorbic acid, naphthionic acid and amino-R-salt are produced. The amino-R-salt was found to be unstable, which probably explains the low levels of amino-R-salt recovered and the appearance of new peaks on the chromatograms. Amino-R-salt is known to oxidise readily to a naphthoquinone (Singh, 1970). That no naphthoquinone was found could be explained by the naphthoquinone condensing with more amino-R-salt or possibly by the naphthoquinone reacting with degradation products of ascorbic acid. The naphthoquinone could also be reduced as below.



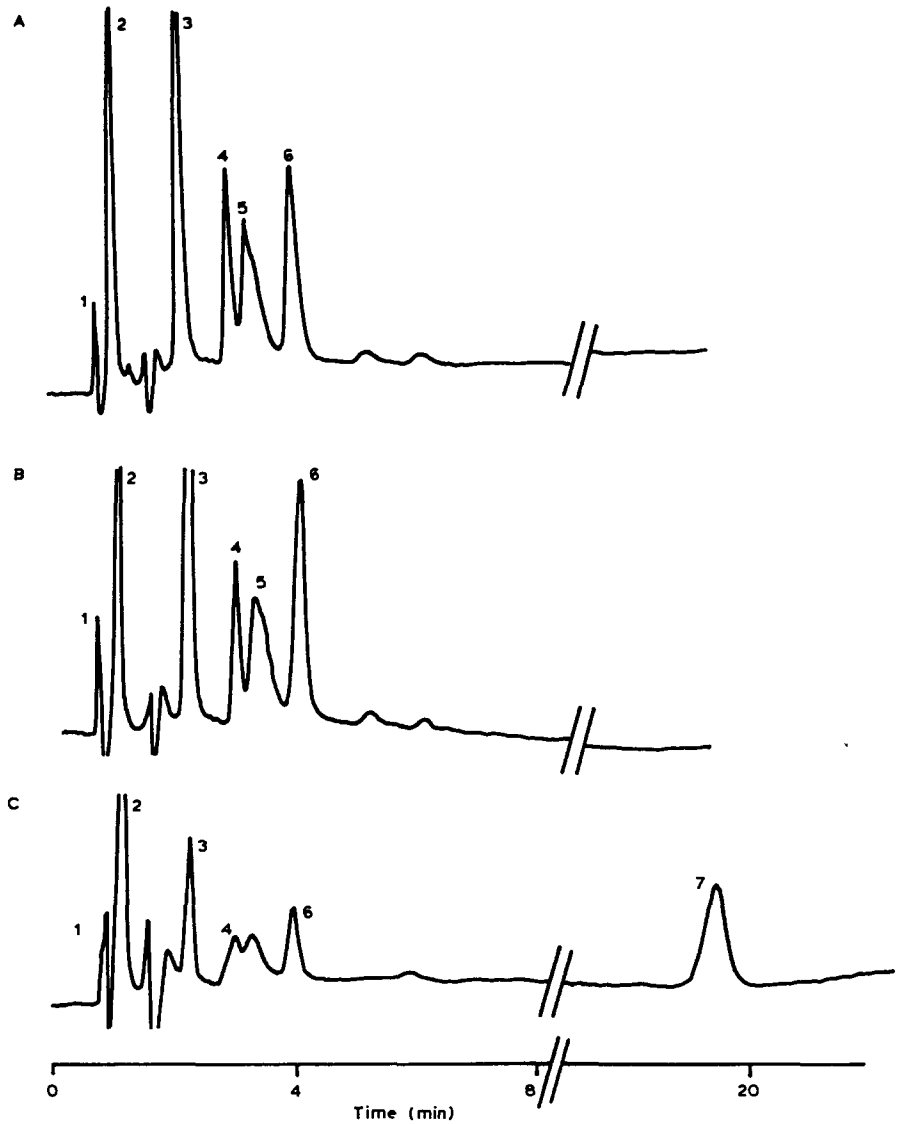


Fig. 2. Degradation of amaranth in the presence of ascorbic acid: (A) amaranth + ascorbic acid (3 months old); (B) amaranth + ascorbic acid + sucrose (3 months old); (C) amaranth + ascorbic acid + sucrose partially degraded (no sodium benzoate) (10 days old). Conditions: column, 12·5 cm, 5 μm ODS-2; mobile phase, (A) methanol and (B) 0·006 M tetra-*n*-butylammonium hydroxide; flow rate, 1 ml/min; detection, 237 nm 0·32 AUFS. Peak identity: 1, citrate; 2, ascorbic acid; 3, napthionic acid; 4, degradation product of amino-R-salt; 5, sodium benzoate (E211); 6, amino-R-salt; 7, amaranth (E123).

TABLE 3
Yield of Naphthionic Acid from Model Solutions (Exposed to Light) With Known Amounts of Dye Added

Amaranth		*Naphthionic acid*	
Added (ppm)	*Recovered (ppm)*	*Found (ppm)*	*Per cent yield*
41	—	13	86
69	—	21	83
85	—	26	83
98	—	30	83
123	—	37	82

TABLE 4
Determination of Amaranth and Naphthionic Acid in Commercial Blackcurrant Juice

Type of juice	*Amaranth recovered (ppm)*	*Naphthionic acid found (ppm)*	*Estimated initial levels (ppm)*	*Per cent decomposition*[a]
1a	5	80	221	98
1b	160	45	282	43
2a	65	180	553	88
2b	150	160	584	74
3a	4	44	123	97
3b	117	2	120	3
4a	35	48	164	79
4b	210	0	210	0
5a	340	11	370	8
5b	350	12	383	9
6	355	—	355	0
7	270	29	349	23
8	19	5	32	39

[a] Based on the assumption that all naphthionic acid is derived from amaranth.
1–7 are commercial concentrates.
8 is ready to drink.
1–4 were stored for a 6-month period.
5–8 were stored for a 2-month period.
a– shop sample (storage history unknown).
b– factory sample.
NB: Samples 1–8 are different brands of blackcurrant juice containing different ingredients.

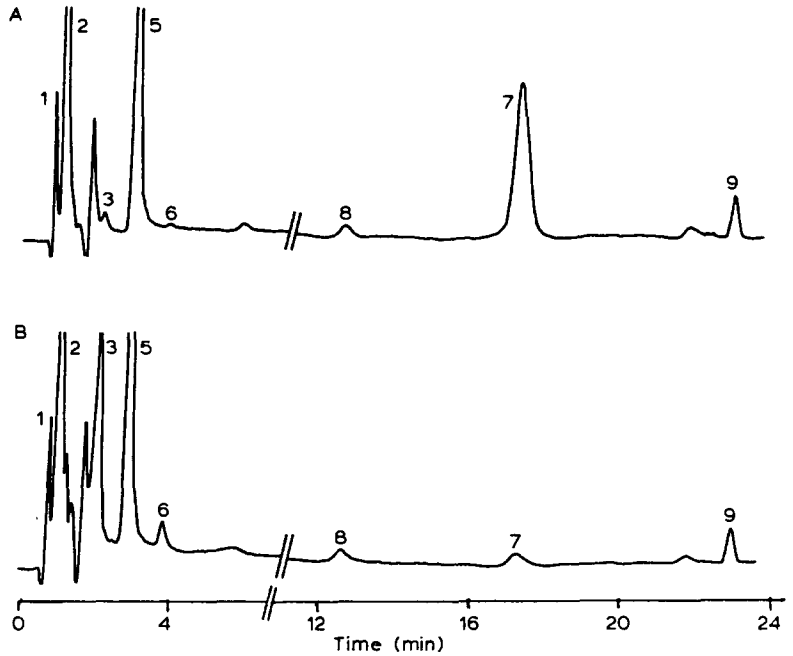


Fig. 3. Degradation of amaranth in stored blackcurrant juice samples (Type 1): (A) factory sample stored 1 month; (B) shop sample stored 6 months. Conditions as for Fig. 2.

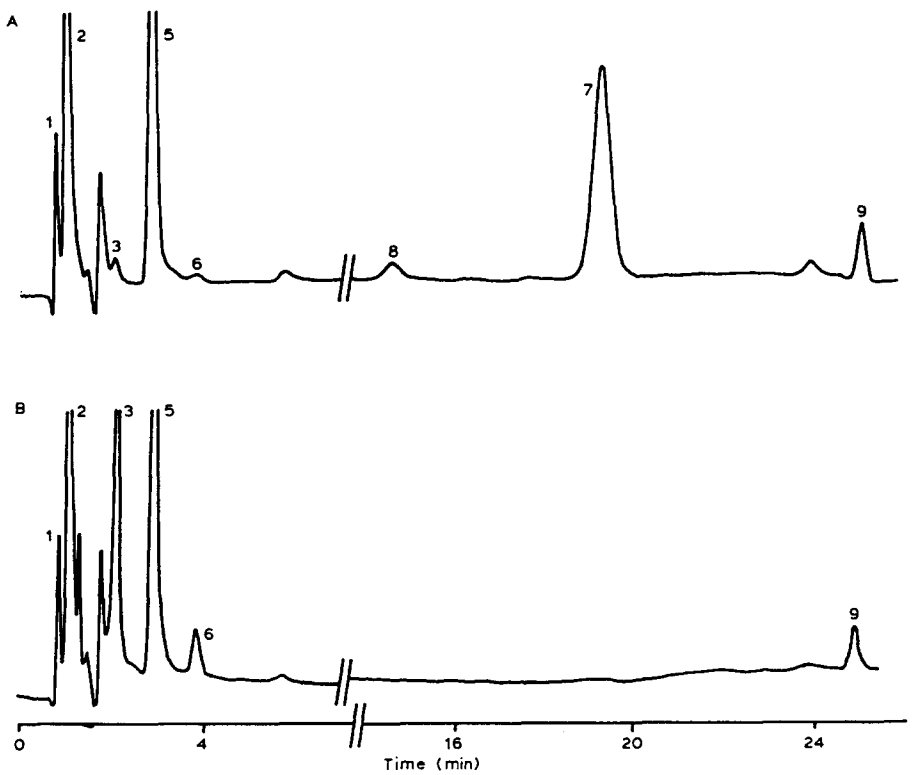


Fig. 4. Degradation of amaranth in stored blackcurrant juice samples (Type 2): (A) factory sample stored 1 month; (B) shop sample stored 6 months. Conditions as for Fig. 2.

TABLE 5

Stability of Blackcurrant and Strawberry Juices. Change in the Levels (ppm) of Amaranth, Naphthionic Acid, Ascorbic Acid and Sodium Benzoate with Time

Type of drink	*Time (months)*						
	0	*1*	*2*	*3*	*4*	*5*	*6*
9a	210	175	112	49	24·2	9·3	4·5
b	7	22	35	56	65	72	80
c	3705	2810	2785	2585	2530	2470	2390
d	502	502	503	493	491	491	497
10a	250	219	207	163	121	83	35
b	7	10	13	21	30	39	47·5
c	3215	3010	2850	2660	2580	2520	2350
d	610	603	601	602	595	595	592
11a	500	286	235	194	127	76	65
b	63	77	89	111	136	155	180
c	4015	3800	3710	3535	3217	3080	3005
d	685	688	681	679	675	675	671
12a	52	52	51	50	49	47	47
b	0	0	0	0	0	0	0
c	—	—	—	—	—	—	—
d	153	153	151	152	147	143	143
13a	150	150	150	147	146	143	141
b	0	0	0	0	0	<2	<2
c	0	0	0	0	0	0	0
d	261	260	261	259	255	254	252

9–11 are blackcurrant concentrates.
12 ready to drink blackcurrant juice, carbonated.
13 strawberry syrup concentrate.
a—amaranth, b—naphthionic acid, c—ascorbic acid and d—sodium benzoate.
NB: 9–13 are different brands of drinks.

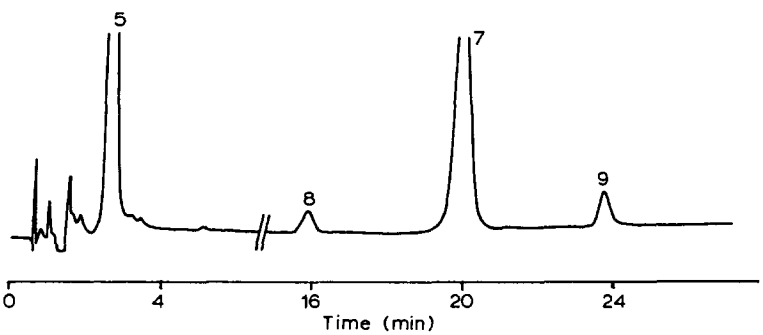


Fig. 5. Stability of amaranth in carbonated blackcurrant juice (shop sample stored 6 months).

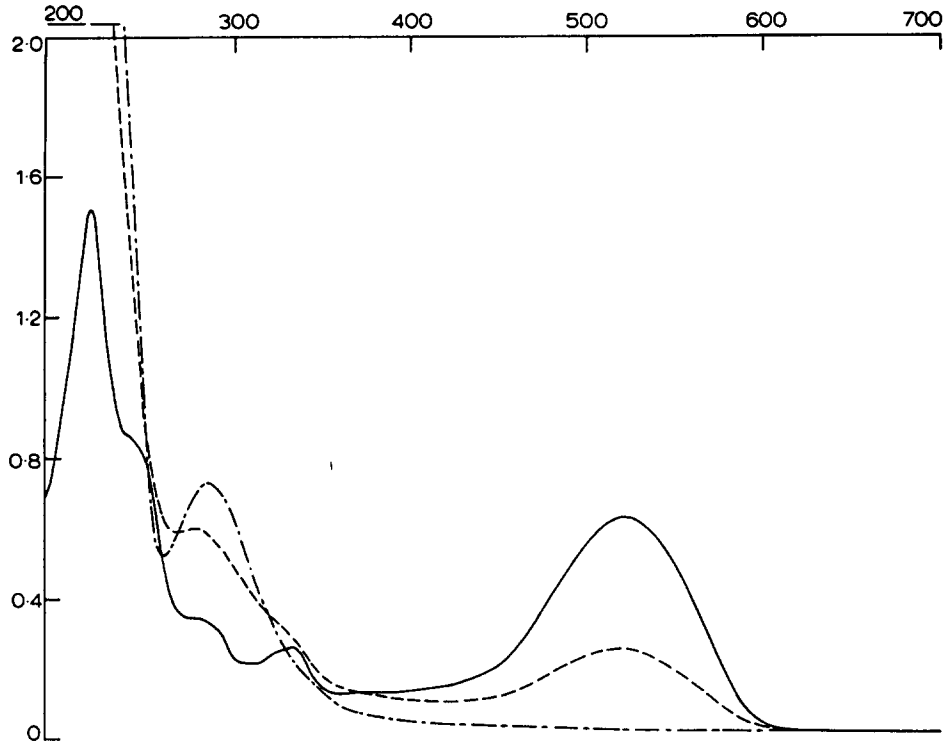


Fig. 6. Effect of ascorbic acid on the λ_{max} for amaranth: —, amaranth fresh; – – – –, amaranth + ascorbic acid (3 months old); — – – – —, amaranth + ascorbic acid (3 months old) + sucrose. Amaranth concentration 10 ppm; cell thickness 1 cm; solvent, water; reference, water.

The fate of the amino-R-salt and the naphthoquinone is still under investigation. Naphthionic acid was found to be relatively stable under the conditions of the experiment. More than 93% naphthionic acid was recovered in model solutions containing naphthionic acid and buffer after 3 months' storage under the same conditions as defined for the dyes. Table 3 shows the percentage yield of naphthionic acid found in model solutions.

The results of the study correlating dye degradation and production of naphthionic acid, in the model systems, were then used to determine the initial levels of amaranth in commercial drinks from the amounts of naphthionic acid found analytically. These results are shown in Table 4. One of these samples (2b, Table 4) was known to have been prepared with an initial amaranth content of 582 ppm (in fact, 685 ppm of 85% purity) and this compares very well with the figure derived from the naphthionic acid content (584 ppm). In the other cases there was a good agreement between the estimated levels of amaranth in factory samples and shop

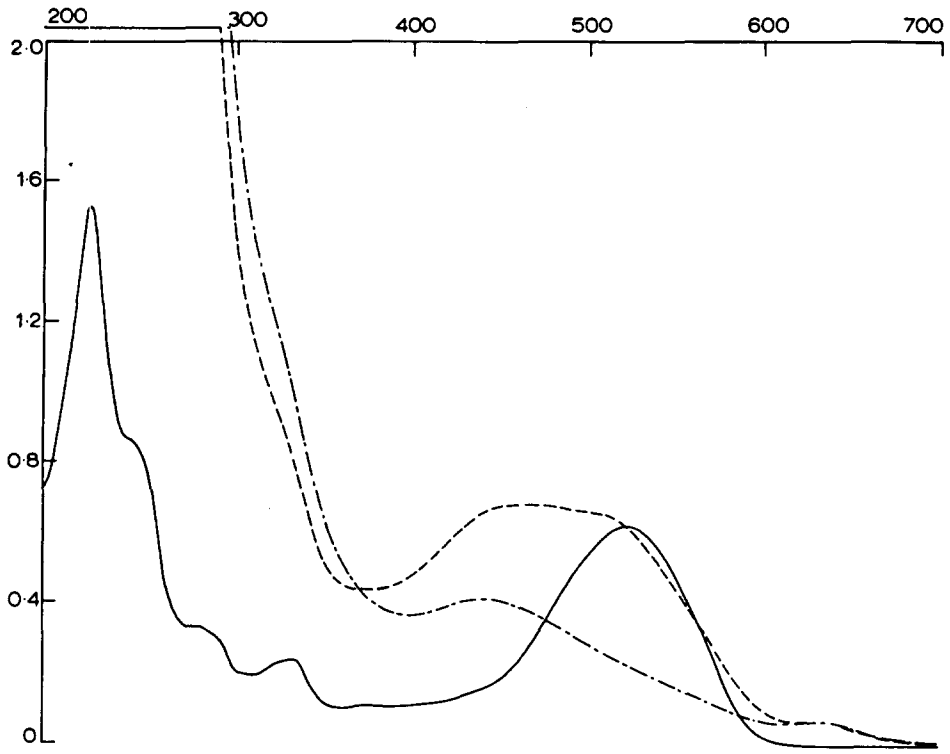


Fig. 7. Change in the visible spectrum of blackcurrant drink Type 1: —, amaranth fresh; – – – –, 1 month stored drink; — — — — —, 6 months stored drink. Conditions as for Fig. 6, with drink diluted 1 in 10.

samples of the same brands. The exact history of these samples was not known and in some cases it is quite clear that the factory samples had been in storage for some time, on account of their significant naphthionic acid levels, e.g. sample 1b, while, in other cases, the factory samples were fresh, showing very little degradation, e.g. samples 3b and 4b. The method is only possible when conditions of storage have been defined and the stability of the degradation products has been tested under the same conditions; for example, naphthionic acid was found to decompose and give off ammonia when using very strong artificial light (Fogg & Summan, 1983, 1984).

Commercial samples of blackcurrant juice are unstable. Table 5 shows the changes in levels of amaranth, naphthionic acid, ascorbic acid and sodium benzoate. Ascorbic acid is either used up in dye reduction or it is oxidised by oxygen. Sodium benzoate, which is used as a preservative, is relatively stable under the conditions of the experiment. Carbonated soft drinks were found to be relatively stable, partly due to the absence of

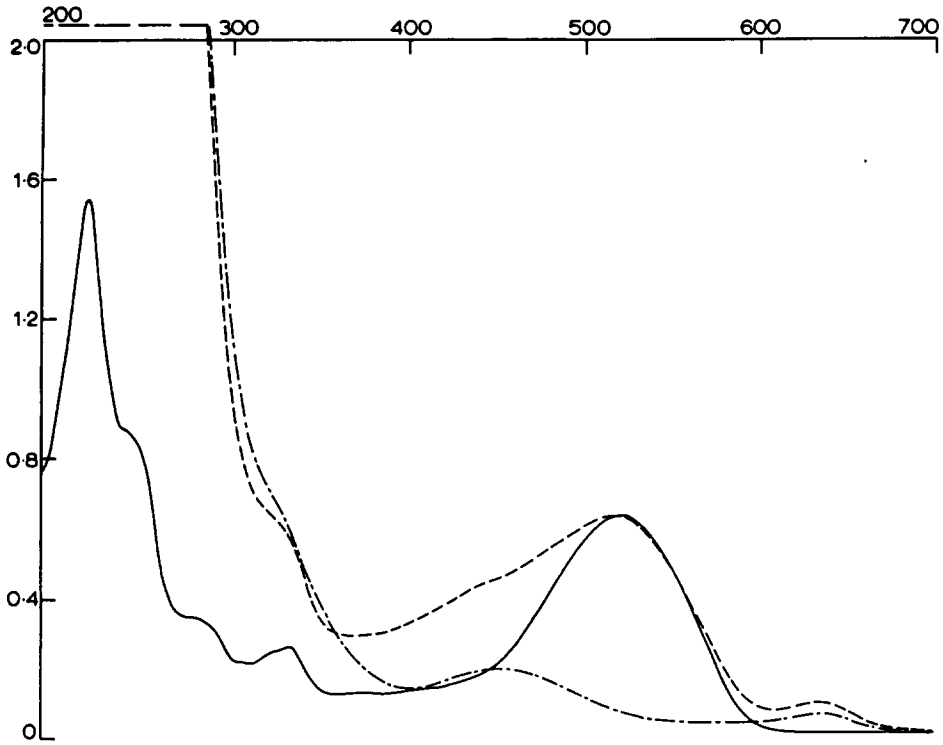


Fig. 8. Change in the visible spectrum of blackcurrant drink Type 2. Key and conditions as for Fig. 7.

ascorbic acid. More than 96% amaranth was recovered in carbonated drinks after 6 months' storage.

Figures 3, 4 and 5 show chromatograms of some stored commercial samples. The appearance of naphthionic acid and amino-R-salt peaks is shown. In this study a number of assumptions were made, but it does show that the degradation products of azo dyes could be used to determine initial levels of dye. The analysis also checks on the levels of aromatic amines such as naphthionic acid, amino-R-salt, sulphanilic acid, etc., which are present in a drink. Stored samples of blackcurrant juice and model solutions, which had been stored, showed browning discoloration which could be due to aromatic amines (from dye degradation) reacting with ascorbic acid or the sugars. In commercial samples ascorbic acid or the sugars could react with amino acids which may be present and cause browning discoloration. Ascorbic acid, when degraded to dehydroascorbic acid and other components, also gives brown coloration (Wedzicha, 1984). The effects of dye degradation may also be seen in the visible spectra of drinks (Figs 6, 7 and 8). There is a clear shift in the λ_{max} resulting in a marked colour change.

CONCLUSION

An attempt has been made to estimate initial levels of amaranth in soft drinks from determinations of levels of naphthionic acid. Further investigations of the fate of the amino-R-salt and ascorbic acid are required to establish the significance of the formation of aromatic amines as a result of dye degradation in commercial soft drinks. Any discussions on the desirability of food colours must consider their degradation products in food systems.

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