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Separation of the coloured reaction products formed in β-carotene and/or phenylalanine model systems†

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Model systems comprising β-carotene or phenylalanine or β-carotene and phenylalanine in liquid paraffin were heated at 210°C for 15 min. The absorbance of the mixture (at 420 nm) was about 15 and 20 times those obtained for the phenylalanine and β-carotene systems, respectively. The methanol extractable components (MEC) of each system represented 17·6, 76·4 and 14·0%, respectively, of the coloured components present after heating (based on absorbance at 420 nm). HPLC of β-carotene MEC revealed no major peaks when monitoring at 420 nm, while TLC gave only one coloured band. In contrast, HPLC chromatograms for the mixture and the phenylalanine systems were complex, and, based on HPLC diode array data, four major peaks were common to both, with two being present in significantly larger amounts in the phenylalanine system. Subsequent HPLC analysis of the TLC bands permitted the detection of further components.

INTRODUCTION

β-Carotene is an important food colouring material as well as a vitamin A precursor. It occurs naturally in many fruits, vegetables and vegetable oils, and it is also added to a number of processed foods, including soups, margarines and flour confectionery items (MAFF, 1987).

Several studies have investigated the thermal degradation products of β-carotene heated in model systems, and attention has often focused on the volatile degradation compounds. Studies of relevance to food systems include those of LaRoe & Shipley (1970), Schreier *et al.* (1979) and Kanasawud & Crouzet (1990). Model system studies involving the identification of the non-volatile β-carotene degradation products can be divided into those investigating the effects of extrusion cooking on β-carotene (Marty & Berset, 1986, 1988, 1990; Guzman-Tello & Cheftel, 1990), and those making use of model systems based on a liquid matrix, such as glycerol (Onyewu *et al.*, 1982, 1986) or water (Kanasawud & Crouzet, 1990). Marty & Berset established that 92% of the β-carotene was degraded on extrusion cooking a corn starch–β-carotene mixture at 180°C, and 25 of the degradation products formed were identified (Marty & Berset, 1986, 1988). Mono- and poly-*cis* isomers of all *trans* β-carotene and mono- and diepoxide derivatives predominated. Extensive losses of β-carotene occur on heating in glycerol at 210°C for 15 min (Onyewu *et al.*, 1982, 1986). Again, 92% of the β-carotene is reported to degrade, and a range of non-volatile degradation products formed, eight of which have been identified (Onyewu *et al.*, 1982, 1986). More recently, the kinetics of formation of the non-volatile degradation products of β-carotene heated in an aqueous system have been investigated (Kanasawud & Crouzet, 1990), and the structures of four of them have been elucidated.

Many of the non-volatile degradation products of β-carotene possess chemically reactive groups, e.g. carbonyl groups, and are likely to react with amino acids and/or their degradation products during the thermal processing of foods. Such reactions could have a bearing on the flavour and colour of heated β-carotene-containing foods, as well as foods containing other carotenoids. No studies have been reported on the reactivity of β-carotene towards other food components, although it is known that β-carotene degrades when subjected to conditions encountered during the thermal processing of foods (Klaui, 1981). It is thus important to understand the nature of the chemical reactions taking place, so that steps may be taken to conserve β-carotene and to optimise the colour of β-carotene-containing processed foods. This study was undertaken to investigate the coloured compounds formed between β-carotene and amino acids during conditions encountered during deep-fat frying.

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MATERIALS AND METHODS

Materials

Light liquid paraffin, phenylalanine, and analytical grade solvents were obtained from BDH Chemicals Ltd, Poole, UK. HPLC grade acetonitrile was obtained from Rathburn Chemicals Ltd, Walkerburn, UK, and HPLC grade water was prepared in the laboratory using a Purite Labwater RO50 unit (Purite Ltd, High Wycombe, UK). Gifts of *all-trans* β-carotene were supplied by Roche Products Ltd, Welwyn Garden City, UK.

Methods

All experimental procedures were performed under subdued lighting.

Preparation of model systems and of the methanol extractable components (MEC)

Liquid paraffin (200 ml) was heated to 210°C in an open 500 ml glass flask. β-Carotene (0·5 g) and phenylalanine (4 g) were added and the mixture was heated, with mechanical agitation, at 210 ± 2°C for 15 min. Following cooling, solvent extraction was carried out with methanol (8 × 50 ml). The solvent was removed under vacuum <40°C, and the residue (MEC) was dissolved in dichloromethane (1 ml) and stored in brown glass vials, under nitrogen at −20°C. MECs were also obtained for model systems prepared using either β-carotene or phenylalanine.

Electronic absorbance spectroscopy

Absorbance measurements and spectra were obtained using a Perkin Elmer Lambda 5 instrument (Perkin Elmer Ltd, Beaconsfield, UK).

TLC

Both analytical and semi-preparative separations were performed on MEC. Aliquots (450 μl) were applied to 20 cm × 20 cm aluminium plates coated with a 0·2 mm layer of unmodified silica gel G60 (BDH Chemicals Ltd, Poole, UK). As a result of numerous trials, the best mobile phase proved to be dichloromethane : ethyl acetate (9 : 1). Separations were monitored in daylight and using a UV lamp. TLC bands which were visible in daylight were recovered by exhaustive elution of the stationary phase with ethyl acetate, removal of the solvent under vacuum <40°C, dissolving in acetone (5 ml) and storing under nitrogen at −20°C.

HPLC

HPLC was performed using a 250 mm × 4·6 mm i.d. Spherisorb ODS2 column connected to a 20 mm × 4·6 mm Spherisorb ODS2 guard column (Hichrom Ltd, Theale, UK), and a linear gradient of water : acetonitrile (40 : 60) to 100% acetonitrile over 90 min, and remaining at 100% acetonitrile until all the components had eluted. The flowrate used was 1 ml min^{-1}. Two HPLC systems were used. The first comprised a Perkin Elmer binary (Model 250) pump (Perkin Elmer Ltd, Beaconsfield, UK) linked to a Kratos Analytical Model 757 absorbance detector, with detection at 420 nm (Kratos Analytical, Manchester, UK), and a Hewlett Packard Model 3396A integrator (Hewlett Packard, Wokingham, UK). The second system comprised a Philips quaternary (Model PU4100M) pump, linked to a Philips PU4120 diode array detector with scanning over the range 190–390 nm (Philips Scientific, Cambridge, UK) and a Dell Systems 310 computer (Dell Computer Corporation, Bracknell, UK), loaded with PU6003 diode array software (Philips Scientific, Cambridge, UK). Aliquots of MEC in dichloromethane (900 μl) were diluted in acetone (1 ml or 5 ml), prior to analysis by HPLC. Components of the recovered TLC bands were also analysed. The injection volume was 20 μl.

RESULTS AND DISCUSSION

The model system chosen represents a deep-fat frying system. Preliminary studies with glycerol as the matrix resulted in the development of a yellow colour and a caramel aroma, which increased in intensity as heating progressed. Liquid paraffin is an inert matrix which can withstand the heating conditions used in this study without change in appearance or odour. The temperature and time of heating of the model system were chosen to imitate conditions encountered during the deep-fat frying of foods. Twenty amino acids (all commonly found in food) were reacted with β-carotene under the experimental conditions. The system containing phenylalanine developed the most colour on heating (based on absorbance at 420 nm), and was therefore chosen for further study.

The β-carotene and β-carotene–phenylalanine model systems were both dark red before heating. After heating, they were yellow and orange-brown, respectively. Degradation of β-carotene, heated alone and with phenylalanine, was confirmed by the absence of its characteristic electronic absorption spectrum after heating. The phenylalanine model system changed from colourless to yellow on heating. The presence of substantially more colour in the β-carotene–phenylalanine system after heating, compared to the systems containing either β-carotene or phenylalanine, indicates that some reaction, involving β-carotene and phenylalanine (or their degradation products), has occurred.

Electronic absorbance spectroscopy

Absorbance readings were taken at 420 nm. No clear λ_{max} was observed in the visible region of any of the heated model systems or solvent extracts and so the choice of wavelength was not easily made. However, 420 nm is in the visible region, and corresponds to the region of the visible spectrum where absorbance by yellow compounds occurs. In addition, the measurement

Table 1. Absorbance readings (at 420 nm) of model systems[a]

Model system	β-Carotene	Phenylalanine	Mixture
After heating	0·559±0·157	0·767±0·135	0·540±0·151[b]
After methanol extraction	0·464±0·155	0·171±0·050	0·463±0·147[b]
Methanol extract[c]	0·074±0·027	0·205±0·068	0·927±0·092
Material extracted by methanol (%)[d]	17·6±4·7	76·4±3·2	14·0±6·2

[a] Values given are the averages obtained from two experiments (β-carotene), four experiments (phenylalanine), five experiments (mixture).
[b] Readings were obtained for a 1:20 dilution of the sample.
[c] Readings were obtained for 0·2 ml residue dissolved in 25 ml dichloromethane.
[d] Material extracted (%) =

$$\frac{(\text{Absorbance after heating} - \text{Absorbance after extraction})}{\text{Absorbance after heating}} \times 100$$

of absorbance at 420 nm is frequently used for monitoring the development of colour in sugar (or carbonyl)-amino compound (Maillard) model systems. Absorbance readings obtained for the model systems and the methanol extracts are shown in Table 1. The absorbance of the β-carotene–phenylalanine system (before methanol extraction) was about 15 and 20 times those obtained for the phenylalanine and β-carotene systems, respectively, indicating that reactions are taking place between β-carotene and phenylalanine (or their degradation products) leading to the formation of coloured components. Differences between the electronic absorption spectra of the three model systems were also apparent (see Fig. 1). No λ_{max} is apparent in the visible region for any of the systems, and tailing into the visible region accounts for the yellow or orange-brown colours. The presence of two

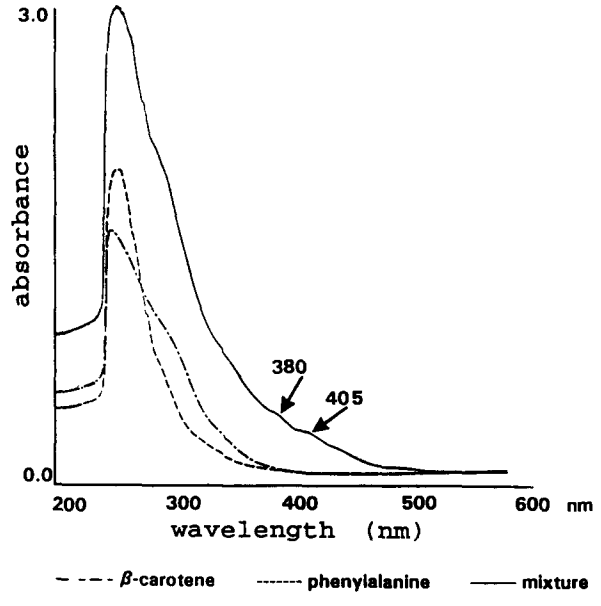

Fig. 1. Electronic absorption spectra of the heated model systems.

shoulders (at 380 and 405 nm) was characteristic of the mixture. The components responsible for these shoulders were not extracted into the methanol, since the shoulders were also observed in the paraffin phase after solvent extraction. Based on absorbance measurements at 420 nm, most of the coloured components present after heating the mixture and β-carotene model systems appear to be relatively non-polar, since the majority of the colour remains in the liquid paraffin and is not extracted into the methanol (see Table 1). In contrast, the majority of the coloured material present in the heated phenylalanine system appears to be extracted by the methanol.

HPLC of MEC

Chromatograms of MEC of the mixture and phenylalanine systems, obtained using the single wavelength detector at 420 nm, are shown in Fig. 2, and the UV spectra of the major peaks, obtained from the diode array data, are shown in Fig. 3. A comparison of the data obtained shows that the major peaks (peaks A, B, C and D) were present in MEC of both the mixture and the phenylalanine systems. Chromatograms were obtained for MEC of five replicate model systems, and the peak area data for the major peaks were analysed using the Student's *t*-test. Amounts of peaks A and D were 2·7 and 3·4 times larger, respectively, in the phenylalanine system. A feature of the chromatogram obtained for the mixture (see Fig. 2) is that the peaks appear to elute superimposed on a background of unresolved components which absorb at 420 nm. Such a background was not observed for the chromatogram of the phenylalanine sample. Another difference between the chromatograms of the two samples was the presence of more minor peaks for the mixture.

HPLC of the methanol extractables from the β-carotene system, using both HPLC systems, showed no large peaks (when monitoring at 420 nm) and no peaks corresponding to peaks A–D could be detected using the diode array system.

TLC of MEC

HPLC revealed that the methanol extracts of the mixture and the phenylalanine model systems were quite complex. Therefore it was decided to attempt to fractionate the methanol extracts by TLC, prior to the proposed isolation and purification of individual reaction products by semi-preparative HPLC, followed by structural analysis.

TLC of MEC from the β-carotene model system resulted in one band observed in daylight (yellow, R_f = 0·00) and one band observed under UV radiation (blue, R_f = 0·59). The TLC data obtained for the mixture and phenylalanine model systems are given in Table 2, from which it can be seen that eight of the nine bands separated are common to both model systems, although the colours of the bands were more intense for the mixture. A feature of the TLC plates of the

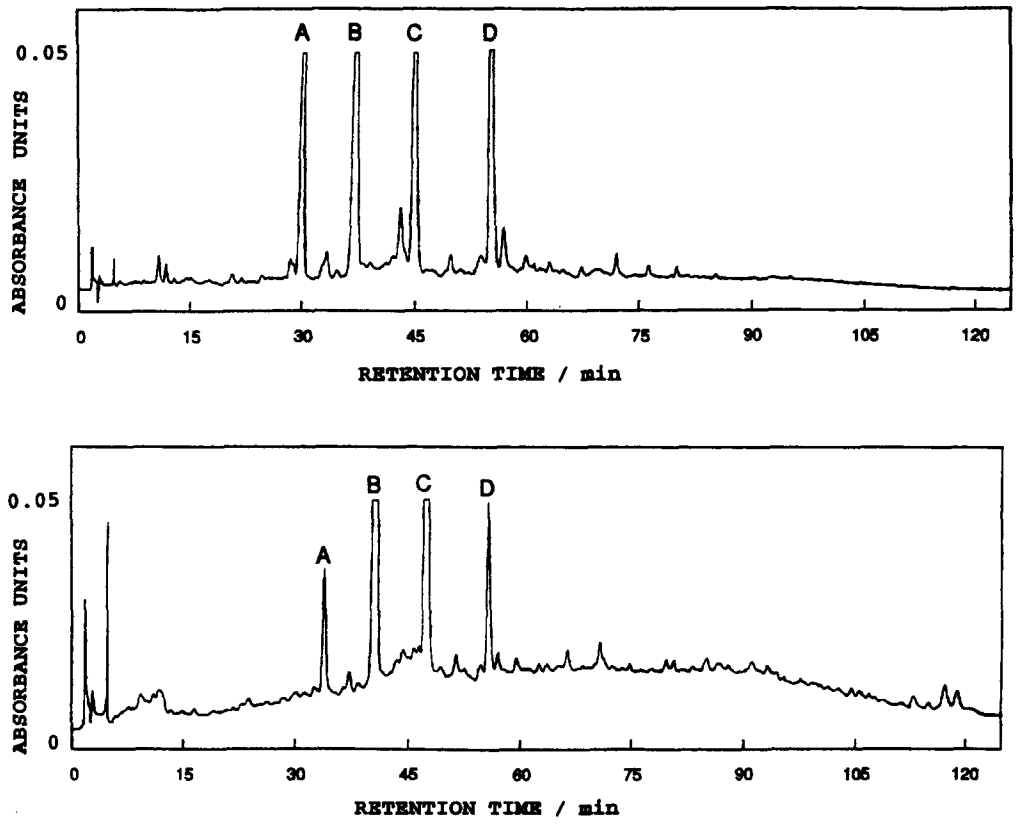

Fig. 2. Representative HPLC chromatograms of MEC obtained from the model systems containing (a) phenylalanine, (b) β-carotene and phenylalanine. (HPLC retention times were not always reproducible, but peak codes could be assigned based on the diode array data. The concentration of the sample injected was 900 μl MEC dissolved in 1 ml acetone.)

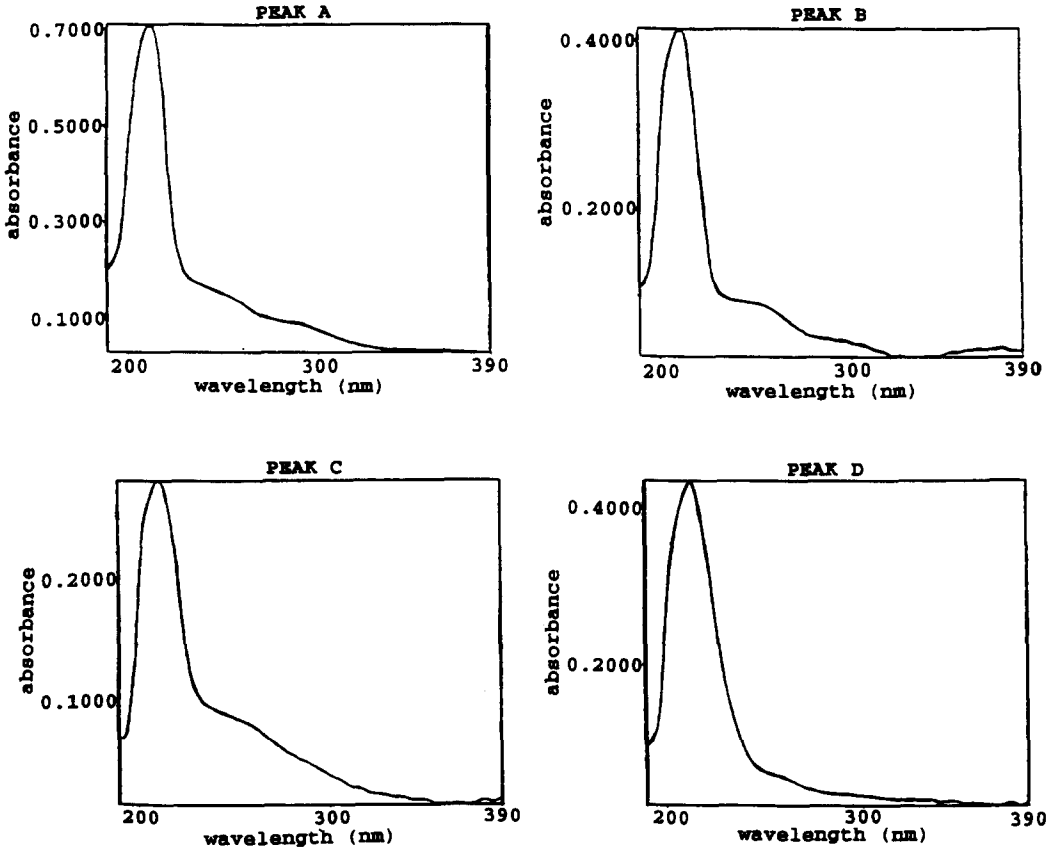

Fig. 3. UV spectra obtained from the diode array data for peaks A–D.

Table 2. TLC data obtained for the methanol extracts

Band	R_f value	Model system			
		Phenylalanine		Mixture	
		Colour		Colour	
		Daylight	UV lamp	Daylight	UV lamp
1	0·2	—[a]	Blue	—	Blue
2	0·3	Yellow	Green	Light brown	—
3	0·4	Yellow	Green	Light brown	—
4	0·45	—	—	Light brown	—
5	0·52	Yellow	Green	Dark yellow	Green
6	0·59	Yellow	Blue	Dark yellow	Dark blue
7	0·68	Yellow	Dark blue	Dark yellow	Dark grey/blue
8	0·75	Yellow	Bright yellow	Dark yellow	Yellow
9	0·85	—	Violet	—	Violet

[a] Indicates that the band could not be detected.

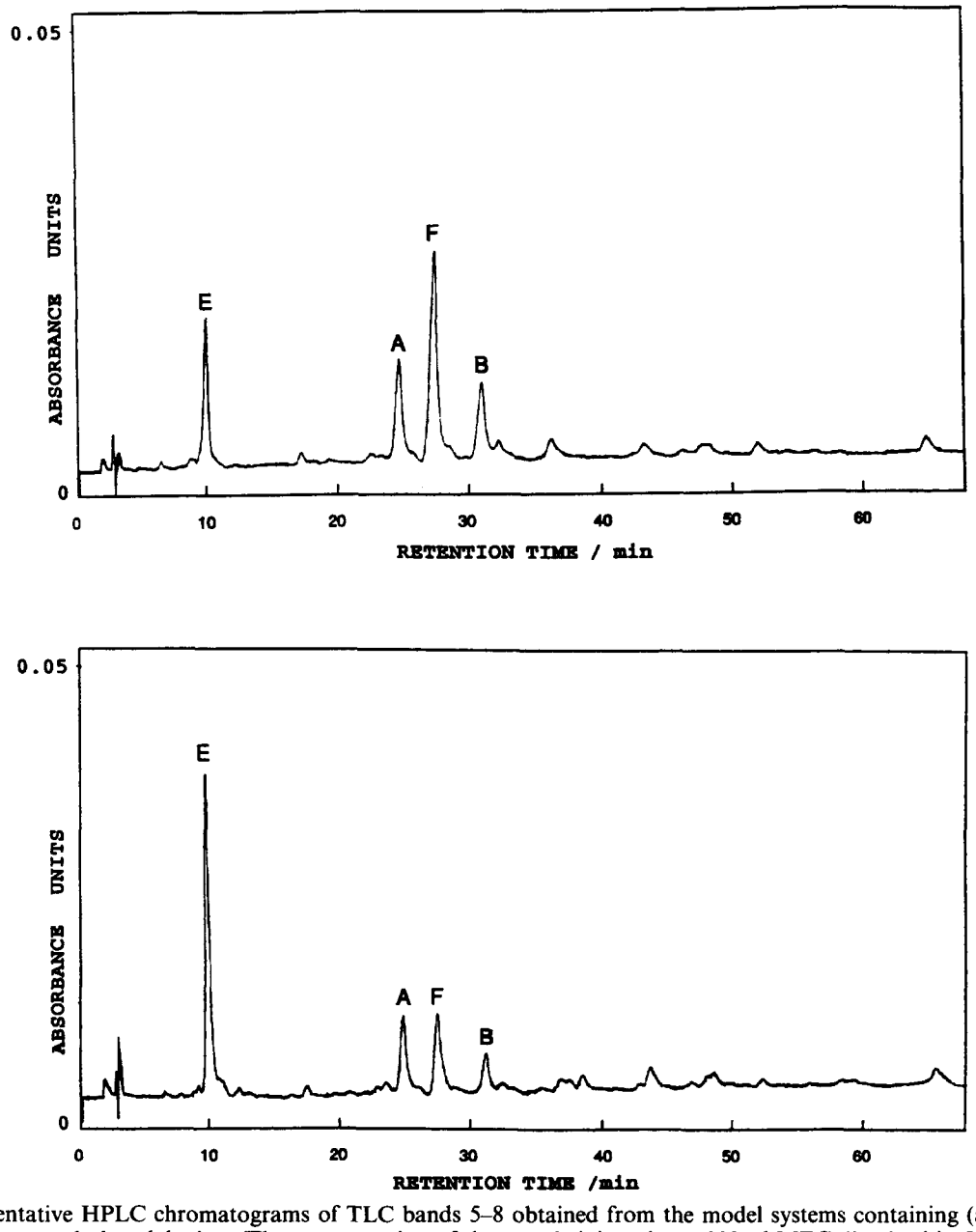

Fig. 4. Representative HPLC chromatograms of TLC bands 5–8 obtained from the model systems containing (a) phenylalanine, (b) β-carotene and phenylalanine. (The concentration of the sample injected was 900 μl MEC dissolved in 5 ml acetone.)

mixture was the presence of a yellow colour over the whole area of the plates, due to poorly resolved components. It is probable that these components were also responsible for the background observed on the HPLC chromatograms of the MEC of the mixture.

Bands 5–8 were poorly resolved from each other and were therefore recovered as one fraction from the mixture and the phenylalanine systems. A fraction was also obtained for the area of the TLC plates corresponding to the R_f values of bands 5–8 for the β-carotene system.

HPLC of the coloured TLC bands

HPLC (with detection at 420 nm) of bands 2 and 3 of the phenylalanine system and bands 2, 3 and 4 of the mixture showed no peaks for any of the samples, under the conditions used for the analysis of bands 5–8.

Analysis of the fraction corresponding to bands 5–8 of the β-carotene model system showed no peaks absorbing at 420 nm. Chromatograms of the corresponding fractions from the mixture and phenylalanine, with detection at 420 nm, are shown in Fig. 4. Analysis of the HPLC diode array data for the mixture and phenylalanine showed the presence (in both systems) of two peaks not detected in the methanol extracts prior to TLC. Peaks E and F, and their UV spectra, obtained from the diode array data, are shown in Fig. 5. Analysis of the chromatograms obtained for five replicate samples of bands 5–8 prepared from both systems showed that amounts of peak E were 2·6 times greater in the mixture than in phenylalanine, while amounts of peak F were 2·4 times greater in phenylalanine than in the mixture. Since neither of these peaks could be detected on HPLC of the MEC without prior TLC, they could form as a result of TLC. TLC

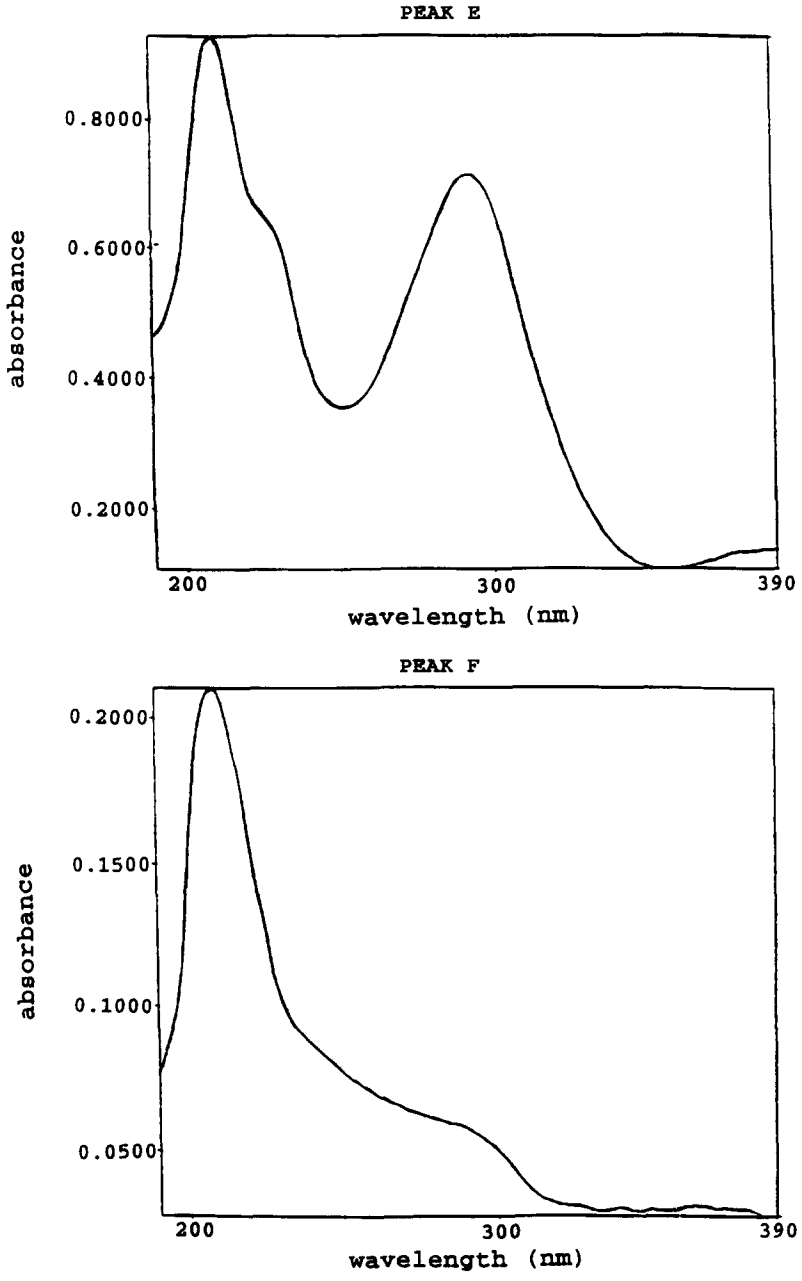

Fig. 5. UV spectra obtained from the diode array data for peaks E and F.

was therefore carried out on aliquots of the MEC from both the mixture and the phenylalanine model systems, with recovery of the entire fractions from the stationary phase, followed by HPLC analysis. Peak E was present in the entire fraction from the mixture and it appears that peak E can form on TLC of the MEC from that system. It seems most likely, therefore, that peak E also forms on TLC of the MEC from the phenylalanine system, but that it could not be detected because it is formed at lower levels in that system. Peak F could not be detected on HPLC analysis of the entire fractions from either the mixture or phenylalanine. Even exposure of the TLC plates to daylight and room temperature prior to recovery of sample components did not result in the detection of peak F. It seems that peak F may be formed on TLC and, if so, it may react with sample components other than those present in bands 5–8. This would account for its presence in bands 5–8 but its absence in the MEC after TLC of the entire fraction. Substantial amounts of peaks A and B were also present in bands 5–8 of both the mixture and phenylalanine (see Fig. 4). The presence of peaks C and D in bands 5–8 could not be confirmed since, if they were present, they existed at levels below those required for the construction of UV spectra from the diode array data.

CONCLUSIONS

Heating mixtures of β-carotene and phenylalanine in liquid paraffin, under the chosen experimental conditions, resulted in about 15 and 20 times as much colour (based on absorbance at 420 nm), compared to heating equivalent systems containing either phenylalanine or β-carotene, respectively. While the majority of the coloured material formed in the phenylalanine system was extracted by methanol, most of the coloured components remained in the liquid paraffin phases of the mixture and the β-carotene systems. Analysis of the MEC of all three systems by HPLC and TLC revealed many similarities between the MEC of the mixture and the phenylalanine, while the MEC of β-carotene showed different separation patterns. Variations between the MEC of the mixture and the phenylalanine systems appear to lie largely in differences in amounts of individual components present rather than the presence of compounds unique to each system. Analysis of the liquid paraffin phase after methanol extraction is likely to reveal further dissimilarities between all three systems.

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