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Kinetics and yields for the formation of coloured and aromatic thermal degradation products of annatto in foods

Michael J. Scotter *, Laurence Castle, Graeme P. Appleton

Ministry of Agriculture, Fisheries and Food, Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK

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Abstract

The thermal stability of annatto food colouring has been studied in model systems and foods. High-performance liquid chromatography with photodiode-array detection has been used to monitor the isomerization products of 9′-*cis*-bixin, the principal colouring component of annatto, and its major coloured C_{17} degradation product. Headspace gas chromatography–mass spectrometry has been used to determine the degradation products toluene and *m*-xylene in the head space of model systems and foods containing annatto heated in situ. Fish and cheese spiked with 9′-*cis*-norbixin produced *m*-xylene whereas control samples did not. Low levels (ca. 10–50 μg/kg) of *m*-xylene were detected in the headspace of annatto-coloured retail samples of custard powder, extruded snacks, margarine and bread crumbs. Higher levels of *m*-xylene were detected in the headspace of kippers (ca. 150–200 μg/kg) and observed in the headspace of Red Leicester type cheese (not quantified). The findings indicated that annatto is readily degraded to form both coloured degradation products and the aromatics *m*-xylene and, to a lesser extent, toluene. In practice however, degradation is slow under heating conditions normal for foods. Neither aromatic species was detected in the headspace of any food studied above 200 μg/kg.

Keywords: Additives; Annatto; Colouring materials; Degradation; Analysis; HPLC; GC

1. Introduction

During commercial oil-extraction of annatto, the principal colouring agent 9′-*cis*-bixin degrades to the *trans*-monomethyl ester of 4,8-dimethyl-tetradecahexaene-dioic acid, otherwise known as C_{17} (McKeown 1963, 1965; Scotter, 1995). Formation of C_{17} is accompanied by the release of *m*-xylene, with lesser amounts of toluene and dihydronaphthalene released corresponding to the formation of C_{18} and C_{13} fragments. These aromatic degradation products are undesirable in colouring materials intended for use in food.

The analysis of 21 samples of commercially produced annatto for colour content (as bixin and norbixin isomers) and coloured thermal degradation products, using high-performance liquid chromatography with photodiode-array detection (HPLC–PDA) has been reported (Scotter, Wilson, Appleton, & Castle, 1998). Detailed analysis of two of the samples, of known production history, revealed only trace amounts of C_{17}-type polyene degradation compounds. However, preliminary analysis for aromatic degradation products detected approximately 20 mg/kg of *m*-xylene in one of the samples.

A follow-on study was carried out to establish the presence of the aromatic thermal degradation products *m*-xylene and toluene (Scotter, Wilson, Appleton, & Castle, 2000). Of the 20 annatto samples analysed, four samples contained 30–88 mg/kg *m*-xylene and a further two contained 160 and 200 mg/kg *m*-xylene. The results were evidence for the thermal degradation of annatto during extraction and processing of the additive. Moreover, it was suggested that further degradation of annatto in food could occur during cooking or other forms of heat processing. These possibilities are explored in this study of annatto degradation in model systems and in foodstuffs.

* Corresponding author. Fax +44-1904-462111.
E-mail address: m.scotter@csl.gov.uk (M. Scotter).

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2. Materials and methods

2.1. Materials

All reagents were of a recognised analytical grade unless specified otherwise. Ethylbenzene (99.8%), toluene (99.8%), *m*-xylene (99 + %), *o*-xylene (98%) and *p*-xylene-d_6 were obtained from Aldrich Chemical Company Limited (Gillingham, UK). Standards of all-*trans*-bixin, 9′-*cis*-bixin, 9′-*cis*-norbixin and all-*trans*-C_{17} were prepared in the laboratory (Scotter, 1995; Scotter et al., 1994).

Samples of vegetable oil, custard powder, dehydrated potato, extruded snacks, fish, cheese, margarine and breadcrumb (coating) were purchased from local retail outlets in and around Norwich, UK.

2.2. Apparatus

2.2.1. HPLC analysis

HPLC analysis was carried out using a Hewlett-Packard 1090M series II DR5 ternary pumping system with integral variable volume autosampler, column oven and model 1040 series II photodiode array detector with an HP Pascal workstation (Hewlett Packard, Bracknell, UK). The column was a 250×4.6 mm HiRPB (C_8/C_{18}) 5 μm (HiChrom Ltd, Reading, UK) and the mobile phase was 65:35 v/v acetonitrile: 0.4% v/v aqueous acetic acid delivered at 1 ml/min. The column was held at 35°C. Detection was at 435 nm with a 60 nm bandwidth. All samples were filtered through a 0.2-μm membrane syringe filter (Anotop 10, Whatman Scientific Ltd., Maidstone, UK) prior to analysis.

2.2.2. Headspace gas chromatography–mass spectrometry (GC–MS) analysis

The system comprised a Carlo Erba model 4160 GC fitted with a model HS800 automated head space sampler (Fisons Instruments, Altrincham, UK) and a 30 m×0.25 mm×0.25 μm film-thickness DB-WAX (polyethylene glycol) capillary column (Jones Chromatography, Hengoed, UK) interfaced to a VG 12–250 quadropole mass spectrometer (Micromass, Wythenshawe, UK). Helium carrier gas was used at 1 ml/min. Analysis was conducted over 8 min at 50°C. The column was then heated to remove solvent (150°C/2 min for dimethylacetamide, 200°C/7 min for propan-1,2-diol). The aromatics were detected by selected ion monitoring; toluene (*m*/*z* 91), *m*-xylene (*m*/*z* 91 and 106) and *p*-xylene-d6 (*m*/*z* 112). The dwell time was 0.15 s with an interscan delay of 0.10 s.

2.3. Methods

2.3.1. Kinetic study of 9′-cis-bixin thermal degradation

Saturated, filtered solutions of purified 9′-*cis*-bixin were prepared at room temperature in a homologous series of *n*-alcohols (methanol, ethanol and 1-propanol). Each test solution (ca. 200 ml) was placed in a 500 ml triple-necked, round-bottomed flask containing several glass beads. The flask was fitted with a thermometer, double-surface condenser, and Teflon 'Mininert' Luer valve (Aldrich Chemical Company Limited, Gillingham, UK). The valve was fitted with a 150 cm×No.12 gauge stainless-steel syringe needle with deflecting tip, adjusted to reach the bottom of the flask. The apparatus was contained in a fume cupboard and experiments were carried out under subdued (60-W fluorescent tube plus diffuse daylight) conditions. The solution was brought to boiling via an electrically heated mantle and at the onset of reflux (time zero), ca. 2 ml of the test solution was withdrawn. Further portions were withdrawn at 10-min intervals (0–60 min) and at 30-min intervals thereafter (60–300 min). An 'infinity' sample was withdrawn after 24 h. All portions were immediately cooled in an ice/water bath and analysed sequentially as a single batch by HPLC.

2.3.2. Product study of 9′-cis-norbixin degradation

A norbixin formulation known to contain 44% 9′-*cis*-norbixin, 9 mg/kg *m*-xylene and trace levels of C_{17} and toluene was treated under the following conditions:

1. 18.5 mg was dissolved in 0.1 M NaOH (ca. 5 ml) and diluted to 100 ml with methanol (control sample);
2. 100 mg dissolved in 0.1 M NaOH (50 ml) was refluxed for 1 h (ca. 100°C);
3. 100 mg suspended in *n*-butyl acetate (50 ml) was refluxed for 1 h (126°C); and
4. 100 mg suspended in ethane-1,2-diol (10 ml) was refluxed for 1 h (198°C).

After each regime, a 1 ml portion was diluted to 10 ml with methanol, filtered and analysed by HPLC.

2.3.3. Head space GC–MS analysis of heated model systems

Solutions of purified *trans*- and 9′-*cis*-bixin were prepared in vegetable oil at ca. 50 and 500 mg/kg. The bixin was pre-dissolved in ca. 1 ml dimethylacetamide to aid dispersion in the oil. A solution of C_{17} was similarly prepared in vegetable oil at ca. 50 mg/kg. Purified 9′-*cis*-norbixin solutions were prepared in propan-1,2-diol at ca. 50 and 500 mg/kg.

Sample (0.5 g) was added to internal standard (40 μl of *p*-xylene-d_6 at 0.0625 mg/ml in ethanol) in 10-ml glass head space vials and sealed with PTFE-faced septa. Calibration standards of toluene and *m*-xylene in oil and ethanol solvent were prepared likewise. The calibrants covered the range 0–2.5 mg/kg initially but this was adjusted later to accommodate sample findings. Since the purpose of this work was to find indicative levels only, full method validation was not performed

for all food matrix-experimental permutations studied. Toluene and *m*-xylene measurements should therefore be considered indicative only.

The head space incubation temperature was set at either 75 or 140°C and the incubation time varied so as to subject the sample to the required heating regime. The following heating regimes were used:

1. *trans*- and 9′-*cis*-bixin in vegetable oil at ca. 50 and 500 mg/kg heated for 2 h at 75°C;
2. *trans*- and 9′-*cis*-bixin in vegetable oil at ca. 50 and 500 mg/kg heated at 140°C for 30 and 60 min;
3. 9′-*cis*-norbixin in propan-1,2-diol at ca. 50 and 500 mg/kg heated for 2 h at 75 and 140°C; and
4. 9′-*cis*-bixin and C_{17} in vegetable oil at ca. 50 mg/kg heated at for 2 h 140°C.

2.3.4. Head space GC–MS analysis of foods

Foods with or without declared annatto were purchased from retail outlets (Table 1). Vegetable oil, dehydrated potato, custard powder, bread crumbs and margarine, were used without further preparation. Extruded snacks were powdered in a pestle and mortar prior to sub-sampling. Fish samples (coley and kipper) were skinned and then chopped to a fine consistency in a food processor. Cheese was chopped roughly with a knife and then finely chopped in a food processor. For spiking experiments, norbixin solution was spread over the chopped samples, which were then further mixed prior to sub-sampling.

Sample (1g) was placed in a head space vial along with glycerol or propan-1,2-diol (1 ml) to aid dispersion. Calibration standards of toluene and *m*-xylene and internal standard (*p*-xylene-d6) were added where appropriate. Samples were prepared and analysed in duplicate using the GC–MS conditions given above. The headspace incubator was set to give the degradation heating regime desired as given in Table 2.

Table 1
Food sample details

Food type	Annatto permitted maximum level (mg/kg)[a]
Annatto-declared	
Custard powder	10
Extruded snack (a)	20
Extruded snack (b)	20
Kippers[b]	10
Cheshire cheese	15
Red Leicester cheese	50
Margarine	10
Bread crumbs	20
No annatto-declared	
Dehydrated potato	
Coley fillets[c]	
Cheshire cheese	
Bread crumbs	

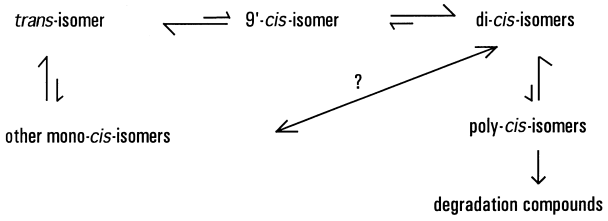
[a] Based on limits in EC Directive (EC, 1994).
[b] Pre-packed, chilled.
[c] Pre-packed, frozen.

3. Results and discussion

3.1. Kinetics of 9′-cis-bixin thermal degradation

A plot of ln (peak area of 9′-*cis*-bixin) against time was linear for the three reaction temperatures studied. The first-order rate constants are given in Table 3 along with the calculated half-lives. Loss of linearity was observed at each temperature beyond ca. 2 h reaction time, suggesting that two or more competing reactions were taking place at different rates. From the rate constants for the initial phase of reaction, the Arrhenius activation energy (ΔEa) for the loss of 9′-*cis*-bixin in refluxing *n*-alcohol solvent was calculated to be 35.7 kJ mol^{-1}.

In these experiments, the rate of *loss* of 9′-*cis*-bixin was measured as a function of time regardless of the isomerisation or degradation pathway. The rate data obtained represent total (summed) values since many similar reaction pathways are available. As well as undergoing irreversible degradation, carotenoids can undergo rapid isomerisation to an equilibrium mixture of forms. This can occur even at ambient temperature and especially in the presence of light. Since various reversible isomerisations and non-reversible degradation reactions may take place concurrently, deviation from first-order kinetics at long observation times is not unexpected.



HPLC was used to monitor the rate of formation of coloured reaction products. Previous studies have shown that several well-characterised isomers of 9′-*cis*-bixin as well as C_{17} can be separated by HPLC (Scotter, 1995; Scotter et al., 1994, 1998). These include *trans*- and other mono- and di-*cis* isomers of bixin. The results from a second degradation experiment carried out on 9′-*cis*-bixin in refluxing ethanol (Table 4) show that the overall loss of 9′-*cis*-bixin proceeded at a rate where $k = 6.8 \times 10^{-5}$ s^{-1} ($t_{\frac{1}{2}} = 2.8$ h) over the first 90 min which then slowed to around 0.3×10^{-5} s^{-1} ($t_{\frac{1}{2}} = 64$ h) over 90–9660 min as equilibrium was approached. Regarding the formation of products, a di-*cis*-isomer formed rapidly over the first 180 min whereas both *trans*-bixin and C_{17}

were formed at a much lower rate (Table 4). For each of the three products, formation slowed with time as isomeric equilibrium was approached.

The 9′-*cis*-configuration is considered to be requisite for electrocyclic elimination from bixin (Scotter, 1995). However, the possibility of degradation via other mono- and di-*cis*-isomers should also be considered since a poly-*cis*- conformation is necessary for formation of the eight-membered cyclic intermediate. It should also be noted that whilst studies on the kinetics of β-carotene degradation in ethanol under illumination (Minguez-Mosquera & Jaren-Galan, 1995) have shown that temperature and light accelerate the degradation reaction without changing the formal aspects of the reaction, the thermodynamic parameters (activation energy and enthalpy of reaction) are dependent upon the conditions used e.g. the reaction solvent. Hence, whilst the calculated 'average' rate constants and activation energy for 9′-*cis*-bixin isomerisation/degradation in alcohols are of a similar magnitude to those reported for β-carotene degradation under similar conditions, an absolute comparison cannot be made.

3.2. *Degradation of* 9′-cis-*norbixin*

These experiments were carried out to see if degradation compounds analogous to C_{17} could be detected in thermally degraded model systems containing 9′-*cis*-norbixin. Fig. 1 shows the chromatograms obtained for each experiment overlaid for comparison. All norbixin isomer peaks other than 9′-*cis*-norbixin and *trans*-norbixin, and all C_{17} isomers other than the all-*trans*- could only be tentatively identified from absorbance spectra, since authentic standards were not available.

Table 2
Heating regimes for foods

Sample type	Heating regime
Dehydrated potato, custard powder and extruded snacks	2 h at 60°C in glycerol
	1 h at 140°C in propan-1,2-diol
Fish: coley and kippers	2 h at 140°C in glycerol
	1 h at 140°C in propan-1,2-diol
Cheese: all types	2 h at 140°C in glycerol
	1 h at 140°C in propan-1,2-diol
Margarine and bread crumbs	1 h at 100°C in glycerol

Table 3
Degradation kinetics of 9′-*cis*-bixin in *n*-alcohols

Solvent	Boiling point (°C)	Rate constant (k, $s^{-1}\times10^5$)	Half-life (h)
Methanol	64.6	0.68	28
Ethanol	78.3	1.08	18
1-Propanol	97.2	2.08	9

1. *Control sample*. The main peak at ca. 9 min was 9′-*cis*-norbixin and traces of other norbixin isomers were present. Several small peaks around 3–4 min exhibited spectral characteristics similar to C_{17} (Fig. 1a).
2. *Degradation in refluxed n-butyl acetate*. The major component was again 9′-*cis*-norbixin but other norbixin isomer peaks were now prominent. These were assigned as *trans*- (6.4 min), di-*cis*- (7 min) and near-to-central mono-*cis*- (11.9 min) isomers of norbixin. A prominent peak at ca. 3 min was also observed which had a spectrum characteristic of C_{17} (Fig. 1b).
3. *Degradation in refluxed ethane-1,2-diol*. All norbixin isomer peaks were reduced to trace levels. The two major peaks at 3.1 and 3.9 min, and several minor peaks which eluted between 3 and 4 min, all exhibited spectra characteristic of C_{17} (Fig. 1c).
4. *Degradation in refluxed 0.1M NaOH*. 9′-*cis*-norbixin remained as the main peak but with a prominent isomer peak at ca. 7 min (di-*cis*-norbixin) and several other isomer peaks at lower levels. Compared to the control there were no observable differences in peaks eluting around 3–4 min (Fig. 1d).

These data provide evidence for the formation of C_{17} analogues from 9′-*cis*-norbixin at 126°C (*n*-butyl acetate) and 198°C (ethane-1,2-diol). At lower temperature (i.e. = ca. 100°C), norbixin isomerization processes appear to predominate. However data from kinetic studies of 9′-*cis*-bixin degradation show that prolonged heating at 100°C should eventually produce C_{17} analogues. The HPLC analysis of a mixture of hydrolysed (i.e. de-esterified) C_{17} isomers clearly shows (Fig. 1e) a series of peaks with identical retention times and spectral characteristics as those observed in degradation mixtures.

Table 4
Kinetic data for the degradation of 9′-*cis*-bixin in ethanol at 78°C

Substance	Rate constant (k, $s^{-1}\times10^5$)	Half-life (h)	Linear observation time (h)[a]
Formation of			
Trans-bixin	0.12	160	0–110
Di-*cis*-bixin	35	0.5	0–3
C_{17}	0.13	148	0–110
Loss of			
9′-*cis*-bixin	6.8 (loss)[b]	2.8	0–1.5
9′-*cis*-bixin	0.3 (loss)	64	1.5–160

[a] In the case of the reaction products, this represents the time in which a first-order relationship was observed.

[b] In this second experiment in refluxing ethanol, the fume cupboard light remained on and this is thought to be why the reaction proceeded six-fold faster (6.8 vs. 1.1×10^{-5} s^{-1}) than the experiment reported in Table 3.

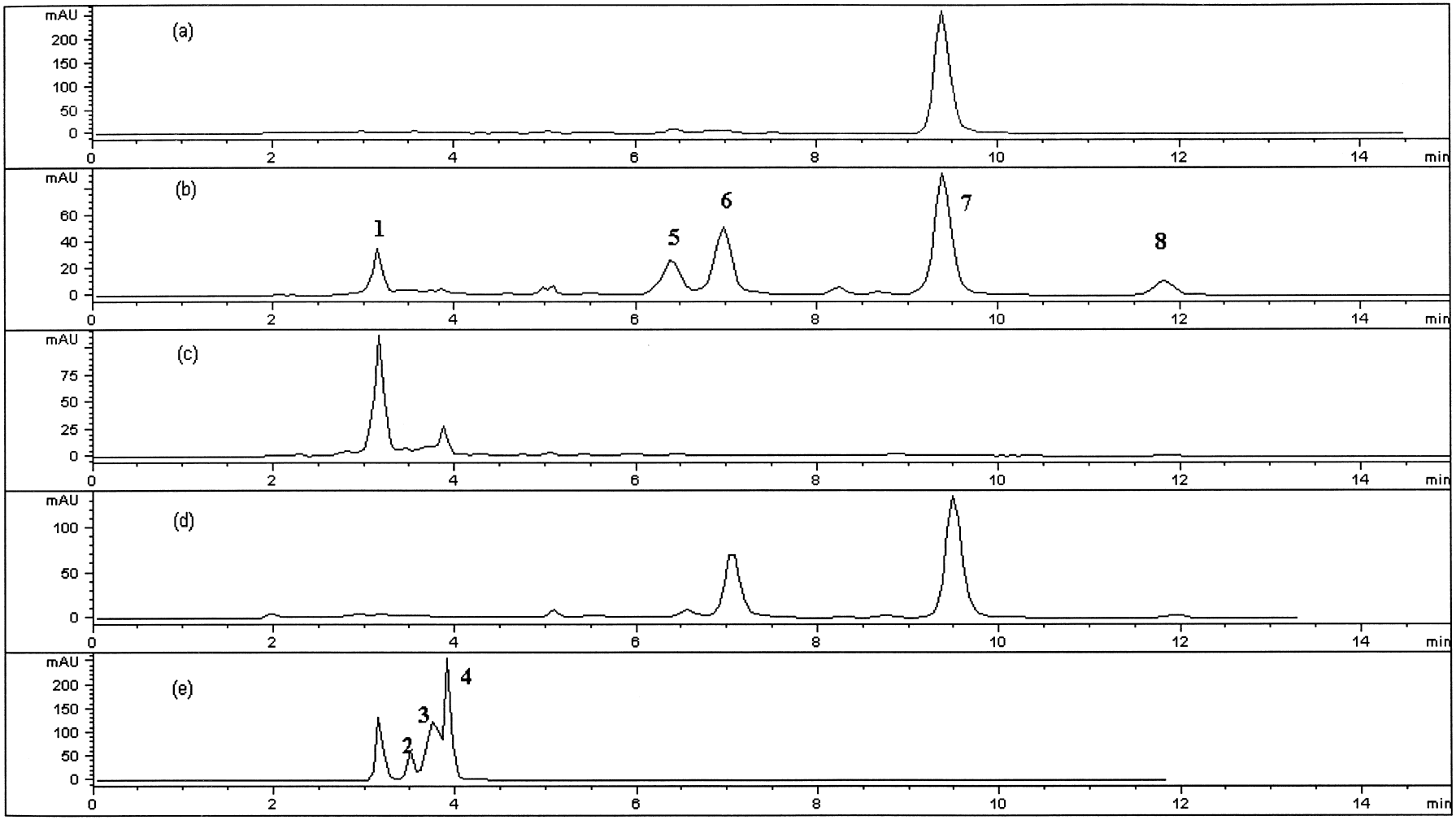


Fig. 1. HPLC chromatograms monitoring the degradation of 9′-*cis*-norbixin standard (a), degraded in (b) *n*-butyl acetate, (c) ethane-1,2-diol and (d) 0.1 M NaOH; (e) isomers of hydrolysed C_{17}. Peaks: 1, C_{17} (de-esterified); 2, 3 and 4, C_{17} isomers; 5, *trans*-norbixin; 6 di-*cis* norbixin; 7, 9′-*cis*-norbixin; and 8, mono (not 9′)-*cis* norbixin.

3.3. Formation of toluene and m-xylene in heated model systems

The heating regimes employed are as detailed in Section 2.

3.3.1. Regime (1)

Fig. 2 shows the response of the m/z 106 channel to peaks corresponding to *m*-xylene from a sample of 9′-*cis*-bixin at 50 mg/kg in vegetable oil heated at 75°C. The results are given in Table 5 and show the formation of *m*-xylene from 9′-*cis*-bixin only, the amount of which was proportional to 9′-*cis*-bixin concentration. The amounts of toluene or *m*-xylene formed from *trans*-bixin, were too low at this temperature to be distinguishable from blank controls. With heating at 140°C, toluene and *m*-xylene were formed from both *trans*- and 9′-*cis*-bixin. Considerably more *m*-xylene was formed compared to toluene, and the yield from 9′-*cis*-bixin was ca. 10-fold higher than from *trans*-bixin (Table 5).

3.3.2. Regime (2)

The results of heating 9′-*cis*- and *trans*-bixin, at 500 mg/kg in vegetable oil, at 140°C for 30 and 60 min are given in Table 6. Negligible amounts of toluene and *m*-xylene were formed from *trans*-bixin. A small amount of toluene was formed from 9′-*cis*-bixin. As expected, relatively large amounts of *m*-xylene (at ca. 60 mg /l) were formed from 9′-*cis*-bixin.

3.3.3. Regime (3)

With heating in propane-1,2-diol at 75°C, no significant amount of toluene was formed from 9′-*cis*-norbixin (Table 7). There was no measurable *m*-xylene formation from the 50 mg/kg 9′-*cis*-norbixin sample and only a low level from the 500 mg/kg 9′-*cis*-norbixin solution. Levels were much lower than seen from 9′-*cis*-bixin solutions at this temperature. With heating at 140°, both toluene and *m*-xylene formation was observed (Table 7).

Table 5
Formation of toluene and *m*-xylene from heated solutions of *trans*- and 9′-*cis*-bixin in vegetable oil

Bixin isomer (time/temp)	Starting concentration (mg/kg)	Toluene found (mg/kg)	*m*-Xylene found (mg/kg)
2 h at 75°C			
9′-*cis*-	50	0.09	0.20
9′-*cis*-	500	0.08	1.18
trans-	50	0.08	0.04
trans-	500	0.07	0.03
2 h at 140°C			
9′-*cis*-	50	0.16	4.7
9′-*cis*-	500	1.3	60.4
trans-	50	0.06	0.43
trans-	500	0.23	4.6

3.3.4. Regime (4)

The formation of toluene and *m*-xylene from 9′-*cis*-bixin and C_{17} was monitored over 2 h at 140°C in order to obtain an estimation of the relative rates of formation. Sample solutions at 50 mg/l were heated for 0, 10, 20 and 30 min in the head space autosampler. Heating times of 45, 60, 90 and 120 min were achieved using a laboratory oven followed by using the autosampler incubator for the final 30 min prior to analysis. The results are displayed in Figs. 3 and 4. They show a rapid formation of toluene and *m*-xylene from both 9′-*cis*-bixin and C_{17} over the first 30–45 min, which eventually slowed or ceased. Both toluene and *m*-xylene have been detected in the thermal degradation products of methyl- and ethyl-esters of C_{17} (McKeown, 1965), where yields were far lower than those from methyl-9′-*cis*-bixin. The results in Fig. 4 indicate that under the given conditions, toluene and *m*-xylene are produced from C_{17} at similar rates and yields.

In the C_{17} molecule there are two possible sites of elimination for toluene compared to just one for *m*-xylene (Fig. 5). This is in proportion to those predicted for 9′-*cis*-bixin (i.e. four versus two, respectively). However, steric and thermodynamic factors are thought to have the greatest influence on the competitiveness of these elimination processes (Scotter, 1995). Hence the differences in relative yields of toluene and *m*-xylene between 9′-*cis*-bixin and C_{17}, may be influenced by molecular size (i.e. conjugate chain length) where *m*-xylene elimination from the relatively shorter C_{17} molecule may be less competitive. Nevertheless, the C_{17}

Table 6
Formation of aromatics from bixin heated in vegetable oil at 140°C

Bixin isomer	30 min Toluene (mg/kg)	60 min Toluene (mg/kg)	30 min *m*-Xylene (mg/kg)	60 min *m*-Xylene (mg/kg)
9′-*cis*-	0.53	0.81	63.1	57.6
trans-	0.19	0.25	0.06	0.09

Table 7
Formation of toluene and *m*-xylene from heated solutions of 9′-*cis*-norbixin in propan-1,2-diol

Time/temperature	Starting concentration (mg/kg)	Toluene found (mg/kg)	*m*-Xylene found (mg/kg)
2 h at 75°C	50	nd[a]	
			nd
	500	nd	
			ca. 0.02
2 h at 140°C	50	0.04	
			0.05
	500	0.08	
			0.43

[a] nd, not detected.

molecule is of sufficient length to undergo similar electrocyclic elimination mechanisms to those proposed for 9′-*cis*-bixin to produce a 9-carbon species and *m*-xylene, or a 10-carbon species and toluene. Both the C_9 and C_{10} species are conjugated systems containing 5 effective double bonds, which predicts a wavelength of maximum absorption of ca. 325 nm in petroleum spirit (Scott, 1964). Allowing for λ max shift differences due to solvent, no measurable levels of compounds exhibiting distinctive maximum absorbance around 325 nm were found in degraded 9′-*cis*-bixin or 9′-*cis*-norbixin solutions by HPLC. However, the separation conditions used were not optimal for the analysis of these shorter-chain compounds which, due to their relatively lower hydrophobicity, were probably eluted amongst other reaction products with the solvent front.

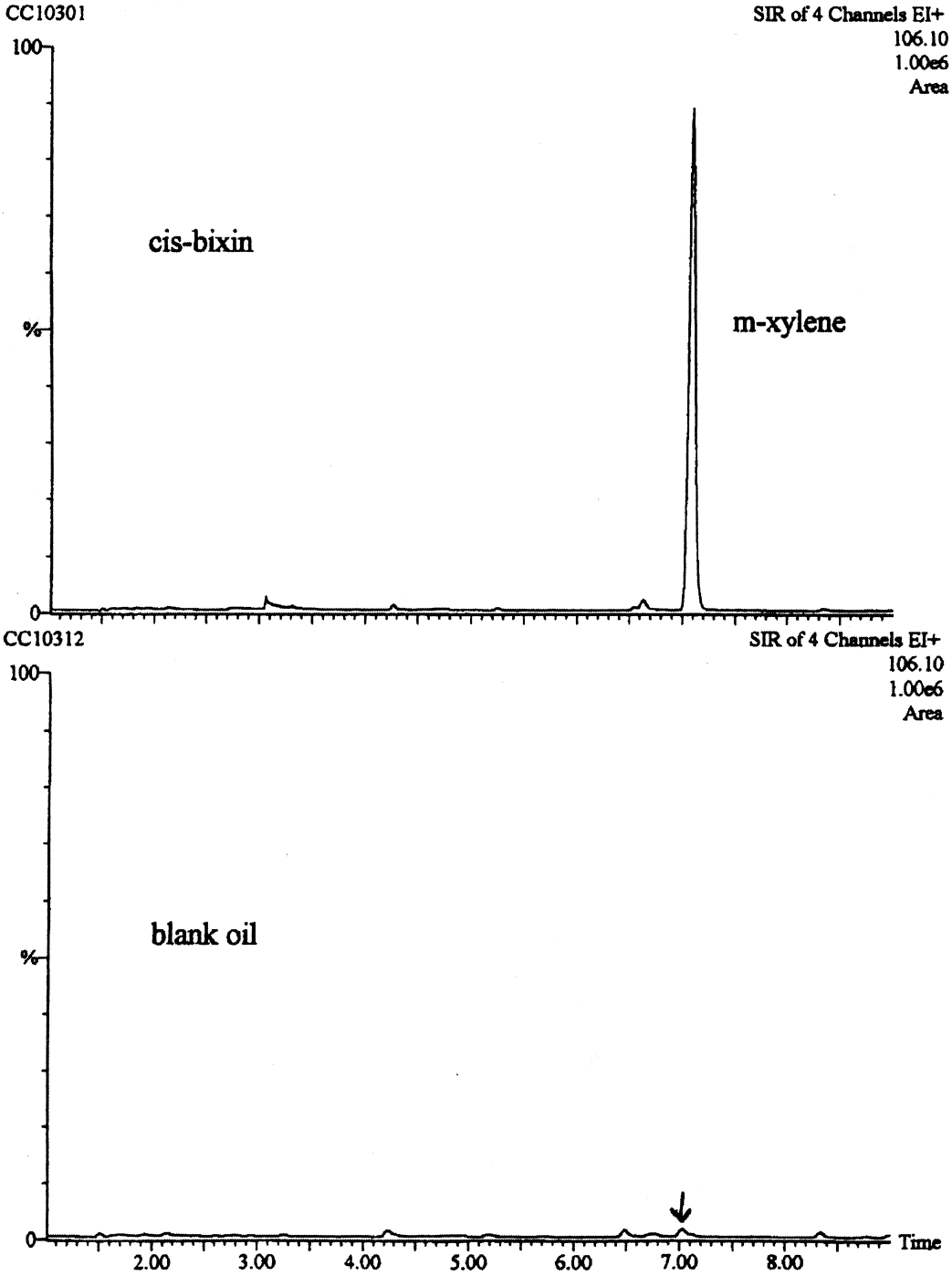


Fig. 2. Reconstructed GC–MS *m*/*z* 106 ion chromatogram showing peaks corresponding to *m*-xylene from a 50 mg/kg sample of 9′-*cis*-bixin in vegetable oil heated at 75°C.

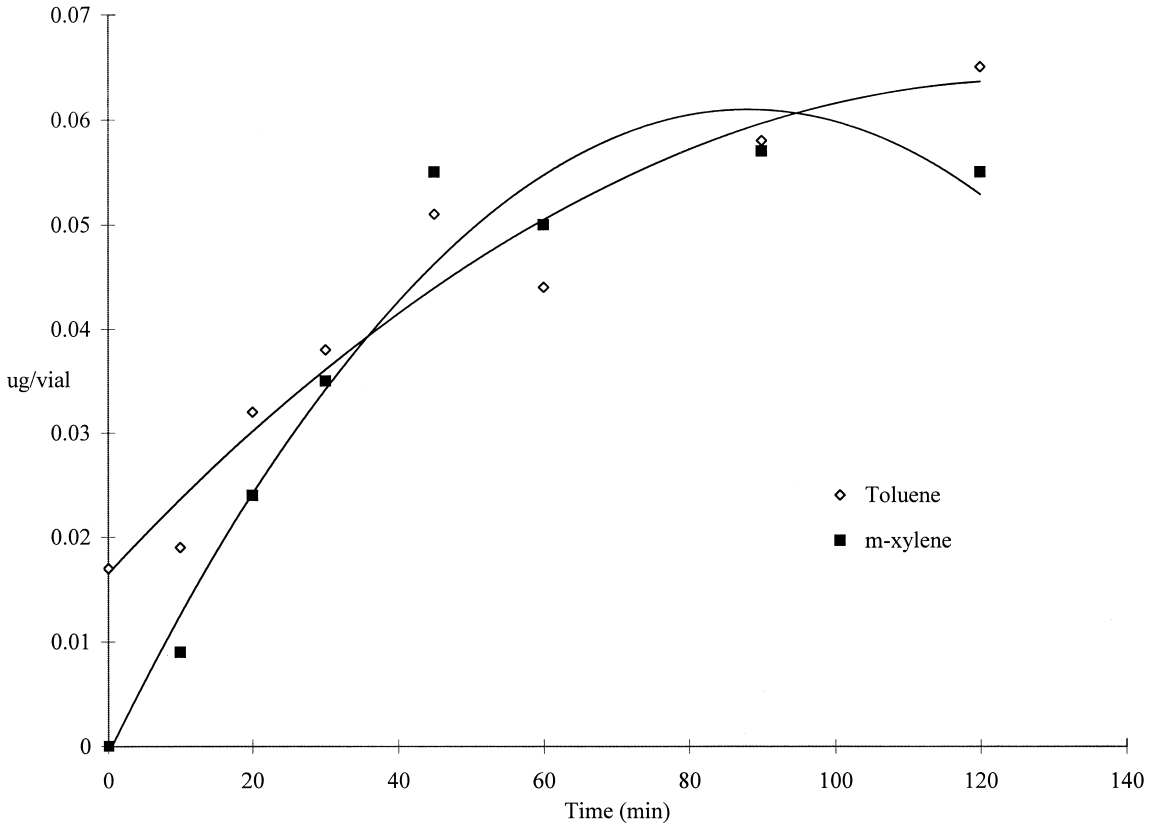


Fig. 3. Formation of aromatics from 9′-*cis*-bixin at 140°C.

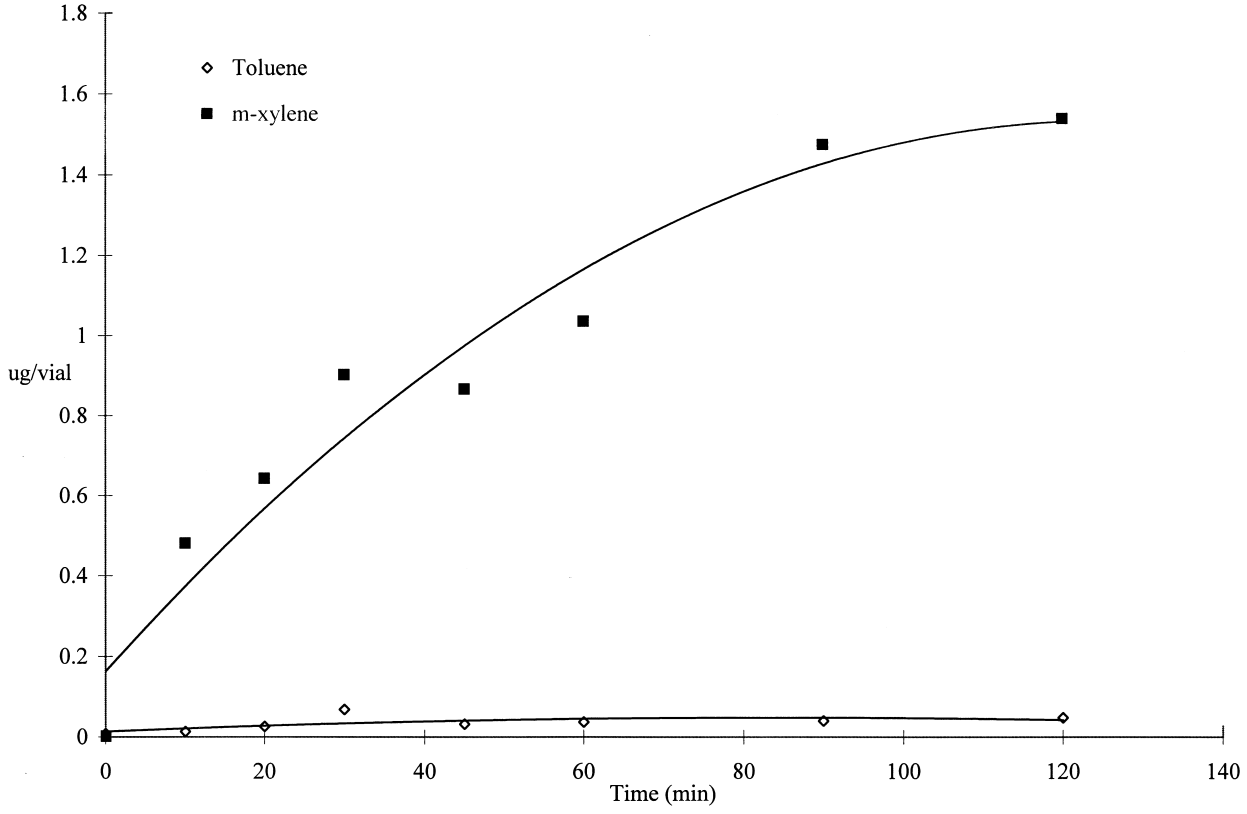


Fig. 4. Formation of aromatics from C_{17} at 140°C.

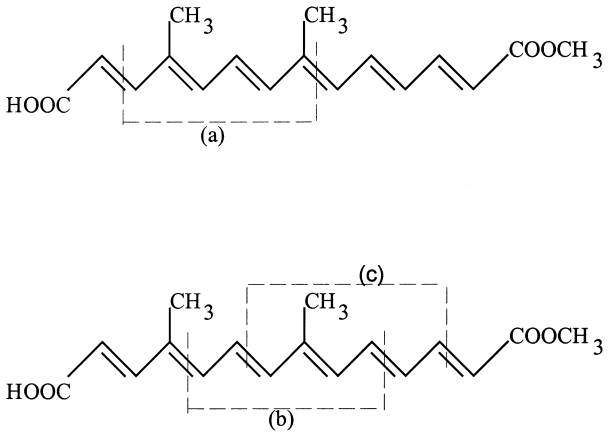


Fig. 5. Elimination sites for *m*-Xylene (a) and toluene (b, c) from C_{17}.

3.4. Aromatics formation in foods

A summary of the heating regimes is given in Table 2 and the results of analysis are given in Table 8.

3.4.1. Custard powder, dehydrated potato and extruded snacks

Samples were heated for 2 h at 60°C. *m*-Xylene was not detected in the headspace from the dehydrated potato control sample. In the headspace from the samples of annatto-containing custard powder and extruded snacks, *m*-xylene was detected and was estimated to be ca. 10–50 μg/kg in the samples.

3.4.2. Fish

Samples were heated for 1 h at 140°C. *m*-Xylene was detected in the headspace at levels corresponding to ca. 50–100 μg/kg in the coloured coley and ca. 150–200 μg/kg in the kipper. Fig. 6 shows the clear absence of *m*-xylene in the headspace from the control coley along with a low background level of toluene. Toluene is a rather ubiquitous environmental contaminant and can be picked up for instance, from exposure to fuel vapours and printing inks (MAFF, 1995).

3.4.3. Cheese

Samples were heated at 140°C in propane 1,2-diol for 1 h. Samples of Red Leicester and Cheshire cheese containing annatto were analysed alongside a sample of uncoloured Cheshire cheese spiked with ca. 50 mg/kg 9′-*cis*-norbixin. Toluene was not detected in the headspace from any of the samples and *m*-xylene was found only in the headspace from the sample of Red Leicester cheese. Compared to the spiked Cheshire cheese sample (containing 50 mg/kg 9′-*cis*-norbixin), the Red Leicester cheese sample had a noticeably stronger orange colour.

When samples were heated at 140°C in glycerol (selected to assist dispersion), the spiked Cheshire cheese was replaced with a sample of coloured Cheshire cheese (as purchased), which was of similar colour intensity. *m*-Xylene was not observed in the headspace from the sample of uncoloured Cheshire, whereas low levels were consistently observed in the headspace from the coloured Cheshire. Higher levels were observed in headspace from the Red Leicester cheese. The amounts of *m*-xylene found in these samples appeared to be consistent with colour intensity.

Table 8
Formation of *m*-xylene from heated annatto-containing foods

Sample	Annatto present (Y/N)	Heating conditions	*m*-Xylene (μg/kg)
Dry foods			
Dehydrated potato	N	2h/60°C	< 3
Custard powder	Y	2h/60°C	10
Extruded snack (a)	Y	2h/60°C	20
Extruded snack (b)	Y	2h/60°C	50
Fish			
Uncoloured Coley	N	1h/140°C	< 1
Coley (+ 50 mg/kg)	Y	1h/140°C	50–100
Kipper	Y	1h/140°C	150–200
Cheese			
Uncoloured Cheshire	N	1h/140°C In propane 1,2-diol	nd[a]
Cheshire (+ 50 mg/kg)	Y		nd
Red Leicester	Y		3
Uncoloured Cheshire	N	1 h/140°C In glycerol	< 3
Coloured Cheshire	Y		5
Red Leicester	Y		20
Miscellaneous			
Bread crumbs	Y	1 h/100°C	27
Margarine	Y	1 h/100°C	9

[a] nd, not detected.

3.4.4. Margarine and bread crumbs

Samples were heated for 1 h at 100 °C. The *m*-xylene content of the headspace from the bread crumbs corresponded to ca. 27 μg/kg in the sample. For the coloured margarine the corresponding level was ca. 9 μg/kg.

4. Conclusions

Thermal degradation of the principal annatto colouring agent, 9′-*cis*-bixin, has been shown to be thermodynamically facile. The degradation is complicated by the many competing isomerisation reactions which proceed at different rates towards equilibrium. This is further complicated by the simultaneous and irreversible formation of C_{17} (with the associated production of *m*-xylene), which itself may degrade further to produce *m*-xylene or toluene.

Norbixin degrades similarly but more slowly and only low levels of degradation products were observed during

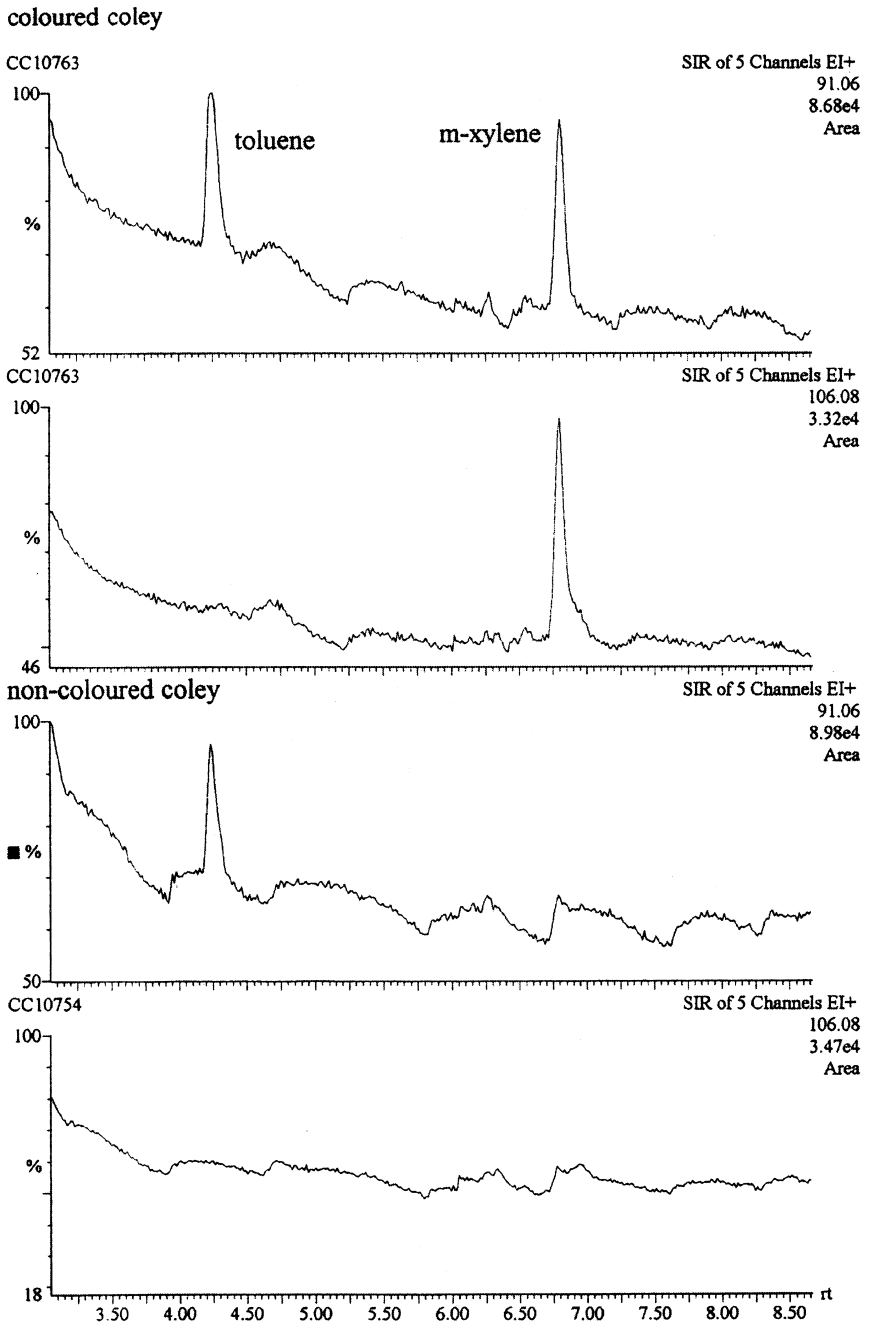


Fig. 6. Reconstructed GC–MS *m*/*z* 91 and 106 ion chromatograms showing peaks corresponding to *m*-xylene and toluene detected in the headspace from coloured and non-coloured coley fish samples.

heating of *trans*-bixin. The formation of toluene and *m*-xylene from the thermal degradation of 9′-*cis*-norbixin has, to our knowledge, not previously been reported in the literature. These results confirm those found for the measurement of C_{17} analogues following the degradation of norbixin in model systems.

m-Xylene has been detected in the head space of heated model systems containing 9′-*cis*-bixin, 9′-*cis*-norbixin and C_{17}. The levels of *m*-xylene formation were consistent with bixin/norbixin concentration. As expected, the formation of aromatic degradation products occurred more rapidly at higher temperatures but other (e.g. oxidative) degradation schemes could not be accounted for. Whilst it was not possible to calculate the actual yield of *m*-xylene from the various foods analysed using head space GC, the very low levels found suggest a low percentage of 9′-*cis*-norbixin loss.

The results indicate that annatto is readily degraded to *m*-xylene and to a lesser extent toluene, both of which may be detected in the head space of heated foods. Theoretically, since one molecule of bixin has the potential to produce a maximum of two molecules of *m*-xylene,

degradation at the maximum permissible level of 50 mg/kg for annatto (e.g. as 9′-*cis*-norbixin in Red Leicester cheese) would equate to ca. 30 mg/kg total *m*-xylene. In practice however, degradation is rather slow under normal heating conditions for foods and toluene and *m*-xylene levels did not exceed 200 μg/kg in any food studied. Moreover, during 'real' cooking situations it is reasonably feasible that these volatile compounds will suffer various fates, depending upon the various food components, the heating conditions employed and where the degradation occurs. For instance, aromatics formed at the surface of the food are more likely to be volatilised and effectively removed, whereas formation of aromatics within the interior of the food may be effectively trapped and solubilised in the lipid phase. The analysis of annatto containing foods for *m*-xylene, toluene and C_{17} both before and after cooking under real conditions may provide the necessary data to give an insight into the significance of annatto degradation and the subsequent exposure to these compounds via the diet.

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