Analysis of Annatto (*Bixa orellana*) Food Coloring Formulations. 2. Determination of Aromatic Hydrocarbon Thermal Degradation Products by Gas Chromatography

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Twenty samples of commercial annatto formulations have been analyzed for *m*-xylene and toluene using ambient alkaline hydrolysis, followed by solvent extraction and capillary gas chromatography. Fifteen of the samples contained <5 mg/kg toluene, four samples contained between 5 and 10 mg/kg toluene, and one sample contained 12 mg/kg toluene. The amounts found of *m*-xylene were 200 mg/kg (one sample), 160 mg/kg (one sample), between 30 and 88 mg/kg (four samples), between 7 and 25 mg/kg (seven samples), and <5 mg/kg (seven samples). Bixin-in-oil formulations contained the highest *m*-xylene concentrations and also gave the largest increase in headspace *m*-xylene concentration when heated in closed systems. The results are evidence for the thermal degradation of annatto during source extraction and processing, resulting in contamination by internal generation of both bixin and norbixin types with aromatic hydrocarbons. Two samples of norbixin of known production history (i.e., thermal versus nonthermal processes) were analyzed specifically to identify possible differences in their degradation component profiles. They were found to differ significantly in *m*-xylene content, which is consistent with their respective production histories.

Keywords: *Annatto; Bixa orellana; bixin; norbixin; coloring materials; additives; degradation; xylene; toluene; aromatic hydrocarbons; gas chromatography*

INTRODUCTION

The *trans*-monomethyl ester of 4,8-dimethyltetradecahexaenedioic acid, otherwise known as C_{17}, and several analogous compounds have been reported to be the main colored thermal degradation products of bixin and norbixin, the principal coloring components of annatto food coloring (McKeown, 1963, 1965; Scotter, 1995). The formation of C_{17} and its analogues is accompanied by the release of aromatic compounds, notably *m*-xylene, toluene, and, to a lesser extent, dimethyldihydronaphthalene and toluic acid and its methyl ester.

Simple aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylenes are common environmental contaminants. Although certain data suggest that benzene is genotoxic and can induce leukemia in some humans exposed to relatively high occupational levels, there is relatively little toxicological data available on toluene, ethylbenzene, and xylenes (MAFF, 1995). U.K. dietary intakes of these compounds have been estimated at 7.7 μg per person per day for toluene, <5 μg per person per day for ethylbenzene and xylenes, and <2.4 μg per person per day for benzene (MAFF, 1995). No U.K. Tolerable Daily Intake (TDI) values have been set for these compounds in foods, but they are nonetheless not desirable in food commodities.

Preliminary studies on the presence of aromatic degradation products in annatto formulations (Scotter et al., 1998) revealed that two samples of norbixin contained approximately 22 and 1 mg/L *m*-xylene, respectively, and both samples contained <1 mg/L toluene. The marked difference in *m*-xylene contents between the two samples was considered to be possible evidence of production history, that is, thermal versus nonthermal procedures. Suitable methods of analysis are required for studies on the effects of storage on annatto stability and the processing of annatto-containing foodstuffs. Furthermore, the EU specifications for annatto (EC, 1995) have separate definition and purity criteria for (i) solvent-extracted bixin and norbixin, (ii) alkali-extracted annatto, and (iii) oil-extracted annatto. Solvent-extracted bixin and norbixin formulations are often referred to as indirectly extracted annatto formulations, whereas alkali- and oil-extracted annatto formulations are termed directly extracted. The JECFA specifications for annatto are similarly separated into annatto extracts (oil- and alkali-extracted) and annatto extracts (solvent-extracted) (Joint Expert Committe on Food Additives, 1996). There is therefore a need to differentiate between these two types of annatto formulations.

The use of food colors in the United Kingdom is controlled by a European Community Directive (EC, 1994), which contains a list of permitted colors, a list of foodstuffs to which these colors may be added, and, where appropriate, maximum limits on the level of addition. Annatto extracts are listed among those colors that may be used singly or in combination in certain foods up to the maximum levels specified (on a ready-for-consumption basis). Specific purity criteria for food colors, including annatto, are given in a separate Commission Directive (EC, 1995). Although this directive lays down purity criteria for solvent residues in solvent-extracted annatto (i.e., acetone, methanol, and hexane at not more than 50 mg/kg, singly or in combi-

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nation; dichloromethane at not more than 10 mg/kg), no general provision is made for the presence of aromatic degradation products.

The aim of this work was to develop an analytical procedure for the determination of the principal aromatic hydrocarbon thermal degradation products *m*-xylene and toluene in annatto formulations that avoids the use of heat, thereby minimizing the degradation of annatto in situ, and to ascertain whether the data are evidence for thermal degradation of annatto during processing.

MATERIALS AND METHODS

Samples comprised various commercial bixin and norbixin preparations in the form of dry powders (or granules), encapsulated powders, oil suspensions/solutions, and aqueous solutions. Samples were acquired over a three year period from several sources (Scotter et al., 1998). All reagents were of recognized analytical grade unless specified otherwise. Ethylbenzene (99.8%), toluene (99.8%), *m*-xylene (99+%), and spectrophotometric grade *o*-xylene (98%) were obtained from Aldrich Chemical Co. Limited (Gillingham, U.K.).

Extraction Procedure. Between 0.1 and 0.2 g of sample was accurately weighed into a 10 mL glass vial fitted with a screw-cap and PTFE-faced septum. Methanolic KOH solution [10% (w/v), 2 mL] and *o*-xylene recovery standard (10 mg/L in pentane, 2 mL) were added. The vial was capped, wrapped in aluminum foil, and roll-mixed overnight. The pentane phase was transferred to a second vial and the methanolic phase re-extracted with 2 × 1 mL portions of pentane by recapping the vial and shaking for ~1 min. If necessary, emulsions were broken by centrifugation at 5000 rpm for ~5−10 min. The pentane extracts were pooled in the second vial and washed with 2 × 1 mL portions of water, which was removed via a Pasteur pipet. The water washings were back-extracted with a fresh 1 mL portion of pentane and the combined pentane extracts dried by passage through a 1 × 5 cm column of anhydrous sodium sulfate. The pentane extract was collected in a 5 mL volumetric flask containing 200 μL of internal standard solution (ethylbenzene in pentane, ~70 mg/L) and the column rinsed with pentane until a total of 5 mL was collected. The extract was analyzed by capillary gas chromatography.

Capillary Gas Chromatography. The system comprised a Carlo-Erba Mega series GC fitted with a model A-200S autosampler and fused silica capillary column of either (i) a 30 m × 0.25 mm i.d. × 0.5 μm film thickness J&W Durabond 210 [(50% trifluoropropyl)methylpolysiloxane) column (Jones Chromatography, Hengoed, U.K.] or (ii) a 30 m × 0.25 mm i.d. × 0.4 μm film thickness Restek Rt-TCEP [1,2,3-tris(2-cyanoethoxy)propane] column (Thames Chromatography, Windsor, U.K.). Hydrogen carrier gas was maintained at ~40 cm/s. Split mode injection (20:1) was used at a temperature of 250 °C, with an injection volume of 8 μL and septum purge flow of 5 mL/min. Flame ionization detection at 250 °C was used with the fuel gas flows adjusted for optimum detection of aromatic hydrocarbons. The columns were temperature programmed at (i) 40−50 °C at 2 °C/min, hold for 10 min, or (ii) 40 °C for 3 min, raised to 100 °C at 6 °C/min, respectively.

Calibration was carried out by injection of mixed standards prepared in pentane over the concentration range ~0.5−5.0 mg/L, containing a fixed amount of ethylbenzene internal standard at 4 mg/L. Detector response of each component was normalized against ethylbenzene, and calibration lines were constructed using response ratios. Detector response was linear for each component over this range (r^2 typically ≥ 0.998).

o-Xylene was added at the hydrolysis stage to calculate recoveries of aromatics. This compound was selected because of its physicochemical similarity to *m*-xylene, and it was well separated from *m*-xylene and the other aromatic peaks under the GC conditions stated. A reagent blank was run with each batch of samples as a check for interferences. Under the conditions given, the limits of determination for toluene and *m*-xylene in annatto formulations were both 5 mg/kg, based on a minimum signal-to-noise ratio of 5:1.

Table 1. Recovery Data ($n = 6$) for Corn Oil Spiked with Toluene and *m*-Xylene

	spike level (mg/kg)	mean	SD	RSD (%)
m-xylene found/added (%)	10.3	101	8.3	8.2
	103	101	2.5	2.5
toluene found/added (%)	11.6	82	1.6	2.0
	116	85	3.8	4.5

Headspace GC-MS Analysis. This series of experiments was undertaken to compare with the results found for ambient hydrolysis and to monitor the in situ production of *m*-xylene and toluene in annatto during heating.

Approximately 0.1 g of sample was accurately weighed into a headspace vial and dispersed in 1 mL of water, except for the oil-based formulations, which were dispersed in 1 mL of olive oil. An internal standard mixture comprising benzene-d_6 and *p*-xylene-d_{10} was added to each vial at a level of 2.5 μg/vial. The samples were mixed on a rocking tube roller for 1 h prior to analysis. Calibration standards comprising benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes were prepared over the range 0−20 μg/vial in both water and olive oil.

Analyses were performed on a Carlo Erba 4160 GC system fitted with a model HS800 automated headspace sampler and a 30 m × 0.32 mm × 0.5 μm film thickness DB-Wax (polyethylene glycol) capillary column (Jones Chromatography, Hengoed, U.K.). This was interfaced to a VG model 12-250 Quadropole mass spectrometer (Micromass, Wythenshawe, U.K.). Helium carrier gas was used at a flow rate of 1 mL/min. The column was operated isothermally at 70 °C with an injection temperature of 250 °C and a split ratio of 20:1. After incubation at 90 °C for 20 min, 1.0 mL of headspace was sampled. The mass spectrometer monitored ions at *m*/*z* 78 for benzene, *m*/*z* 91 for toluene, and *m*/*z* 106 for ethylbenzene and xylenes, with ions at *m*/*z* 84 for benzene-d_6 and *m*/*z* 98 for *p*-xylene-d_{10}. Each ion was monitored for 80 ms with a delay of 10 ms, giving a cycle time of 450 ms.

RESULTS AND DISCUSSION

Analysis of Spiked Corn Oil. Corn oil spiked with toluene and *m*-xylene at levels of approximately 10 and 100 mg/kg were analyzed repeatedly. The results are given in Table 1 and have been corrected for the recovery of the *o*-xylene internal standard (81 ± 3%). The recovery figures for toluene and *m*-xylene were acceptable at the given levels of spiking.

Analysis of Annatto Formulations. Twenty samples of commercial annatto formulations were analyzed. The samples were acquired over a three year period from several sources (Scotter et al., 1998). The results for the analysis of annatto formulations toluene and *m*-xylene are given in Table 2. The results shown in this table are the averages of duplicate portions analyzed, and the agreement between duplicates was excellent at ±4%.

Toluene and *m*-Xylene Content of Samples. Fifteen of the 20 samples analyzed contained <5 mg/kg toluene, four samples contained between 5 and 10 mg/kg toluene, and one sample contained 12 mg/kg toluene. These results do not indicate significant formation of toluene via thermal degradation of annatto but may be indicative of either low-level adventitious contamination from processing or residual contamination from thermal degradation followed by losses during storage and handling.

Eight samples contained ≤5 mg/kg *m*-xylene, and a further six samples contained between 6 and 23 mg/kg *m*-xylene. Four of the remaining six samples (S17, S10,

Table 2. Analysis of Annatto Formulations for Toluene and *m*-Xylene[a]

sample	sample type	extraction method	toluene (mg/kg)	*m*-xylene (mg/kg)
S1	bixin powder	indirect	<5	17
S2	bixin powder	indirect	<5	8
S3	bixin crystalline	direct	<5	5
S4	bixin crystalline	direct	<5	7
S5	bixin oil suspension	direct	6	160
S6	norbixin solution	direct	<5	<5
S7	norbixin granules	direct	<5	9
S8	norbixin granules	direct	12	58
S9	norbixin powder	direct	6	7
S10	norbixin granules	direct	8	38
S11	bixin encapsulated	NA	<5	<5
S12	norbixin solution	direct	<5	<5
S13	bixin water dispers.	direct	<5	88
S15	norbixin granules	direct	<5	23
S16	bixin crystalline	direct	<5	<5
S17	norbixin solution	direct	<5	30
S18	norbixin solution	direct	<5	<5
S19	bixin oil solution	NA	5	200
S20	norbixin spray-dried	NA	<5	<5
S21	bixin oil suspension	NA	<5	<5

[a] All figures are corrected for recovery. Results are averages of duplicate portions analyzed; the agreement between duplicates was excellent at ±4%. NA, not available.

S8, and S13) contained 30, 38, 58, and 88 mg/kg *m*-xylene, respectively. Samples S8, S10, and S17 were all norbixin formulations, and sample S13 was a water-dispersible bixin formulation. Sample S19, a bixin-in-oil solution, contained 200 mg/kg *m*-xylene (Table 2).

Comparison of Samples S17 and S18 of Known Production History. Sample S17 was an aqueous solution of norbixin obtained by an indirect extraction method, that is, solvent extraction followed by alkaline hydrolysis. S17 contained 30 mg/kg *m*-xylene (Figure 1). Sample S18 was obtained by direct aqueous alkaline extraction and contained <5 mg/kg of both toluene and *m*-xylene. These results indicate that contamination of annatto formulations with *m*-xylene is not restricted to oil-based bixin formulations but may also be significant in various norbixin formulations. Furthermore, there is evidence for the discrimination between annatto formulations produced via different production methods on the basis of their aromatics (and colored components) content. In a previous study (Scotter et al., 1998), these samples were subjected to detailed HPLC analysis to identify possible differences in their colored and degradation component profiles. The samples differed significantly in their *all-trans*- and *di-cis*-norbixin isomer contents. Because direct aqueous alkaline extraction may employ temperatures of up to 70 °C, there is scope for the formation of *trans*-norbixin via thermally driven isomerization. The isomer profiles obtained by HPLC analysis support this because some 6 times as much *trans*-norbixin was found in sample S18 compared to sample S17 (normalized figures). The different extraction procedures may, however, give rise to different isomer profiles due to their different solubilities and/or stabilities in the extraction medium, which may be further complicated by the effects of light and oxygen during extraction and handling and by the nature of the source material. The presence of C_{17}-type polyene degradation products is indicative of the use of higher extraction temperatures. It is possible that such processes could occur during solvent extraction of annatto prior to hydrolysis to norbixin. Furthermore, study into the mechanism of the thermal degradation of bixin (Scotter, 1995) has revealed that it may be possible for

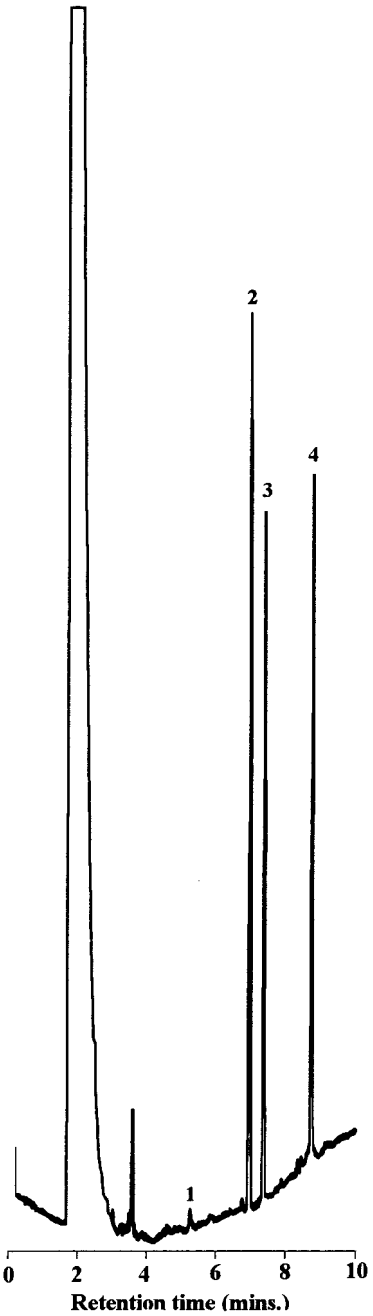

Figure 1. GC-FID chromatogram of commercial norbixin sample extract S17 containing ~30 mg/kg *m*-xylene. Peaks: (1) toluene; (2) ethylbenzene (internal standard); (3) *m*-xylene; (4) *o*-xylene (internal standard).

C_{17}-type polyenes to similarly degrade to shorter chain analogues accompanied by the further release of *m*-xylene or toluene. This may explain the tentative presence of *m*-xylene and the absence of C_{17}-polyenes in sample S17. The possibility that *m*-xylene may be present as a contaminant from an alternative route such as an impurity in the extraction solvent cannot, however, be ruled out.

It is not possible to draw firm conclusions about the relationship between the isomer and degradation product profile of annatto formulations and their production history from such limited analytical data. Although some differences in these parameters were apparent between samples S17 and S18, the analysis of further samples of annatto of known detailed production history is required to obtain sufficient data.

Table 3. Comparison of *m*-Xylene Contents Obtained by Ambient Hydrolysis GC-FID and after Heating by Headspace GC-MS Procedures

sample	ambient hydrolysis GC-FID (mg/kg)	increase following headspace incubation at 90 °C for 20 min (mg/kg)
S5	160	+270
S11	<5	+30
S13	88	<10
S17	30	<10
S19	200	+250
S20	<5	+40
S21	<5	+100

Samples of Oil-Based Formulations S5 and S19. Two samples of oil suspensions of bixin (S5 and S19) contained ca. 160 and 200 mg/kg *m*-xylene, respectively. These samples also contained 0.06 and 0.16% of the C_{17} degradation product, respectively, by HPLC (Scotter et al., 1998). There is therefore clear evidence that these samples had undergone degradation during processing.

Headspace GC-MS Analysis of Samples after Heating. Several samples were submitted for headspace GC-MS analysis to monitor the effect of heating the formulations in a closed controlled environment. The results of samples previously found to contain significant levels of *m*-xylene by ambient hydrolysis analysis are given alongside their corresponding results from headspace GC-MS analysis in Table 3. Figure 2 shows the chromatogram obtained from analysis of sample S19 extract. The effect of heating the samples was manifested in an observed increase in the corresponding levels of *m*-xylene; bixin-in-oil formulations showed the highest rise in *m*-xylene concentration on heating. It is anticipated that headspace GC-MS techniques could therefore be developed for the in situ monitoring of the thermal degradation of annatto in food systems.

CONCLUSIONS

A method for the determination of the aromatic degradation compounds toluene and *m*-xylene in annatto formulations has been developed. The method uses an ambient alkaline hydrolysis procedure, thus avoiding the possibility of toluene and *m*-xylene forming as artifacts in situ. Quantitative measurement was by capillary gas chromatography. Twenty samples of various annatto formulations have been analyzed. Toluene levels were generally low (≤12 mg/kg). Six samples comprising both bixin and norbixin formulations contained *m*-xylene in the range of 30−200 mg/kg. The highest levels were found in oil-based bixin formulations, which is not unexpected because bixin may be extracted from annatto seeds using hot oil. The presence of *m*-xylene in norbixin formulations has not previously been reported in the literature. Two samples of norbixin of known production history were analyzed specifically to identify possible differences in their degradation component profiles, in support of earlier HPLC data on the colored component profiles, that is, C_{17}. The samples were found to differ significantly in their *m*-xylene contents, which appear to be consistent with their respective production histories.

Although the estimated intakes of xylenes from dietary sources are considered to be relatively low compared with those estimated from environmental sources (MAFF, 1995), the levels found in annatto food color preparations, as reported here, do merit some consideration.

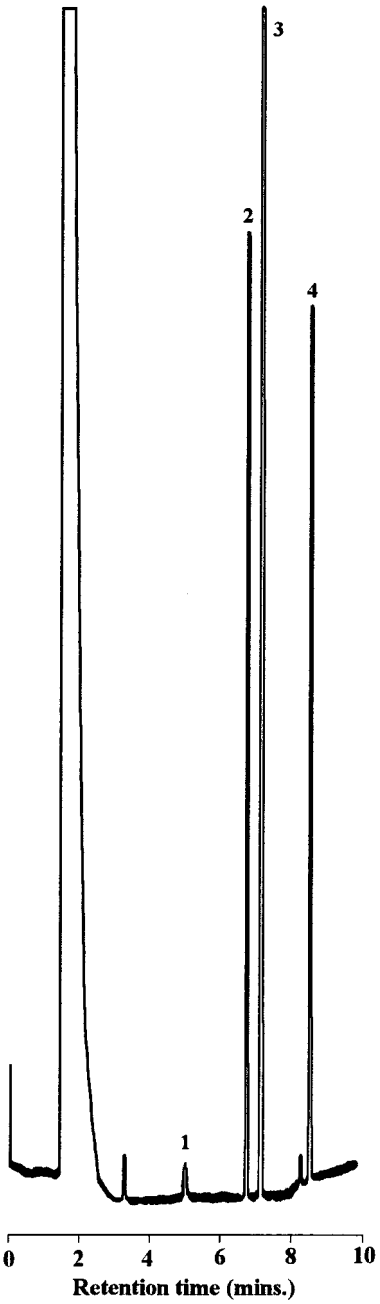

Figure 2. GC-FID chromatogram of commercial norbixin sample extract S19 containing ~200 mg/kg *m*-xylene. Peak numbers refer to those given in Figure 1.

The results from this study showed that *m*-xylene was present in certain annatto formulations up to a level of 200 mg/kg. If this is taken as a "worst case", then, because the annatto content of the formulation has been determined at 2500 mg/kg, the concentration ratio of *m*-xylene to annatto is 200/2500 = 0.08, that is, for every milligram per kilogram of annatto added to a food, 0.08 mg/kg of xylene will be present. A typical maximum annatto content of a foodstuff requiring this type of formulation is 25 mg/kg, which equates to 2 mg/kg *m*-xylene carried over. The significance of this level of *m*-xylene in a foodstuff, however, is not clear; hence, there is an obvious need for reliable analytical data on the annatto and *m*-xylene contents of foods. Moreover, the dietary intake of xylenes could be estimated more reliably if consumption data on annatto-containing foodstuffs were available.

With an analysis method thus established, investigations into the generation of toluene and *m*-xylene in foods and model food systems containing annatto are anticipated.

LITERATURE CITED

EC. European Parliament and Council Directive 94/36/EC on colours for use in foodstuffs. *Off. J. Eur. Communities* **1994**, *No. L237/13* (June 30).

EC. European Commission Directive 95/45/EC laying down specific purity criteria concerning colours for use in foodstuffs. *Off. J. Eur. Communities* **1995**, *No. L226/1-45* (July 26).

Joint FAO/WHO Expert Committee on Food Additives. *Compendium of Food Additive Specifications*; FAO Food and Nutrition Paper 52, Addendum 4, 46th Session; Geneva, Switzerland, Feb 6−15, 1996.

MAFF Joint Food Safety and Standards Group. *Benzene and Other Aromatic Hydrocarbons in Food—Average UK Dietary Intakes*; Food Surveillance Information Sheet 58; U.K. Ministry of Agriculture, Fisheries and Food: London, U.K., March 1995.

McKeown, G. G. Composition of oil-soluble annatto food colours. II. Thermal degradation of bixin. *J. Assoc. Off. Anal. Chem*. **1963**, *46* (5), 790−796.

McKeown, G. G. Composition of oil-soluble annatto food colours. III. Structure of the yellow pigment formed by the thermal degradation of bixin. *J. Assoc. Off. Anal. Chem*. **1965**, *48* (4), 835−837.

Scotter, M. J. Characterisation of the coloured thermal degradation products of bixin from annatto and a revised mechanism for their formation. *Food Chem*. **1995**, *53*, 177−185.

Scotter, M. J.; Wilson, L. A.; Appleton, G. P.; Castle, L. Analysis of annatto (*Bixa orellana*) food coloring formulations. 1. Determination of coloring components and colored thermal degradation products by high-performance liquid chromatography with photodiode array detection. *J. Agric. Food Chem*. **1998**, *46*, 1031−1038.

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