Measurement of Safrole and Isosafrole in Ham

M.P. ZUBILLAGA and G. MAERKER

ABSTRACT

Safrole, isosafrole, and mixtures of the two compounds were added to boiled or cooked ham at the 100 ppm level to simulate products that might be obtained in a smoking process in which sassafras wood is used. Samples of the ham were extracted with mixtures of hexane:ethyl acetate by a modified "dry column" procedure and were freed from the bulk of the accompanying neutral lipids by HPLC or by preparative TLC. Quantitation was by capillary GC with FID detection. Recoveries ranged from 85–95% depending on the procedure used.

INTRODUCTION

SAFROLE (4-allyl-1,2-methylenedioxybenzene) is a natural constituent of the sassafras plant and is a major component (approximately 80-90%) of its oil (Guenther, 1949). Safrole is also a minor constituent of other essential oils such as those from star anise, camphor, nutmeg, cinnamon leaf, and mace oil (Guenther, 1952). Isosafrole, 1,2-(methylenedioxy)-4-propenylbenzene, an isomer of safrole that is known to exist both in a *cis*-and a *trans*-form, has not been reported in oil of sassafras but may exist in other oils such at that of ylang ylang and perhaps some others (Guenther, 1952). Dihydrosafrole has not been reported to occur in nature. As a result of experimental findings that safrole and related compounds are carcinogens, the Food & Drug Administration published an order in 1960 (Anonymous, 1960) prohibiting the food additive use of safrole, isosafrole, dihydrosafrole and oil of sassafras. Recently there has been some concern that the use by some meat processors of sassafras wood in the smoking process might cause the addition of safrole or isosafrole to processed meat.

A number of analytical procedures are available for the determination of safrole and related compounds in alcoholic and nonalcoholic beverages (AOAC, 1984; Curró et al., 1987; Galli et al., 1984; Liddle and De Smedt, 1978); however, they appear to be less suitable for the analysis of meat, since they depend on an initial steam distillation step to extract the safrole, which is lipid soluble.

The objective of this study was the development of a method for the detection and estimation of safrole and isosafrole in ham at a contamination level of 100 ppm. The envisioned method consisted of three steps: (1) separation of the essential oils from the bulk sample by a modified dry column extraction procedure (Maxwell et al., 1980), (2) isolation of the analytes from co-extracted lipids, and (3) measurement of the analytes by suitable means.

MATERIALS & METHODS

Materials

Safrole (97%) and isosafrole (90%) were purchased from Aldrich Chemical Company, Milwaukee, WI, and m-tolyl acetate from C.T.C. Organics, Atlanta, GA. Isosafrole, as purchased, contained 15% *cis*-isomer and 85% *trans*-isomer as determined by GC and NMR. Celite 545 was purchased from Fisher Scientific, Malvern, PA. Other chemicals were of reagent grade quality. Domestic cooked or smoked and boiled ham sliced Deli style (2 mm thick) was purchased from retail stores. All solvents used were "distilled in glass grade" and for HPLC were degassed by vacuum filtration through a 0.2 μm filter. TLC plates, high efficiency, hard layer (HETLC-GHL) and prescored (20 × 20 cm) GHL (250 microns) were purchased from Analtech, Newark, DE. The solvent used throughout this study was hexane:ethyl acetate (95:5, v/v) unless stated otherwise and will be referred to as hexane:ethyl acetate or "the solvent".

Stock solutions

Safrole or isosafrole (50 mg weighed accurately) was diluted to 10 mL total volume with hexane:ethyl acetate.

Model solutions

Stock solutions of safrole or isosafrole (100 μL) were diluted to 25 mL total volume with hexane:ethyl acetate. Model solutions of 90/10 and 50/50 safrole/isosafrole were prepared from the appropriate volumes (total 100 μL) of safrole and isosafrole stock solutions diluted to 25 mL with the same solvent.

Dry column extraction of safrole/isosafrole from spiked meat

The procedure reported previously for the extraction of lipids (Maxwell et al., 1980) was modified for the current purpose. Lean ham, from which visible fat had been trimmed, was slightly crushed in a mortar to break its initial structure. The ham, (5.0g weighed accurately) was transferred to a porcelain mortar and to its surface was added 100 μL of a stock solution (500 μg) of either safrole or isosafrole or of 100 μL total of appropriate volumes of the two stock solutions to give 90/10 or 50/50 (by volume) safrole/ isosafrole mixtures. Anhydrous sodium sulfate (20g) and Celite 945 (15g) were added to the mortar and were ground with the meat as described previously (Maxwell et al., 1980). The resulting dry powder was transferred quantitatively to a glass column containing a glass wool plug and a mixture of calcium acid phosphate/Celite 545 (1:9 ratio, w/w, 10g). The powder was wet carefully with 60 mL hexane:ethyl acetate and eluted with an additional 60 mL of the same solvent mixture. The first 25 mL eluate containing all the extractable safrole/ isosafrole, was collected, and the balance was discarded. The receiving flask was stoppered immediately after the eluate was collected, mixed thoroughly and stored for use in the TLC or HPLC isolation procedure.

Thin-layer chromatography

Prior to use, plates were washed by development with chloroform:methanol (2:1, v/v) and activated overnight in an air oven at 115°C. Plates used for preparative TLC were 250 micron, 20 × 20 cm prescored GHL plates. Dry column eluate (1 mL) was applied with a band applicator to three sections of the plate, while further column eluate (100 μL), spiked with safrole or isosafrole, was applied to the fourth section, which was to be used for visualization. The plate was developed in hexane:ethyl acetate, and the spiked section was cut off, sprayed with 50% H_2SO_4 and charred. The safrole/isosafrole band of main section of the plate was scraped, the scrapings were extracted with 4 mL hexane:ethyl acetate, and internal standard (20 μL), m-tolyl acetate (1 mg/mL in hexane:ethyl acetate), was added. The extract was centrifuged at 2000 rpm for 2 min, and the supernatant was passed through a 0.45 μm filter.

HETLC-GHL plates were used to monitor extraction of the column effluent. They were developed with hexane:ethyl acetate, sprayed with 50% H_2SO_4 and charred.

Liquid chromatography (HPLC)

Analytical HPLC was performed with an Autochrom, Inc. (Milford, MA). Model M500A pump, Model M112 CIM for three-solvent gradients, and a model M320 gradient workstation softward/hardware. The system included a Waters Associates (Milford, MA) model R-401 differential refractometer, a Rheodyne (Cotati, CA) model 7125 injector with a 20 μL loop, and a Spectra-Physics (Piscataway, NJ) SP 4290 computing integrator. The normal phase separation was performed on a 3.9 mm × 30 cm μ-Porasil column. The flow rate was 1 mL/min. The mobile phase consisted of hexane:ethyl acetate.

Concentration of HPLC fractions

Fractions (1-2 mL) collected from HPLC were placed in a Kuderna-Danish (K-D) concentrator equipped with 10 mL collection tube and a 150 mm Snyder distilling column. After addition of a boiling chip, the HPLC fraction was evaporated on the steam bath to a final volume of 500 μL, after the Snyder column and neck had been rinsed with a few drops of hexane:ethyl acetate, and internal standard (10 μL) m-tolyl acetate 40 μg/mL had been added.

Gas chromatography

Gas chromatography was performed on a Varian Model 3600 gas-chromatograph with flame ionization detector and a temperature programmable on-column capillary injector. The column was a 0.2 mm i.d. × 25 m bonded phase 5% phenylsilicone column with 0.33 μm film thickness (Hewlett-Packard Ultra #2). Helium was the carrier gas at 1 mL/min. The initial injector temperature of 70°C was held for 1 min and then programmed at 50°C/min to 250°C. Initial column temperature of 100°C was held for 2 min, programmed at 5°C/min to 115°C, held for 10 min. Detector temperature was 325°C. Samples (1-2 μL) of TLC extracts or of K-D concentrates were injected. Quantitation was by comparison of the area counts of safrole or isosafrole with those of internal standard.

RESULTS & DISCUSSION

THE METHOD DEVELOPED during the course of these studies consisted of the following steps (Fig. 1): extraction of safrole and/or isosafrole, together with neutral lipids, from spiked ham by a "dry column" procedure. This was followed by one of two alternative procedures for isolating and measuring the aromatic compounds: (1) thin layer chromatographic separation of components, scraping of the appropriate TLC band, extraction of the band and quantitation by GC or (2) HPLC separation of components, collection of the appropriate HPLC fraction, concentration of that fraction and quantitation by GC.

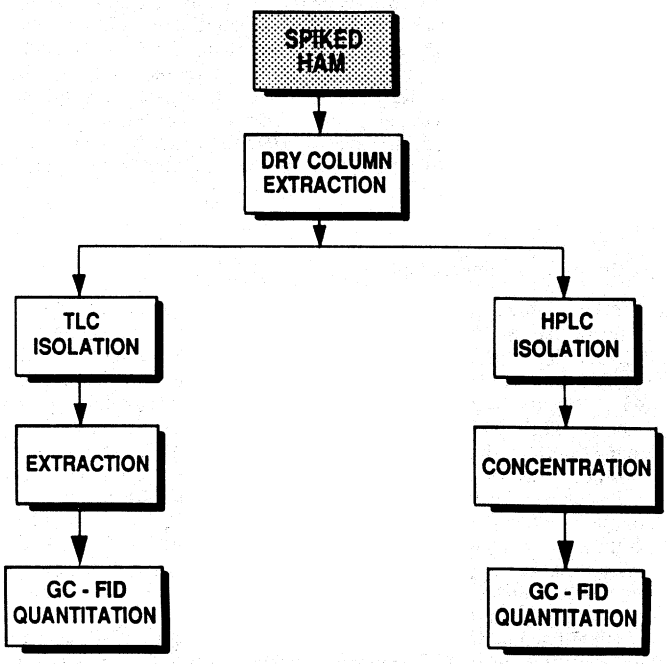

Fig. 1—Schematic presentation of the analytic method.

Extraction of safrole/isosafrole from meat

The "dry column" method for the extraction of lipids (Maxwell et al., 1980) was used because it is faster and more convenient than the Folch procedure (Folch et al., 1957) or the Bligh and Dyer (1959) method and permits separate isolation of neutral and polar lipids. It has proved reliable in the extraction of lipids from various food including meat (Adnan et al., 1981; Maxwell, 1984; Zubillaga and Maerker, 1984; Zubillaga et al., 1984).

The "dry column" procedure derived its name from the fact that water-containing matrices, e.g. meat, are ground with anhydrous sodium sulfate to capture their water content. After further grinding with Celite 545, a dry powder results that can now be extracted with nonaqueous solvents without the formation of emulsions. Since it was not known whether the essential oils were extractable with the neutral lipids or polar lipids, or whether they would be retained on the column, exploratory experiments were necessary. These experiments indicated that both safrole and isosafrole, added to meat at 100 ppm (500 μg/5g), could be removed from the column with a number of nonpolar solvents and that they eluted together with the neutral lipids. In the interest of a later quantitation step, it was desirable to maximize the concentration of the analytes in the dry column eluate. Although methylene chloride was an acceptable solvent because only 25 mL were required to recover the spike from a 5g meat sample, mixtures of hexane and ethyl acetate (95:5 or 97:3, v/v) were preferred because 25 mL of either of these mixtures gave complete extraction of safrole/isosafrole from the dry column and both of them were suitable as mobile phases in a subsequent HPLC isolation. On the other hand, hexane was less desirable because 100 mL were required. Completeness of extraction was judged by high efficiency TLC which permitted visualization of 50 ng (0.01% of the spike).

Isolation of safrole/isosafrole by HPLC

The dry column eluate containing the essential oils also carries relatively large amounts of neutral lipids from which they must be freed before safrole or isosafrole can be measured by GC. Excellent separation between safrole and triglycerides was obtained when the mobile phase was either 95:5 or 97:3 hexane:ethyl acetate (v/v) as shown in Fig. 2 for a mixture of safrole and triolein. However, safrole and isosafrole were not resolved in this system. The analyte peak was collected in a 2 mL eluate fraction in preparation for quantitation by GC. The GC analysis, however, could not be carried out without prior concentration of the sample, because the sample had been diluted 100 fold (20 μL → 2 mL) during HPLC isolation. As a result, since the safrole spike was 500 μg/5g in the ham, the HPLC eluate fraction contained not more than 0.2 ng/μL safrole, an amount which was insufficient for accurate GC measurement.

Concentration of HPLC eluate

Enrichment of the HPLC eluate presented some problems related to the volatility of safrole (and of isosafrole). Evaporation of the eluate at room temperature under a stream of nitrogen led to complete loss of the analyte in spite of its relatively high boiling point (232°C). Addition of a 5-fold (based on safrole) excess by weight of methyl oleate to the solution before evaporation did not have the intended effect of retarding safrole evaporation. Regardless of the rate of solvent removal, all or nearly all of the safrole was lost.

Use of a Kuderna-Danish concentrator gave better retention

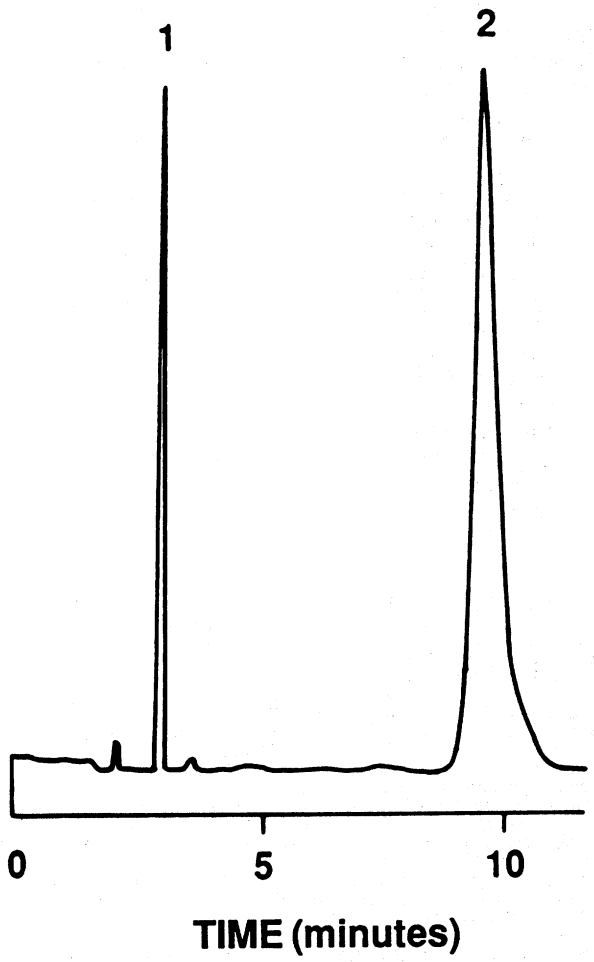

Fig. 2—HPLC separation of safrole and triglycerides. Mobile phase:hexane:ethyl acetate (95:5, v/v). Peak (1) safrole, peak (2) triolein.

of safrole, but reproducibility was only fair. Known amounts of safrole in 5 mL hexane:ethyl acetate were subjected to gentle concentration in a steam bath to a final volume of 500 μL. In seven replicate attempts the recovery of safrole was 92.2 ± 6%. When the original solution was taken to a final volume of 250 μL, the recovery of safrole was 85 ± 6% in five replicates. Problems with reproducibility among replicates were attributed to the difficulty in reading exact volumes remaining in the receiver of the concentrator after volume reduction, as well as to the high volatility of safrole. Similar difficulties with reproducibility were also encountered when isosafrole was subjected to the concentration step prior to GC analysis.

Isolation of safrole/isosafrole by TLC

Preparative TLC was examined as an alternative to HPLC in the isolation of safrole or isasafrole. The amount of neutral lipid present in the dry column eluate was 100 to 500 times that of the safrole that had been added to the meat at the 100 ppm level. Exploratory TLC experiments indicated that polar lipids were not present in the dry column eluate containing the safrole, that a good separation between neutral lipids and safrole/isosafrole could be achieved with the use of hexane:ethyl acetate as the developing solvent, that either of the essential oils applied to a silica plate could be recovered quantitatively by extraction of the scraped silica powder with the developing solvent and that the two isomers could not be resolved by TLC with any of the several developing solvents tried. The TLC procedure described in the Experimental section of this paper

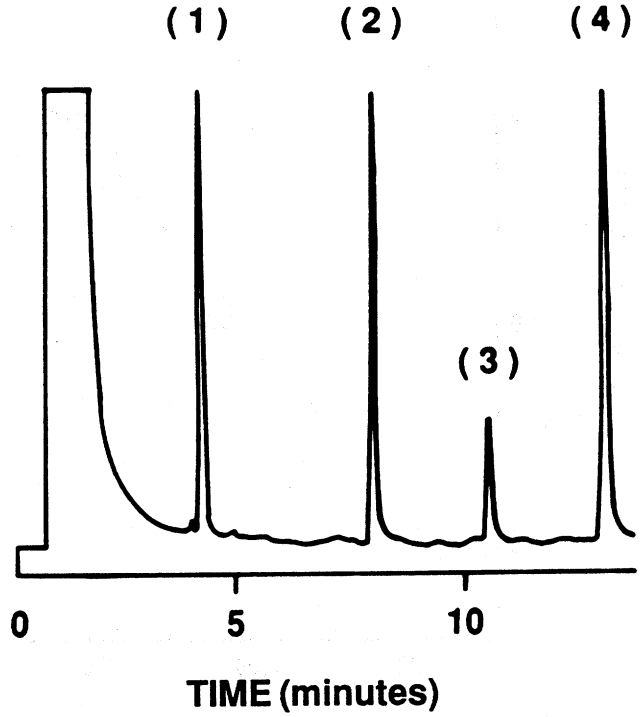

Fig. 3—GC separation of tolyl acetate (internal standard), safrole and isosafrole. (1) m-tolyl acetate, (2) safrole, (3) cis-isosafrole, (4) trans-isosafrole.

was followed in isolating the safrole or isosafrole contained in 1 mL of dry column elute or 1 mL of model solution. In each case the amount of essential oil applied to three sections of the TLC plate was 20 μg (assuming complete recovery from the dry column procedure) so that after extraction of the silica the safrole concentration of the extract should have been 5ng/μL, which is substantially greater than that obtained after HLPC isolation.

GC determination of safrole/isosafrole

In contrast to the lack of resolution by TLC or HPLC, excellent separation was obtained when mixtures of safrole and isosafrole were analyzed by GC (Fig. 3). The cis-and trans-isomers of isosafrole were also resolved by GC. Use of an internal standard, m-tolyl acetate, or direct comparison of peak areas, allowed calculation of safrole and/or isosafrole content. For studies of linearity of response, a stock solution containing safrole, isosafrole and m-tolyl acetate was prepared in hexane:ethyl acetate and then serially diluted as needed. Concentrations employed in this study ranged from 50 ng/component per injection to 5 ng/component per injection. Linearity of response data are shown in Fig. 4.

Recovery of safrole/isosafrole added to ham

Safrole, isosafrole and mixtures of the two compounds were added to ham at 500 μg per 5g meat and were isolated and measured by the procedures described above. Results are shown in Table 1. Recoveries were measured by GC by comparison of the peak areas to that of the internal standard as well as by comparison of the peak areas of the appropriately diluted "stock solution," the vehicle by which the spike had been added to the ham, with the peak areas of the recovered material.

The data indicate that the TLC isolation route, although it was somewhat more labor-intensive than the HPLC/concentration pathway, led to higher recovery. This was believed to be due to the losses incurred during the concentration step as

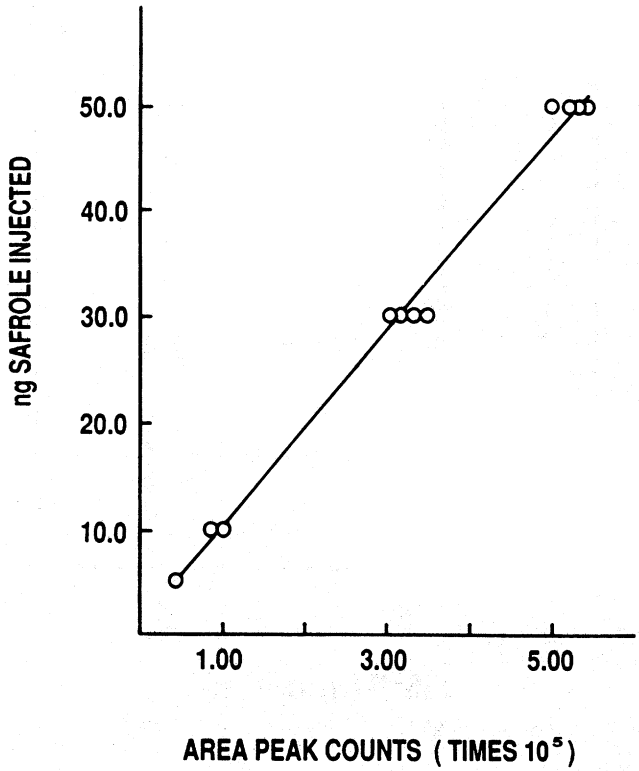

Fig. 4—Concentration vs. GC detector response of safrole.

Table 1—Recovery of safrole/isosafrole added to ham (quantitated by GC)

Amount added (ppm)		Amount recovered (%)[a]	
Safrole	Isosafrole	TLC isolation	HPLC isolation
100	0	97 ± 4 (5)[b]	87 ± 5 (4)
90	10	91 ± 2 (5)	83 ± 3 (4)
50	50	96 ± 4 (4)	84 ± 1 (3)
0	100	95 ± 3 (4)	85 ± 3 (3)

[a] Total of both components.
[b] Figures in parentheses indicate the number of spiked samples analyzed.

mentioned above. However, although high volatility of both safrole and isosafrole contributed to their loss, this might not be the only factor responsible. To determine whether ambient laboratory temperature contributed to safrole/isosafrole loss during the process of injecting the "stock solution" unto the meat, that part of the procedure was carried out in a cold room (4°C). No significant improvement in recovery was observed.

In several tests of a procedure blank, 500 μg of safrole in "model solution" was applied to the dry column materials in the absence of meat, and the entire procedure was then carried out. Recoveries via the TLC isolation route were consistently close to 100%. Since recoveries from meat seemed to be somewhat lower, a component in meat might contribute to the loss of safrole.

Cooked or boiled ham from a variety of commercial sources was employed in our experiments. All ham samples were taken through the procedure without addition of safrole or isosafrole to test them for possible interfering substances, perhaps originating in spices. No interferences were detected in the ham samples used.

Recently we have begun to explore the question whether safrole and isosafrole can be resolved by modifications of normal phase HPLC and whether HPLC with UV detection can be utilized to quantitate these components in a matrix containing substantial amounts of triglycerides. Success in these experiments might lead to a shorter and more direct procedure than the one that is reported here.

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