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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–FLUOROMETRIC DETERMINATION OF SAFROLE IN PERFUME, COLOGNE AND TOILET WATER

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SUMMARY

A spectrophotofluorometric method for the determination of safrole (1,2-methylenedioxy-4-allylbenzene) in fragrances (perfume, cologne, toilet water) is described. The fragrance is diluted with ethanol and injected onto a reversed-phase high-performance liquid chromatographic (HPLC) column interfaced to a spectrophotofluorometer. The fluorescence is measured and the safrole determined using external standardization. Recoveries from samples spiked with safrole at levels ranging from 0.01 to 0.4% varied from 98 to 102% with an average of 100%. The minimum amount of safrole detected was 10 ng. After separation of the fragrance components by preparative HPLC, safrole was verified in those samples spiked at the lowest level (0.01%) by gas chromatography–mass spectrometry.

INTRODUCTION

Safrole (1,2-methylenedioxy-4-allylbenzene) is a naturally occurring constituent found in a wide range of concentrations in over 70 essential oils derived from botanical sources[1]. It is produced in a relatively pure state by isolation from several oils in which it occurs as the principal constituent. These sources are primarily Brazilian Ocotea and Formosan–Japanese–Chinese camphor oils and, to a lesser extent, North American sassafras oil[2]. Safrole and sassafras oil were used in flavorings until their use in foods was banned in the United States by the Food and Drug Administration in 1958[3]. Although safrole and essential oils in which safrole is a constituent had been used in perfumes and other fragrance products[2,4], their present use, if any, in such products is minimal[5]. The average use level of safrole in perfumes is 0.2% and is correspondingly less in the more dilute fragrance products[4]. Safrole is also a constituent of oils used to prepare topical medicinal preparations such as balms and ointments[6].

In toxicity studies, malignant tumors have been induced in experimental animals by safrole[7–9]. According to Borchert *et al.*[10], the metabolized product of safrole, 1′-hydroxysafrole, is also an active carcinogen.

Various analytical methods for detecting and determining safrole have been reported in the literature. A colorimetric test detects safrole in root-beer and root-beer flavors[11].

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Fishbein *et al.*[12] reviewed the thin-layer chromatographic (TLC) and gas–liquid chromatographic (GLC) literature on the detection of safrole and many other naturally occurring and synthetic compounds which, like safrole, contain the methylenedioxyphenyl group. Forrest *et al.*[13] investigated those TLC spray reagents which optimize the sensitivity and selectivity in the TLC identification of safrole and many of its naturally occurring analogues. Gubitz[6] discussed the TLC and GLC detection of safrole and many other naturally occurring compounds of diverse structures occurring in oils used in various medicinal balms and ointments. Lukaszewski[14], in a study of the impurities in an amphetamine synthesized from isosafrole, separated safrole from other components, using GLC. Wulf *et al.*[15] used high-performance liquid chromatography (HPLC) to separate and detect safrole and other similar compounds.

Safrole has been determined by a variety of techniques. Gunner[16] separated safrole from other compounds and determined it by *in situ* densitometry on a TLC plate. Tanaka and Ono[17] determined safrole in red camphor oils by scraping it off a TLC plate and monitoring the intensity of selected IR bands. Moryashchev and Voronin[18] determined safrole in Chinese sassafras oil by GLC. Larry[19] determined safrole and related compounds in flavors and non-alcoholic beverages with GLC. Using a gas chromatograph coupled to a mass spectrometer, Liddle and DeSmedt[20,21] determined safrole and other compounds after fractionation of an alcoholic beverage such as vermouth. Bowman and Beroza[22] investigated the spectrophotofluorometric properties common to the many commercially available carbamate and pyrethroid insecticide synergists containing the methylenedioxyphenyl group. They determined many of these synergists (excluding safrole) in these insecticides by direct fluorescent measurement in a quartz cell.

The method reported here for the detection and determination of safrole in fragrances is based on its fluorescence when excited by short wavelength UV light.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph equipped with a variable wavelength fluorescence detector (see below) and gradient elution capability was used. Determinations were performed with a Waters 204 liquid chromatograph equipped with two 6000A solvent pumps, a U6K universal injector and a 660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.).

The operating conditions for safrole determination were: column, Zorbax ODS, 10 μm, 250 × 4.6 mm I.D. (P.N. 850952702; DuPont, Wilmington, DE, U.S.A.); mobile phase, methanol–water (6:4); flow-rate, 1.5 ml/min, isocratic.

The operating conditions for safrole isolation were: column, Partisil M-9 10/50 PAC, 500 × 9.4 mm I.D. (4230.226; Whatman, Clifton, NJ, U.S.A.); mobile phase components, A = isooctane, B = isooctane–methylene chloride (400:50); solvent program, linear, 20 min, 25%B to 95%B; flow-rate, 4 ml/min.

The HPLC guard column was 75 × 4.6 mm I.D. with 10-μm frits, packed with 37–50 μm silica gel.

A Model MPF-3 fluorescence spectrophotometer equipped with a recorder and an N-063-0576 ultra-micro flow cell accessory was used (Perkin-Elmer, Norwalk, CT,

U.S.A.). The detector operating conditions were: direct mode; sample sensitivity range, x1–x30; excitation wavelength, 295 nm; excitation slit, 10 nm; emission wavelength, 323 nm; emission slit, 10 nm; emission filter, 310 nm.

A Hewlett-Packard Model 5992A gas chromatograph–mass spectrometer system (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used. The gas chromatograph was equipped with a 183 cm × 2.0 mm I.D. glass column packed with 3% OV-101 on Supelcoport 80–100 mesh. The GC conditions were: column temperature, 150–170°C at 5°/min; injection temperature, 250°C; flow-rate (helium), 20 ml/min.

The MS conditions were: MS peak detection threshold, 2000; sample/0.1 a.m.u., 4; electron multiplier voltage, 2000 V; total abundance from 45 to 250 a.m.u.

Reagents

HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ, U.S.A.), HPLC grade water and spectrophotometric or HPLC grade isooctane and methylene chloride, distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), were used.

Safrole (98%) (S20-8; Aldrich, Milwaukee, WI, U.S.A.) was used to prepare the stock solutions.

Preparation of stock and working solutions

Stock solution 1 (1.0 mg/ml) was prepared by weighing approximately 100 mg of safrole into a 10-ml beaker, dissolving in ethanol and diluting to volume with ethanol in a 100-ml volumetric flask.

Stock solution 2 (0.1 mg/ml) was prepared by pipetting an appropriate aliquot of stock solution 1 into a 10-ml volumetric flask and diluting to volume with ethanol.

Working solutions A (0.04 mg/ml), B (0.02 mg/ml), C (0.01 mg/ml) and D (0.001 mg/ml) were prepared by pipetting appropriately selected aliquots of either stock solution and diluting to volume with ethanol in 100-ml volumetric flasks.

Preparation of sample

A fragrance sample (perfume, cologne or toilet water) (1.0 ml) was pipetted into a 100-ml volumetric flask, diluted to volume with ethanol and mixed. For toilet water and cologne, the final volume should be 10–25 ml, depending on oil concentration.

Determination

The C_{18} analytical HPLC column was installed in the HPLC/fluorometric system and equilibrated for 20 min using methanol–water (6:4) at 1.5 ml/min or until a stable baseline on the recorder was established. Working solution A (20 μl) was injected, and the sensitivity was adjusted to keep the peak on scale. This procedure was repeated until reproducible retention times were obtained. Working standard solutions A, B, C and D (20 μl each) were each injected in duplicate. For each injection the sensitivity was adjusted to obtain maximum scale response.

Using the above procedure, each fragrance sample was then injected three times. If there was any doubt as to the location of the safrole peak, an aliquot of the sample was spiked with the standard and analyzed as above to identify the safrole peak.

For each working standard, the peak heights (fluorescence emission) from the duplicate injections were measured and averaged. All standard data were adjusted to a common attenuation (sensitivity) and the concentration (mg/ml) plotted *versus* peak height. The three sample peak heights were averaged and adjusted to attenuation of the standard curve, and the concentration of safrole in the sample was obtained. The safrole content was calculated as follows

$$\text{Safrole (\%, w/v)} = (C_s \times V_x)/(V_s \times 10)$$

where C_s = concentration (mg/ml) of safrole in sample, V_x = volume (ml) of prepared sample and V_s = volume (ml) of sample. If the safrole content was to be determined on a w/w basis, the pipetted sample was weighed before dilution. The above expression for the calculation of safrole content holds only if the volumes injected are the same for the standards and samples.

GC–MS confirmation

The sample (*ca.* 5 ml) was added to a 100-ml separatory funnel containing 20 ml of water and extracted with two 15-ml portions of diethyl ether. The combined ether extracts were washed with three 20-ml portions of water. The ether extract was dried for a minimum of 2 h over anhydrous sodium sulfate, transferred to a 50-ml beaker and carefully evaporated to *ca.* 5 ml on a steam-bath. The extract was taken up in a 10-ml syringe and passed through a silica gel Sep-Pak cartridge (51900, Waters Assoc.) into a 50-ml tapered centrifuge tube. Isooctane (*ca.* 1 ml) was added and the remaining ether was evaporated on a steam-bath. If the remaining isooctane solution was cloudy (water), a small amount of anhydrous sodium sulfate was added.

The preparative column was installed in the HPLC/fluorometric system. Using the operating conditions given above, the column was equilibrated with 120 ml of the initial solvent composition. Several micrograms of safrole dissolved in isooctane were injected and the gradient run was initiated. The retention time of safrole was determined and the analysis repeated with 20 μl of sample. The safrole peak was identified on the analytical chromatogram generated from the preparative HPLC procedure. Approximately 200 μl of sample were injected and the safrole was collected at its predetermined retention time. To keep the safrole peak on scale, it may be necessary to reduce both the excitation and emission slit widths as well as to reduce sensitivity. The collected eluate was concentrated to *ca.* 200 μl under a gentle stream of air on a steam-bath.

An aliquot of the concentrated eluate containing 100–200 ng of safrole was injected into the GC–MS system using the conditions given above. Safrole was confirmed by comparing the mass spectrum and retention time with those of a standard.

RESULTS AND DISCUSSION

Ten commercial fragrance oils were used for recovery studies. The fragrance compositions were prepared as ethanolic solutions containing 10% fragrance blends. Each solution was analyzed by HPLC/fluorometry and was free of safrole and interferences. A known amount of safrole corresponding to 0.01% was added to aliquots of each of the ten samples. Five samples were prepared that contained safrole at the

0.1% level and five at the 0.4% level. The safrole content of each sample was determined. Recoveries (Table I) ranged from 98 to 102% with an average of 100%.

Fragrance compositions are complex mixtures of natural and synthetic ingredients that typically contain over 100 compounds. The determination of safrole in these products requires an analytical system of relatively high resolution and selectivity. Analyses done by GLC or HPLC in combination with relatively non-specific detectors are not reliable due to the possibility of direct interferences from other ingredients. Since safrole is highly fluorescent, fluorescence detectors, which have the sensitivity and selectivity required, can be used in combination with HPLC to determine this compound. Because the excitation and emission bands of safrole (295 and 323 nm) lie close together and are of relatively short wavelengths, a fluorescence detector containing variable excitation and emission monochromators and a xenon lamp excitation energy source are required to obtain adequate band selectivity and excitation energy. Detectors that use fluorescent tubes for excitation sources and excitation and emission filters are not suitable because of their extremely weak excitation energy output at 295 nm and their inadequate separation of the close-lying excitation and emission bands.

Initial studies indicated that safrole could not be accurately determined in fragrances by direct fluorescence measurements. Several commercial fragrance blends containing no safrole were spiked with known levels of safrole, and the fluorescence emission intensities of safrole in ethanol-diluted samples were measured. In these

TABLE I

RECOVERY OF SAFROLE FROM FRAGRANCES

Sample	*Safrole added (mg/ml)*	*Safrole found (mg/ml)*	*Recovery (%)*
1	0.10	0.10	100
	1.00	1.02	102
2	0.10	0.098	98
	1.00	1.02	102
3	0.10	0.10	100
	1.00	1.00	100
4	0.10	0.10	100
	1.00	0.99	99
5	0.10	0.10	100
	1.00	1.01	101
6	0.10	0.10	100
	4.00	4.00	100
7	0.10	0.10	100
	4.00	4.00	100
8	0.10	0.10	100
	4.00	4.00	100
9	0.10	0.102	102
	4.00	3.90	98
10	0.10	0.10	100
	4.00	4.00	100
Mean			100
S.D.			1.1

cases the measured emission intensity was two to four times greater than that of the corresponding standards. To eliminate these positive interferences, as well as any possible interference from *cis*- or *trans*-isosafrole, an HPLC column was interfaced with the fluorescence detector. Although different types of HPLC columns may be suitable for the analysis, a reversed-phase C_{18} column was used to simplify sample preparation. Reversed-phase columns, usually used with polar eluents, are less susceptible to a decrease in efficiency and reproducibility after being exposed to repeated injections of samples dissolved in polar organic solvents or water.

In preparing the standard calibration curve, emission values deviated from linearity when safrole concentrations exceeded 0.04 mg/ml. At 0.08 mg/ml, for example, the emission value was *ca.* 6% below the extrapolated value. This indicates that samples containing relatively large amounts of safrole should be diluted to concentrations of 0.04 mg/ml or less.

The minimum detectable amount of safrole was *ca.* 10 ng. Using the dilution volumes in the method, this would correspond to a safrole content of 0.005%. Determination of safrole at lower concentrations can be achieved, if necessary, by altering final dilution volumes and the fluorometer attenuation.

Fragrance oils (perfume bases) are prepared by blending essential oils, synthetics and other natural isolates. The fragrance is prepared by dissolving the fragrance oil in ethanol. The concentration range of the base in the ethanol determines the type of fragrance product: perfume, 10–30%; cologne, 2–4%; and toilet water, 1–2%.

Due to the complexity of fragrance blends, the direct GC–MS verification of safrole is not ordinarily possible. For this reason, fractionation of the sample by preparative HPLC to remove interfering compounds was necessary. Successful isolation of safrole or any compound from a preparative HPLC column depends on the resolution of the column and the reproducibility of the compound's retention volume. The use of a normal bonded phase partition column, such as the PAC preparative column used in this work, with a relatively non-polar eluent system requires the exclusion of injected aliquots containing large amounts of polar solvents such as water or alcohol. The injection of such solvents could adversely affect the column activity, with a subsequent alteration of resolution and decrease in retention volume. For this reason, fragrance samples, which contain alcohol and water diluents, cannot be injected directly. By dissolving the sample in diethyl ether and washing it several times with water, most of the ethanol in the sample is removed. After drying and concentration, the ether extract is passed through a Sep-Pak silica cartridge to filter suspended material and to remove traces of water and other polar compounds. Despite these precautions, some variation in the retention volume for safrole may be observed. If there is doubt as to which of several fluorescent peaks is derived from safrole, the ambiguity can be resolved by adding safrole to an analytical sample and comparing its chromatogram with that of an unspiked sample.

After several preparative runs, the PAC column may exhibit decreased retention volumes, an unstable baseline and some loss of resolution due to an accumulation of polar fragrance ingredients. At this point the column can be completely reconditioned according to the manufacturer's instructions. We have found, however, that complete reconditioning is necessary only after long use. Initial efforts at partial reconditioning involved the use of moderately polar solvents containing low

levels of methyl or isopropyl alcohol. Although these solvents are effective for removing contaminants, restoration of column performance was difficult, even after prolonged equilibration at initial gradient conditions. We found that this column could be restored to good working condition by washing with 250–300 ml of methylene chloride (6–8 ml/min) followed by 300–350 ml of the initial solvent.

The volume of collected eluate must be reduced to obtain a safrole concentration of *ca.* 100 ng/μl to assure GC–MS confirmation. Seven of the ten samples spiked at the 0.01 % level were randomly selected for GC–MS to check the confirmation procedure. In all cases safrole was confirmed.

In conclusion, a rapid, sensitive method for the determination of safrole in fragrances has been developed. Although the method is fairly specific, a procedure for the GC–MS verification of safrole was also developed. The method can be easily extended to the determination of safrole in other products that contain water and/or alcohol diluents. For example, safrole in undiluted beverages could be determined by this procedure.

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