Identification of Some C_{19}– Δ^{16} Steroids Contributing to Sex Odor in Pork

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Evidence is presented confirming the contribution of 5α-androst-16-en-3-one to sex odor in pork. Combined data from tlc, glc, and mass spectrometry indicate that 5β-androst-16-en-3-one is present in the nonsaponifiable material in appreciable quantities and may also contribute to sex odor. There is also evidence that the 5α- and/or 5β-androst-16-en-3-ols contribute to sex odor.

The undesirable odor frequently associated with the cooking of meat from many uncastrated sexually mature male pigs (boars) has long been a problem to the hog raising/meat packing industry. Various aspects of the problem have been discussed in a review by Pearson *et al.* (1969).

Craig and Pearson (1959) and Craig *et al.* (1962) established that the odor was associated with the nonsaponifiable fraction of boar fat, and was not present in the saponifiable material nor the lean tissues. Sink (1967) postulated that the odor was due to C_{19}–Δ^{16} sterols and that they may function as sex pheromones in pigs. Patterson (1968, 1969) isolated the C_{19}–Δ^{16} steroid, 5α-androst-16-en-3-one, by vacuum distillation of heated boar fat and identified it as contributing to sex odor.

The present work was undertaken to confirm Patterson's (1968) findings by employing a different procedure of isolation, namely thin-layer chromatography (tlc). Attempts were also made to determine if substances other than 5α-androst-16-en-3-one contributed to sex odor, and to ascertain if sow fat contains any of the undesirable compounds.

MATERIALS AND METHODS

Samples. Experimental samples were obtained from the backfat of a 450-lb boar possessing strong sex odor, whereas the control samples were from a 500-lb sow, which possessed no sex odor, as determined by smelling heated portions of fat.

Saponification. Pork fat (60 g) was saponified under a nitrogen atmosphere as recommended by Cocks (1933) with appropriate alterations in the volume of reagents according to the amount of lipid saponified. The ethereal extract was reduced to dryness at room temperature under a stream of nitrogen gas and redissolved in 300 μl of tetrahydrofuran. The general scheme of the subsequent fractionation and study of the nonsaponifiable material is illustrated in Figure 1.

Thin-Layer Chromatography. The nonsaponifiable material and steroid standards were applied to tlc plates coated with silica gel G (750 μm thick). The tlc plates were not heat activated, but instead were stored in a desiccator containing anhydrous calcium sulfate (W. A. Hammond Drierite Company, Xenia, Ohio) for 72 hr before use, because this procedure gave more reproducible tlc separations. The plates were developed in benzene–ethyl acetate (9:1, v/v) in a tank under saturated conditions, and visualized at room temperature by spraying with a sulfuric–acetic acid mixture (1:1, v/v).

The center portion of the tlc plate was spotted with a known steroid mixture to serve as markers (Figure 1). Then, 22 mg of boar fat nonsaponifiable material, dissolved in 80 μl of tetrahydrofuran, was streaked on one end of the tlc plate, while the same amount of sow fat nonsaponifiable material was streaked on the other end of the same tlc plate. This procedure permitted the simultaneous tlc separation of boar and sow nonsaponifiable material, along with authentic steroids for comparison.

Following development, the fractionated nonsaponifiable materials on both ends of the tlc plate were covered with glass plates to protect them from the sulfuric–acetic acid mixture, which was then sprayed on the center section containing the authentic steroids. Several variously colored spots appeared after 5 min at room temperature. This permitted the division of the plate into four regions. Each region was composed of two zones, one corresponding to boar nonsaponifiable material and one corresponding to sow nonsaponifiable material. The eight zones of the tlc plate were removed individually with a spatula, and eluted with benzene–methanol (3:1, v/v) and chloroform–methanol (3:1, v/v) as outlined by Masaracchia and Gawienowski (1968). The samples were taken to dryness at room temperature under a stream of nitrogen and redissolved in 100 μl of tetrahydrofuran before gas chromatographic analysis.

Fourteen different authentic steroids were chromatographed under the same conditions. This helped in characterizing the tlc regions in terms of the types of steroids. The relative mobility (RR_f) was computed assigning the mobility of authentic 5α-androst-17-one a value of unity (Table I).

Gas Chromatography. Gas–liquid chromatography (glc) was performed with a Beckman GC-4 gas chromatograph equipped with a flame ionization detector. A 15 ft aluminum column (1/8 in. o.d.) was packed with 5% OV-101 on 100–120 mesh Gas Chrom Q. The column was operated at 250°C with a flow rate of high-purity helium of 20 ml per min. The on-column inlet was at 265°C and the detector oven was at 260°C. A 5 μl calibrated Hamilton syringe was used to apply the samples onto the column. All samples were dissolved in tetrahydrofuran. A 1:1 post-column stream splitter was installed to permit smelling of the column effluent while simultaneously detecting and recording the compounds as they were eluted.

Mass Spectrometry. Mass spectrometric analysis was made with a single focusing, rapid magnetic-scanning mass spectrometer (LKB 9000, LKB Produkter AB, Stockholm) coupled through molecule separators with a gas chromatographic system.

The coiled glass glc column was 2 m in length by 3 mm (i.d.) and was packed with 3% SE-30 on 100 to 120 mesh, acid-washed and silanized Gas Chrom S (Applied Science Laboratories, Inc., State College, Pa.). Isothermal analysis was carried out at a column temperature of 190°C. Mass spectra measurements were recorded at 70 eV electron energy

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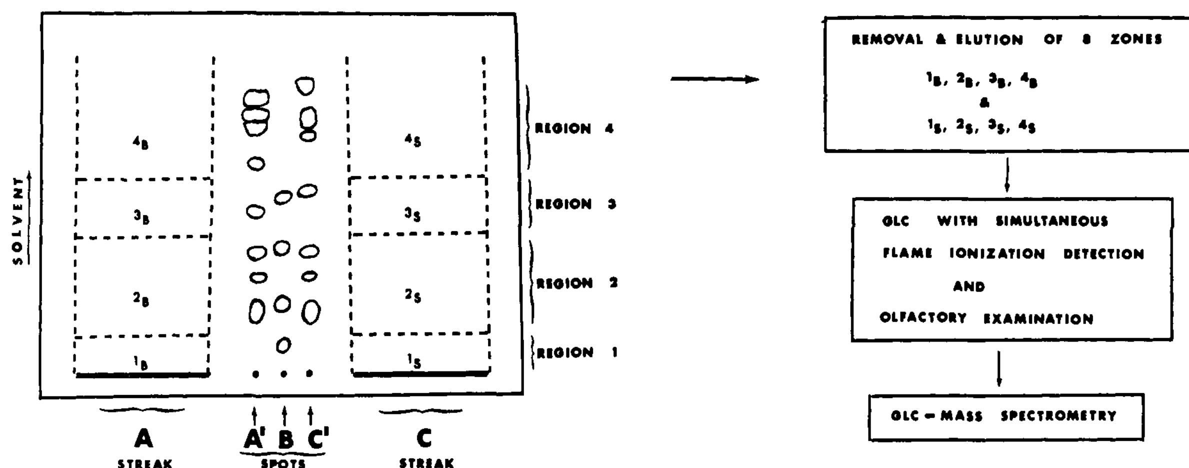

Figure 1. Flow diagram for tlc separation, sample recovery, and identification of sex odor substances from the nonsaponifiable material of boar and sow fat. [a]Application of samples to tlc plate: A = streak of 22 mg boar fat nonsaponifiable material dissolved in 80 μl of tetrahydrofuran. A′ = spot of 2.2 mg boar fat nonsaponifiable material dissolved in 8 μl of tetrahydrofuran. B = spot of authentic steroid mixture, 80 μg of each, in order of increasing mobility: pregnenolone, 5α-androst-16-en-3β-ol, androst-4,16-dien-3-one, and 5α-androst-16-en-3-one dissolved in 8 μl of tetrahydrofuran. C′ = spot of 2.2 mg sow fat nonsaponifiable material dissolved in 8 μl of tetrahydrofuran. C = streak of 22 mg sow fat nonsaponifiable material dissolved in 80 μl of tetrahydrofuran. Development: solvent, benzene–ethyl acetate (9:1, v/v); distance, 15 cm

with 3500 V accelerating voltage; the filament emission current was 60 μA.

RESULTS AND DISCUSSION

A summary of results of olfactory examination of the four tlc regions of nonsaponifiable material is shown in Table II. Neither tlc zone 1_B nor 1_S (RR_f 0 → 0.22) contained any traces of sex odor. In contrast, tlc zone 2_B (RR_f 0.22 → 0.65) did contain sex odor, but it was not detected in the corresponding 2_S zone.

While no differences were observable by tlc, differences were easily found by glc upon olfactory examination of the column effluent. Glc analysis revealed (Figure 2) that zone 2_B contained (a) substance(s) that possessed the same glc retention time and the same objectionable boar-like odor as the authentic Δ^{16}-androstenols, 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol. The corresponding glc peak in the sow nonsaponifiable zone 2_S was much smaller and did not possess a boar-like odor. Unfortunately, the amount of material isolated from zone 2_B was insufficient for positive mass spectral identification. However, tlc mobility, glc retention time, and odor evaluation provide strong preliminary evidence that zone 2_B contained 5α-androst-16-en-3α-ol and/or 5α-androst-16-en-3β-ol. This suggests that one or both of the Δ^{16}-androstenols is/are responsible for sex odor in zone 2_B.

The most significant difference between boar and sow fat nonsaponifiables was found in zones 3_B and 3_S. Glc analysis of zone 3_B (Figure 3) showed that it contained only one major component, which had the same strong sex odor and the same glc retention time as authentic 5α-androst-16-en-3-one. Mass spectral analysis of the unknown showed that its spectra compared very closely with that of authentic 5α-androst-16-

Table I. Relative R_f Values for tlc Separation of Authentic Steroids[a]

Steroid	RR_f	Region[b]
5α-Androstane-3β,17β-diol	0.07	1
Testosterone	0.09	1
Androsterone	0.12	1
Androstanolone	0.17	1
Pregnenolone	0.18	1
Progesterone	0.24	2
5α-Androst-16-en-3β-ol	0.30	2
Androsta-5,16-dien-3β-ol	0.32	2
Cholesterol	0.36	2
5α-Androst-16-en-3α-ol	0.48	2
Pregnane-3,20-dione	0.49	2
Androsta-4,16-dien-3-one	0.60	2
5β-Androst-16-en-3-one	0.78	3
5α-Androst-16-en-3-one	0.82	3
5α-Androst-3-one	0.85	3
5α-Androst-17-one	1.00	3

[a] All RR_f values are based on an arbitrary assignment of a value of 1.00 for 5α-androst-17-one. [b] Region numbers refer to the separation on tlc shown in Figure 1.

Table II. Olfactory Characterization of Boar and Sow Nonsaponifiables Separated by tlc

tlc Zones[a]	Range in RR_f values[b]	Sex odor[c]	Intensity of odor[d]	Characterization of sex odor[e]
1_B	0.00–0.22	None	...	...
1_S	0.00–0.22	None	...	...
2_B	0.22–0.65	Yes	+++	Musk-like
2_S	0.22–0.65	None	...	...
3_B	0.65–1.00	Yes	++++	Typical sex odor
3_S	0.65–1.00	Yes	tr	Typical sex odor
4_B	1.00–solvent front	Yes	+	Musk-like
4_S	1.00–solvent front	None	...	...

[a] The zones are shown on the tlc diagram shown on Figure 1. [b] RR_f values are relative to 5α-androst-17-one, which was assigned a value of 1.00. [c] Present or absent. [d] Intensity: ++++ = very strong, +++ = strong, + = evident but not strong, tr = traces only. [e] Sex odor was characterized as typical (strong perspirative odor) or as musk-like (weaker).

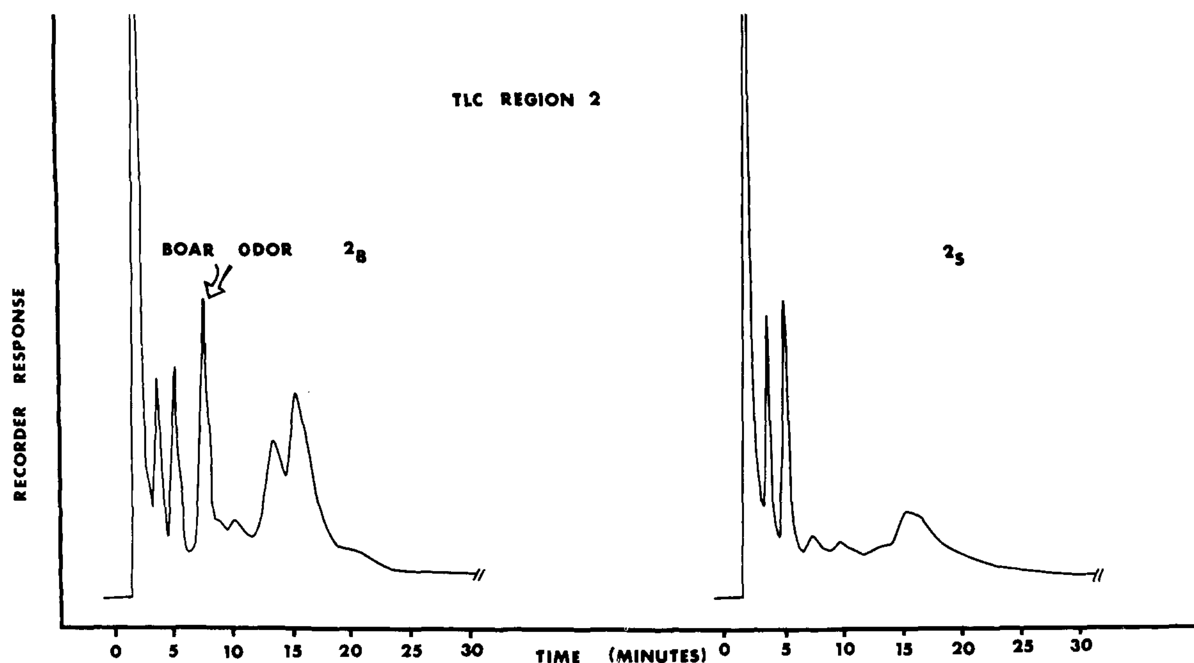

Figure 2. Chromatograms showing results of glc analysis of boar and sow fat nonsaponifiable material taken from region 2 of the tlc plate. 2_B = tlc region 2 of boar nonsaponifiable material. 2_S = tlc region 2 of sow nonsaponifiable material

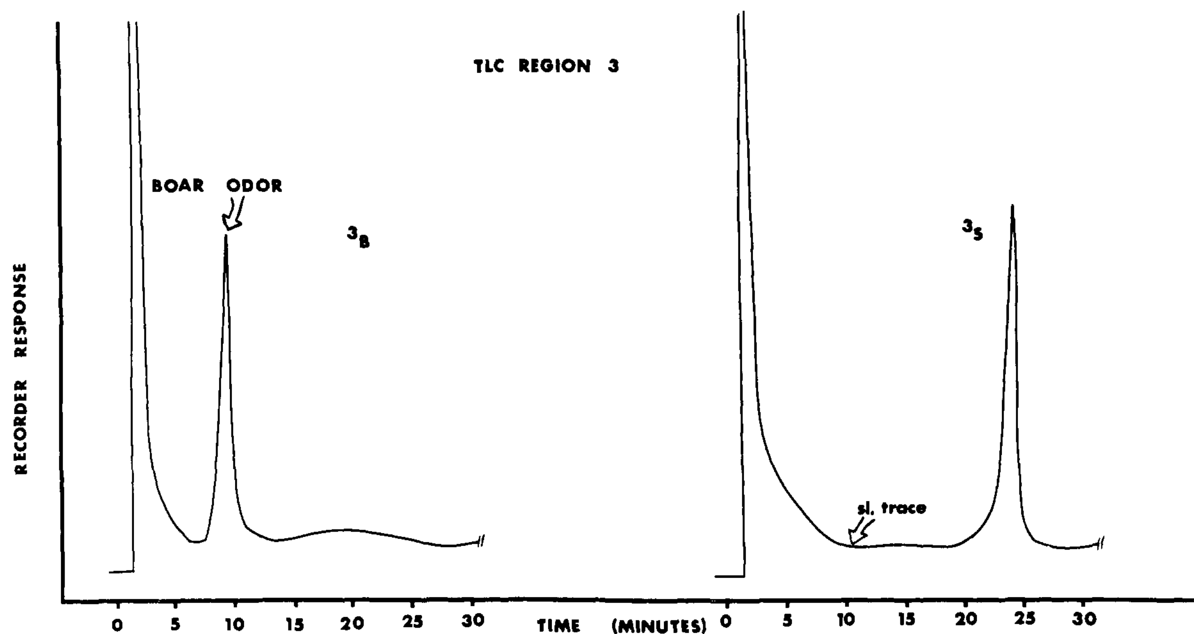

Figure 3. Chromatograms showing results of glc analysis of boar and sow fat nonsaponifiable material taken from region 3 of the tlc plate. 3_B = tlc region 3 of boar fat nonsaponifiable material. 3_S = tlc region 3 of sow fat nonsaponifiable material

en-3-one (Figures 5 and 6). Both substances had a molecular weight of 272, indicated by an abundant molecular ion at *m/e* 272, and both had major ions at *m/e* 257, 239, 149, 147, 107, 105, 94, and 79. Therefore, on the basis of glc retention time, odor, and mass spectra, it appeared that the unknown responsible for the strong sex odor in zone 3_B was 5α-androst-16-en-3-one. However, tlc observations were not consistent with this.

Only one spot was visible on the tlc plate in zone 3_B after the plate was visualized by spraying with the sulfuric–acetic acid mixture (Figure 1). Likewise, only one peak was found in the glc analysis of zone 3_B after it was eluted and recovered from the silica gel layer (Figure 3). The single glc peak in zone 3_B appeared to be 5α-androst-16-en-3-one because of similar odor, retention time, and mass spectra. However, the single spot seen by tlc (RR_f 0.78) did not compare with authentic 5α-androst-16-en-3-one (RR_f 0.82, over a wide range of concentration) in its tlc mobility. Therefore, the strong sex odor substance isolated from zone 3_B had the same molecular weight, mass spectra, retention time, and odor as 5α-androst-16-en-3-one; but it had a slower tlc mobility than 5α-androst-16-en-3-one (Figure 1).

From these data it appeared probable that the A/B cis epimer of 5α-androst-16-en-3-one, namely 5β-androst-16-en-3-one, had been isolated. The authentic 5β epimer was found to have the same glc retention time, similar mass spectra

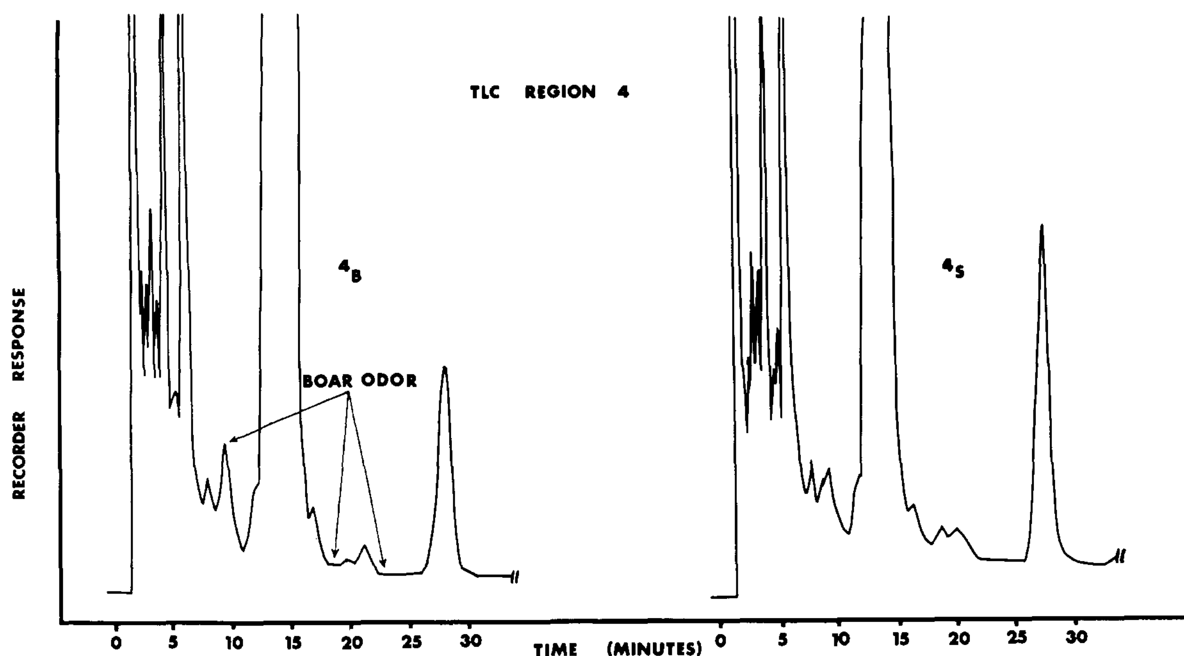

Figure 4. Chromatograms showing results of glc analysis of boar and sow fat nonsaponifiable material taken from region 4 of the tlc plate. 4_B = tlc region 4 of boar fat nonsaponifiable material. 4_S = tlc region 4 of sow fat nonsaponifiable material

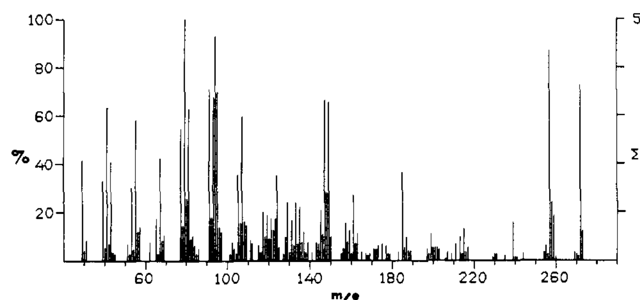

Figure 5. Mass spectra of unknown with strong sex odor isolated from boar fat nonsaponifiable material in tlc region 3

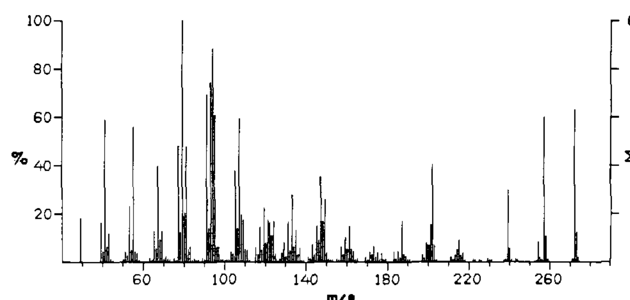

Figure 7. Mass spectra of 5β-androst-16-en-3-one

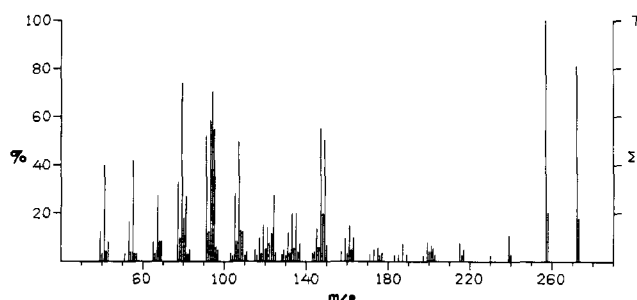

Figure 6. Mass spectra of 5α-androst-16-en-3-one

(Figure 7) and the same odor (although considerably less intense) as 5α-androst-16-en-3-one. More importantly, the tlc migration of the authentic 5β epimer (RR_f 0.78) corresponded with the migration of the single spot in zone 3_B.

These data suggested that both 5α- and 5β-androst-16-en-3-one had been separated simultaneously from zone 3_B of boar nonsaponifiable material. The two had cochromatographed in the glc column and were simultaneously introduced into the ion source of the mass spectrometer, resulting in a mixed mass spectrum (Figure 5).

It was apparent from the tlc plate (Figure 1) that the quantity of 5β-androst-16-en-3-one present in the fat was greater than the quantity of 5α-androst-16-en-3-one because the 5β epimer was visible and the 5α epimer was not visible. However, the small quantity of 5α-androst-16-en-3-one in the fat is probably more important, because it has a more intense sex odor.

The corresponding sow zone (3_S) also contained a trace of sex odor as it emerged from the glc column (Figure 3). Although there was no discernible glc peak associated with the odor, it was found that the time of elution corresponded to the retention time of 5α- and 5β-androst-16-en-3-one.

Zone 4_B of tlc region 4 (RR_f 1.04 → solvent front) contained several substances that had a sex odor, but which were absent from the corresponding sow nonsaponifiable zone (4_S) as judged by olfactory examination of the glc column effluent (Figure 4). These substances were present in small amounts. The identity was not determined, but they cannot be free C_{19}–Δ^{16} steroids because of their fast tlc mobility. Further work is being carried out in an attempt to clarify the nature of these component(s).

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Production of Volatile Compounds in the Ripening Banana

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Sequential samples of air swept through a chamber containing ripening bananas and incorporating internal standards were trapped on Porapak Q, and recovered for gas chromatographic analysis. Peak areas, determined with digital integration and corrected for the internal standard(s), show that both the acetate esters and the butyrate esters are produced at rates that vary in a cyclic manner. The two cycles are out-of-phase, and it appears that they compete for some limiting reagent which first one and then the other reaction arrogates. Ripening fruit is shown to be a highly dynamic system, with short-term variations and wide fluctuations in the relative amounts of individual volatile compounds.

There have been many gas chromatographic studies on the volatile compounds produced by ripening fruits; some of these studies utilized sequential sampling (Paillard, 1968; Heinz *et al.*, 1965; Romani and Ku, 1966; Brown *et al.*, 1966; Drawert *et al.*, 1971). Methods that utilize extraction techniques suffer from the disadvantage that the biological system is upset and the sample destroyed by the extraction; sequential sampling then demands a number of individual fruits, and one must assume these are all at the same stage of development. Direct gas chromatographic analysis of headspace vapors has also been utilized, but without some means of preconcentration the amount of many of the volatiles injected is below the limits of detection. The method employed by Paillard (1968)—trapping of headspace volatiles on activated carbon, followed by elution and analysis—surmounts some of these difficulties, but observations in our laboratory have lately left in doubt the question of how completely adsorbed volatiles are recovered from a trapping charcoal. Additionally, quantitative comparisons of chromatographic analyses demand a reliable method for incorporating internal standards.

Jennings *et al.* (1971) recently employed traps containing a porous polymer, Porapak Q, to obtain samples of headspace volatiles for gas chromatographic analysis. Close examination indicates the method is quantitatively and qualitatively reproducible. It was employed here, together with a method of incorporating internal standards, to study the volatile emanations of ripening banana.

METHODS AND PROCEDURE

Trapping Techniques. 10 cm of an 11-mm × 14-cm borosilicate glass tube, with machined Teflon adaptors (Figure 1), was filled with 100–120 mesh Porapak Q between glass wool plugs. An iron-constantan thermocouple was incorporated, and the assembly wrapped with heating tape. The trap was conditioned for 48 hr at 180°C with a flow of 300 cm^3/min of nitrogen, which was prepurified by passage through a 0.25-in. o.d. × 20-ft stainless steel column packed with 80–100 mesh uncoated firebrick and immersed in Dry Ice–acetone. Fruit volatiles and internal standards were trapped for 2 hr at 25°C from an air stream as described below. The gas was then changed to purified nitrogen, and the column developed (Jennings *et al.*, 1971) for 15 min at 25°C to remove the air and most of the water. The direction of flow was then reversed, and in a 200 cm^3/min nitrogen stream the column was heated to 100°C, and the eluting volatiles were trapped in a thin-walled glass capillary chilled with Dry Ice, which was then flame sealed until analyzed. When air was used as the eluting gas at 150°C, severe degradation of both the volatiles and of the Porapak was experienced.

Sampling. Green Valerie bananas were purchased at a retail outlet, and 2 kg placed in a 5-l./glass chamber (desiccator), as shown in Figure 1. Purified breathing air was passed through a 0.25-in. o.d. × 20-ft column, packed with uncoated 60–80 mesh firebrick, immersed in a Dry Ice–acetone bath, and swept through the fruit chamber at a rate of 300 cm^3/min. Except for the gas purifier, all components of the system were constructed of glass or Teflon and housed in a 25°C constant temperature chamber. The effluent gas, with its entrained volatiles, was passed through the internal standards container, thence to the Porapak trap, a flow meter, and discharged to atmosphere. The internal standard selected for this study was heptadecane.

Gas Chromatography. Glc analyses utilized a 1.4-mm i.d. × 5.3-m glass column containing 3.0% Carbowax 20M on 60–70 mesh HMDS A/W Chromosorb G. The chromatograph was of our own design, with glass-lined injectors and FID. The signal was fed through a Hewlett-Packard 5771 A

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