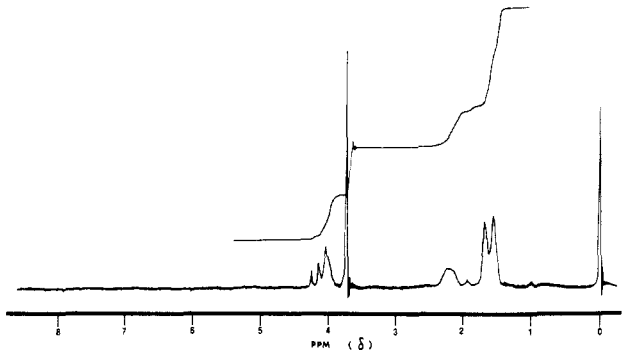

Figure 5. Nuclear magnetic resonance spectrum of methyl 3,4-dimethyl-5,6-dihydro-α-pyran-6-carboxylate.

111 (loss of $-COOCH_3$) are characteristic peaks of the $-COOCH_3$ functional group.

The infrared spectrum of MDDPC is shown in Figure 4. The absorption band at 1740 and 1130 cm^{-1} clearly indicates the presence of an ester group.

In the NMR spectrum of MDDPC (Figure 5), the two singlets at δ 1.56 and 1.67 are due to methyl groups at C-3 and C-4, respectively. The broad singlet at δ 2.23 is due to two protons at C-2. The sharp singlet at δ 3.74 is the absorption of the methyl group in $-COOCH_3$. The peak between δ 3.85 and δ 4.28 is due to protons at C_5 and C_6.

Sensory Characteristics of MDDPC. Unfortunately, the synthesized MDDPC did not possess an odor which could be described as beeflike. Instead, it was described as an interesting green note. Nevertheless, MDDPC represents a new class of heterocyclic compounds in food aroma and may have an application in food flavorings.

The identification of other compounds in these three fractions of roast beef flavor will be reported in the future.

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Concentration and Fractionation of Aromas on Reverse-Phase Adsorbents

Thomas H. Parliment

A new procedure for concentration and fractionation of volatile aroma chemicals has been devised. The dilute aroma-bearing aqueous solution is passed over a reverse-phase packing and concentration of the volatiles is achieved. A solvent gradient is generated and the aroma chemicals are desorbed. Retention of the aromatics is proportional to the number and type of hydrocarbon groups present; thus fractionation occurs. Fractions are obtained which may be subjected to organoleptic analysis, GC, HPLC, or GC–MS. Recoveries are generally over 80%. Experimental details are discussed, and the technique is applied to a homologous series of methyl esters, a mixture of flavor chemicals, a peppermint oil, and a carbonated beverage.

The volatile chemicals in a food are important since they are responsible for the aroma of the food. These aromatic chemicals are normally present at exceedingly low levels and are frequently present as complex mixtures. Numerous works have been published on techniques for isolating and concentrating these volatile chemicals prior to gas chromatographic analysis (Bemelmans, 1979; Weurman, 1969). The isolation procedures generally fall into one of two classes. In one case, the aromatic chemicals are trapped on a porous polymer (e.g., Tenax or Porapak) and subsequently desorbed by heat into a gas chromatograph. This procedure was the subject of a recent ACS symposium (Charalambous, 1978). The other common technique involves distillation at atmospheric or reduced pressure to produce an aqueous distillate free of nonvolatile materials. The organics are then concentrated by some form of solvent extraction, often by continuous liquid–liquid extraction. Frequently, the Likens–Nickerson apparatus (Likens and Nickerson, 1966) or some more efficient modification (Schultz et al., 1977) is used to concurrently distill and concentrate the aromatics. The essence may then be analyzed by gas chromatography.

Often, such concentrates are still too complex for gas chromatographic separation and further fractionation is required. Preparative scale gas chromatography has been used for this purpose (Merritt, 1971). Column chromatography on silica or alumina can be used to produce fractions based on polarity; vacuum distillation can be used to yield fractions based on boiling points.

The purpose of this paper is to describe a new technique which permits both the concentration and subsequent fractionation of volatile aromatic chemicals from dilute aqueous solutions. The concentration and fractionation are both accomplished using reverse-phase packing materials.

Trace enrichment of the organics from dilute aqueous solution on reverse-phase adsorbents was first proposed by Kirkland (1974) and has since been used in analysis of

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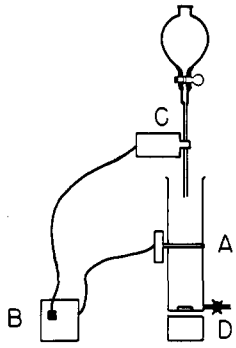

Figure 1. Device used to generate the solvent gradient.

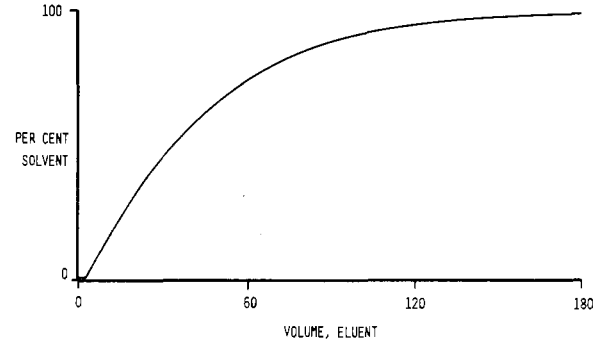

Figure 2. Gradient generated when constant volume = 60 mL.

various types of aqueous materials such as drinking water (Ogan et al., 1978) and sea water (May et al., 1975). Although high-pressure liquid chromatographic columns containing reverse-phase bonded material have been used in the flavor field, these packings have not found wide use for concentrating and fractionating volatile aroma chemicals.

The procedure we have devised can be summarized as follows: dilute aqueous solutions containing the aroma chemicals are passed over a reverse-phase packing material and the organics are retained. A solvent gradient is generated (by a device described) and the organics are desorbed in a concentrated form. Fractionation occurs as the solvent polarity in the gradient increases. Solute retention is generally proportional to the number and type of hydrocarbon groups on the solute, which means the most polar molecules elute early while the least polar, least water soluble components are most retarded. Fractions are collected by using an automated fraction collector. Aliquots can now be analyzed by chromatography or GC–MS or evaluated by aroma on a sniff strip.

The advantages of this system are that the volatile aroma chemicals from large volumes of dilute solution can be concentrated, fractionation is achieved, and the recoveries are generally near 100%. Reverse-phase packings are quite useful for this purpose. These adsorbents equilibrate rapidly, do not irreversibly retain chemicals, and can be used with a variety of organic solvents such as methanol, acetone, acetonitrile, etc.

EXPERIMENTAL SECTION

Apparatus and Materials. *Liquid Chromatographic Concentrating System.* A Michel-Miller column measuring 22 × 300 mm (ACE Glass, Vineland, NJ) was dry packed with ~50 g of C-18 reverse-phase Hi-Flosil, 80–100 mesh (Applied Science Labs, State College, PA). This packing has a silica gel backbone to which octadecyl groups are chemically bonded. Large volumes of dilute aqueous solutions were passed through this column by using a FMI Model RRP pump (Fluid Metering Inc., Oyster Bay, NY) at a rate of ~60 mL/min which concentrated the organics on the column. The pump used for desorption of the organics was an FMI Model RPSY, which produces a relatively smooth pulseless output of constant volume. The usual flow rate of solvent for desorption was 5 mL/min.

Desorption Procedure. The organics were desorbed from the reverse-phase packing by use of a gradient which progressed from water to organic solvent. A number of simple gradient devices was investigated. Ultimately the system shown in Figure 1 was chosen. This is a modified constant volume type mixer (Bock and Ling, 1954). The gradient generated is convex, easy to prepare, and highly reproducible; its shape is shown in Figure 2. It has the advantage that the total volume of the system (and thus the slope of the gradient) is easily adjustable. In our experiments we generally use a constant volume of 60 mL. Solvent level is controlled by a Thermo-watch (Instruments for Research and Industry, Cheltenham, PA) controller (B). Water is placed in the mixing chamber and the level is monitored by the sensing head (A). As liquid is drawn out of the mixing chamber, the level falls and the solenoid valve (C) opens, admitting the solvent from the separatory funnel. Thus, the total volume is held constant. The system is continuously stirred by magnetic stirrer (D). The solvent we normally use for desorption work is acetone, since it is water soluble and relatively low in polarity. The gradient is continued until all volatile components have eluted. Other solvents such as methanol or tetrahydrofuran may also be used. Possible reactivity of the organic solvent with the solutes should be kept in mind.

Fraction Collection and Analysis. Fractions were collected by an ISCO Model 1200 fraction collector (Instrumentation Specialties Co., Lincoln, NE). Fractions were collected by constant volume, normally between 5 and 8 mL.

The collected fractions were analyzed for solute composition by gas chromatography. The methyl ester model system was analyzed on a Perkin–Elmer 3920 gas chromatograph by using a $^1/_8$ in. × 6 ft column packed with a 10% OV-101 (methyl silicon) liquid phase. The column was held 2 min at 80 °C and then programmed to 290 °C at 16 °C/min.

The peppermint sample was analyzed on a Perkin-Elmer 3920 gas chromatograph by using a $^1/_8$ in. × 10 ft column packed with 10% SP-1000 (Carbowax 20M terminated with terephthalic acid; Supelco, Belefonte, PA). The column was temperature programmed from 80 to 220 °C at 8 °C/min.

The flavor chemical model system and the cola sample were quite complex and required a capillary column for satisfactory resolution. We used a Perkin-Elmer Sigma 2 gas chromatograph with an all-glass linear splitter (split 80:1) and a helium carrier gas linear velocity of 23 cm/s. The fused silica column was made by Perkin-Elmer, measuring 0.235 mm i.d. × 25 m, coated with an OV-101 liquid phase. The column was temperature programmed from 70 to 180 °C at 4 °C/min. The effluent at the end of the column was combined with helium makeup gas and split three ways (1:1:1) to a flame ionization detector, a nitrogen-selective (alkali flame) detector, and a sulfur-selective (flame photometric) detector.

The liquid chromatographic fractions were also evaluated by odor on perfume blotters. With many odorous materials, this is the detection procedure of choice.

Quantitation of each fraction and total recovery studies were performed on a Perkin-Elmer Sigma 10 data system. Percent of each component in each fraction was calculated by comparison of relative area counts in each fraction to total area counts of each component. Total recovery was

Table I. Composition of Synthetic Flavor Mixture

GC peak no.[a]	component	contains N or S
1	*cis*-3-hexenol	
2	2,4-dimethylthiazole	N, S
3	2-methyl-3-methoxypyrazine	N
4	2-methyl-6-methoxypyrazine	N
5	octanal	
6	hexyl acetate	
7	heptyl mercaptan	S
8	3,6-dimethyl-2-ethylpyrazine	N
9	3,5-dimethyl-2-ethylpyrazine	N
10	2-nonanone	
11	dibutyl sulfide	S
12	methyl octanoate	
13	nonanol	
14	phenylethyl acetate	
15	anethole	
16	methyl anthranilate	N
17	eugenol	
18	8-mercapto-*p*-methan-3-one	S
19	impurity in no. 18	S

[a] In Figure 5.

determined by combining all fractions and comparing the area of the individual peaks to that of an appropriate aliquot of the original material.

Source of Materials. The methyl esters and the flavor chemicals were all obtained from standard commercial sources. The peppermint oil used was a U.S.P. redistilled Yakima (Citrus and Allied Essences, Floral Park, NJ). The carbonated beverage investigated incorporated a commercial cola flavor. Small amounts of ethanol were used to disperse the chemicals, as necessary.

Experimental. *Methyl Ester Model System.* The purpose of this experiment was to study the fractionation of a homologous series of compounds which differ in polarity. In this case 1 L of water containing 100 ppm each of methyl butyrate, hexanoate, octanoate, decanoate, and dodecanoate was pumped on the reverse-phase column. The column was washed with 100 mL of water and the organics were desorbed with an acetone gradient. Eight-milliliter fractions were collected.

Peppermint Oil. The objective here was to study the adsorption and fractionation of an actual natural material. In addition, we wanted to assess the result using low levels of organics and larger volumes of water. In this case the organics from 5 L of 50 ppm of aqueous peppermint solution were adsorbed on the column. The column was washed with 100 mL of water and desorbed with an acetone gradient. Fractions of 6.2 mL were collected.

Flavor Chemical System. The purpose of this experiment was to study the adsorption and fractionation of a complex synthetic mixture of organic compounds. The compounds chosen are all naturally occurring flavor chemicals which exhibit a wide variety of molecular weights, functional groups, and polarities. The compounds used are listed in Table I; also indicated is whether the molecule contains N or S. In this case 150 mg of each was dispersed in 5 L of water to give a final concentration of 30 ppm. This material was adsorbed on the reverse-phase column and subsequently desorbed with an acetone gradient. Approximately 7-mL fractions were collected.

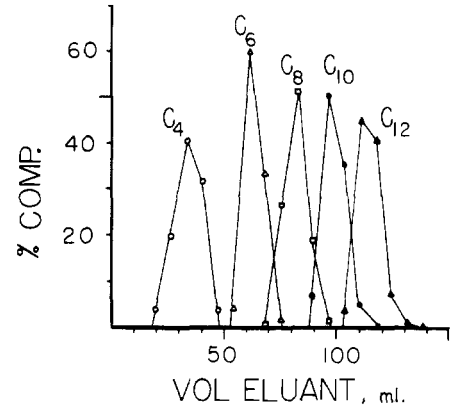

Figure 3. Fractionation of the methyl ester model system.

Carbonated Beverage. In this case we wished to study the effect of soluble solids (e.g., caramel color, caffeine, and sugar) on concentration and fractionation of the flavor chemicals. For this experiment, 3 L of commercial cola was decarbonated under vacuum. To this were added 13 ppm of methyl octanoate (as internal standard) and 100 ppm of a commercial cola flavor. The pH was raised to 6.12 with base. This solution was passed over the reverse-phase column. The column was washed with 100 mL of water and the organics were desorbed with an acetone gradient. Fractions of 4.5 mL were collected.

The soluble solids of the beverage before and after treatment were determined by refractive index measurement. The starting material was 11.5%; the column effluent measured 12.5%.

RESULTS

Methyl Esters. The results of the methyl ester experiments are plotted in Figure 3 as percent ester as a function of elution volume. From this, it is apparent that quite satisfactory resolution is obtained. The order of elution is from most polar to least polar.

Total recovery of each ester is as shown.

	methyl ester				
	C_4	C_6	C_8	C_{10}	C_{12}
total % recovery	93	85	85	105	104

All recoveries are over 80%. The high recoveries show the practical utility of this technique for concentrating a range

Table II. Percent of Each Peppermint Component in Each Fraction

peak no.[a]	identity	% in fraction no. (elution volume, mL)										
		1-10 (59)	11 (65)	12 (72)	13 (78)	14 (84)	15 (90)	16 (96)	17 (103)	18 (109)	19 (115)	20 (121)
1	limonene								13	52	31	3
2	1,8-cineole			22	47	25	4					
3	menthone			8	41	39	11	1				
4	menthofuran						36	50	14			
5	isomenthone			48	39	10	2					
6	menthyl acetate						15	45	34	6		
7	neomenthol				46	39	14					
8	menthol		4	42	40	11	2					

[a] In Figure 4.

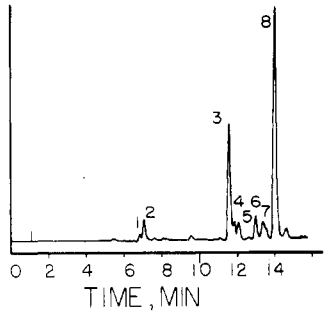

Figure 4. Gas chromatogram of peppermint oil.

Table III. Total Percent of Each Component Recovered

component	%
limonene	47
1,8-cineole	100
menthone	108
menthofuran	70
isomenthone	103
menthyl acetate	80
neomenthol	108
menthol	104

of chemicals from dilute aqueous solutions.

Peppermint Oil. A typical chromatogram of the peppermint oil is shown in Figure 4. Table II shows the composition of the fractions recovered by acetone elution from the reverse-phase column. Table III shows the actual recovery of each component.

The menthone, menthol, and 1,8-cineole tend to elute together with only minimal resolution in the liquid chromatographic system. On the other hand, the menthofuran and the menthyl acetate are somewhat retarded and the least polar component, limonene, is most retained.

The overall recoveries of the components are nearly quantitative, with two exceptions. Limonene was only partially recovered. The reduced recovery of menthofuran probably occurs since it does not resolve well (by gas chromatography) from menthone and the data reduction system does not quantitate it accurately.

From this experiment we conclude that large volumes of quite dilute (50 ppm) solutions may be concentrated and then fractionated with a high percent recovery.

Flavor Chemical Mixture. The test mixture was chosen to possess a variety of functional groups and polarities. Figure 5 is the capillary chromatogram of this test mixture which shows that not all of the components are resolved. Superimposed above this curve are the nitrogen- and sulfur-selective responses which were recorded.

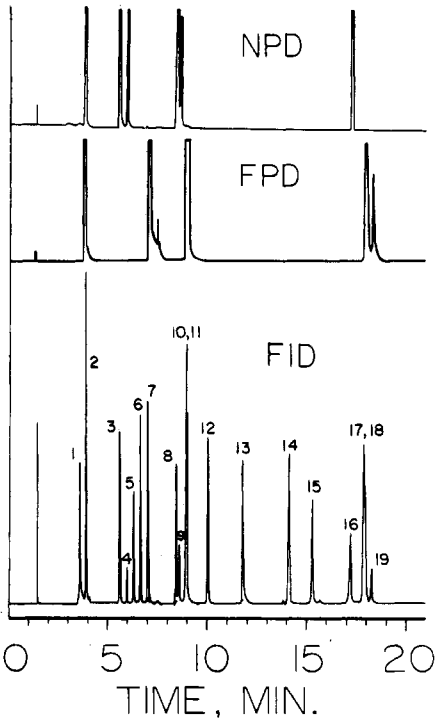

Figure 5. Capillary chromatogram of the flavor chemical model system.

Composition of the respective fractions is presented in Table IV. The chemicals in this table represent a number of naturally occurring compounds which are important in food aromas. The lower molecular weight, more polar components elute early—examples are hexenol and the alkylalkoxypyrazines. Components of intermediate polarity such as hexyl acetate, nonanal, and anethole elute at moderate volumes. The last eluting components are those of least polarity and include heptyl mercaptan and dibutyl sulfide.

The value of element specific detectors in monitoring composition is demonstrated nicely in a number of these fractions. A good example of this is shown in Figure 6, where the gas chromatographic patterns of two fractions

Table IV. Percent of Each Flavor Chemical in Each Fraction

GC peak no.	component	% in fraction no. (elution volume, mL)													
		1 (4)	2 (11)	3 (17)	4 (24)	5 (31)	6 (38)	7 (45)	8 (52)	9 (59)	10 (66)	11 (73)	12 (80)	13 (87)	14 (94)
1	*cis*-3-hexenol	5	12	44	31	5									
2	2,4-dimethylthiazole	3	16	49	26	5	1								
3	2-methyl-3-methoxypyrazine	6	16	46	24	4									
4	2-methyl-6-methoxypyrazine	7	17	46	24	4									
5	octanal								5	45	50	1			
6	hexyl acetate								5	70	21	1			
7	heptyl mercaptan											2	65	32	1
8	3,6-dimethyl-2-ethylpyrazine			4	46	39	5								
9	3,5-dimethyl-2-ethylpyrazine			6	53	34	4								
10	2-nonanone								1	48	46	4			
11	dibutyl sulfide												44	50	4
12	methyl octanoate									2	44	48	5		
13	nonanol									46	54				
14	phenylethyl acetate						22	63	13	1					
15	anethole								2	58	36	1			
16	methyl anthranilate			2	19	52	24	4							
17	eugenol					11	65	23	1						
18	8-mercapto-*p*-menthan-3-one									54	41	3			
19	impurity in no. 18								11	65	18				

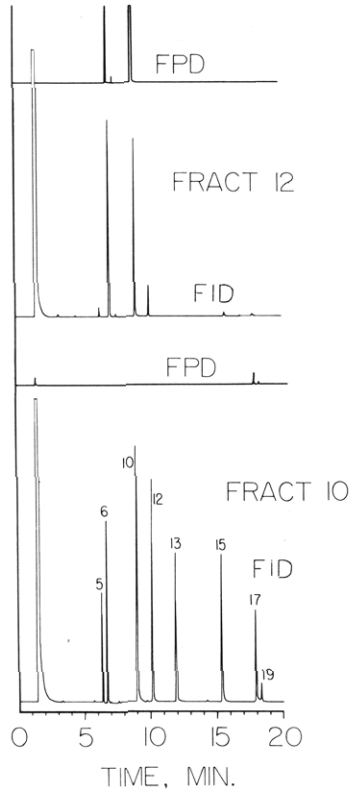

Figure 6. Comparison of flavor chemical fraction 10 vs. fraction 12.

are compared. Fraction 10 represents the components which elute between 59 and 66 mL, while fraction 12 represents those eluting between 73 and 80 mL. Both curves show a major gas chromatographic peak eluting at retention time of 9 min. Superimposed above each flame ionization detector response is the respective flame photometric detector (FPD; sulfur) response. The FPD shows that the peak which elutes at 9 min in fraction 12 (peak 11) contains sulfur; the corresponding peak in fraction 10 (peak 10) does not. These selective detectors are also useful where slight chromatographic resolutions of peaks is apparent. For example, 2-methyl-6-methoxypyrazine (peak 4) is barely separated from octanal (peak 5); the NPD shows that the earlier component contains nitrogen. At a gas chromatographic retention of 18.0 min, two components coelute. The FPD shows that LC fractionation resolves two components and that the one with the larger retention volume possesses sulfur.

Overall recoveries of the majority of the chemicals in this mixture were in the 85–110% range. Two components had recoveries of only 50% and these were *cis*-3-hexenol and heptyl mercaptan; it is quite possible that these two components reacted with the solvent. Recovery of methyl octanoate was 80%.

One other useful feature of this fractionation technique should be noted. It is possible to dip perfume blotters in the liquid chromatographic fractions and evaluate the odors of these fractions. Although we did not evaluate all LC fractions in a rigorous fashion, it was easy to pick out eugenol (clove) aroma at 38 mL, phenylethyl acetate (honey–rose) at 45 mL, methyl anthranilate (grape-like) at 31 mL, and various pyrazine-type aromas at 24 mL.

The complimentary nature of our technique and gas chromatography is nicely shown on this series. The gas chromatograph separates primarily by boiling point (especially on a methyl silicon column) whereas a reverse-phase column separates by polarity. One example of this

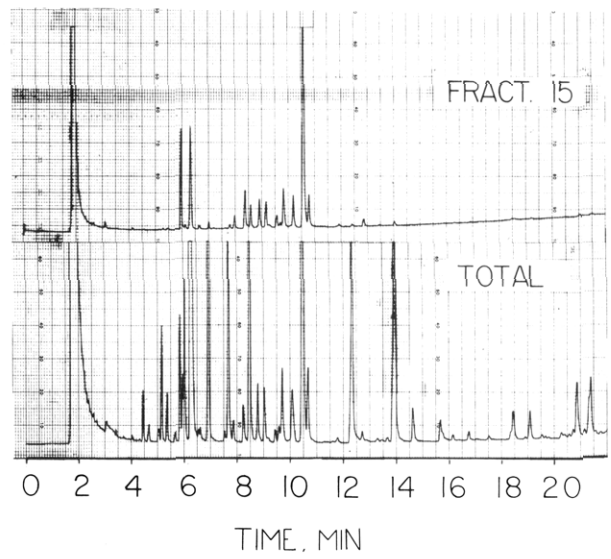

Figure 7. Gas chromatogram of cola volatiles—total sample vs. fraction 15.

complimentary nature is shown by the relationships of three compounds in Table IV, specifically 3-hexenol, methyl anthranilate, and hexyl acetate. The hexenol and the anthranilate elute from the liquid chromatographic column relatively early while the hexyl acetate is retarded. Yet when we compare the boiling points of these compounds, we find both the hexenol and hexyl acetate boil at ~160 °C, while the value for the anthranilate is 237 °C.

Carbonated Beverage Mixture. This mixture was chosen to evaluate the technique in a more realistic situation, as well as to study the effect of soluble solids. Carbonated colas generally contain ~10% sugar as well as caramel color, caffeine, and phosphoric acid. On the basis of refractive index measurements, the resin does not adsorb the sweetener since the RI in ≅ RI out.

The effluent was collected in 27 fractions and each of these was analyzed via capillary gas chromatography. In addition, the composition of each fraction was calculated with our data system. The overall recovery of methyl octanoate, the internal standard which elutes at 8.6 min, is 115%. From this we can conclude that the presence of significant quantities of soluble solids does not effect recovery of the aroma chemicals.

As an example of the simplification achieved, Figure 7 shows two superimposed chromatograms. The lower curve represents the capillary chromatogram of the total sample; the upper curve presents the pattern found for fraction 15.

In order to further demonstrate the separation obtained, we presented the 27 samples to our creative flavorists. They evaluated the odors on perfume blotters and the results of this experiment are presented in Table V. It is apparent that these experts were able to detect subtle nuances in consecutive fractions. The value of odor descriptions in apparent. Such information can be used by flavorists in compounding efforts; alternatively, fractions possessing useful organoleptic properties can be further studied by standard isolation and identification techniques. For example, if the flavorist wished a flavor note to contribute a "fresh lime" or "citrus" note, he would study the composition of fraction 15.

CONCLUSION

The advantages of this technique can be summarized as follows. (1) Volatile aromatic chemicals can be concentrated from dilute aqueous solutions. (2) These chem-

Table V. Odor Properties of Cola Fractions

fraction no.	character
2	weak
3	sweet, vanillin
4	vanillin
5	phenolic, smokey, coumarin-like
6	tonka bean, sweet
7	tonka bean
8	cinnamic aldehyde, resinoid
9	clean cinnamon
10	cassia-like, cinnamic aldehyde
11	cola lime, cinnamon
12	limey, cinnamic, piney
13	piney, musty, oily
14	citral, lemon
15	fresh lime, citrus, nootkatone-like
16	harsh, floral, petitgrain-like
17	citrus, orange
18	orange, aldehydic
19	cola-citrus, orange, woody
20	ginger, lime
21	carrot seed oil
22	carrot note
23	tomato, vegetable
24	green tomato, metallic, rosemary
25	vegetative, green
26	herbaceous
27	weak

icals can then be fractionated by a solvent gradient. (3) Overall recoveries of aroma chemicals are generally over 80%. (4) The fractions can be evaluated on sniff strips as well as by standard (GC, GC–MS, and HPLC) methods. (5) The presence of significant levels of soluble solids does not cause interference. (6) The procedure is inherently nondestructive, since the catalytic activity of silica gel and the heat associated with gas chromatography are eliminated.

The value of this procedure has been shown with a methyl ester model system, a synthetic flavor mixture containing a variety of functional groups, a peppermint oil, and a carbonated beverage.

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Effects of Plant Growth and Air Curing on Amounts of *trans*-Coniferyl and *trans*-Sinapyl Alcohols in Midveins of Burley Tobacco

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A quantitative gas chromatographic method was developed for the estimation of *trans*-coniferyl and *trans*-sinapyl alcohols in leaf midveins of field-grown Ky 14 burley tobacco. It was confirmed by gas chromatography–mass spectrometry that both alcohols were major phenylpropanoids in hot-water extracts of the leaf midvein samples. Concentrations of both alcohols increased as the maturity of plants increased. Greater quantities of alcohols were obtained after air curing than at harvest. Leaf position on the stalk also affected the amounts of the alcohols recovered, but the magnitude and direction of this effect depended on plant maturity and air curing parameters. Levels of *trans*-sinapyl alcohol among all the samples were only about one-third those of *trans*-coniferyl alcohol; the amounts found ranged from 7.3 to 70.5 mg of coniferyl alcohol and 0 to 24.6 mg of sinapyl alcohol per kg dry weight of sample.

Coniferyl alcohol [3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol] and sinapyl alcohol [3-(-hydroxy-3,5-dimethoxyphenyl)-2-propen-1-ol] are considered to be precursors of the monolignol building blocks of plant lignin (Freudenberg, 1966), and they were recently identified as phenolic phenylpropanoids in hot-water extracts of burley tobacco stalk (Andersen et al., 1980). These alcohols were thought to be weakly bound to non-cross-lined regions of lignin or polysaccharide and capable of release by hydrolysis with hot water. Coniferyl alcohol has been identified as a constituent in a phenolic fraction of cigarette tobacco smoke condensate; its concentrations were twice as high in midvein condensate as in leaf lamina condensate (Ishiguro et al., 1976). Since tobacco leaf midveins are used in smoking tobacco (Moshy, 1967), it is important to learn whether contents of these phenolic phenylpropanoids in tobacco leaf midveins are influenced by plant growth environment, stage of plant development, and curing.

In this report we identify *trans*-coniferyl alcohol and *trans*-sinapyl alcohol in superheated water extracts of burley tobacco leaf midveins by gas chromatography–mass spectrometry (GC–MS). The effects of plant population

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