Identification of Alcohols and Volatile Organic Acids from Natural Orange Essence

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Alcohols and volatile organic acids were isolated from natural orange essence and identified. The alcoholic constituents were removed from carbonyl-free aqueous essence by solvent extraction and column chromatography, while the organic acids were separated by steam distillation. Programmed temperature gas chromatography (PTGC), infrared analysis, and paper chromatography of the alcohols and alcohol derivatives, and PTGC and paper chromatography of the acids furnished confirmations of component identities. The following alcohols and acids were found in natural orange essence: methanol, ethanol, *n*-propanol, isobutanol, *n*-butanol, isopentanol, *n*-pentanol, *n*-hexanol, 3-hexen-1-ol, linaloöl, *n*-octanol, terpinen-4-ol, *n*-nonanol, α-terpineol, *n*-decanol, citronellol, acetic acid, propionic acid, butyric acid, caproic acid, and capric acid. The presence of the following alcohols and acids was indicated but not confirmed: isohexanol, methyl heptenol, 2-nonanol, nerol, geraniol, carveol, isovaleric acid, valeric acid, isocaproic, and caprylic acid.

In recent years, the use of chromatography and supplementary analytical techniques has greatly accelerated interest in the identification of specific flavor-producing compounds in fruit and fruit products. Several investigators in this field have studied the oxygen-containing flavor components of citrus juices and oils. Of especial interest is the work of Kirchner and Miller (*6*), who made specific identifications of *n*-hexanol, 3-hexen-1-ol, linaloöl, *n*-octanol, α-terpineol, *n*-decanol, and carveol as alcoholic constituents, and acetic, propionic, butyric, and isovaleric acids as acidic constituents of California Valencia orange juices. In addition, Bernhard (*4*) used gas liquid chromatography to identify tentatively 3-hepten-1-ol, isopulegol, borneol, and citronellol among the constituents of California Valencia orange oil.

An earlier report from this laboratory (*10*) showed tentative identification assignments for a number of alcohol components of orange essence based on PTGC retention temperature studies using two separate column phases, and these results were used in comparing volatile flavor components of fresh juices from three established varieties of Florida oranges (*11*). Also, Attaway *et al.* (*1*, *2*) described a method suitable for the identification of small quantities of gas chromatographically separated alcohols through formation of their *o*- and *m*-nitrophenyl- and *p*-phenylazophenylurethan derivatives. This method is based on paper chromatographic and infrared analysis of the derivatives and has been found useful in some cases where direct infrared analysis was not practical.

The present paper reports the isolation and specific identification of the alcoholic and volatile acidic constituents of Florida Valencia orange essence extracts utilizing both gas and column chromatography for separation and preliminary identification, followed by infrared spectroscopy and derivative formation for definite identification.

Experimental

Apparatus and Reagents. The orange essence used was described previously (*3*, *10*).

Programmed temperature gas chromatography (PTGC) of the alcohols was conducted on an F & M Model 502, dual-column instrument equipped with thermal conductivity detection. The columns were 10 feet in length, 1/4-inch o.d., and packed with 20% Carbowax 20M on 40- to 60-mesh Chromasorb regular. A 60 ml. per minute helium flow was used.

PTGC of the volatile acids was carried out with an F & M Model 720, dual-column instrument also equipped with thermal conductivity detection. The columns were 12 feet in length, 1/4-inch o.d., packed with 20% Carbowax 20M on 60- to 80-mesh Gas-Chrom Z. A helium flow of 150 ml. per minute was used.

A Beckman IR-4 recording infrared spectrophotometer was used for the determination of infrared spectra.

Procedure. Prior to the analysis of alcohols, carbonyl components were removed from the aqueous essence using sodium bisulfite, according to the procedure of Wolford *et al.* (*10*), or from essence extracts by Girard's "T" reagent, according to the procedures described by Teitelbaum (*9*) and Stanley, Ikeda, Vannier, and Rolle (*8*). The carbonyl-free extract was passed through columns of either activated alumina or silicic acid, with both materials being used as a check for possible alteration on the columns. Elution from the columns was accomplished by using either Skellysolve B or isopentane followed by progressively more polar solvent combinations utilizing benzene, diethyl ether, and methanol. The order of elution from the column was terpene hydrocarbons, esters, and alcohols, with no organic acids being found in the eluate. The last fractions from the column were shown by infrared analyses to consist entirely of alcoholic components, and were subsequently analyzed by PTGC over a range of 60° to 250° C. at a programmed rate of 4° C. per minute.

Identifications of individual alcohol peaks were based upon retention temperature comparison by the enrichment technique (*10*), paper chromatographic analysis of *o*- and *m*-nitrophenyl- and *p*-phenylazophenylurethan derivatives (*1*, *2*), and infrared analysis of both derivatives and pure alcohols. The paper chromatographic analysis of the urethans was done on the entire spectrum of alcohols prior to gas chromatographic separation, and on individual or groups of individual alcohols separated gas chromatographically and condensed in glass U-tubes as described previously (*2*).

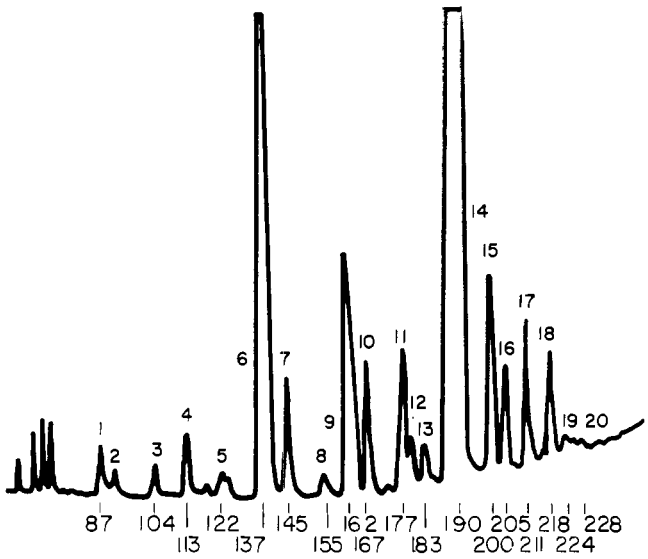

Figure 1. Gas chromatogram of alcohols, with retention temperatures (°C.)

Peak 1, methanol; 2, ethanol; 3, *n*-propanol; 4, isobutanol; 5, *n*-butanol; 6, isopentanol; 7, *n*-pentanol; 8, isohexanol (?); 9, *n*-hexanol; 10, hexen-1-ol; 11, methyl heptenol (?); 12, unknown; 13, 2-nonanol (?); 14, linaloöl and *n*-octanol; 15, terpinen-4-ol; 16, *n*-nonanol; 17, α-terpineol; 18, *n*-decanol and citronellol; 19, nerol (?); 20, geraniol (?) and carveol (?)

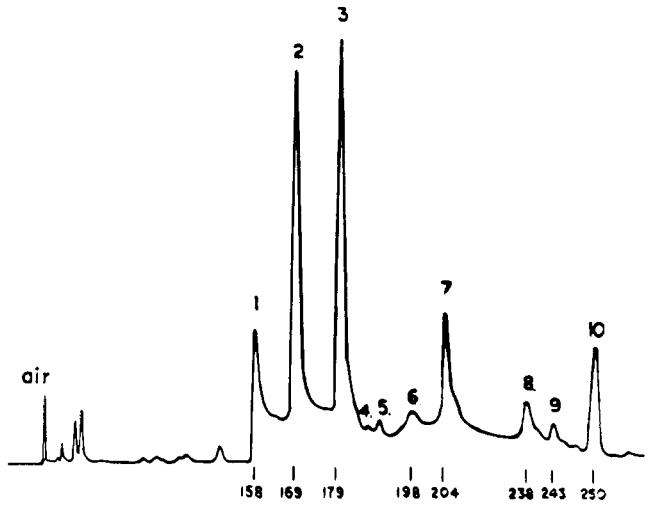

Figure 2. Gas chromatogram of acids, with retention temperatures (°C.)

Peak 1, acetic; 2, propionic; 3, butyric; 4, isovaleric (?); 5, valeric (?); 6, isocaproic (?); 7, caproic; 8, caprylic (?); 9 ,unknown; 10 ,capric

Table I. Paper Chromatographic R_f Values of *m*-Nitrophenylurethans[a]

Peak No.	Corresponding Alcohol	R_f of Unknown	R_f of Known	R_f of Known + Unknown
1	Methyl	0.09	0.09	0.09
2	Ethyl	0.20	0.21	0.21
3	Propyl	0.37	0.37	0.38
4	Isobutyl	0.54	0.55	0.54
5	Butyl	0.54	0.55	0.54
6	Isopentanol	0.68	0.68	0.68

[a] Papers dipped in methanol–propylene glycol (80–20 v./v.) developed with methanol-saturated heptane.
[b] Represents a mixed R_f value used in a similar manner as mixed melting points for identification.

Table II. Paper Chromatographic R_f Values of o-Nitrophenylurethans[a]

Peak No.	Corresponding Alcohol	R_f of Unknown	R_f of Known	R_f of Known + Unknown
7	Pentanol	0.68	0.66	0.67
9	Hexyl	0.56	0.57	0.57
10	3-Hexen-1-yl	0.65	0.63	0.64
14[b]	Linalyl +	0.49	0.49	0.49
	octyl	0.39	0.39	0.39
15	Terpinen-4-yl	0.41	0.42	0.41
16	Nonyl	0.32	0.33	0.32
17	Terpinyl	0.38	0.38	0.37
18[b]	Citronellyl +	0.45	0.44	0.45
	decyl	0.23	0.24	0.23

[a] Papers dipped in 7% solution of petroleum jelly in heptane, developed with methanol-water (95–6 v./v.).
[b] Dual component peak.

The preparation of a sample for PTGC analysis of acids presented a sizable problem because of the high concentration of all other volatile components in the aqueous essence. Various procedures utilizing steam distillation and ion exchange chromatography were tried. The following procedure was selected as the most straightforward and effective for producing a good acid sample relatively free from neutral contaminants. Two liters of pH 4 essence were made to pH 9.0 by addition of 10% aqueous Na_2CO_3 and slowly evaporated to dryness on a hot water bath. The basic residue was dissolved in 20 ml. of distilled water and steam distilled to remove unevaporated neutral volatiles. After 250 ml. of steam distillate had been collected, the residue was cooled in an ice bath and acidified to pH 1.0 with 18*N* H_2SO_4, following which it was again steam distilled and a second 250 ml. collected. The receiver was cooled in an ice water bath and contained a few milliliters of 10% Na_2CO_3 solution to maintain basicity. The resulting solution was evaporated to dryness and redissolved in 10 ml. of distilled water. It was cooled in an ice water bath, saturated with NaCl, and extracted with two 10-ml. portions of ethyl bromide. The extracts were combined in a 25-ml. beaker and allowed to evaporate at room temperature until the solvent was removed. About 0.1 ml. of free acids remained for chromatographic analysis. The gas chromatogram was programmed from 100° to 260° C. at 4° C. per minute.

To confirm the major peaks the acids were paper chromatographed as diethylamine (DEA) salts. Two solvent systems were used; butanol–water–DEA, 100:15:1, as described by Block, Durrum, and Zweig (*5*), and butanol–cyclohexane–propylene glycol–DEA–water, 30:30:10:0.5:3.7, a modification of system III of Osteaux, Guillaume, and Laturaze (*7*). Double acid-washed paper, SS 589, was used for all of the acid paper chromatograms. The first system works well for the higher acids in question, but the second system was necessary to separate the salts of formic and acetic acids. Bromophenol blue (*5*) was used for detecting the spots.

Results and Discussion

Sixteen alcohols and five acids were definitely identified as constituents of aqueous orange essence. The alcohols

Table III. Criteria for Identification of Alcohols Isolated from Orange Essence

Alcohol	Chromatography: Gas, retention temp.	Chromatography: Paper, R_f value	Infrared spectrum
Methanol	+	+	
Ethanol	+	+	+
Propanol	+	+	
Isobutanol	+	+	
Butanol	+	+	
Isopentanol	+	+	+
Isohexanol (?)	+[a]		
Hexanol	+	+	
3-Hexen-1-ol	+	+	
Methyl heptenol (?)	+		
2-Nonanol (?)	+		
Linaloöl	+	+	+
Octanol	+	+	+[b]
Terpinen-4 ol	+	+	+
Nonanol	+	+	
α-Terpineol	+	+	+
Citronellol	+	+	
Decanol	+	+	
Nerol (?)	+		
Geraniol (?)	+		
Carveol (?)	+		

[a] Estimated. [b] Spectrum of derivative.

Table IV. DEA Salts of Acids from Orange Essence Chromatographed with Butanol–Water–DEA

Acid	R_f of Sample	R_f of Known	Relative Spot Intensity
Unknown	0.22	...	Weak
Formic and acetic	0.34	0.34	Strong
Propionic	0.46	0.47	Strong
Butyric	0.59	0.59	Strong
Valeric and/or isovaleric	0.69	0.69	Weak
Caproic	0.75	0.76	Strong
Capric	0.86	0.87	Weak

Table V. DEA Salts of Acid from Orange Essence Chromatographed with Butanol–Cyclohexane–Propylene Glycol–Water–DEA

Acid	R_f of Sample	R_f of Known	Relative Spot Intensity
Unknown	0.33	...	Weak
Formic	0.47	0.47	Strong
Acetic	0.54	0.54	Moderate
Propionic	0.70	0.69	Strong
Butyric	0.81	0.81	Strong
C_5–C_{10}	0.90–0.98	0.90–0.98	Moderate

were methanol, ethanol, *n*-propanol, isobutanol, *n*-butanol, isopentanol, *n*-pentanol, *n*-hexanol, 3-hexen-1-ol, linaloöl, *n*-octanol, terpinen-4-ol, *n*-nonanol, α-terpineol, *n*-decanol, and citronellol. The acids were acetic, propionic, butyric, caproic, and capric. In addition, six other alcohols and three acids were indicated as possible components. These were isohexanol, methyl heptenol, 2-nonanol, nerol, geraniol, carveol, isovaleric acid, valeric acid, and caprylic acid. The identification of these constituents as concentrated in a natural orange essence provides additional basis for a future understanding of the role of these constituents in producing the flavor of orange juice.

Alcohols. A typical gas chromatogram of the separated alcohols with peak identities is shown in Figure 1. The first five components had relatively minor peaks and could not be condensed for individual study. However, after tentative identifications had been obtained by retention temperature studies, the components were collected as a group, converted to their *m*-nitrophenylurethans, and paper chromatographed on propylene glycol–impregnated Whatman No. 3MM paper according to the directions of Attaway *et al.* (*1*) to confirm the identities as shown in Table I.

Component 6 was present in substantial quantity and gave a prominent peak. It was condensed and studied through its infrared spectrum and *m*-nitrophenylurethan derivative. All evidence confirmed it as isopentanol. Further substantiation as isopentanol was achieved by paper chromatographic separation of the mixed *m*-nitrophenylurethans of the alcohols as obtained from the alumina or silicic acid columns. Table I also contains the data obtained for confirmation as the *m*-nitrophenylurethan.

Component 7, identical to *n*-pentanol by retention temperature, was condensed in sufficient amount to form an *o*-nitrophenylurethan. The derivative was studied paper chromatographically on Whatman No. 3MM paper impregnated with petroleum jelly (*1*). Its R_f value confirmed the identification as *n*-pentanol as shown in Table II.

Component 8 could possibly be isohexanol because of its location on the chromatogram between *n*-pentanol and *n*-hexanol.

Component 9 was identical to *n*-hexanol by retention temperature. A small quantity was condensed, and reacted with *o*-nitrophenylisocyanate, and the product was paper chromatographed. The R_f value corresponded to known *n*-hexyl *o*-nitrophenylurethan as shown in Table II.

Component 10 was identical to 3-hexen-1-ol gas chromatographically. It was condensed, converted to the *o*-nitrophenylurethan, and paper chromatographed. The R_f value corresponded to that of the known derivative. In addition, a *p*-phenylazophenylurethan of 3-hexen-1-ol was isolated from the unseparated alcohols obtained from the alumina column by reaction with *p*-phenylazophenylisocyanate and prep scale paper chromatography of the product.

Component 11 was tentatively identified as methyl heptenol by its retention temperature. However, attempts to prepare a confirmatory derivative were unsuccessful.

The minor component designated 12 could not be identified, while the minor component numbered 13 was gas chromatographically identical to 2-nonanol.

Peak 14, the major peak, corresponded to linaloöl and *n*-octanol. Condensate was converted to *o*-nitrophenyl- and *p*-phenylazophenylurethans and analyzed paper chromatographically. Two spots obtained from each type urethan corresponded to spots of the known linaloöl and octanol derivatives. The mixtures were separated by prep scale paper chromatography and the individual derivatives analyzed through their infrared spectra. Again they corresponded to the known derivatives of linaloöl and octanol.

Component 15, which corresponded gas chromatographically to terpinen-4-ol, was condensed in sufficient quantity for both derivative formation and direct determination of the infrared spectrum of the free alcohol. Both the derivative and the spectrum confirmed the identification.

Peak 16 corresponded to *n*-nonanol on the gas chromatogram. An *o*-nitrophenylurethan was prepared, paper chromatographed, and shown to be *n*-nonanol *o*-nitrophenylurethan.

Component 17 corresponded to α-terpineol on the chromatogram. It was condensed in sufficient quantity for both derivative formation and infrared spectral determination, both of which confirmed the identification.

Peak 18 corresponded to *n*-decanol and citronellol. Both were confirmed by paper chromatography of the *o*-nitrophenylurethan mixture.

Peak 19 corresponded to nerol gas chromatographically, while peak 20 corresponded to geraniol and carveol. These components were not present in sufficient quantity for derivative formation or infrared analysis.

Table III summarizes criteria used for the identifications of the alcohols.

Acids. This high helium flow rate was necessary to elute the higher molecular weight acids at a reasonably low temperature on the programmed instrument. For example, capric acid emerged at a temperature of 250° C. at the high flow, but at 60 ml. per minute, a temperature of 290° C. was required to elute this acid. Less tailing of the acid peaks was also observed at the higher rate of flow.

A typical gas chromatogram of the acids is shown in Figure 2. The retention temperatures strongly indicate

the identities of the important acids, and consequently the paper chromatographic data shown in Tables IV and V are sufficient for confirmation of acetic, propionic, butyric, caproic, and capric.

Although the paper chromatographic data show formic acid to be present in substantial quantity, it was not detected on the gas chromatograms. Known formic acid solutions also gave no peak. Apparently either decomposition of formic acid takes place in the gas chromatograph, or else the acid combines with the liquid stationary phase (Carbowax 20M) and therefore never elutes from the column.

The acids in low concentrations, as indicated by the minor peaks 4, 5, 6, and 8, received only tentative identification by retention temperature coincidence on one column, although Table IV does show evidence for the presence of a 5-carbon acid. Peak 9 remains as an unknown.

At least seven components not previously identified as constituents of oranges or processed orange products have been reported. These newly identified compounds are *n*-propanol, isobutanol, *n*-butanol, isopentanol, terpinen-4-ol, *n*-decanol, and *n*-caproic acid. Also, 10 compounds previously tentatively identified (*10*) were confirmed.

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FEED ADDITIVE RESIDUES

Determination of Trace Amounts of Nitrofurazone in Milk

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A method has been developed for quantitative determination of nitrofurazone in milk with a sensitivity of 0.01 p.p.m. The procedure is based on conversion of nitrofurazone to 5-nitrofurfuraldehyde phenylhydrazone, followed by extraction and concentration on a chromatographic column. Final estimation depends upon development of a blue color with Hyamine base.

NITROFURAZONE is used for the treatment of mastitis in cattle. Methods previously available for determination of nitrofurazone residues in milk include those of Paar (*7*) and of Cox and Heotis (*5*). Paar's method determines nitrofurazone by direct photometric measurement at the 375 mμ maximum after precipitation of milk proteins with sodium tungstate and sulfuric acid. The method is very simple and suitable for levels of 1 p.p.m. or more. The limit of detection is 0.5 p.p.m. The method of Cox and Heotis involves conversion of nitrofurazone to 5-nitrofurfural phenylhydrazone with subsequent toluene extraction and chromatography, finally measuring the phenylhydrazone compound at 430 mμ. The procedure is sensitive to 0.25 p.p.m. Colorimetric estimation of the phenylhydrazone is also the basis of methods for determination of nitrofurazone in feeds (*1*), plasma (*4*), and tissues (*6*).

A method for determining nitrofurazone in milk sensitive to 0.01 p.p.m was needed to meet a Food and Drug Administration requirement. The procedure developed consists of converting any nitrofurazone present to 5-nitrofurfural phenylhydrazone followed by extraction in toluene as in the Cox and Heotis method (*5*). A sensitivity of 0.01 p.p.m. is attained by use of the following modifications. A large sample of milk, 120 ml., is used for each determination. Sodium chloride is added to prevent the formation of gel in the toluene extract. This permits recovery of a larger portion of extract. Column chromatography is used as a means of concentrating the phenylhydrazone compound. A blue color is developed; thus the wave-length of color measurement is out of the range of milk pigment interference.

Materials and Methods

Reagents and Instrument. The reagents used include phenylhydrazine hydrochloride, 1% in water, freshly prepared for each set of samples; aluminum oxide, Merck chromatographic grade; hydroxide of Hyamine 10× (Rohm & Haas, Reg. Trade Mark), 1 molar solution in methanol as used in preparation of samples for scintillation counting (available from Packard Instrument Co., Lagrange, Ill.); crystalline nitrofurazone standard (Hess & Clark). A Beckman Model DU spectrophotometer was used for all absorbance measurements.

Preparation of Standard. Prepare the standard curve by assay of samples of control milk with known amounts of nitrofurazone added. Dissolve 100 mg. of pure nitrofurazone in 100 ml. of dimethylformamide. Dilute 1 ml. of this solution to 100 ml. with water. Each milliliter of this standard contains 10 μg. of nitrofurazone. Measure 200-ml. portions of control milk