Methods for the Determination of Oxygenated Terpene, Aldehyde, and Ester Concentrations in Aqueous Citrus Essences

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Three new methods were developed for functional group analysis of water-soluble components of citrus juice essences. These included techniques for estimating oxygenated terpenes as $C_{10}H_{18}O$, saturated aliphatic aldehydes as octanal, and α,β-unsaturated aldehydes as citral. In addition, a standard procedure was adapted for the determination of esters as ethyl butyrate. Analyses of 15 citrus juice essences are given.

For several years this laboratory has been engaged in the study of aqueous citrus juice essences, chiefly orange essences, and their possible use as flavor-enrichment agents in processed products such as frozen concentrated orange juice. The term "orange essence" as used in this paper refers to aqueous solutions of volatile flavor components recovered following their removal from fresh juice or from the evaporator condensate during the production of concentrated orange juice. They usually contain aldehydes, alcohols, esters, ketones, and organic acids as well as azeotropes of terpene hydrocarbons. Past publications have dealt with the identification of many of these components by chromatographic techniques (Attaway *et al.*, 1964a; Attaway and Wolford, 1965; Attaway *et al.*, 1964b; Attaway *et al.*, 1962; Wolford *et al.*, 1963; Wolford *et al.*, 1962; and Wolford *et al.*, 1965.)

The usual procedure for analysis of essences has involved solvent extraction using methylene chloride, diethyl ether, or isopentane, followed by solvent removal and gas-liquid chromatographic separation of the remaining organic fraction. While this approach is satisfactory to determine individual compounds, it cannot be readily adapted for estimating the blending qualities of an essence to be used for flavor enrichment on a commercial scale. The chemical oxygen demand (COD) method (Dougherty, 1964; McNary *et al.*, 1957) has been used with some success for this purpose but is too general because of its applicability to all types of oxidizable compounds. Accordingly, the new methods described in this paper were developed to provide rapid colorimetric techniques for evaluating various functional groups of essence samples in the quality control laboratory without the use of time-consuming techniques requiring solvent extraction and sophisticated instrumental analyses.

Experimental

Apparatus. Colorimeters used were either the Fisher Electrophotometer II with 425-, 525-, and 585-mμ filters and 10-mm. cuvettes, or the Bausch and Lomb Spectronic 20 with $^1/_2$-inch square cuvettes. Volumetric pipets were used in all liquid transfers and dilutions.

For locating best absorption maxima the Beckman DK-2 recording UV-visible spectrophotometer was used.

Reagents. FOR $C_{10}H_{18}O$. Vanillin, 2% w./v. (Eastman White Label) in H_2SO_4 (reagent grade), 80% ethanol, citronellal (K & K Laboratories), linalool (Eastman White Label), terpinen-4-ol (L. Light & Co., Ltd.), and α-terpineol (Felton Chemical Co.).

FOR SATURATED ALIPHATIC ALDEHYDES. Aqueous p-phenylenediamine (0.25%, Aldrich Chemical Co.), 3% aqueous H_2O_2 (Fisher reagent grade), 80% ethanol, and octanal (K & K Laboratories).

FOR UNSATURATED ALDEHYDES. o-Dianisidine (2.5% w./v., Eastman technical grade) in glacial acetic acid (reagent grade), and citral (Eastman practical grade).

FOR ESTERS. Aqueous hydroxylamine hydrochloride (13.9%, Eastman practical), 10% aqueous $FeCl_3 \cdot 6H_2O$ (Fisher reagent grade), 3N NaOH, and 4N HCl, and ethyl butyrate (synthesized from ethyl alcohol and butyric acid).

Procedure. FOR $C_{10}H_{18}O$. A standard $C_{10}H_{18}O$ solution was prepared by mixing equal parts of citronellal, linalool, terpinen-4-ol, and α-terpineol. One gram of this mixture was dissolved in 1 liter of 80% ethanol to give a stock solution of 1000 p.p.m., which was then diluted to the 25-, 50-, 100-, 150-, and 200-p.p.m. $C_{10}H_{18}O$ levels for preparation of the standard curve (Figure 1).

For analysis, 1 ml. of essence or standard solution was added to 10 ml. of water in a 25-ml. Erlenmeyer flask, following which 5 ml. of the vanillin–H_2SO_4 reagent were added with swirling. The solution was allowed to stand for approximately 5 minutes, after which the absorbance was read using the 585-mμ filter in the Fisher Electrophotometer II, or at 600 mμ using the Bausch and Lomb Spectronic 20. A blank solution with 1 ml. of H_2O instead of essence or standard solution was used to set the colorimeter to 100% transmission (0.00 absorbance). Readings were calculated as parts per million of $C_{10}H_{18}O$.

FOR SATURATED ALIPHATIC ALDEHYDES. A 1000-p.p.m. octanal solution was prepared by dissolving 1 gram of octanal in 1000 ml. of 80% ethanol. This mixture was subsequently diluted to the 150-, 250-, 350-, 450-, and 650-p.p.m. levels for preparation of the standard curve (Figure 2).

In the analysis of saturated aliphatic aldehydes 1 ml. of essence of standard solution was added to 10 ml. of
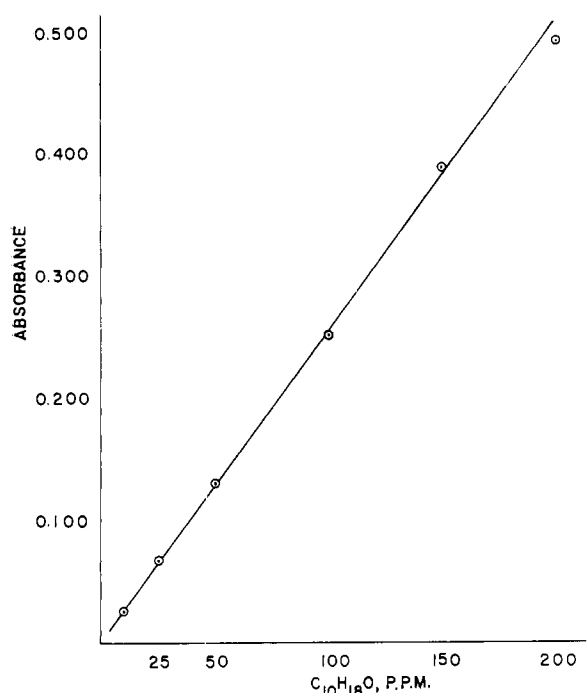
Figure 1. Standard curve for calculation of parts per million of oxygenated terpenes

water in a 25-ml. Erlenmeyer flask, following which 2 ml. of 3% H_2O_2 were added with swirling. Finally, 1 ml. of the p-phenylenediamine reagent was added. The reagent blank, prepared by using water instead of essence, was orange in color, while solutions containing saturated aliphatic aldehydes became progressively darker with increasing concentration. In exactly 15 minutes the absorbance was read using the 425-mμ filter of the Fisher Electrophotometer II, or at 435 mμ with the Bausch and Lomb Spectronic 20. When several samples are run in series, the p-phenylenediamine must be added to each in a predetermined order and the absorbance measurements read in the same order without interruption to allow a similar reaction time for each sample. Values were expressed as parts per million of octanal.
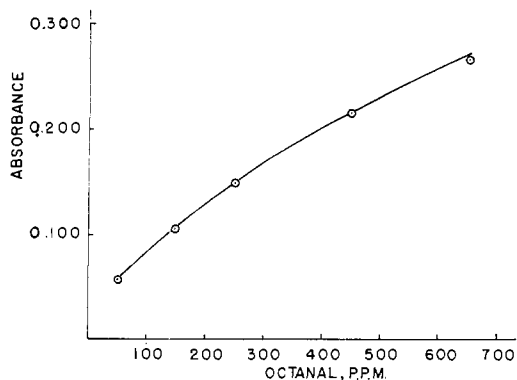
For Unsaturated Aldehydes. Standard citral solutions of 5, 10, 20, 30, 40, and 50 p.p.m. were prepared from a 1000-p.p.m. stock solution, made by dissolving 1 gram of citral in 1 liter of 80% ethanol, and used to prepare the standard curve (Figure 3). The analysis consisted of adding 1 ml. of the o-dianisidine solution to 10 ml. of essence in a 25-ml. Erlenmeyer flask. The sample solutions turn light to dark yellow with concentration while the blank remains pale blue or lavender. Readings were taken at 385 mμ using the Bausch and Lomb Spectronic 20 colorimeter and expressed as parts per million of citral.

For Esters. Standard solutions of 5, 10, 25, 50, and 100 p.p.m. of ethyl butyrate in 80% ethanol were prepared in the usual manner and the analysis was carried out as follows: Two milliliters of hydroxylamine hydrochloride solution were added to 5 ml. of essence in a 25-ml. Erlenmeyer flask. Immediately, 2 ml. of NaOH were added, and the solution was allowed to stand for 5 minutes, following which 2 ml. of HCl were added. Finally, 2 ml. of $FeCl_3$ solution were added, and the absorbance was read at 525 mμ using either colorimeter. The blank was yellow, while the ester solutions became progressively more orange with increasing concentration. The results were calculated as parts per million of ethyl butyrate.

Development of Methods

$C_{10}H_{18}O$ Method. The procedure for oxygenated terpenes was based on their reaction with strongly acid vanillin solutions to produce colored reaction products as were described on thin-layer chromatoplates in previous publications (Attaway and Wolford, 1965; Attaway et al., 1965). Orange essences gave a blue-green to blue color in solution.

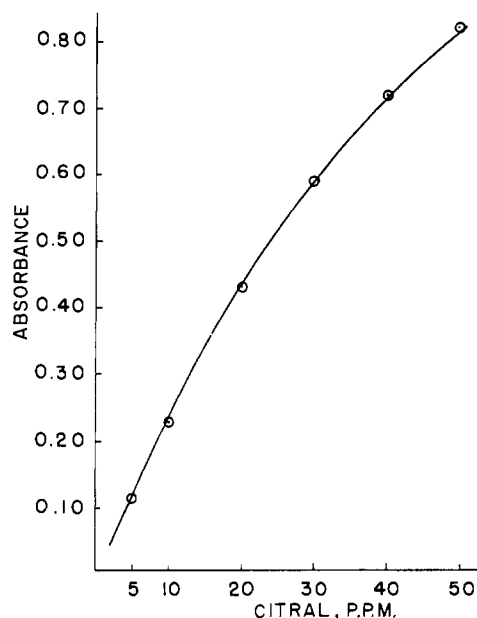Figure 2. Standard curve for calculation of parts per million of saturated aliphatic aldehydes
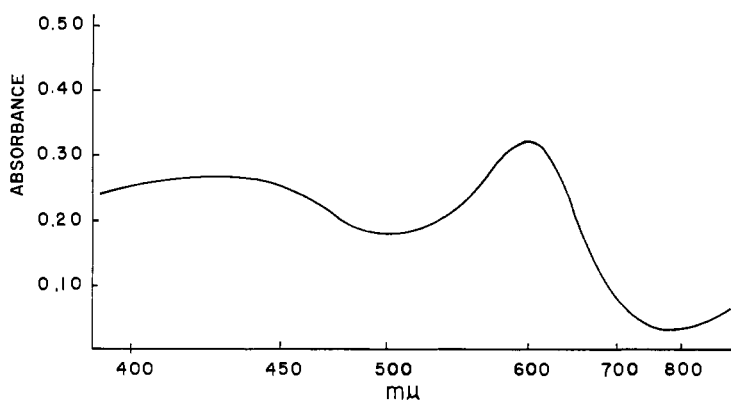

Figure 3. Standard curve for calculation of parts per million of unsaturated aldehydes

Figure 4. Visible spectrum of the reaction product of vanillin–H_2SO_4 and standard oxygenated terpene mixture
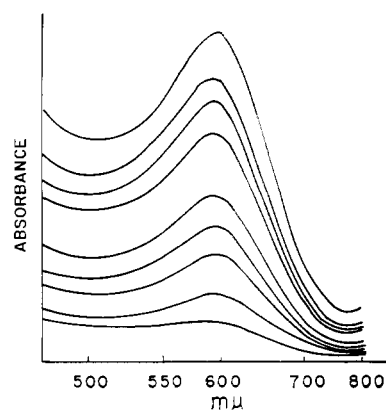

Figure 5. Effect of stepwise dilution of essence on height of oxygenated terpene peak

After extensive experimentation with vanillin solutions of varying concentrations and degrees of acidity and heating in the water bath at various temperatures to drive the reaction to completion, the use of a solution of 2 grams of vanillin in 100 ml. of concentrated H_2SO_4 was ideal. The intensity of the blue-green color produced was excellent for colorimetric measurement, and the heat of mixing between the H_2SO_4 and the aqueous essence drove the reaction to completion within 5 minutes without the use of supplementary heating. Determination of the visible spectra of the reaction products of essence and vanillin–H_2SO_4 showed an excellent analytical peak near 600 mμ (Figure 4). An equal mixture of citronellal, linalool, terpinen-4-ol, and α-terpineol, when diluted to the proper concentration in 80% ethanol, gave a reaction product with an almost identical curve. Consequently, these compounds were selected as the components of the standard $C_{10}H_{18}O$ mixture. No single compound alone was as satisfactory. The change in peak height with concentration is shown by Figure 5, which was prepared by diluting an orange essence stepwise with water.

Saturated Aliphatic Aldehyde Method. This procedure was based on the ability of aldehydes to catalyze the oxidation of p-phenylenediamine by hydrogen peroxide to produce a black product known as Bandrowski's base (Feigl, 1946, p. 345). The dark solution which results from reaction with both standard aldehydes and essence has a broad absorption maximum in the region of 425 to 475 mμ (Figure 6). Unsaturated and aromatic aldehydes did not appreciably increase the absorbance at this frequency, and carvone and methyl heptenone, ketones known to occur in citrus (Attaway and Wolford, 1965), also did not interfere. Unfortunately, different aldehydes produced different degrees of response. As can be seen in Table I, the shorter chain aldehydes were more effective catalysts than the longer chain aldehydes. Since the results of these analyses were based on standard solutions of octanal, one of the major saturated aldehydes of citrus, it is possible that the values may be high because

hexanal and acetaldehyde are known to occur in orange essence (Attaway *et al.*, 1962).

The time of reaction is very important in this procedure. All samples, including the blank, will eventually darken on standing, so each sample must be made to react for the same length of time. Consequently, a separate standard curve should be prepared for each set of samples. This was done by selecting three standard solutions of strengths equal to those of the essences being analyzed and including them in the particular set of determinations.

Unsaturated Aldehyde Method. This technique was based on the ability of aldehydes to combine with

Table I. Comparative Effects of Equal Strength Aldehyde Solution on Reagents

| Aldehyde	Absorbance
Saturated	
Formaldehyde	0.392
Acetaldehyde[a]	0.640
n-Butyraldehyde	0.552
n-Valeraldehyde	0.403
α-Ethylbutyraldehyde(C6)[a]	0.349
1-Octanal[a]	0.170
1-Nonanal[a]	0.120
1-Decanal[a]	0.133
1-Undecanal[a]	0.078
1-Dodecanal[a]	0.172
Unsaturated	
2-Hexenal[a]	0.000
2,4-Hexadienal	0.020
Citronellal[a]	0.082
Citral[a]	0.000
2-Nonen-1-al	0.000
Furfural[a]	0.042
Aromatic	
Phenylacetaldehyde	0.050

[a] Has been reported in orange essence.
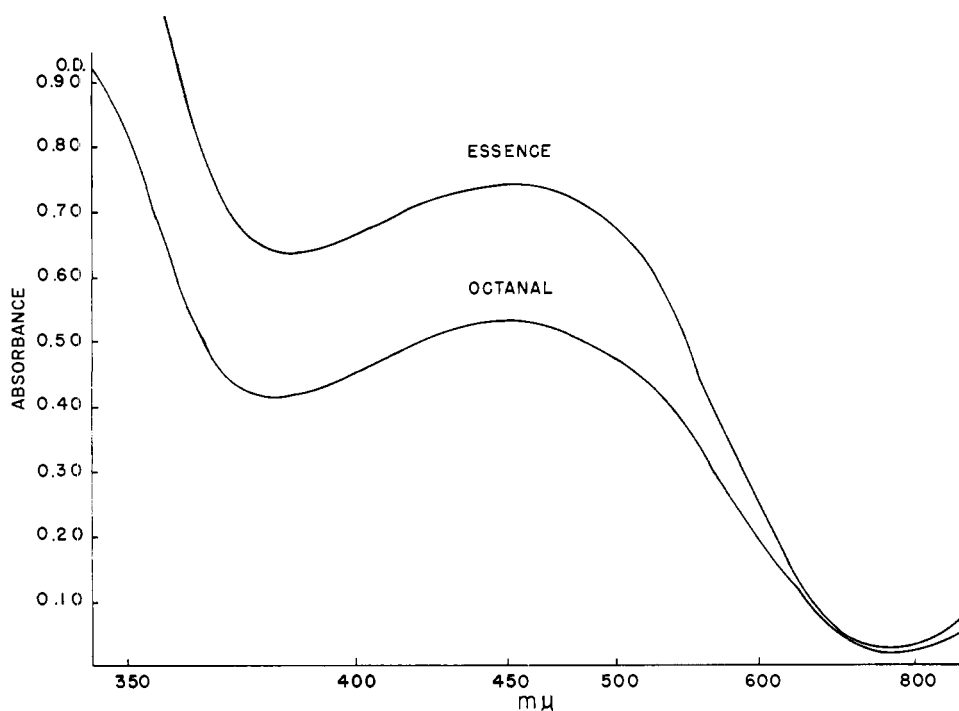
Figure 6. Visible spectra of reaction products of essence and standard octanal solutions with p-phenylenediamine and hydrogen peroxide

primary aromatic amines to form colored Schiff bases (Feigl, 1946, p. 340). Under the conditions of the analysis, a lavender color was obtained with the blank and with saturated aldehydes, while α,β-unsaturated aldehydes gave yellow colors. In Table II are shown the varying responses by approximately 10-p.p.m. solutions of various α,β-unsaturated aldehydes. According to the spectrum obtained with the Beckman DK-2 spectrophotometer, there was an absorption maximum near 380 mμ. Consequently, this frequency was used with the Spectronic 20 only, there being no corresponding filter for the Fisher Electrophotometer II.

Ester Method. This procedure is now being used by juice processors to estimate ethyl acetate (Kelly, 1958). It was adapted to ethyl butyrate simply by using standard solutions of ethyl butyrate.

Results and Discussion

The methods described above have been used to analyze 15 samples of citrus essence from several varieties and different stages of maturity (Table III). In a typical analysis of a blended essence (sample 8) values of 168, 165, 170, and 166 p.p.m. $C_{10}H_{18}O$; 552, 574, 534, and 544 p.p.m. octanal; 19, 17, 17, and 18 p.p.m. citral; and 22, 21, 23, and 21 p.p.m. ethyl butyrate were obtained. The standard deviation was ± 1.9 p.p.m. oxygenated terpenes, ±14 p.p.m. saturated aldehydes, ±0.5 p.p.m. unsaturated aldehydes, and ±0.5 p.p.m. esters. The relative standard deviations were 14, 25, 28, and 23 parts per thousand, respectively.

These values are well within the necessary limits of accuracy for blending essences into concentrated citrus juices.

Flavor components in Hamlin oranges increased with maturity. For example, in the four time-of-harvest experiments (samples 10 to 13) the esters increased from only a trace on Nov. 16, 1965, to 10 p.p.m. on Jan. 19, 1966, while the saturated aldehydes increased from 0 to 195 p.p.m. Oxygenated terpenes and unsaturated aldehydes also increased but with less regularity.

Two essences (samples 4 and 5), both of which were fairly dilute and equivalent in concentration as determined by COD gave an interesting comparison by the new methods. Sample 5 gave much higher values in all categories, actually giving the highest parts per million of $C_{10}H_{18}O$ tabulated.

Table II. Comparative Responses of 10-P.P.M. Solutions of Unsaturated Aldehydes to o-Dianisidine Reagent

Aldehyde	Absorbance, 380 mμ
2-Hexen-1-al[a]	0.090
2,4-Hexadienal	0.620
Citral[a]	0.260
2-Nonen-1-al	0.100
Furfural[a]	0.040
Phenylacetaldehyde	0.000
Cinnamaldehyde	0.200

[a] Has been reported in orange essence.

Table III. Comparative Data from Analysis of 15 Essences Representing Five Citrus Varieties

Sample No.	Fruit Variety Used as Source of Essence	COD, P.P.M.	$C_{10}H_{18}O$, P.P.M.	Esters as Ethyl Butyrate, P.P.M.	Aldehydes Saturated aliphatic as octanal, p.p.m.	Unsaturated as citral, p.p.m.
1	Valencia	56,500	163	22	523	18
2	Valencia, 1965	25,500	87	14	115	10
3	Valencia, 1965	22,800	65	13	195	10
4	Valencia, 1965	14,400	88	9	160	11.5
5	Pineapple, 1965	15,150	183	19	451	19.5
6	Pineapple	55,000	156	20	523	44.5
7	Pineapple	42,000	177	13	408	26.5
8	Valencia and pineapple	57,700	167	22	551	18
9	Hamlin	36,500	98	32	398	25
10	Hamlin, picked 11/16	8,000	35	Trace	0	1.5
11	Hamlin, picked 11/30	8,000	79	2	95	6
12	Hamlin, picked 12/21	25,000	68	4	130	11.5
13	Hamlin, picked 1/19	18,000	98	10	195	10
14	Temple	3,300	106	7	115	11
15	Grapefruit	4,340	24	6	0	1.5

Conclusions

The development of these methods and their adaptation to orange essence analysis have enabled us to replace a broad general technique with four specific techniques. This provides greater accuracy in the comparison and evaluation of different citrus essences. However, these techniques are not specific to citrus essences. They should be applicable to other fruits and natural products as well.

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