pensating in nature. The simplified procedure was adopted as part of the assay method.

The method described above has been in use in these laboratories for 3 years. The analysis of over 1000 samples has permitted the segregation of high and low yielding varieties from a collection of 63 types. One operator can assay from 12 to 24 samples per day. A single determination usually gives a result which is within ±5% of the mean value of triplicate determinations run by the most accurate and precise method available. The results obtained by different laboratories have been in very close agreement. Three analysts assayed a tuber that contained 6.2% diosgenin and reported the following results: (*a*) 5.8, 6.0, 6.1%; (*b*) 5.9, 6.1, 6.1%; (*c*) 5.9, 5.9, 6.2%.

A recent investigation by Peal (*4*) demonstrated that diosgenin loses a molecule of water when refluxed in 4*N* hydrochloric acid for 2 hours, and that maximum yields of diosgenin are obtained with 2*N* hydrochloric acid and a reflux period of 2 hours. These observations are in complete agreement with the data presented herein.

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CITRAL DETERMINATION

Specific Quantitative Colorimetric Method of Analysis for Citral in Lemon Oil

W. L. STANLEY, R. C. LINDWALL, and S. H. VANNIER

Fruit and Vegetable Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Pasadena, Calif.

The aldehyde, citral, is the component in lemon oil responsible for the typical lemon aroma. Methods of analysis used in the past for determining the citral content of lemon oils, however, were nonspecific and measured only total carbonyl content. A specific method for citral in the presence of other aldehydes and ketones has been developed. It is based on the discovery that citral with a reagent mixture of vanillin and piperidine in absolute alcohol forms an alcohol-soluble green comp'ex (absorption maximum 605 mμ). Other carbonyls produce yellow, orange, or red colors. Only dihydrocitral and pseudoionone interfere. The method provides a highly sensitive and selective objective tool for evaluation and standardization of lemon oil quality and should be useful in following the effects of process variables and agronomic conditions on the composition of lemon oils.

The typical fragrant aroma of lemon peel oil has been ascribed to the terpene aldehyde citral (*2*). However, other aldehydes (octanal, nonanal, decanal, lauryl aldehyde, citronellal, and an unknown aldehyde, $C_{10}H_{18}O$) and a ketone (methyl heptenone) have also been reported in lemon oil (*2*). The relative concentrations of carbonyl compounds in lemon oils are not known, but it has been assumed among oil processors and essential oil dealers that citral represents about 80% of the total carbonyl content.

In evaluating the quality of samples of lemon oil and folded or concentrated lemon oils, the practice has been to determine total carbonyl with hydroxylamine (*7*) or phenylhydrazine (*4*) reagents and to report the results as per cent citral. Methods of analysis specific for citral in the presence of other aldehydes and ketones have been sought, particularly by processors who are interested in determining the effects of processing changes on the quality of lemon products.

A method which eliminates interference from ketones, proposed by Chace (*1*), employed the fuchsin aldehyde reagent and was reported to be best adapted for lemon extracts. It was of limited value, because the other aldehydes interfered. More recently a method employing benzidine was reported to be specific for citral (*8*). However, positive tests with this reagent also were obtained in this laboratory with crotonaldehyde and 2-hexenal. This evidence led to the assumption that the benzidine method could serve only as a general test for the α,β-unsaturated aliphatic aldehydes.

A general test for the detection of aldehydes and ketones was reported by Levine and Taterka (*6*). They found that the saturated aliphatic aldehydes and ketones gave yellow or red solutions when heated with vanillin and potassium hydroxide in aqueous or alcoholic solution. Testing the reaction with a series of known aldehydes, it was found that saturated aldehydes including citronellal gave yellow solutions and that α,β-unsaturated aldehydes, including citral, gave red solutions. Unfortunately, the method was not adaptable to quantitative colorimetric analysis of higher molecular weight aldehydes, because in alcohol-water mixtures suitable for dissolving the higher molecular weight aldehydes the addition of alkali caused an undesirable cloudiness.

The difficulty was overcome by substituting piperidine for the potassium hydroxide and using absolute alcohol (methanol or ethyl alcohol) as solvent. The vanillin-piperidine system in alcoholic solution produced yellow solutions with saturated aldehydes and red solutions with the α,β-unsaturated aldehydes as obtained previously with the vanillin-potassium hydroxide reagent. Citral, dihydrocitral, and pseudoionone, on the other hand, developed an intense emerald green color. These latter compounds are unique in having a branching methyl group on the terminal carbon atom of a double bond system conjugated with the carbonyl group. Citronellal, having no double bond at the position

α,β to the aldehyde group, gives the same color reaction as the saturated aldehydes. These color reactions are summarized in Table I.

The specific nature of the reagents was then determined by testing the various aromatic aldehydes and organic bases available as substitutes for vanillin and piperidine. The organic bases are listed in Table II and the aromatic aldehydes in Table III. Of the aldehydes tested, only protocatechuic aldehyde could be substituted for vanillin. Of the bases tested, only 1,2,3,4-tetrahydroisoquinoline could be substituted for piperidine. The contaminants normally encountered in synthetic vanillin—i.e., isovanillin, vanillic acid, guiacol—and other phenols were tested and found not to produce the green color reaction with citral and piperidine (Table IV).

In contrast to the reaction of aldehydes with vanillin and potassium hydroxide, the reaction of citral with vanillin and piperidine is carried out at room temperature. Maximum color development is obtained in 50 to 55 minutes (Figure 1). A large excess of vanillin over citral is required (about 20,000 to 1 molar ratio). Maximum color development is obtained with a 10 to 7 molar ratio of vanillin to piperidine. The effect of vanillin and piperidine concentration on color development is shown in Figure 2. Dilution with water and acidification cause conversion of the green coloration to yellow.

Once the color has been developed, subsequent dilution with alcohol causes rapid fading, which levels off to a constant rate after about 20 minutes. Consequently, it is important to select suitable dilutions and aliquots of the original sample to be analyzed and to avoid any further dilution once the color has been developed.

The spectral absorption curve for the green citral-vanillin-piperidine complex has a major absorption peak of 605 mμ. No change in absorbance is observed when alcohol is substituted for the reagent blank; consequently a reagent blank is not necessary.

The standard curve for citral was obtained with aliquots from a 0.0005*M* solution of a special grade synthetic citral (98 to 100% purity). To test recovery of citral, weighed amounts of citral were added to a sample of lemon oil that had been analyzed previously for citral content. The oil samples containing added citral were analyzed and the results corrected for citral present prior to addition (Table V).

It is important to prepare fresh vanillin solutions for each day's run of analyses.

In studying the specificity of the method, it was found that geraniol, nerol, geranyl acetate, neryl acetate, linalyl acetate, and pseudoionone gave green solutions of varying color intensity with vanillin and piperidine in ethyl alcohol. The color intensity produced by different samples of geraniol and nerol also varied considerably.

These alcohols and esters were tested for carbonyls by treating with 2,4-dinitrophenylhydrazine–sulfuric acid reagent, followed by separation of the de-

Table I. Compounds Tested with Vanillin-Piperidine Reagent and Resulting Colors

Compound[a]	Color
Hydrocarbons	
Terpinolene	Medium red
α-Pinene	Light orange
Alcohols	
Citronellol	Yellow
Geraniol[b]	Yellow-orange
Nerol[b]	Yellow
Isopulegol	Light brown-orange
Linalöol	Light red-brown
Nopol	Yellow
Esters	
Geranyl acetate	[c]
Neryl acetate	[c]
Terpinyl acetate	Orange
Linalyl acetate	[c]
1-Cyclohexene carboxylic acid, ethyl ester	Orange
Aldehydes	
Propanal	Yellow
Pentanal	Yellow
Hexanal	Yellow
Heptanal	Yellow
Octanal	Yellow
Nonanal	Yellow
Decanal	Yellow
Undecanal	Yellow
Dodecanal	Yellow
Stearic aldehyde	Yellow
Crotonaldehyde	Red to red brown
Hexen-2-al	Red
Cinnamic aldehyde	Yellow
Citronellal[d]	Yellow
Citral[d]	Dark green
Dihydrocitral[d]	Dark green
Ketones	
Acetone	Dark red
Diethyl ketone	Yellow
Methyl heptenone	Red
Diacetone alcohol	Red
Methyl ethyl ketone	Orange
Methyl *n*-propyl ketone	Orange
Methyl isobutyl ketone	Orange
Piperitone	Orange
Carvone	Yellow-brown
Pseudoionone	Dark green
Peroxides	
Ascaridole	Light red

[a] Samples not purified prior to use, except where indicated.
[b] Traces of citral removed prior to test by separation on chromatostrips (*3*).
[c] Pale green, variable in intensity, experimental section.
[d] Synthetic samples kindly supplied by G. F. Siemers, Hoffman-La Roche Co.

Table II. Colors Developed by Organic Bases with Citral and Vanillin

Organic Base	Color
Ethylamine	Yellow
tert-Butylamine	Yellow
Diethylamine	Yellow
Benzylamine	Yellow
Collidine	Yellow
Ethanolamine	Yellow
Triethanolamine	Yellow
Morpholine	Yellow
Pyridine	Yellow
Benzidine	Red-brown, depositing yellow solid
Sodium ethoxide	Yellow, depositing white solid
1,2,3,4-Tetrahydroquinoline	Yellow
1,2,3,4-Tetrahydroisoquinoline	Green
Piperidine	Green

Table III. Colors Developed by Aromatic Aldehydes with Piperidine and with Piperidine and Citral

Aromatic Aldehyde	Color with Piperidine	Color with Piperidine and Citral
Isovanillin	Amber	No change
Salicylaldehyde	Yellow	No change
m-Hydroxybenzaldehyde	Light brown	No change
p-Hydroxybenzaldehyde	Light red brown	No change
2,4-Dihydroxybenzaldehyde	Yellow brown	Yellow orange
4-Anisaldehyde	Colorless	No change
2,4-Dihydroxyacetophenone	Colorless	No change
Protocatechuic aldehyde	Light yellow	Green
Vanillin	Yellow	Green

Table IV. Colors Developed by Allowing Mixture of Citral and Piperidine to React with Compounds Commonly Encountered as Contaminants in Vanillin and with Certain Phenols

Compound	Color
Isovanillin	Amber
Vanillic acid	Yellow
Guiacol	Yellow to orange-red
Phenol	Yellow to orange-red
Catechol	Yellow to orange-red
Phloroglucinol	Yellow to orange-red

Table V. Recovery of Added Citral by Vanillin-Piperidine Method of Analysis

Citral, Gram		
Added	Found	% Error
0.01046	0.01053	0.7
0.01048	0.01025	2.2
0.02098	0.02093	0.2
0.02237	0.02257	0.9
0.03236	0.03223	0.4
0.04140	0.04094	1.3
		Av. 1.0

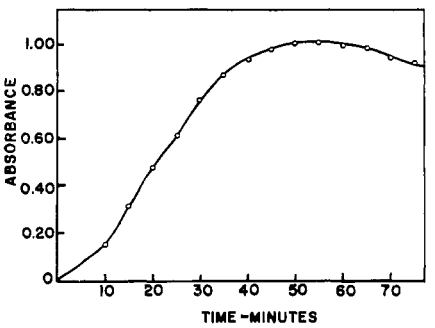

Figure 1. Effect of time on color development of citral-vanillin-piperidine complex

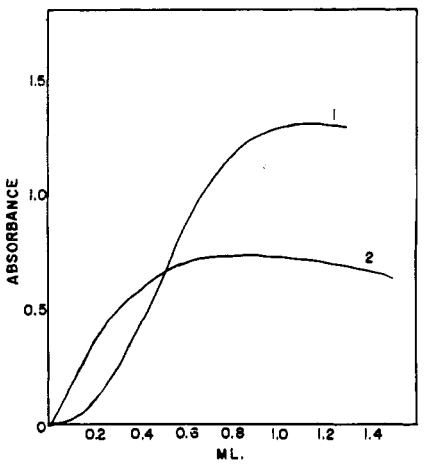

Figure 2. Effect of varying quantities of reagents on color development of the citral-vanillin-piperidine complex

1. Holding piperidine constant and varying amount of vanillin (1 M in absolute alcohol)
2. Holding vanillin constant and varying amount of piperidine (10% v./v. in absolute alcohol)

rivative from the reagent on chromatostrips (*3*) using benzene as the developing solvent. The presence of carbonyl compounds was demonstrated in all these materials. The original materials were then chromatographed on chromatostrips using 15% ethyl acetate in hexane as the developing solvent. With the exception of pseudoionone, several spots were obtained from each. The spots were detected by spraying the strips with fluorescein-bromine reagent (*3*) and by viewing them under ultraviolet illumination. The individual spots were cut from the strips extracted with alcohol and the extracts treated with vanillin-piperidine reagent. In all the chromatostrip tests, except that for pseudoionone, the main spot on each strip gave a negative test, and a minor spot having the same R_f value as citral gave the positive green coloration for citral. The results with geraniol and nerol showed conclusively that geraniol and nerol in themselves do not react with vanillin-piperidine and that the samples of these alcohols were contaminated to varying degrees with, among other things, citral or a similar carbonyl compound. The green color produced with pseudoionone-vanillin-piperidine is intense and, because the pseudoionone appeared to be homogeneous on chromatostrips, it is assumed that either the α- or β- or both isomers of this unsaturated ketone give a positive test with vanillin-piperidine reagent.

Difficulties were encountered in separating the impurities in geranyl acetate, neryl acetate, and linalyl acetate. The results are, therefore, not considered conclusive. However, allowing a large excess of these esters to react with the vanillin-piperidine reagent produced only light green colorations. For this reason, it is believed that these esters were contaminated with traces of citral or a citrallike compound.

Another specific method of analysis for citral, based on the formation of an adduct with barbituric acid, was described by Laughton and Levi, (*5*) who presented citral data obtained with the barbituric acid method and the vanillin-piperidine method on the same samples of citrus oils. There was good agreement between the two methods.

Procedure

Apparatus. The accuracy of the method is limited primarily by the accuracy of pipetting and preparation of dilutions, and by the purity of the citral used as a standard. The reaction is conveniently carried out in 15-cm. test tubes. Spectral absorption measurements are made with a Beckman DU spectrophotometer at 605 mμ using 1-cm. Corex cells and the tungsten lamp.

Reagents. The 1.00M vanillin reagent solution was prepared by diluting 15.215 grams of U.S.P. grade vanillin to 100 ml. with absolute ethyl alcohol in a volumetric flask. Lesser quantities may be prepared to meet requirements. Fresh vanillin reagent should be prepared for each series of analyses, if runs are more than 4 to 8 hours apart. The 7% by volume piperidine reagent solution is prepared by transferring 7 ml. of purified grade piperidine by pipet to a 100-ml. volumetric flask and diluting to volume with absolute ethyl alcohol or methanol. The two reagent solutions may be added separately to the aliquot of sample solution containing citral, the piperidine solution being added last, or equal volumes of the vanillin and piperidine solutions may be combined and the required amount of the mixture added to the sample. If the mixed reagent is used, the over-all time for completing a series of analyses must not exceed $1^3/_4$ hours. Nine samples can be run in duplicate conveniently in this length of time with the mixed reagent.

The choice of methods will depend on the number of samples to be run and the number of analysts available. Using the technique of adding the two reagents separately, one analyst can handle a series of 11 samples in duplicate. More samples can be handled when two analysts are available, one adding reagents and one taking readings with the spectrophotometer. A 3-minute time interval between samples is adequate to allow for pipetting, rinsing the sample cells, and manipulating the spectrophotometer.

Standard Curve. To prepare the standard curve, add from 0.20- to 2.00-ml. aliquots of the 0.0005M standard citral solution (equivalent to 15.2 to 152 γ of citral) to a series of 15-cm. test tubes. To each tube add sufficient absolute alcohol to bring the volume to 2.00 ml. If the mixed reagent is to be used, add to each tube exactly 4.00 ml. of mixed reagent, spacing the addition at 3-minute intervals. Stopper and shake each tube. If the two reagents are added separately, add 2.00 ml. of the 1.00M vanillin reagent to each tube and then add 2.00 ml. of the 7% v./v. piperidine reagent at 3-minute intervals. After 55 minutes have elapsed from the time that the final reagent was added to the first tube in the series, read the absorbances consecutively at 3-minute intervals in a 1-cm. Corex cell at 605 mμ using absolute alcohol as blank.

Analysis of Oils. For determining the citral content of lemon oil use a 2-ml. aliquot of a 500 to 1 dilution; for orange oil use a 2-ml. aliquot of a 25 to 1 dilution. If smaller aliquots are used, add alcohol to bring the volume to 2.00 ml. To the 2.00 ml. of suitably diluted sample in a 15-cm. test tube add 4.00 ml. of the mixture of equal volumes of the 1.00M vanillin and the 7% piperidine or, if the reagents are added separately, add 2.00 ml. of the 1.00M vanillin solution and then add 2.00 ml. of the 7% piperidine solution. Stopper and shake the tube and after 55 minutes read the absorbance of the mixture at 605 mμ in a 1-cm. Corex cell.

Calculation

$$\% \text{ citral} = \frac{R \times F}{D} \times 10^{-4}$$

where

R = reading from standard curve in micrograms of citral
D = density of citrus oil analyzed
F = dilution factor (for lemon oil, 250, and for orange oil, 12.5, on basis of dilutions recommended in text)

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