oil molecules. The loops which project into the aqueous phase are potential sites for interaction with flavor compounds. Their presence may shift the equilibrium away from any denatured protein which is suspended in the emulsion. This shift in equilibrium would result in less adsorption of the flavor compound to the denatured protein. The protein–lipid complex might be said to correspond to the lipoprotein membranes found in most foods. The fact that such structures are not present in synthetic foods could explain the decrease in flavor potential attributed to "binding" in these foods.

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High-Performance Liquid Chromatographic Determination of Some Coumarins and Psoralens Found in Citrus Peel Oils

James F. Fisher* and L. Anthony Trama

Citrus peel oil was removed hypodermically from the oil glands of grapefruit *Citrus paradisi* Macf. cv. Duncan, sweet orange *C. sinensis* (Linn.) Osbeck cv. Valencia, sour orange *C. aurantium*, and lemon *C. limon* (Linn.) Burm. cv. Eureka and dissolved in acetonitrile. These oils were examined by high-performance liquid chromatography for their coumarin and psoralen content. This resolution and identification was carried out on μ CN, C-18, and Porasil columns. The coumarins and psoralens were detected at 320 and 305 nm, respectively.

The coumarin compounds reported in citrus peel oil are usually obtained by methods which do not discriminate between components in the oil glands and the parenchymous tissue. These procedures generally involve rupturing the oil gland and surrounding tissue with pressure (cold pressed) to obtain the peel oil (Kesterson et al., 1971). This is followed by concentration and open column or thin-layer chromatography to resolve the components (Fisher and Nordby, 1965, and references therein). These methods of isolation subject the components to conditions conducive to artifact formation.

Peyron and Trêfouël (1963) or Peyron (1963) obtained citrus peel oil directly from the oil glands and examined the oil by thin-layer chromatography.

In the method described below only the contents of the oil glands were examined by LC, showing the application of this technique in resolving citrus coumarins.

MATERIALS AND METHODS

Apparatus. A Model ALC 202 high-performance liquid chromatograph with a Model 6000A pump and U6K injector (Waters Associates, Milford, MA) was used. The recorder was a two-pen Soltec Model B-281 (Soltec Corp., Encino, CA). A Schoeffel UV-visible liquid chromatography analyzer Model SF770 with a wavelength scan unit 338/SFA 501 (Schoeffel Instrument Corp., Westwood, NJ), a Perkin-Elmer Model 204A fluorescence spectrophotometer with a 20-μL flow cell, and an Aminco Fluoro-Monitor equipped with an excitation 7-60 narrow pass filter (λ max 360 nm) and a 2-A emission filter which excepts wavelengths greater than 415 nm were the detectors. An ultrasonic cleaner (Cole-Parmer, Model 8845-6), a 25 gauge hypodermic needle, and a Wild dissecting microscope were used.

Standard Samples Employed as Criteria of Identity. Where practical the authentic compounds were recrystallized. In all cases, a LC examination showed one major peak along with from one to three minor peaks. The major peak was assumed to be the desired substance. Limited amounts of material made an extensive purification impractical.

Columns and Eluting Systems. Waters Associates 10-μm Porasil and 10-μm C-18 30 cm × 4 mm i.d. columns were used. Also, a 5-μm CN Dupont 25 cm × 4.6 mm i.d. Zorbax column was employed. The organic solvents were Burdick and Jackson and LiChrosolv (Scientific Products). The water was distilled and deionized. The solvent systems were degassed with the ultrasonic cleaner.

Eluting systems employed with the μ Porasil column were (A) heptane–ethyl acetate (90:10), (B) heptane–ethyl acetate (95:5), and (C) heptane–ethyl acetate (80:20). Eluting systems used with the 5-μm CN column were (D) water–acetonitrile (65:35), (E) heptane–ethyl acetate (95:5), (F) water–acetonitrile (70:30), and (G) water–acetonitrile (75:25). Eluting systems used with the μ C-18 column were (H) water–acetonitrile (50:50), (I) water–acetonitrile (65:35), and (J) water–acetonitrile (70:30).

Sample Preparation. A slice of citrus peel (grapefruit, sweet orange, sour orange, or lemon) consisting of the flavedo and some albedo was viewed under the dissecting microscope. A 25 gauge hypodermic needle and syringe were used to remove the contents from approximately 50 oil glands (20–50 μL). This peel oil was dissolved in about 3.0 mL of acetonitrile.

High-Performance Liquid Chromatographic Resolution of Some Coumarins and Psoralens Present in

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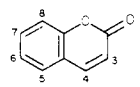
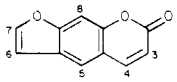
Table I. Some Coumarins and Psoralens Identified in Citrus Peel Oils

	basic structure	column and eluting system
grapefruit		
coumarins		
7-geranoxycoumarin		A, D, H
osthol (7-methoxy-8-isopentenyl)		A, D, I
marmin (7-dihydroxygeranoxy)		D, F, I
psoralens		
bergaptol (5-hydroxypsoralen)		C, D, G, I, J
bergapten (5-methoxy)		A, D, G, I
bergamottin (5-geranoxy)		A, B, E
lemon		
coumarins		
limettin (5,7-dimethoxy)		A, B, E
5-geranoxy-7-methoxycoumarin		A, B, E
psoralens		
phellopterin (5-methoxy-8-isopenteneoxy)		A, B, E
8-geranoxypsoralen		A, B, E
bergamottin		A, B, E
valencia orange		
psoralen		
bergaptol		D, F, G
sour orange		
coumarin		
osthol		D, F, G
psoralens		
bergaptol		D, F, G
bergapten		D, F, G

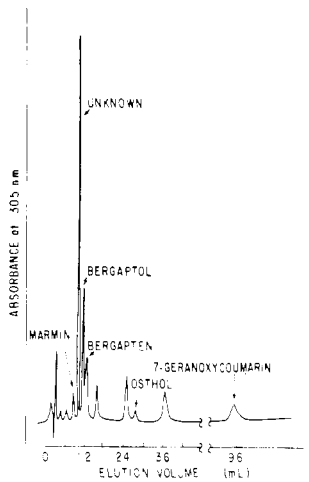


Figure 1. Resolution in system D of some coumarins and psoralens in grapefruit peel oil. Marmin appears at a k' of 2.0, begaptol 3.1, bergapten 3.4, osthol 8.0, and 7-geranoxycoumarin 30.8.

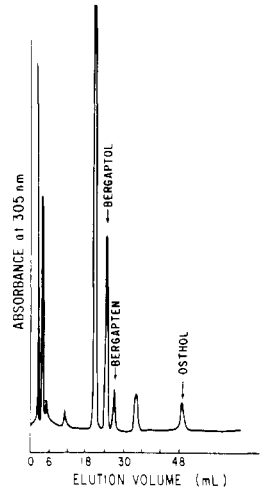


Figure 2. Resolution of coumarin compounds in sour orange oil using system G. Bergaptol appears at a k' of 7.2, bergapten 8.0, and osthol 15.2.

the Above Peel Oils. A 10–50-μL sample (depending on the concentration of peel oil) of the above acetonitrile-/citrus peel oil solutions was examined by LC using the systems shown in Table I. Detection was accomplished at an attenuation of 0.04 aufs with one injection at 320 nm for the coumarins and a second injection at 305 nm for the psoralens. The UV and fluorescence detectors were operated in series. The recorder chart speed for all samples was 15 cm/h. All experiments were conducted between 20 and 23 °C. All grapefruit and orange samples were run at 1.5 mL/min. The lemon samples were run at 1.0 mL/min. The resolution of grapefruit peel oil in system D, sour orange in system G, and lemon oil in system A is shown in Figures 1, 2, and 3, respectively. The injection of acetonitrile, as a blank, into the chromatographic systems did not produce any of the chromatographic peaks.

Identification of Coumarins and Psoralens. The identity of the compounds in the citrus oils listed in Table I was established by peak enrichment, showing coincident retention volumes, with the authentic compounds in different LC systems as shown. Stop-flow fluorospectroscopic analysis was also employed for those compounds which fluoresced.

An example of the stop-flow analysis is bergaptol using system D and grapefruit peel oil. The standard bergaptol displayed an excitation of 330 nm and an emission of 389 nm. The "bergaptol peak" (Figure 1) showed an excitation of 328 nm and an emission of 391 nm. The peak widths at half the emission peak heights of the standard bergaptol and the "bergaptol peak" were the same. Additional verification of identity was obtained by the absorbance ratios

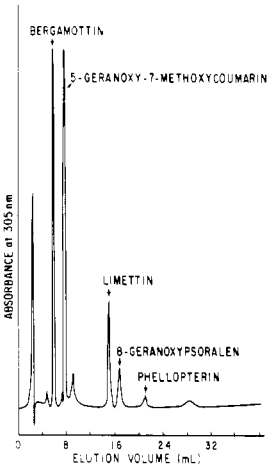


Figure 3. Resolution in system A of some coumarins and psoralens in lemon oil. Begamottin appears at a k' of 1.2, 5-geranoxy-7-methoxycoumarin 1.9, limettin 4.6, 8-geranoxypsoralen 5.3, and phellopterin 6.8.

(Yost et al., 1976). An example of this method is osthol in sour orange oil using system D. The ratios of the absorbance at 320 nm to 305 and 330 nm for authentic osthol was 2.2 and 1.4, respectively. The ratios at the same wavelengths of the "osthol peak" in the sour orange oil was 2.1 and 1.4, respectively.

RESULTS AND DISCUSSION

No new coumarins or psoralens are reported. However, some compounds previously reported in grapefruit peel oil such as two formylated coumarins (Fisher and Nordby, 1965) were not found and are now known to be artifacts. An LC examination (systems A, B, D, E, and H) of grapefruit peel oil failed to detect these two formylated coumarins. However, when the grapefruit peel oil was fortified with the formylated coumarins they were readily resolved. When a sample of the unfortified grapefruit oil was passed through an open 5 cm × 80 cm glass column containing 500 g of silica gel (adsorption, Woelm) using hexane–ethyl acetate (90:10) as the eluant (conditions similar to those used by Fisher and Nordby, 1965), the two formylated coumarins were readily found in the effluent by LC. Therefore, it is clear these aldehydes are artifacts, perhaps formed as pointed out by Dreyer (1967) on the open silica gel column.

Bergamottin could not be identified in grapefruit peel oil using system D (Figure 1); however, when system A was employed, bergamottin was readily identified with a retention volume of 7.2 mL and a k' of 1.4. The 6,7-dimethoxycoumarin reported in citrus peel but not always detected in fresh picked fruit (Tatum and Berry, 1977) was not observed in grapefruit peel oil using systems A, D, and I, but was readily resolved when added to grapefruit peel oil. Limettin reported in grapefruit peel oil (Fisher and Nordby, 1965) was not found under the above conditions. Figure 1 shows the absence of limettin in the native grapefruit peel oil. Figure 4 shows the resolution of grapefruit peel oil fortified with limettin. Limettin appears between the unknown and bergaptol peaks. Osthol, previously reported by Stanley et al. (1963), in the Seville (sour) orange was confirmed by comparative chromatography with known osthol; however, osthol was not observed in Valencia, a sweet orange. When Valencia peel oil was fortified with osthol, this coumarin appeared at a retention volume of 26.3 mL and a k' of 7.8 in system D. This is an area devoid of UV detectable compounds with high loading of unfortified Valencia peel oil. Also, bergaptol and bergapten were found in the peel oil of sour orange while the

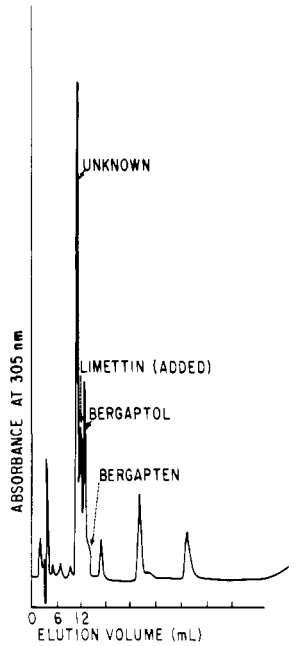


Figure 4. Resolution of limettin (k' = 2.2) in system D using grapefruit peel oil which was fortified with limettin.

Valencia peel oil contained only bergaptol. Therefore, while the sour orange peel oil contains osthol, bergaptol, and bergapten the Valencia (sweet orange) peel oil contained only bergaptol (Table I). This could be of some help in the chemical taxonomy of oranges.

Imperatorin reported in lemon (Stanley, 1963) was not detected in the native oil under our conditions. Lemon peel oil, fortified with imperatorin, showed a peak at a retention volume of 20 mL and a k' of 5.3 in system A. This is just prior to where phellopterin elutes (Figure 3). The occurrence of isoimperatorin in lemon (Stanley, 1963) appeared likely but not definite due to unsatisfactory resolution of peaks in the isoimperatorin area. The retention volume of isoimperatorin was determined with both pure isoimperatorin and lemon oil fortified with isoimperatorin. These compounds, while not found in the peel oil, could be present in the parenchymous tissue around the oil glands. The parenchyma was not examined because a technique selective enough to assure oil-free tissue was not developed.

In order to show that coumarins were not in the cuticle and, therefore, not a source of coumarins collected by the hypodermic needle as it passed through the cuticle to the oil gland, the cuticle was removed with a sharp razor and extracted with ethyl acetate. Examination of this extract in systems A, E, and D showed no evidence of coumarins or psoralens.

All the excitation and emission wavelengths and absorbance ratios of standards and identified compounds were within ±10% of each other. Peak shapes, as determined by peak widths at half-heights, were within ±10% of the authentic compounds.

The importance of this technique to citrus science is in the fine resolution obtainable as is apparent in Figure 4. The conditions used in this method are such as to minimize the formation of artifacts. If high-performance liquid chromatography had been available and applied earlier, the formylated coumarins (Fisher and Nordby, 1965) would not have been reported.

ACKNOWLEDGMENT

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ley, CA, for the authentic samples used in this work.

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Composition of Essence Oil from Overripe Oranges

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Analysis of essence oil from overripe oranges yielded 41 compounds, 14 of which are being reported for the first time as components of orange essence oil. We identified none of the phenolic ethers that had been found in products from oranges treated with abscission chemicals and judged to contribute an overripe flavor. A sensory panel preferred essence oil from ripe fruit (control) to that from overripe fruit, but showed no preference between control oil and a blend of oils from control and overripe fruit. Essence oil from overripe oranges contained more valencene than that from control fruit. Thus, it appears it is the best available source of valencene, a desirable flavor-related compound.

Recent studies by Moshonas et al. (1976, 1977) showed that abscission agents used to loosen citrus fruit prior to mechanical harvesting affect the quality of the processed orange juice and cold-pressed essential oil. In 1978, Moshonas and Shaw isolated and identified six phenolic ethers present in fruit that had been treated with abscission chemicals. The compounds were shown to be unnatural components formed by a change in metabolic pathways brought about by the chemicals. Since a majority of an expert taste panel indicated that products from oranges treated with abscission chemicals had an overripe flavor (Moshonas et al., 1976, 1977), we undertook to determine whether the phenolic ethers are synthesized when the natural metabolic processes are allowed to continue beyond the normal harvesting period for oranges.

The primary byproduct obtained during the concentration step in the preparation of frozen orange juice concentrate is aqueous essence. Part of the essence is subsequently returned to the concentrate to give it a fresh orange aroma. A second byproduct, essence oil, separates from the aqueous essence and is also used as a flavoring agent. Essence oil contains most of the oil-soluble flavoring components of orange juice. Thus, if present in overripe oranges, the phenolic ethers should be recovered in the essence oil of those fruit.

Orange essence oil has been analyzed by several workers. In 1965, Hunter and Brogden analyzed the hydrocarbons in essence oils. Coleman et al. (1969) compared composition of mid- and late-season orange essence oils and found quantitative and qualitative differences. Veldhuis et al. (1972) compared composition of orange essence oil with that of an aroma oil prepared by distillation of aqueous discharge from centrifugation of orange peel oil. Shaw and Coleman (1971) analyzed the highly volatile compounds distilled from essence oil and Coleman and Shaw (1971) reported quantitative and qualitative analysis of essence oil constituents. However, in none of these studies was essence oil from overripe fruit analyzed.

The current study reports major components of essence oil from overripe Valencia oranges, including 14 compounds not found previously in essence oil, and compares the composition of essence oils from overripe and normal fruit. An aroma panel was used to compare aromas of essence oils from overripe fruit and normal fruit and to determine affects of blending essence oils from overripe fruit with that from normal fruit.

EXPERIMENTAL SECTION

Essence oils from Valencia oranges harvested after the regular season (late July) and from Valencia oranges harvested in March, and known to have good flavor and aroma qualities, were obtained from Redd Laboratories, Safety Harbor, FL, and stored at 5 °C.

Whole Oil Analyses. Each of the essence oils was injected directly into the gas chromatograph (GLC) for separation and purification of major constituents so that they could be identified and quantitated. A Hewlett-Packard Model 3380-A computing integrator coupled to the gas chromatograph measured GLC peak areas for the quantitative work.

Separation Procedure. Essence oil from overripe Valencia oranges (84.5 g) was distilled at a bath temperature of 35 °C and 0.5 mmHg from a rotary evaporator until most of the hydrocarbons (99% limonene) were removed (81.5 g). A liquid nitrogen trap located between the receiver and vacuum pump contained 0.4 g of liquid. The distillation residue (2.6 g) was transferred into an ice–water jacketed column (1 × 15 in.) containing 60/80 mesh Florisil (Fisher Scientific Co.) deactivated with 6% water (Lund and Coleman, 1977). Fractions were eluted with 300 mL

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