

Coumarin compounds are generally distributed throughout the citrus species. The highest amounts in number and concentration are found in lime oils, which contain about 7% by weight in the cold-pressed oil; the lowest are found in orange oils, which contain less than 0.5%. The physiological role of these compounds in the plants is not yet known, although there is reason to believe that they

may be of importance. Closely related compounds, seselin and xanthyletin, were recently found in citrus roots and reported to inhibit certain enzyme systems. Substituted furocoumarins have been shown to sensitize the skin to sunlight and to be toxic to fish. In screening tests, derivatives of umbelliferone were found to exhibit antimicrobial activity primarily against yeasts and molds.

Knowledge of the identity and distribution of substituted coumarins and psoralens in the cold-pressed (cp) citrus peel oils has contributed greatly to developing sensitive and specific methods of analysis for detecting adulteration of the oils.

The better quality citrus oils which are used in perfumery and for flavoring candies, soft drinks, and baked goods are pressed directly from the peel. Poorer grades used in soaps and other less demanding applications are recovered from spent peel or other processing plant streams by steam distillation. An example of the latter is stripper oil, recovered from the orange juice concentrate process. The distilled oils are less stable and lack the true bouquet of the cold-pressed oils and, therefore, command a much lower price in the trade. The one exception is distilled lime oil, in which, through long usage, the reverted flavor has become the accepted standard of quality (Guenther, 1952).

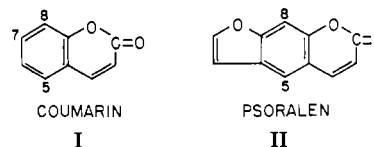
The coumarin and furocoumarin (psoralen) compounds present in the cp citrus peel oils are essentially nonvolatile and, consequently, are not found in the distilled oils. They exhibit strong absorption in the ultraviolet region (Figures 1 and 2). The absorption peak of cp lemon oil at about 315 nm was the basis of Sale (*et al.*, 1953) for estimating the dilution of cp lemon oil with distilled oil. Riganeis (1955) used a similar approach examining the ultraviolet spectra of distillation residues. Both grapefruit and lime oil exhibit much more intense absorption than lemon or orange oil and can be added in small amounts to a diluted lemon oil, *e.g.*, to bring the uv absorption within the required range. Now that it is known that 7-geranoxycoumarin is present in the grapefruit and isopimpinellin is present in lime, and neither are present in lemon oil (Stanley, 1963), reliable methods have been developed for detecting this type of "sophistication" (Stanley and Vannier, 1957; Vannier and Stanley, 1958).

The new, more precise analytical procedures make use of thin-layer and column chromatography combined with spectral absorption and fluorescence measurements and spot tests. These, augmented with gas chromatographic analysis of the volatile constituents, provide the discerning buyer and user of citrus oils with much more reliable information for establishing standards and make the work of the manipulator considerably more difficult than it once was (Stanley, 1962).

The wide variation in intensity of uv absorption of the citrus oils is a consequence of the variation in their dissolved solids. The amount of dissolved solids recovered by column chro-

matography of representative commercially processed oils is given in Table I. The amounts initially present in the freshly pressed oils prior to clarification by the "winterizing" process is higher; in lime oils it may be over 10%. It should be noted that most of the material recovered from bitter orange oil was flavonoids (tangeretin, nobiletin, and heptamethoxyflavone) (Stanley, 1963).

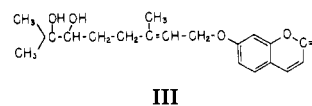
The presence of coumarin compounds is widespread in plants of the *Rutaceae* family (Dean, 1963) to which the commercial citrus varieties belong. Many new citrus compounds have been identified in recent years. All but a few exceptions are derivatives of the following basic structures.



The numbered positions may be substituted with hydroxyl, methoxyl, isopentenyl (prenyl), isopentenoxyl, geranoxyl, and oxygen containing modifications of the terpenoid side chain. Those that have been identified are listed in Tables II and III.

Four compounds were found to have unique modifications in the side chain worthy of special note. Three were minor constituents in grapefruit oil: 7-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]coumarin (III), 7-methoxy-8-(2-formyl-2-methylpropyl)coumarin (IV), and 5-[(3,6-dimethyl-6-formyl-2-heptenyl)oxy]psoralen (V). The fourth was a minor constituent in Seville orange oil, 7-methoxy-8-(2-hydroxy-3-methyl-3-butenyl)coumarin (auraptinol) (VI).

Compound III (Fisher *et al.*, 1967) appears to be identical



with marmin first isolated from the trunk bark of *Aigle marmelo* Correa (Chatterjee *et al.*, 1967). It is the hydration product of the epoxide at the terminal double bond of 7-geranoxycoumarin, the most abundant coumarin in grapefruit oil.

Compounds IV and V (Fisher and Nordby, 1965, 1966) are the only known naturally occurring coumarins having an aldehyde moiety in the side chain although several having keto groups are known, *i.e.*, mamein (Djerassi *et al.*, 1959), glabralactone (Hata and Nitta, 1956), and geijerin (Lahey and Wluka, 1955). The tertiary carbon atom bonded to four other carbon atoms in IV and V is a departure from the usual

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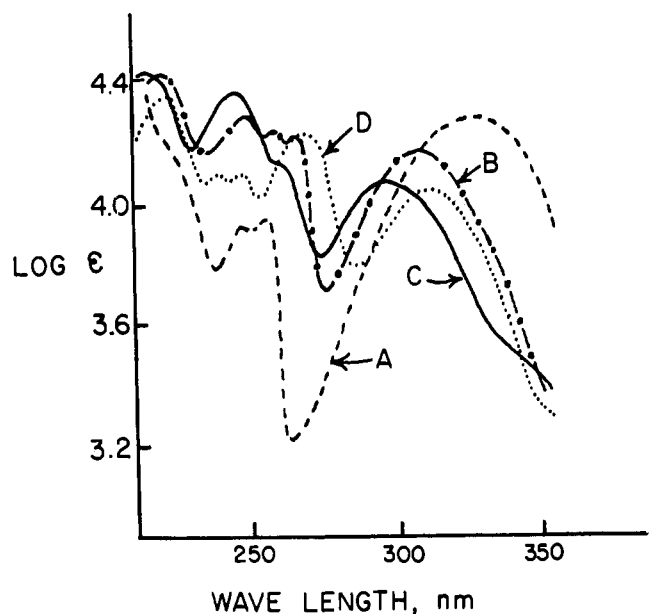


Figure 1. Typical ultraviolet spectral curves of: A. 5,7-dialkoxy-coumarins (Limettin); B. 5-alkoxy-psoralens (Bergapten); C. 8-alkoxy-psoralens (Xanthotoxin); D. 5,8-dialkoxy-psoralens (Isopimpinellin)

terpenoid side chain structure. However, as Dreyer (1967) has pointed out, this can be the result of bond migration analogous to the pinacol rearrangement, the precursors being diols similar to marmin III and byakangelicin (VII). These particular precursor diols so far have not been reported in the literature.

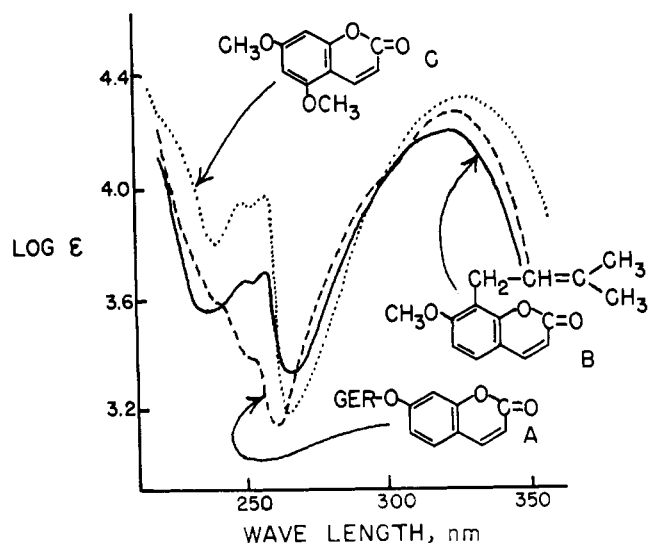
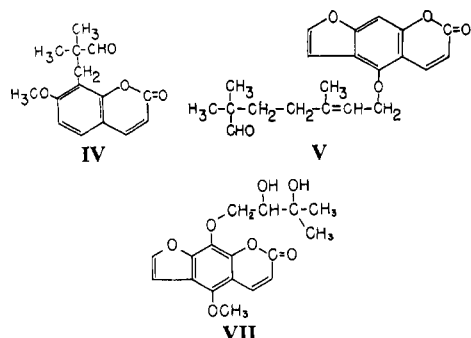


Figure 2. Typical ultraviolet spectral curves of: A. 7-alkoxy-coumarins (7-geranoxycoumarin); B. 7-alkoxy-8-alkylcoumarins (Osthol); C. 5,7-dialkoxy-coumarins (Limettin)

Table I. Solids Recovered from cp Citrus Peel Oils by Column Chromatography on Silicic Acid

Oil	Wt %
Lime, Mexican	6.67
Grapefruit	1.37
Bergamot	0.56
Lemon	0.47
Bitter orange	0.23

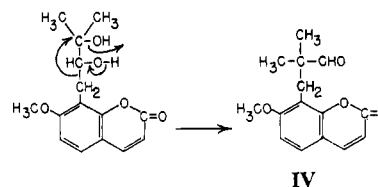
Table II. Substituted Coumarins in Citrus Oils

Coumarin	Citrus Oil				
	Lemon	Lime	Ber-gamot	Grape-fruit	Seville orange
7-Geranoxy-	-	-	-	X	-
7-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy-] (Marmin)	-	-	-	X	-
7-Methoxy-8-isopentenyl- (Osthol)	-	-	-	X	X
7-Methoxy-8-(2-formyl-2-methyl-propyl)-	-	-	-	X	-
7-Methoxy-8-(2-hydroxy-3-methyl-3-butenyl)- (Auraptanol)	-	-	-	-	X
5,7-Dimethoxy- (Limettin)	X	X	X	X	-
5-Isopentenoxy-7-methoxy-	X	-	-	-	-
5-Geranoxy-7-methoxy-	X	X	X	-	-

Table III. Substituted Psoralens in Citrus Oils

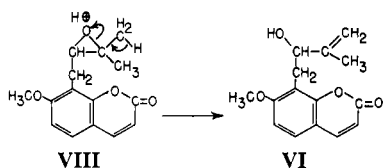
Psoralen	Citrus Oil				
	Lemon	Lime	Ber-gamot	Grape-fruit	Seville orange
5-[(3,6-Dimethyl-6-formyl-2-heptenyl)oxy]-	-	-	-	X	-
5-Methoxy- (Bergapten)	-	X	X	X	X
5-Isopentenoxy- (Isoimperatorin)	X	X	-	-	-
5-Geranoxy- (Bergamottin)	X	X	X	X	-
5-Dihydroxyisopentanoxy- (Oxypeucedanin hydrate)	X	X	-	-	-
5-Hydroxy- (Bergaptol)	-	-	-	X	-
9-Isopentenoxy- (Imperatorin)	X	X	-	-	-
8-Geranoxy-	X	X	-	-	-
5,8-Dimethoxy- (Isopimpinellin)	-	X	-	-	-
5-Methoxy-8-isopentenoxy- (Phellopterin)	X	X	-	-	-
5-Methoxy-8-dihydroxy-isopentanoxy- (Byakangelicin)	X	-	-	X	-
5-Geranoxy-8-methoxy- (?)	X	X	-	-	-

The suggested pinacol arrangement is as follows.



Compound VI (auraptanol) from Seville orange oil (Stanley *et al.*, 1965) is unique in that the 8-position terpenoid side chain contains a single hydroxyl group with the double bond shifted to the isopropyl end group. This presumably results from initial protonation of an epoxide, ring opening to the

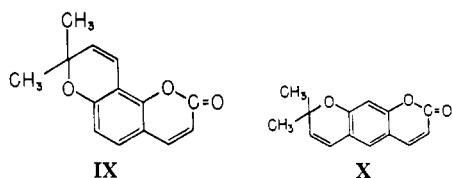
tertiary carbonium ion, and elimination of a proton to form the ethylenic compound. The precursor epoxide here is auraptene (VIII) found by Bohme and Pietsch (1939) in deposits from orange peel oil.



Isomerization of auraptene according to Bohme and Pietsch (1939) with 20% sulfuric acid converted it to isoauraptene in which the side chain has no carbon-carbon double bond and a keto group in the position of the hydroxyl of aurapteneol.

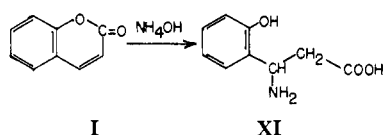
Very little is known about the distribution of coumarins in citrus plant material. Bernhard (1958) tentatively identified, at very low concentration, five of the substituted coumarins in lemon juice that had been identified in the peel oil: bergamottin, 8-geranoxypsoralen, limettin, oxypeucedanin hydrate, and byakangelicin. Three presumed coumarin compounds that fluoresced under uv light were unidentified.

Two pyranocoumarins, seselin (IX) (Tomer *et al.*, 1969) and xanthyletin (X) (von Reisch *et al.*, 1969; Goren and Stanley, 1970), were found in the roots of citrus trees, seselin and xan-



thyletin in Shamouti orange, sour orange, Palestine sweet lime, and Marsh seedless grapefruit, and xanthyletin in Eureka lemon. Xanthyletin, was also detected in the stems of sour orange seedlings. So far, these two pyranocoumarins have not been found in the fruit of any of the commercial citrus varieties.

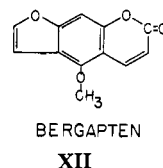
Seselin and xanthyletin exhibit inhibitory activity in both indole acetic acid oxidase and peroxidase enzyme systems (Goren, 1969). The inhibition by coumarin of the growth of lettuce and clover and of germination of barley and radish seeds has been ascribed to the addition of the sulfhydryl groups of the enzymes to the 3,4-double bond of coumarin. We have found that the highly water soluble β amino acid, 3-aminodihydrocoumaric acid (XI) and its amide are formed when coumarin is allowed to react with aqueous ammonia solutions (Bergot and Stanley, 1966).



Hydroxylamine was shown to react with coumarin in a similar manner (Posner, 1909). Possibly, free amino groups in enzymes can also react in this way with coumarin compounds.

Coumarins have been reported to exhibit a wide variety of physiological activities (Bose, 1958). Peucedanin has been reported to accelerate the alcoholic fermentation of yeast (Dean, 1952). Peucedanin and several others are poisonous to or have a narcotic action on fish (Dean, 1952). Certain methoxy psoralens in contact with the skin sensitize the skin to sunlight (Pathak and Fitzpatrick, 1959; Musajo and Rodighiero, 1962). Oil of bergamot and the sour orange

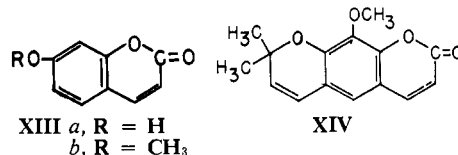
contain bergapten (XII), which is one of the more active of these so-called photodynamic compounds. The presence of bergapten in oil of bergamot which is used in perfumery in



eau de Cologne has been shown to be responsible for the so-called eau de Cologne dermatitis, an erythema and blistering of the skin. Psoralen itself is the most active of these compounds and 5- and 8-methoxypsoralen are almost as active. The corresponding phenols are inactive, as is 5,8-dimethoxypsoralen (isopimpinellin in lime oil) (Musajo *et al.*, 1953).

We have recently identified bergapten in deposits from lime oil (Stanley and Wu, 1971). This would account for skin sensitivity of lime pickers. The 5- and 8-methoxy compounds can be prepared readily from the corresponding geranyl ethers in lemon and lime oils (Stanley and Vannier, 1959).

Natural coumarins, including scopoletin, furocoumarins, and umbelliferone (XIIIa), a constituent (Peyron, 1963) of the oleiferous cells of citrus fruits, appear to play important roles as natural protective agents in plants (Wain, 1969). In fungi it has been shown that coumarin itself inhibits growth of the yeast *Saccharomyces cerevisiae* (De Greef and Van Sumere, 1966) and at higher concentrations (100-1000 ppm) it inhibits or retards germination of the spores of *Aspergillus niger* and *Penicillium glaucum* (Knypl, 1963). Chakraborty *et al.* (1957, 1961) have studied the activity of umbelliferone and of 16 other natural coumarins at concentrations of $2 \times 10^{-2} M$ to $2 \times 10^{-5} M$ against *A. niger* and *Curvularia lunata* and found that umbelliferone was virtually inactive against these fungi at lower concentrations. Seselin (IX), xanthyletin (X), psoralen (II), and luvangetin (XIV), however, markedly inhibited growth of *C. lunata* and, to a lesser extent, of *A. niger* at concentrations between 30 and 300 ppm.



Umbelliferone has been reported to be active against *Brucella* species. However, from recent examination of the effects of this compound and other natural coumarins on the growth of gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus lysodeicticus*, and *Bacillus magatherium*) and on gram-negative bacteria (Dadák and Hodák, 1966) observed that umbelliferone and its 7-geranyl ether are completely inactive against all of these organisms. Ostruthin (XV), the C-geranyl isomer of 7-geranoxycoumarin, however, strongly inhibited growth of the gram-positive microorganisms at concentrations of only $10^{-4} M$ to $2.5 \times 10^{-4} M$, and it was also effective (Dadák, 1967) against *Saccharomyces cerevisiae*. These authors suggested that the antibacterial activity of ostruthin requires both the free 7-hydroxyl group and the long geranyl side chain, since methylation of the hydroxyl or elimination of the side chain resulted in considerable loss of

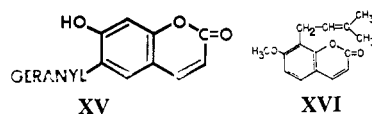


Table IV. Effect of 500 ppm of Umbelliferone Derivatives on Growth of Microorganisms

Microorganisms	Umbelliferone derivatives ^a							
	1	2	3	4	5	6	7	8
Yeasts								
<i>Zygosaccharomyces japonicus</i> C124	-	+	+	+	-	-	-	-
<i>Zygosaccharomyces barkeri</i> C293	-	+	+					
<i>Candida tropicalis</i> C147	-	+	+	+	-	-	-	-
<i>Candida chalmersi</i> C127	-	+	+					
<i>Pichia chodatii</i> /var. <i>fermentans</i> C238	-	+	+	+	-	-	-	-
<i>Hansenula anomala</i>	-	-	+	-	-	-	-	-
<i>Saccharomyces cerevisiae</i> /var. <i>ellipsoideus</i> Y44	-		+	-	-	-		
<i>Saccharomyces rosei</i> 59-4	-	+	+					
<i>Saccharomyces millis</i>	-	+	+					
<i>Torula utilis</i> NRRLY 660	-			±	-	-	-	-
<i>Hanseniaspora melligeri</i> C23	-	+	+					
Molds								
<i>Aspergillus flavus</i> NRRL 3145	-	+	+	-	-	-	-	-
<i>Aspergillus flavus</i> NRRL 2999	-	+	+					
<i>Aspergillus niger</i> A-7705	-	+	+	-	-	-	-	-
<i>Aspergillus oryzae</i>	-	+	+					
Bacteria								
<i>Bacillus cereus</i> 2006	-	+	+	-	-	-	-	-
<i>Sarcina lutea</i>	-	+	-	±	-	-	-	-
<i>Staphylococcus aureus</i> SG8A	-	-	-	±	-	-	-	-
<i>Streptococcus lactis</i>	-	-	+	-	-	-	-	-
<i>Alcaligenes faecalis</i> B170	-	+	+	-	-	-	-	-
<i>Escherichia coli</i> ML30	-	-	+	-	-	-	-	-
<i>Pseudomonas aeruginosa</i> 111	-	-	-	-	-	-	-	-
<i>Salmonella typhimurium</i> Tml	-	-	+	-	-	-	-	-
<i>Serratia marcescens</i>	-	-	-	-	-	-	-	-

^a 1, Umbelliferone; 2, Herniarin; 3, 7-Ethoxycoumarin; 4, 7-Propoxycoumarin; 5, 7-Allyloxycoumarin; 6, 7-Isopentenylloxycoumarin; 7, 7-Isopentenylloxycoumarin; 8, 7-Geranylloxycoumarin; +, inhibits growth; -, no growth inhibition; ±, slight growth.

activity. In contrast to this observation, however, it was recently reported (Kuznetsova *et al.*, 1966) that osthol (XVI) is very active at only 25 ppm against a number of species of gram-positive bacteria, as are the furocoumarins, alloimperatorin, and imperatorin. Martin *et al.* (1966) found that mixtures of furocoumarins (pimpinellin, isopimpinellin, isobergaptin, and sphondin), isolated from the leaves of several plant species, including lime (*Citrus aurantifolia* Swingle) are toxic to the fungi (*Gloeosporium limeticola*, *Botrytis cinerea*, *Sclerotinia fructicena*, and *Stereum purpureum*) at 200 ppm or less in nutrient media.

With the exception of ostruthin it is noteworthy that the umbelliferone derivatives which have been reported to be active possess a protected 7-hydroxyl group. More recent studies (Jurd *et al.*, 1970) have provided additional data on the effects of alkylation on the inhibitory activity of umbelliferone. Thus, with the exception of *Byssoschlamys fulva*, umbelliferone proved to be ineffective against a wide variety of yeasts and molds at a concentration of 500 ppm. Herniarin (XIIIb), the 7-methyl ether of umbelliferone, however, completely inhibited the growth of five *Aspergillus* species, of *Byssoschlamys fulva* and *Penicillium chrysogenum*, and of eight of the nine species of yeasts examined. The antifungal activity of 7-ethoxycoumarin was similar to that of herniarin. However, increasing length of the 7-Oalkyl group diminished the activity and 7-geranoxycoumarin, for example, was com-

Table V. Effect of 500 ppm Lime Oil Precipitate, Derivatives, and Lemon Sludge Derivative on Growth of Microorganisms

Microorganisms	Citrus derivatives ^a					
	1	2	3	4	5	6
Yeasts						
<i>Zygosaccharomyces japonicus</i>	±	+	+	+	+	+
<i>Candida tropicalis</i>	-	+	+	-	+	+
<i>Pichia chodatii</i>	-	-	-	-	-	+
<i>Hansenula anomala</i>	-	-	-	-	-	-
<i>Saccharomyces cerevisiae</i>	-	-	-	-	-	-
<i>Torula utilis</i>	-	-	-	-	-	-
Molds						
<i>Aspergillus flavus</i>	-	-	-	-	-	-
<i>Aspergillus niger</i>	-	-	-	-	-	-
<i>Penicillium chrysogenum</i>	±	±	-	-	-	-
<i>Rhizopus senti</i>	-	NG	NG	-	NG	-
<i>Botrytis cinerea</i>	+	+	+		+	
<i>Byssoschlamys fulva</i>	±	+	+	±	±	-
<i>Alternaria</i>	-	±	+	-	-	-
Bacteria						
Gram-Positive						
<i>Bacillus cereus</i>	+	+	+	+	+	-
<i>Sarcina lutea</i>	-	-	±	-	-	-
<i>Staphylococcus aureus</i>	-	-	±	±	-	-
<i>Streptococcus lactus</i>	-	-	±	±	+	+
Gram-Negative						
<i>Acaligenes faecalis</i>	-	-	-	-	-	-
<i>Escherichia coli</i>	-	+	±	-	±	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	+
<i>Salmonella typhimurium</i>	-	-	-	-	-	-
<i>Serratia marcescens</i>	-	-	-	-	-	-

^a 1, Lime oil precipitate; 2, Hydrogenated precipitate; 3, Phenols by hydrolysis; 4, Propionylated phenols; 5, Neutral mat. from hydrolysis; 6, Lemon oil sludge methylated phenols; +, inhibits growth; -, no growth inhibition; ±, slight growth; NG, control and test cultures did not grow.

pletely inactive (Table IV). As indicated in Table IV, the antibacterial activities of herniarin and 7-ethoxycoumarin were also more pronounced than those of umbelliferone.

Because of the availability of large quantities of solids from cp lime oils (Table I), tests were made to see if this material or simple derivatives of it might have microbiological activity. A portion of the crude precipitate was hydrogenated and another portion hydrolyzed in glacial acetic acid with sulfuric acid and the mixed phenolic products separated from neutral material with base. The phenols were recovered by acidification and a portion was converted to propionate with propionyl chloride. In addition, a sludge recovered from lemon oil processing was hydrolyzed with acid and the recovered phenolic material was methylated with dimethyl sulfate. The various products from lime precipitate and lemon sludge were tested for activity against yeasts, molds, and bacteria with the results shown in Table V.

It is readily apparent that the crude mixture from lime oil and its derivatives and the methylated lemon mixture do not have the broad spectrum of activity against microorganisms found for herniarin and 7-ethoxycoumarin, and so could only be useful in limited microbiological control applications.

ACKNOWLEDGMENT

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