being used to study geographic and seasonal influences on citrus oil composition.

We found the stationary phase SE-54 to be superior to the widely used Carbowax 20M for analysis of citrus oils. It not only affords good separation of aldehydes, but its greater chemical and thermal stability also provides longer column life and shorter analysis time.

CONCLUSION

Existing data on the quantitative composition of cold-pressed lemon oil probably suffered from the inaccuracies due to the relatively low resolution and high residual activity of packed gas chromatography columns. The application of glass capillary gas chromatography allows quantitation of major, minor, and trace components in a single chromatographic run when used with a calibration mixture for determination of detector response factors.

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Reduction of Limonin Bitterness in Navel Orange Juice Serum with Bacterial Cells Immobilized in Acrylamide Gel

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Limonin debittering of navel orange juice serum was successfully demonstrated with *Arthrobacter globiformis* cells immobilized in acrylamide gel. Treatment of 30 mL of serum (10–27 ppm of limonin), for instance, on a 1.5 cm diameter column packed with 1.6 g of immobilized cells (16-mL bed volume) reduced limonin content by 70% or more. This column was used 17 times without losing its effectiveness. The treatment did not affect juice composition as measured by total acids, total soluble solids, pH, and sugars. 17-Dehydrolimonoate A-ring lactone was identified as the major metabolite, showing the involvement of limonin D-ring lactone hydrolase and limonoate dehydrogenase in the debittering process.

Limonin (I; see Scheme I) is a bitter member of a group of limonoids which are chemically related triterpene derivatives found in Rutaceae and Meliaceae. Bitterness due to I in certain citrus processed products such as navel orange juice is one of the primary determinant factors for product acceptability and has significant economic impact on the industry. The intact fruits do not normally contain I but rather a nonbitter precursor, limonoate A-ring lactone (II), and this nonbitter precursor converts to bitter I under acidic conditions after extraction of juice (Maier and Beverly, 1968; Maier and Margileth, 1969). This conversion is also accelerated by action of limonin D-ring lactone hydrolase which has been shown to be present in citrus (Maier et al., 1969). This phenomenon is referred to as delayed bitterness.

In dealing with this bitterness problem one of the approaches taken at our laboratory is to develop a process which converts I and II in the juice to nonbitter compounds with limonoid-metabolizing enzymes. During the course of this study, we have isolated from soil several species of bacteria which metabolize limonoids (Hasegawa et al., 1972a,b; Hasegawa and Kim, 1975), and have established that limonoids are metabolized in bacteria and citrus through at least two pathways: one via 17-dehydrolimonoids (Hasegawa et al., 1972b, 1974) and the other via deoxylimonoids (Hasegawa et al., 1972a, 1980).

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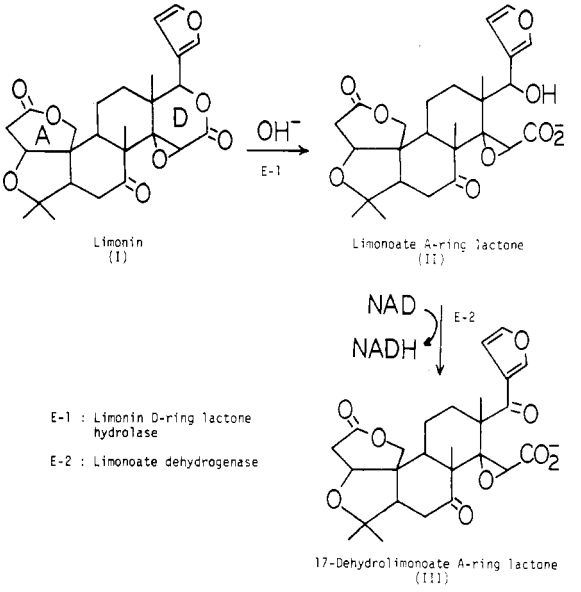
Scheme I. The Major Limonoid-Metabolizing Enzyme System in *A. globiformis* Cells Immobilized in in Acrylamide Gel



Among the species of bacteria isolated, *Arthrobacter globiformis* produces limonoate dehydrogenase, which catalyzes the conversion of II to nonbitter 17-dehydrolimonoate A-ring lactone (III) (Hasegawa et al., 1972b). Possible uses of limonate dehydrogenase enzymes of *A. globiformis* and *Pseudomonas* 321-18 for removal or prevention of limonin

Table I. Reduction of Limonin Content in Navel Orange Juice Sera with *A. globiformis* Cells Immobilized in Acrylamide Gel[a]

time after immobilized, days	column 1,[b] limonin content			column 2,[c] limonin content			column 3,[d] limonin content		
	control, ppm	treated, ppm	reduction, %	control, ppm	treated, ppm	reduction, %	control, ppm	treated, ppm	reduction, %
1	15.2	1.9	88	22.0	4.3	81	27.7	4.4	84
2	15.0	3.0	80	22.0	4.0	82			
3	15.1	2.8	82				8.9	1.1	87
4							13.3	3.3	75
6	27.0	5.2	81						
7				21.3	4.0	81	8.5	3.1	64
8	27.0	4.8	82	22.0	4.7	79	11.0	3.8	65
9	26.5	4.0	85	22.3	4.0	82	14.7	4.3	71
10				22.3	4.3	81			
13	10.5	2.5	76						
14	10.2	3.0	71	22.0	0.7	97	9.7	2.3	75
15	10.3	2.7	74	22.0	4.7	79	10.0	2.3	77
16				22.0	0	100	9.3	1.0	89
17	10.5	0.5	95	22.0	1.3	94	10.0	2.3	77
18				22.0	5.7	74	14.3	3.0	79
20	15.2	3.2	79						
21				22.0	11.3	49	20.0	4.7	77
22	15.0	2.8	81				20.0	5.3	74
23				22.0	12.3	44	20.0	5.7	72
24	14.9	3.5	77				7.2	1.7	76
28	26.9	6.5	76				16.7	9.0	46
29	26.8	5.9	78				17.7	9.7	45
30							12.8	5.7	55
31							12.2	6.0	51
32							12.2	5.7	53
35							16.1	7.7	52
36	22.2	5.7	74				16.1	13.3	17
37							17.7	14.0	21
38							16.7	10.6	36
42	21.8	12.0	45	16.1	10.6	34	16.7	10.6	36
43	22.0	18.7	15	16.7	16.1	4			

[a] 30 mL of juice serum was passed once through a 1.5 cm diameter column packed with immobilized cells at a rate of 1 mL/min and at room temperature. [b] Column 1: 1.6 g of cells; 16-mL bed volume after immobilized; serum pH 3.5-3.7. [c] Column 2: 1.5 g of cells; 15-mL bed volume after immobilized; serum pH 3.6. [d] Column 3: 1.5 g of cells; 15.5-mL bed volume after immobilized; serum pH 3.5-3.6.

bitterness in navel orange juice were demonstrated previously (Hasegawa et al., 1973; Brewster et al., 1976). However, since the dehydrogenase enzymes have optimal activity at a high pH, either adjustment of juice pH with alkali before treatment with enzyme or use of large amounts of enzyme is required to reduce limonin content of the juice to below the bitterness threshold. Recently, limonin debittering in navel orange juice serum was successfully demonstrated with *A. globiformis* cells immobilized in acrylamide gel. This paper reports the results of such experiments.

EXPERIMENTAL SECTION

Materials. Navel oranges were grown at University of California at Riverside and harvested during Nov and Dec 1980. The juices were extracted with a Sunkist juicer, and the sera were obtained from the juices by centrifugation at 2500 g for 10 min and kept in a freezer until used. *A. globiformis* cells were prepared by the procedures described previously (Hasegawa et al., 1972b) and kept in a freezer until used.

Immobilization of Cells. *A. globiformis* cells were immobilized in acrylamide gel by the procedures reported by Tosa et al. (1974). The resulting gel was then blended with a Polytron.

Treatment of Sera with Immobilized Cells. Routinely, about 1.5 g of *A. globiformis* cells were immobilized, blended, and packed in a 1.5 cm diameter column (15-mL bed volume). A 30-mL portion of the serum was passed through the column once at a rate of 1 mL/min at room temperature. The control was treated similarly on a column packed with acrylamide gel without bacterial cells. The column was then washed thoroughly with 30 mL of 0.05 M of potassium phosphate buffer at pH 7.0 and kept at 3 °C until the next use. Limonoids were analyzed by the TLC method described by Maier and Grant (1970) after extraction with CH_2Cl_2.

Limonin D-Ring Lactone Hydrolase Activity. The hydrolase was prepared from *A. globiformis* cells by the procedures described previously (Hasegawa, 1976). Hydrolyzing activity, the hydrolysis of I to II, was assayed in a reaction mixture consisting of 5×10^{-3} M limonin, 0.1 M Tris buffer at pH 8.0, 20% acetonitrile, and an approprite amount of enzyme in 0.3 mL. After 5 min of incubation at 30 °C, 5-μL portions of the reaction mixture were spotted on a silica gel plate which was developed with toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer). Limonoids were revealed by spraying the plate with Ehrlich's reagent and exposing to HCl gas (Dreyer, 1965).

Analyses of Other Constituents and Parameters. Total acids, total soluble solids, and total and reducing sugars were analyzed by the procedures reported by Vandercook et al. (1975).

RESULTS AND DISCUSSION

Table I shows the results of treatment of navel orange juice sera with *A. globiformis* cells immobilized in acrylamide gel. The limonoid-metabolizing enzyme system in the immobilizing cells very actively reduced limonin (I) content of the juice serum. It was found also that the enzyme system was so stable that the immobilized cells could be used many times without losing their effectiveness. In the column 1, for instance, 1.6 g of cells immobilized, blended, and packed in a 1.5 cm diameter (16 mL

Table II. Effects of Treatment of Navel Orange Juice Serum with *A. globiformis* Cells Immobilized in Acrylamide Gel on Limonin and the Other Juice Constituents and Parameters[a]

constituents and parameters	control	treated
limonin, ppm	22.0	4.0
total acids, mequiv/100 mL	25.3	25.3
total soluble solids, g/100 mL	10.7	10.7
total sugars, g/100 mL	8.5	8.4
reducing sugars, g/100 mL	5.2	5.1
nonreducing sugars, g/100 mL	3.3	3.3
pH	3.6	3.6

[a] 30 mL of the serum was treated with a 1.5 cm diameter column packed with 1.5 g of cells immobilized in gel (15-mL bed volume). The control was treated with the same column packed with acrylamide gel without bacterial cells.

bed volume) column converted 70% or more of I in 30 mL of serum, which contained 10–27 ppm of I, by passing the serum through the column only once. Compound I was completely metabolized when the serum was treated with the column twice. This column was used 17 times before losing its effectiveness.

The major metabolite in the treated serum was acidic, negative to Ehrlich's reagent, and identified as 17-dehydrolimonoate A-ring lactone (III) with NMR spectra and TLC by the procedures described previously (Hasegawa et al., 1972b). The formation of this metabolite during the treatment clearly shows the involvement of limonoate dehydrogenase. This enzyme attacks only the open D ring of limonoids such as limonoate A-ring lactone (II) (Hasegawa et al., 1972b). Since the dehydrogenase cannot attack I, compound I has to be converted to II before being attacked by the dehydrogenase by the action of limonin D-ring lactone hydrolase, which catalyzes the hydrolysis of the D-ring lactones of limonoids (Maier et al., 1969; Hasegawa, 1976). Analyses of cell-free extracts of *A. globiformis* showed the presence of this hydrolase activity in the cells. Like the hydrolase of *Pseudomonas* 321-18 (Hasegawa, 1976), this enzyme catalyzed the hydrolysis of the D-ring lactone of I at pH 7.0 or higher with its optimal activity at pH 8.0. The presence of this enzyme explains why I is converted to III. This conversion via II suggests very strongly that the enzyme system in the immobilized cells is functioning in a compartment where the pH is 7.0 or higher regardless of treatment with low pH serum. Under these conditions, limonoate dehydrogenase of *A. globiformis* very actively converts II to III (Hasegawa et al., 1972b). It is of interest to note that the immobilized cells metabolized I in the serum faster than limonoate in pH 7.5 potassium phosphate solution even though limonoate (both the A- and D-ring lactone open) is the best substrate for the limonoate dehydrogenase. Addition of the cofactor NAD to the buffer solution did not affect the rate. Thus, the difference in rates of metabolism between the two systems is most likely due to I being able to penetrate the cell wall and reach the enzyme faster than limonoate.

In addition to III we observed a minor metabolite which has not yet been identified. This compound was positive to Ehrlich's reagent, showing that it is not a metabolite of the 17-dehydrolimonoid pathway. TLC analyses indicated also that it is not any of the known metabolites of the deoxylimonoid pathway. The presence of this minor metabolite, however, suggests strongly that the enzyme system is functioning in dead cells because we have never observed such metabolites in the media of living cells. The following experiment provided an additional evidence to support the above hypothesis. When fresh cells were immobilized right after being harvested, the enzyme system in the immobilized cells had an activity of only about 50% of its maximal level, and it required a few days of aging to reach its maximal activity level.

Treatment of the serum with the immobilized cells gave no adverse effect on juice composition as measured by total acids, total soluble solids, pH, and sugars (Table II). There apppeared to be no detectable change in organoleptic properties between the control and treated samples except that the treated serum was significantly less bitter.

This study shows that *A. globiformis* cells immobilized in acrylamide gel were capable of reducing the I content in navel orange juice serum. The cell system was not only active at natural juice pHs but also stable. Treatment of navel orange juice instead of serum seems to give no serious technical problems such as blockage of the column by juice particles and channeling of the column. Recently, we successfully treated navel orange juice with a column (2.5 × 50 cm) packed with acrylamide gel beads in which the juice was pumped upward through a bed of beads. Further studies would be necessary for the process to be practical, but this study shows that the process has potential for commercial application.

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