Table III. Mean Recoveries of Metalaxyl Added to Sunflower Foliage before Extraction

metalaxyl added, ppm	recovery from sunflower foliage, %[a]
0.05	89 ± 2.2 (5)
0.1	98 ± 2.5 (5)
0.2	103 ± 3.1 (4)
1.0	105 ± 1.9 (5)
5.0	91 ± 2.9 (4)

[a] Mean ± standard error (number of determinations in parentheses).

fair content of organic matter.

Sweep codistillation as a purification process for routine determination has yielded good results. With regard to the lifetime of silanized glass bead column, we find that it can be used for three sunflower foliage analyses or for ten soil analyses. The silica gel microcolumn has been used successfully to clean up Metalaxyl extracts from soils and sunflower foliage. The efficiency of the analytical methods for sunflower foliage is indicated in Table III by the recovery of the fungicide added to untreated samples.

When the chromatograms of the numerous blanks tested are taken into account (five from soils; seven from sunflower foliage), the limit of sensitivity was 0.05 ppm. Typical chromatograms obtained by injecting extracts of fortified samples are shown in Figure 2.

As the operator need not be present during the 6 h of Soxhlet extraction of Metalaxyl from the soil, this method is less time consuming than the Ramsteiner (1976) procedure.

The most significant advantage of the method presented here is that it is simple, straightforward, and reproducible.

LITERATURE CITED

Bor, N. A.; Wierstema, W. P. *Int. Symp. Crop Prot.* **1979**, *31*, 63.
Cohen, Y.; Reuveni, M.; Eyal, H. *Phytopathol. News* **1979**, *69*, 645.
De Baets, A. *Int. Symp. Crop Prot.* **1979**, *31*, 47.
Garibaldi, A.; Morando, A. *Atti Giornate Fitopatol., Catania* **1978**, *1978*, 233.
Ramsteiner, K., Ciba Geigy, internal method, 1976.
Thornburg, W. W. In "Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives"; Zweig, C., Ed.; Academic Press: New York, 1963; Vol. I, p 99.
Urech, P. A.; Schwinn, F.; Staub, T. *Br. Crop Prot. Conf.—Pests Dis.* **1977**, 623.

Francesco Tafuri*
Cesare Marucchini
Maurizio Patumi
Mario Businelli

Centro di Studio sulla Chimica degli Antiparassitari del C.N.R.
Istituto di Chimica Agraria dell'Università
06100 Perugia, Italy

Received for review April 27, 1981. Accepted August 20, 1981.

Hydrolysis of High Levels of Naringin in Grapefruit Juice Using a Hollow Fiber Naringinase Reactor

Grapefruit juice that has excessive naringin bitterness is generally unmarketable. This paper describes a procedure for reducing the naringin content of grapefruit juice containing very high levels of naringin using a commercially available naringinase restrained in a hollow fiber reactor. Juice containing 885 μg/mL naringin was debittered to a level acceptable to a sensory evaluation panel sensitive to bitterness. Single-strength unclarified juice from a frozen concentrate was recycled through the hollow fiber reactor at temperatures up to 45 °C to achieve different levels of naringin hydrolysis. Parameters affecting naringin hydrolysis to prunin and naringenin such as flow rates, hollow fiber membrane surface area, temperature, and enzyme loadings were investigated in order to improve debittering rates. The 4-ppm limonin content of the juice did not contribute to the excessive bitterness and was found to be unchanged by the debittering process. Juice containing 885 μg/mL naringin could be debittered to 285 μg/mL at a rate of 14 mL/min at 45 °C, with no adverse effects from the particulate matter in the juice.

Grapefruit juices with high levels of naringin are extremely bitter, and a method of lowering the naringin concentration to a more acceptable level would permit the blending of these juices into a product without excessive bitterness. This is dependent upon a suitably low level of limonin (Guadagni et al., 1973, 1974), which at normal levels contributes a small amount to total grapefruit bitterness.

The use of hollow fiber immobilized naringinase (from *Aspergillus niger*) has been shown to be a feasible method for reducing the naringin content of grapefruit juice from 290 μg/mL to any desired lower concentration (Olson et al., 1979). In this work, juice with an extremely high, almost 900 μg/mL, naringin level was used to explore debittering parameters. In addition, the objective was to obtain the highest debittering rate possible and to determine the acceptability of the treated juice.

EXPERIMENTAL SECTION

Hollow Fiber. The Romicon HF 1.1-43-PM10 hollow fiber (HF) cartridge, manifolds, and pumping system for flow rates up to 1.0 L/min used in this work are the same as those reported previously (Olson et al., 1979). Plumbing was entirely Eastman semirigid EVA-66 food-grade tubing. The large (up to 10.0 L/min) pumping capacity unit used was the Romicon HFXS MKII Ultrafiltration System incorporating the PM10 HF cartridge. This system employed two centrifugal pumps: a $^3/_4$-hp stainless steel

pump which circulated the grapefruit juice through polypropylene tubing to the HF cartridge, past inlet and outlet control valves, pressure gauges, and a thermometer; a small $^1/_{15}$-hp pump which circulated fluids from a small fiberglass tank into the HF cartridge shell region, past control valves and a pressure gauge for the shell ports of the HF cartridge.

HF reactor temperatures above ambient in the laboratory were achieved by insulating the HF reactor and placing the substrate reservoir in a thermostated water bath. The HFXS system was not temperature controlled. Flow rates expressed in this work are for continuous recycle operation.

Enzyme Loading and Hollow Fiber Cleaning. The naringinase (Sigma Chemical Co., N-1753) used was identical with that reported previously (Olson et al., 1979). It contained 27.8% protein (Kjeldahl nitrogen × 6.25) and was solubilized and filtered as before. In the laboratory system, the enzyme solution was pumped into the shell through 0.45-μM Millex filters. In the HFXS system, the shell was loaded with enzyme by back-flushing the nearly clear enzyme solution into the shell, followed by 1.0 L of tap water to rinse the enzyme solution completely into the shell. Enzyme leakage across the fiber membranes was checked by collecting the ultrafiltrate from enzyme loading and incubating it with substrate or by incubating an aliquot of grapefruit juice after HF naringinase processing and analyzing it for naringin content decrease.

The HF cartridges were cleaned by successively pumping 0.5% NaOH (in cold tap water), warm tap water, and 20% aqueous ethanol through the systems in both ultrafiltration and back-flush modes. Before enzyme loading, the 20% ethanol bacteriocide and storage solution were rinsed out of the system with 20–40 L of cold tap water.

Substrate. Naringin substrate solutions were prepared in two concentrations. The less concentrated solution was prepared by adding 1.2 g of naringin (Grade II, Sigma Chemical Co.) to 4.0 L of tap water, bringing the slurry to near boiling, and then cooling it to room temperature. The solution was then adjusted to pH 3.2 by the addition of citric acid (Mallinkrodt) and filtered (no. 604 paper, Schleicher & Schuell) to remove any insolubles. This produced a solution of 270–300 μg/mL naringin. More concentrated naringin solutions were prepared for comparison to grapefruit juice with a high level of naringin, by adding 3.2 g of naringin to 4.0 L of tap water containing 190 g of sucrose and 1 g of citric acid and heating to near boiling. After the mixture was cooled to room temperature, the pH was adjusted to 3.2 with either additional citric acid or 4 N NaOH and the solution filtered to remove residual insolubles. This produced a solution containing 700 μg/mL naringin which was stable for ~24 h, after which it required reheating to solubilize naringin crystals.

The grapefruit juice used as the naringinase substrate was obtained from Sunkist Growers, Inc., Ontario, CA 91761, as a 58 °Brix concentrate. It was reconstituted 1:4.5 (w/w) with cold tap water to yield a 10.0 °Brix single-strength juice with 885 ± 15 μg/mL naringin concentration and pH 3.2.

Analysis. High-pressure liquid chromatographic (HPLC) analysis of naringin in single-strength grapefruit juice, and the identification of naringenin 7-β-rutinoside, prunin, and naringenin, was performed by the method of Fisher and Wheaton (1976). Enzyme activities or reactor activities were determined from the slope of the decreasing naringin concentration vs. time between approximately 10 and 40% hydrolysis and were reported on the basis of micromoles of naringin hydrolyzed per minute per milligram of protein or micromoles of naringin hydrolyzed per minute.

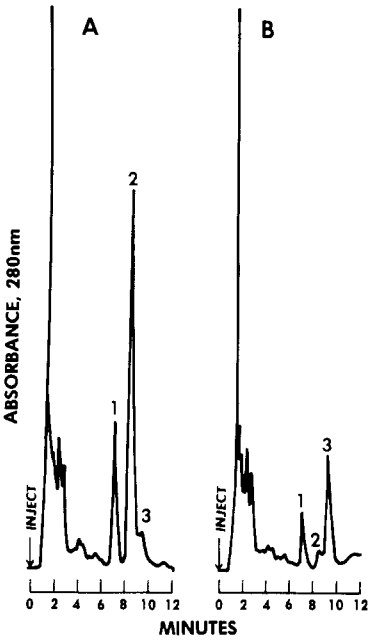

Figure 1. High-pressure liquid chromatogram of grapefruit juice. (A) Untreated juice, 885 μg/mL naringin; (B) Naringin, 95% hydrolyzed. Peak 1, naringenin 7-β-rutinoside; peak 2, naringin; peak 3, prunin.

Limonin content of single-strength grapefruit juice was determined by the HPLC method of Fisher (1975). Limonin content was also determined by a new procedure using a μBondapak C_{18} column (Waters Associates) and isocratic elution with a 28:6:66 mixture of CH_3CN (Waters Associates), CH_3OH (Burdick and Jackson), and H_2O (prepared by a Milli-RO/Milli-Q-System, Millipore Corp.) pumped at 2.0 mL/min with an M6000A solvent delivery system (Waters Associates). Detection was accomplished by monitoring column effluent at 207 nm with a Model 450 variable-wavelength detector (Waters Associates). Quantitation was accomplished by adding known amounts of limonin to the samples to generate a standard curve. Extrapolation of the plot to the base line yielded the native amount of limonin in the sample.

Sensory Evaluation. Samples for the taste panel were prepared by recycling the single-strength juice through a two-cartridge, HF naringinase reactor at 1000 mL/min and 45 °C until a desired level of naringin was obtained. The juice was then frozen until used. A 42-member panel, experienced in grapefruit juice testing, was used to determine the effect of HF naringinase treatment on bitterness in this juice. The juice was rated on a Hedonic scale of 1 to 9, with "1" as "dislike extremely", "5" as "neither like nor dislike", and "9" as "like extremely".

RESULTS AND DISCUSSION

Extremely bitter high naringin level single-strength unclarified juice was debittered by recycling it through the HF naringinase reactor until a desired level of naringin hydrolysis was obtained. No plugging of the fibers with the juice pulp was observed. After use, the HF reactor and system were easily cleaned of used enzyme and adsorbed compounds. The previous method (Olson et al., 1979) for cleaning the HF system preparatory to reloading with enzyme was discontinued when it was found that warm tap water and aqueous ethanol (following the occasional dilute NaOH solution) were more effective in removing flavors and odors accumulated on the hollow fibers during use. Naringin content of the grapefruit juice (before treatment

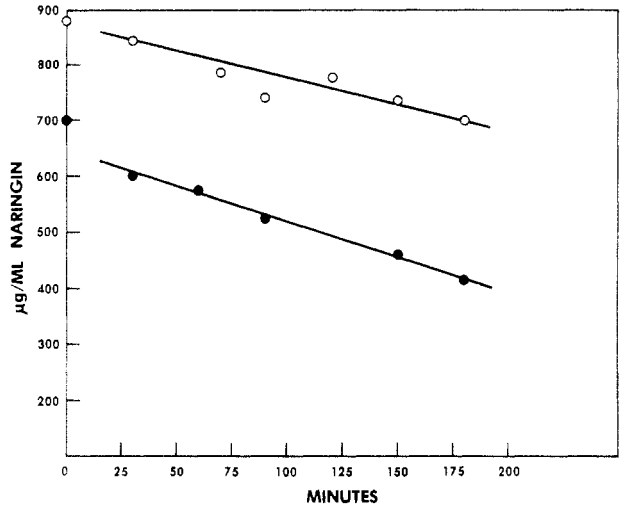

Figure 2. Hydrolysis curves of naringin in grapefruit juice (○) and in water solution (●).

was 885 ± 15 μg/mL. A chromatogram of untreated and HF naringinase reactor treated grapefruit juice with 45 μg/mL naringin (95% hydrolysis) is shown in Figure 1. In the untreated juice (A), naringin 7-β-rutinoside, peak 1, and naringin, peak 2, levels were much higher than those for the juice used in previous work (Olson et al., 1979). The debittered juice (B) shows the dramatically reduced amounts of naringenin 7-β-rutinoside and naringin remaining and also shows an increase in peak 3, the retention time of prunin.

A comparison of enzyme activities in the HF reactor using 700 μg/mL naringin solution and 885 μg/mL naringin containing grapefruit juice substrates resulted in activities of 0.04 and 0.03 μmol min^{-1} (mg of protein)$^{-1}$, respectively, at 25 °C in an HF reactor containing 112 mg of enzyme (shell full of liquid) with a 2000-mL substrate solution recycled at 1000 mL/min (Figure 2).

Enzyme Load. Incubation of the ultrafiltrate from enzyme loading with grapefruit juice resulted in no hydrolysis of the naringin. Similarly, the naringin content of grapefruit juice after debittering in the HF reactor did not change either with incubation or after refrigerator storage for several weeks. Therefore, no detectable enzyme activity was leaked from the shell during enzyme loading or during use of the HF reactor. Enzyme loadings varied from 400 to 3000 mg of enzyme preparation (110 to 850 mg of protein). The amount of enzyme used vs. the hydrolysis rate was described in previous work; there was no deviation from the previous conclusion of decreased enzyme activity above about 50 mg of protein applied. For example, tripling the amount of enzyme in the HF reactor from 115 to 350 mg (protein) gave 60% more HF reactor activity (at 25 °C and a 1000 mL/min flow rate of 2000 mL of grapefruit juice substrate for 3 h) but dropped the enzyme activity from 0.030 to 0.016 μmol min^{-1} (mg of protein)$^{-1}$.

In our previous work, after the enzyme solution had been back-flushed into the HF shell, air was pumped into the shell to force the enzyme into the sponge region of the fibers until no visible liquid remained; the assumption was that the enzyme would be more efficiently utilized in the small volume immediately adjacent to the membrane forming the inner walls of the fibers. For investigation of the effects of having the shell volume empty or filled with the enzyme solution, experiments were performed where the HF reactor was operated with the shell first empty and then filled with permeate by ultrafiltration and the reverse, where the shell was left full of liquid after enzyme loading, the reactor operated and then emptied by back-flushing with air, and the operation started again with fresh substrate. For an enzyme loading of 112 mg of protein and 2000 mL of 270 μg/mL naringin substrate solution recycled at 1000 mL/min, reactor activity for both configurations was 3.4 μmol min^{-1} at 25 °C; there was no difference in reactor activities between having the enzyme solution in the sponge region of the fibers or in the ca. 127-mL volume of the HF shell.

Temperature. Naringin hydrolysis rates were increased 60% by increasing the temperature from 25 to 35 °C and 25% more by increasing the temperature to 45 °C in an HF reactor containing 225 mg of enzyme protein.

The enzyme activity in the soluble form using 885 μg/mL naringin grapefruit juice substrate was 0.04 μmol min^{-1} (mg of protein)$^{-1}$ at 25 °C and was increased to 0.08 μmol min^{-1} (mg of protein)$^{-1}$ at 45 °C.

Surface Area. For determination of the effect of membrane surface area on hydrolysis rate, two HF reactors were connected in series with the enzyme load split equally between the two reactor shells. This gave a 40% increase in reactor activity at 45 °C and a 1.0 L/min flow rate compared to that from a single HF reactor with the same (824 mg of enzyme protein) load. This less than 2× activity may be related to changes in pressure drop and flow characteristics across the extended reactor length resulting in lowered transmembrane diffusion and less effective use of the enzyme in the second reactor.

Flow Rate Effects: HFXS Unit. For ultrafiltration use, flow rates high enough to cause turbulent flow are suggested by the manufacturer to minimize concentration polarization and membrane fouling. The debittering rate dependence on reactor recycle flows at 10.0 L/min was studied with the use of the Romicon HFXS unit incorporating an identical PM-10 HF cartridge. The HFXS system exhibited a temperature rise of 20 °C due to heat transfer from the pump to the system, using a substrate juice volume of 8.0 L. Comparison between the HFXS and the laboratory (1.0 L/min) HF naringinase reactors showed that the HFXS system increased reactor activity 70%; in the HFXS, the enzyme activity at 44 °C was 0.05 μmol min^{-1} (mg of protein)$^{-1}$ (120 mg of enzyme protein, 8.0 L of juice substrate) compared to 0.03 μmol min^{-1} (mg of protein)$^{-1}$ at 44 °C in the same reactor at a 1.0 L/min flow using 112 mg of enzyme protein. The higher flow rate in the HFXS system gave increased reactor activity because of better enzyme utilization as shown by the increased enzyme activity despite higher loading. This comparison of enzyme activities was important because increased enzyme loading in the 1.0 L/min flow system led to lowered enzyme activity as mentioned previously. A comparison of activities of 14 μmol min^{-1} for the HFXS and 3.5 μmol min^{-1} for the laboratory system was encouraging, but it should be recognized that the increase was due to both the higher flow and the increased enzyme load.

Calculated Reynolds numbers for 1.0 and 10.0 L/min recycle flow rates were 290 and 2900, respectively. Reynolds numbers less than 2100 indicate laminar flow conditions and above 2100 indicate turbulent flow (Bennett and Myers, 1962). Thus, at the lower flow rate the reactor was functioning under laminar substrate flow conditions, while at the higher flow rate the increased hydrolysis rate was probably due to turbulent substrate flow within the hollow fibers, reducing the concentration gradient at the membrane surface, resulting in improved substrate–product diffusion across the hollow fiber membrane walls. On the basis of the overall debittering rate from the HFXS, grapefruit juice containing 885 μg/mL naringin could be

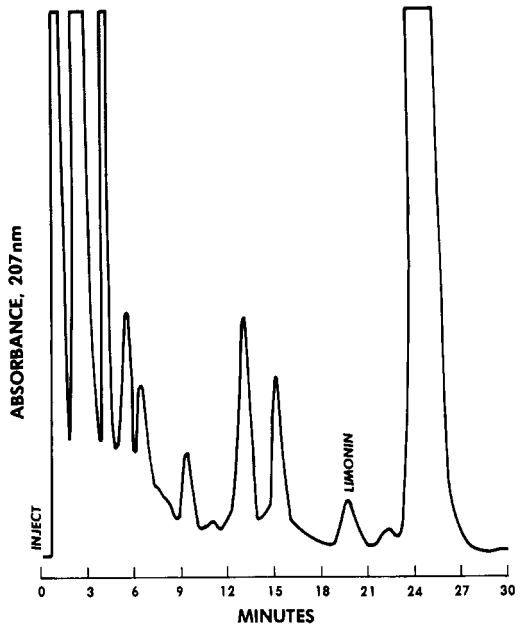

Figure 3. High-pressure liquid chromatogram of limonin in the chloroform–acetonitrile extract from single-strength grapefruit juice.

debittered to 285 μg/mL naringin at 45 °C at a rate of 130 mL min^{-1} (M^2 of HF surface area)$^{-1}$.

Limonin Content of Juice. The limonin contribution to the bitterness in this grapefruit juice was checked by comparing the limonin concentrations of samples of grapefruit juice taken before and after HF naringinase reactor debittering.

Figure 3 shows a typical chromatograph of the chloroform–acetonitrile extract without added limonin. No corrections for unresolved interfering limonoids were made; therefore, the results should be maximum values. The plot of detector response vs. amount of limonin added produced a straight line which was extrapolated back to zero detector response; the limonin content of both untreated and treated juice was 4 μg/mL. This level of limonin was not considered high enough to be a significant contributor to grapefruit juice bitterness. In addition, the results show that limonin levels were not changed by the process procedure.

Sensory Evaluation. Grapefruit juice tasting panels may be divided into three subgroups: A, those who liked grapefruit juice no matter how bitter; B, those whose ratings of taste increased as the bitterness was reduced; C, those who disliked grapefruit juice.

Approximately 50% of the panel of 42 persons were in group B (there were none in group C). This group of panelists assigned an average score of 4.8 ("neither like nor dislike") to the untreated juice containing 885 μg/mL naringin and an average score of 7.3 ("like moderately") to juice partially debittered with the HF naringinase reactor to a level of 275 μg/mL naringin. This preference for debittered juice was highly significant, with a confidence of $P < 0.01$. In addition, those panelists who like bitter juice, group A, did not dislike debittered juice and rated the untreated juice at 6.1 and the HF naringinase treated juice at 6.7.

ACKNOWLEDGMENT

We thank D. Guadagni for the taste panel evaluations. We also express our appreciation to Sunkist Growers, Inc., for the bitter grapefruit juice, without which this work would have been impossible.

LITERATURE CITED

Bennett, C. O.; Myers, J. E. "Momentum, Heat, and Mass Transfer"; McGraw Hill: New York, 1962; p 14.
Fisher, J. F. *J. Agric. Food Chem.* **1975**, *23*, 1199.
Fisher, J. F.; Wheaton, T. A. *J. Agric. Food Chem.* **1976**, *24* (4), 898.
Guadagni, D. G.; Maier, V. P.; Turnbaugh, J. G. *J. Sci. Food Agric.* **1973**, *24*, 1277.
Guadagni, D. G.; Maier; V. P.; Turnbaugh, J. G. *J. Sci. Food Agric.* **1974**, *25*, 1349.
Olson, A. C.; Gray, G. M.; Guadagni, D. G. *J. Food Sci.* **1979**, *44*, 1358.

Gregory M. Gray
Alfred C. Olson*

Western Regional Research Center
Science and Education Administration
U.S. Department of Agriculture
Berkeley, California 94710

Received for review January 16, 1981. Revised manuscript received May 26, 1981. Accepted August 10, 1981. Paper presented at the Second Chemical Congress of the North American Continent, Las Vegas, NV, Aug 25–29, 1980. Reference to a company and/or product named by the U.S. Department of Agriculture is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Microbiological Release of Unextracted (Bound) Residues from an Organic Soil Treated with Prometryn

Soil-bound ^{14}C-labeled residues were released by microbes from an organic soil treated with ^{14}C-ring-labeled prometryn [2-(methylthio)-4,6-bis(isopropylamino)-*s*-triazine]. The compounds that were extractable from the soil after incubation (27% of the total ^{14}C) were identified as prometryn and small amounts of hydroxypropazine [2-hydroxy-4,6-bis(isopropylamino)-*s*-triazine] and deisopropylprometryn [2-(methylthio)-4-amino-6-(isopropylamino)-*s*-triazine].

In recent years there has been a growing concern about the release of bound pesticide residues from soil. A number of studies have demonstrated the potential availability of the bound residues to plants (Fuhremann and Lichtenstein, 1978; Helling and Krivonak, 1978; Fuhr and Mittelstaedt, 1980; Khan, 1980) and earthworms (Fuhremann and Lichtenstein, 1978). Thus, soil-bound pesticide residues are not excluded from environmental interactions (Fuhremann and Lichtenstein, 1978).

Previously we reported that a considerable portion of the bound ^{14}C-labeled residues in an incubated soil treated with ^{14}C-ring-labeled prometryn [2-(methylthio)-4,6-bis-

0021-8561/81/1429-1301$01.25/0