This means that the pronase procedure appears to exaggerate damage of lysine in beef muscle processed in the absence of sugar, although other forms of lysine damage may have occurred. Only 7% of the total lysine from the beef muscle heated for 12 h in the absence of glucose was not recovered after acid hydrolysis (Table I). This indicates that the fall in lysine released by pronase is mainly due to the formation of inter and intra peptide bonds and not reactions between ϵ amino groups of lysine and breakdown products of other amino acids such as cystine. Serine, which was also released at a low level by pronase from this treatment, may form an ester linkage with aspartic acid and glutamic acid or may have been destroyed. These bonds may be resistant to enzymic attack (Mauron, 1972) and this may explain the low release of aspartic and glutamic acid by pronase.

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LITERATURE CITED

Amadi, S. C., Hewitt, D., *Proc. Nutr. Soc.* **34**, 26A (1975).
Association of Official Agricultural Chemists, "Official Methods of Analysis", 9th ed, Washington, D.C., 1960, p 284.
Bjarnason, J., Carpenter, K. J., *Br. J. Nutr.* **24**, 313 (1970).
Boctor, A. M., Harper, A. E., *J. Nutr.* **94** 289 (1968).
Booth, V. H., *J. Sci. Food Agric.* **22**, 658 (1971).
Carpenter, K. J., Booth, V. H., *Nutr. Abstr. Rev.* **43**, 423 (1973).
Carpenter, K. J., Woodham, A. A., *Br. J. Nutr.* **32**, 647 (1974).
Finlayson, A. J., *Can. J. Plant Sci.* **45**, 184 (1965).
Hefferan, P. M., Goodnight, K. C., *J. Food Sci.* **34**, 353 (1969).
Hurrell, R. F., Carpenter, K. J., *Br. J. Nutr.* **32**, 589 (1974).
Hurrell, R. F., Carpenter, K. J., Sinclair, W. J., Otterburn, M. S., Asquith, R. S., *Br. J. Nutr.* **35**, 383 (1976).
Lipton, S. H., Bodwell, C. E., *J. Agric. Food Chem.* **24**, 32 (1976).
Mauron, J., in "International Encyclopaedia of Food and Nutrition", Vol. 2, Bigwood E. J., Ed., Pergamon Press, Oxford, 1972, p 417.
Moore, S., *J. Biol. Chem.* **238**, 235 (1963).
Provansal, M. M. P., Cuq, J. L. A., Cheftel, J. C., *J. Agric. Food Chem.* **23**, 938 (1975).
Rao, M. N., Screenivas, H., Swaminathan, M., Carpenter, K. J., Morgan, C. B., *J. Sci. Food Agric.* **14**, 544 (1963).
Rayner, C. J., Fox, M., *J. Sci. Food Agric.* **27**, 643 (1976).
Roach, A. G., Sanderson, P., Williams, D. R., *J. Sci. Food Agric.* **18**, 274 (1967).
Sgarbieri, V. C., Amaya, J., Tanaka, M., Chichester, C. Q., *J. Nutr.* **103**, 1731 (1973).
Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.* **30**, 1190 (1958).
Taverner, M. R., Rayner, C. J., *Aust. J. Exp. Agric. Anim. Husb.* **15**, 626 (1975).

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An Improved Method for the Quantitation of Limonin in Citrus Juice by High-Pressure Liquid Chromatography

James F. Fisher

Microgram quantities of limonin were resolved from a chloroform extract of citrus juice by high-pressure liquid chromatography using a micro CN column and eluting with a water–methyl alcohol system. The limonin was detected at 210 nm.

Various methods have been published for the determination of limonin (Fisher, 1975, and references therein). The high-pressure liquid chromatographic procedure for the quantitation of limonin in grapefruit juice employing a refractometer reported by Fisher (1975) has the inherent difficulties associated with refractive index detection.

In the method described below, detection is accomplished with an ultraviolet spectrophotometer. The method is more sensitive and factors such as temperature, flow rate, and pressure changes are less critical.

MATERIALS AND METHODS

Apparatus. A Model ALC 202 high-pressure liquid chromatograph (HPLC) with a Model 6000 A pump and U6K injector (Waters Associates, Milford, Mass.) was used. The recorder was a Soltec Model B-281 (Soltec Corp., Encino, Calif.). A Schoeffel UV-visible liquid chromatography analyzer Model SF 770 (Schoeffel Instrument Corp., Westwood, N.J.) was the detector. A Spectra-Physics integrator (minigrator, Spectra-Physics, Santa Clara, Calif.) was used. An International Clinical Centrifuge (Model CL), a Buchi Rotovapor R evaporator, and an ultrasonic cleaner (Cole-Parmer, Model 8845-4) were used.

Column. A Waters Associates 30 cm × 4 mm i.d. μ Bondapak CN column (cyanopropylsilane chemically bonded to a 10 μm porous silica support) was used.

Reagents. The eluting systems were methyl alcohol–water 35:65 or 40:60, v/v. The water was distilled and deionized. The methyl alcohol was Mallinckrodt Spectr AR 3018. Also, a 70:30 isopropyl alcohol–hexane or heptane (Burdick and Jackson) system was used. The solvent systems were degassed with the ultrasonic cleaner.

Sample Preparation. Processed single-strength orange or grapefruit juice or reconstituted concentrate was centrifuged for 5 min at a RCF of 2050 (top speed). A 10-g sample of the supernatant was extracted with 3 × 10 mL of chloroform. The combined chloroform layers were evaporated to dryness in a 50-mL round-bottomed flask, using the rotovapor. The residue was redissolved in 2000 μL of methyl alcohol with the aid of the ultrasonic cleaner.

High-Pressure Liquid Chromatographic Resolution and Quantitation of Limonin. A 20-μL aliquot of the above methyl alcohol solution was injected onto the col-

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umn. The flow rate was 1.5 mL/min. Detection was accomplished at 210 nm and 0.04 aufs. Integration was conducted at an attenuation of 1.0, peak width setting of 72, and slope sensitivity of 300. The recorder chart speed was 15 cm/h.

The quantity of limonin in unknown samples was determined from a linear regression equation ($r = 0.992$). This equation was obtained from six standard samples of limonin over the range of 0.2 to 1.2 μg. These samples were eluted and detected under the above conditions.

Percent Recovery and Precision. The reliability of the procedure was determined by a series of recovery experiments in which a base sample of grapefruit juice was fortified with known amounts of limonin. The native limonin in the base sample was previously determined by this liquid chromatographic procedure. Individual samples were fortified with sufficient limonin to provide a concentration of 2 to 8 ppm of limonin in 1-ppm increments.

The precision of the method was determined by analyzing three different grapefruit juice samples, one each from fruit collected during the months of October, November, and December of 1976. Each of the three samples were divided into three 10-g fractions. The resulting nine samples were each carried through the above sample preparation procedure. Five 20-μL injections were made from each of the nine samples. Peak areas were determined using the above conditions. The amount of limonin (in ppm) corresponding to the peak areas was calculated from the above linear regression equation.

RESULTS AND DISCUSSION

Previous work (Fisher, 1975) indicated that detection by ultraviolet (UV) absorption was possible but impractical. However, subsequent study showed that a reverse-phase column such as a C-18 (octadecyltrichlorosilane) or, as in this method, a CN column could be used to analyze for limonin in citrus juice. A reverse-phase column allows the use of a methyl alcohol–water solvent system having a low UV cut off. The maximum optical absorbance of the methyl alcohol was 0.70 at 210 nm. A 20:80 acetonitrile–water system (0.3 maximum absorbance at 210 nm for acetonitrile) will also resolve limonin on a CN column. However, the expense of quality acetonitrile rendered it unattractive for prolonged routine use. The isopropyl alcohol–hexane or heptane system will be discussed elsewhere (Rouseff and Fisher, 1978). These conditions permit the resolution of limonin and detection at 210 nm. Limonin has a maximum absorbance at 207 nm; however, because of solvent absorbance we chose 210 nm.

Depending upon the polarity of the solvents used, one obtains normal or reverse-phase separations with a CN column.

In this system, a 10 μ CN column separated limonin in citrus juice better than a 10 μ C-18 column. While both the C-18 and CN columns gradually lose overall resolving power, the CN column lost resolution faster.

The solubility of silica in water is not serious until a pH of 8 or above. The methyl alcohol–water employed in this work had a pH of 4.5. Both the C-18 and CN columns were in continuous contact with the methyl alcohol–water system for approximately 2 months with about the same solvent volume passing through each column. Therefore, it does not seem likely that dissolution of the silica support followed by collapse of the cyanopropylsilane coating would explain the preferential loss of resolution by the CN column.

The eluting system was placed in an ultrasonic bath each day prior to pumping through the column. This should

Table I. Precision Showing Peak Areas and (ppm Limonin)

Sample	Peak areas[a] (ppm Limonin)[b]								
	October			November			December		
	A	B	C	A	B	C	A	B	C
	24174 (7.2)	24103 (7.2)	24332 (7.3)	23153 (6.9)	22502 (6.8)	20694 (6.2)	25678 (7.7)	25792 (7.7)	25264 (7.5)
	24657 (7.4)	23430 (7.0)	23487 (7.0)	23258 (7.0)	21704 (6.5)	22046 (6.6)	26455 (7.9)	26078 (7.8)	25186 (7.5)
	24422 (7.3)	23566 (7.1)	24753 (7.4)	22863 (6.9)	20471 (6.2)	20919 (6.3)	25174 (7.5)	26541 (7.9)	25054 (7.5)
	24323 (7.3)	24565 (7.3)	23973 (7.2)	22872 (6.9)	21389 (6.4)	21725 (6.5)	25818 (7.7)	26139 (7.8)	24537 (7.3)
	24272 (7.3)	24360 (7.3)	23842 (7.1)	23265 (7.0)	21228 (6.4)	20929 (6.3)	26646 (7.9)	26336 (7.8)	25332 (7.6)
Mean	24370 (7.3)	24004 (7.2)	24077 (7.2)	23082 (6.9)	21459 (6.5)	21263 (6.4)	25954 (7.7)	26177 (7.8)	25075 (7.5)
Standard deviation	184 (0.07)	493 (0.13)	484 (0.16)	201 (0.05)	739 (0.22)	587 (0.16)	599 (0.17)	282 (0.07)	318 (0.11)
Standard error of mean	82 (0.03)	221 (0.06)	216 (0.07)	90 (0.02)	330 (0.10)	263 (0.07)	268 (0.07)	126 (0.03)	142 (0.05)

[a] Integrator counts from 20-μL injections. [b] Calculated from the linear regression equation.

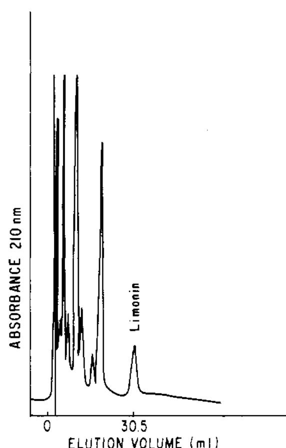

Figure 1. This chromatogram represents grapefruit juice containing 10 ppm of limonin. For experimental details, see text.

help remove dissolved oxygen, which may contribute to the deterioration of an active coating such as cyanopropylsilane.

Attempts to regenerate the CN column by washing with acetonitrile, Me_2SO or chloroform failed to return the column to its original resolving ability. The first sign of CN column deterioration was a lowering of limonin retention time. This loss of resolution causes some confusion in the integrator, because of changes in peak shape and bunching, which results in erratic peak areas. However, the peak heights appeared to be less influenced.

Several procedures were employed to clean the column of retained or late eluting material which may eventually elute at a retention volume of interest, thus, giving erroneous and erratic peak areas. If time is not a serious factor, you can wait until the "last" compound has eluted (about 45 min). The injection of 20 to 100 μL of acetonitrile onto the column and flushing with the eluting system at the established or slower flow rate will usually clean the column. In this procedure, we cleaned the column after the third injection of citrus juice. The number of injections prior to cleaning will vary with the samples.

It is also possible to calculate the point of injection for a sample that will allow the interested peak of that sample to occur at a retention volume isolated from the late eluting peaks of a previous run. A 5 μ C-18 column gave good resolution but required excessive pressure and a low flow rate with increased analysis time.

Particulate material, which aids in the formation of annoying emulsions, were removed by centrifuging the juice.

Since all processed citrus juice is heated and at the pH of grapefruit juice (3.5), the limonoate A-ring lactone, a nonbitter limonin precursor present in grapefruit juice (Maier and Grant, 1970, and references therein), should be converted to the bitter limonin. Therefore, the processed juice samples used in this analysis will contain the total juice limonin. An investigation of the pellet resulting from the centrifugation showed that limonin was not lost in the pellet. Maier and Grant (1970) and Brewster et al. (1976) also found that this was not a problem. The processed juice may also be filtered through glass wool or Celite.

The peak labeled limonin in Figure 1 was identified as previously reported (Fisher, 1975). Isocratic elution required 20.3 min (30.5 mL).

The use of a UV detector for limonin is preferred over the refractometer. While temperature control is a definite consideration with UV detection, the extreme temperature control required for RI is eliminated.

In one experiment, which was typical of many, the temperature in the laboratory gradually increased over a range of 5 °C (21–26 °C) during the course of an 8-h period. The retention times gradually decreased over a range of 2 min (1210 to 1090 s). The column pressure gradually dropped from about 1800 to 1500 psi during the same period. Stabilization of retention times and column pressure was improved by insulating the column and maintaining a more constant laboratory temperature.

The UV detector is less influenced by flow and pressure changes than the RI detector.

The amount of native limonin in the base sample was found to be 1.0 ppm. The recoveries of limonin from the seven fortified base samples were all within ±15% of the total limonin with a mean of 8.8%.

Table I shows the precision of the method. The precision is ±14.1% at the 95% confidence level.

Under the conditions of this procedure, a sample with a concentration of 1.0 ppm limonin gave a S/N of 2/1.

A standard limonin sample was injected daily to assure validity of the regression equation.

The number of theoretical plates for the column, using limonin as the reference peak, was 1600 equivalent to a plate height of 0.19 mm. The column capacity factor k' was 15.6.

The average time required for 12 complete analyses was 8.0 h. Either orange or grapefruit juice can be used in this procedure. The amount of limonin in Florida orange juice is considerably lower than grapefruit juice.

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LITERATURE CITED

Brewster, L. C., Hasegawa, S., Maier, V. P., *J. Agric. Food Chem.* **24**, 21 (1976).
Fisher, J. F., *J. Agric. Food Chem.* **23**, 1199 (1975).
Maier, V. P., Grant, E. R., *J. Agric. Food Chem.* **18**, 250 (1970).
Rouseff, R. L., Fisher, J. F., in press (1978).

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