HPLC Profile of Amino Acids in Fruit Juices as Their (1-Fluoro-2,4-dinitrophenyl)-5-L-alanine Amide Derivatives

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Many lemon, orange, apple, and white grape juice samples, and a few samples of several other kinds of juices, were profiled after being adjusted to pH 8.7 ± 0.2, cleaned up via a Waters C-18 Sep Pak, and reacted with FDAA reagent for 1 h at 55 °C. The resulting diastereomeric amino acid derivatives were then chromatographed in a gradient HPLC run from 10 to 40% acetonitrile containing triethanolamine phosphate buffer, pH 3.0. The addition of D-proline or glycine to a juice was easily spotted. Data are presented and discussed for asparagine, serine, aspartic acid, arginine, glutamic acid, proline, and γ-aminobutyric acid (GABA). Despite relatively high variations in the data for each type of fruit juice, the patterns are distinct enough to differentiate juices of biologically dissimiliar fruits from each other and thus can be used to detect adulteration of orange with apple within 30%. On the other hand, lemon and grapefruit cannot be distinguished from each other. Tentative identification and semiquantitation of the major amino acids are given.

Marfey's reagent [(1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide] was reported to form diastereomeric derivatives of optically active amino acids that could be separated by gradient HPLC. L-Amino acids always eluted before D isomers (Marfey, 1984). The value of separating L- and D-amino acids in a fruit juice "aminogram" was pointed out by Sandra et al. (1984), who used a chiral GC phase to separate fluorinated esters of the amino acids. The preparation was complex but showed that the addition of racemic amino acids to falsify a juice could be detected. It is validly assumed that economic factors prevent the addition of optically pure amino acids for the purpose of concealing adulteration.

Sandra's discussion spurred us to begin a search for simplified chromatographic method that could spot the presence of D-amino acids in fruit juices and at the same time provide the valuable amino acid profile needed to serve as the basis of authenticity. William's review (1982) indicated stereospecific technology was lacking in the food area, probably due to most methods being based on ion exchange, which has no chirality, or precolumn derivatization for HPLC with nonstereospecific reagents. Many other authors used these methods to accumulate valuable data on the occurrence of various amino acids in fruit juices. Depending on the object of the work, conclusions vary from that of Bieling and Hofsommer (1982) who said that no mean or limiting values can be set for apple juice to that of Ooghe (1983) who considers it necessary to set standard values for amino acid content in fruit juices. He reports that many European countries have set amino acid specifications for individual types of juices. Beiling et al. (1985) provides an excellent review of many analytical criteria used to regulate and define fruit juices by the EEC and lists their amino acid specifications for orange and grapefruit.

This paper will present a new and interesting way to profile amino acids in fruit juices that can signal the addition of D isomers, as would occur if an adulterer added racemic amino acids to mask an aminogram. We feel the other advantages of this method are the ease of sample preparation, quantitation of primary and secondary amino acids, stability of derivatives, and elimination of postcolumn derivatization apparatus. Disadvantages are a relatively long run time (105 min) and the occasional coelution of some peaks. Those peaks are summed when they do not coelute for the purpose of keeping the data constant.

MATERIALS AND METHODS

Most samples were concentrates submitted by various manufacturers representing lots currently for sale, and others were reputable retail products. It is possible that a few adulterated samples were included in this database, but since it is not known which these might be, all data are included. All concentrates were diluted to standard Brix or acid values before analysis.

Apparatus. A gradient HPLC, composed of two Waters M-45 pumps, a Waters 720 system controller, a Waters U6K injector, a Kratos Spectroflow 757 variable-wavelength UV detector set at 340 nm, and a HP 3390A recording integrator, was used in conjunction with a Rainin 5-μm, spherical, fully endcapped C-18 column (4.6 × 250 mm) to effect the separation.

Reagents. HPLC-grade acetonitrile and water were blended, accordingly, to prepare 1 L each of 10 and 40% acetonitrile mobile phases. Before final adjustment of volume, 3.36 g of reagent-grade triethanolamine and enough orthophosphoric acid to buffer the pH to 3.0 ± 0.1 were added to each. The mobile phases were then adjusted to final volume with water. FDAA reagent was obtained from Pierce Chemical Co., Rockford, IL, and diluted to 0.1% in reagent acetone. L-Amino acid oxidase and optically pure amino acids were obtained from Sigma Chemical Co., St. Louis, MO, including D-isoleucine, which was diluted to 0.05% in water for use as an internal standard.

Procedure. The method was derived from a Pierce Chemical Co. technical bulletin (1985) illustrating derivatization and liquid chromatography of pure D- and L-amino acids. The following procedure was developed from it to optimize separation and quantitation of the individual amino acids in orange juice, which has the most complex chromatogram studied.

The single-strength juice was first diluted to 10%, and a 25-mL aliquot was adjusted to pH 8.7 ± 0.2 with first 20% and then 1% sodium hydroxide. The sample was then immediately diluted to 50 mL and passed through a prewetted Waters C-18 Sep Pak, discarding the first 7 mL of effluent and then collecting the next 3 mL for analysis. A 400-μL portion of the cleaned up sample, 5 μL of the D-isoleucine internal standard, 200 μL of Marfey's reagent solution, and 40 μL of 2 N sodium bicarbonate

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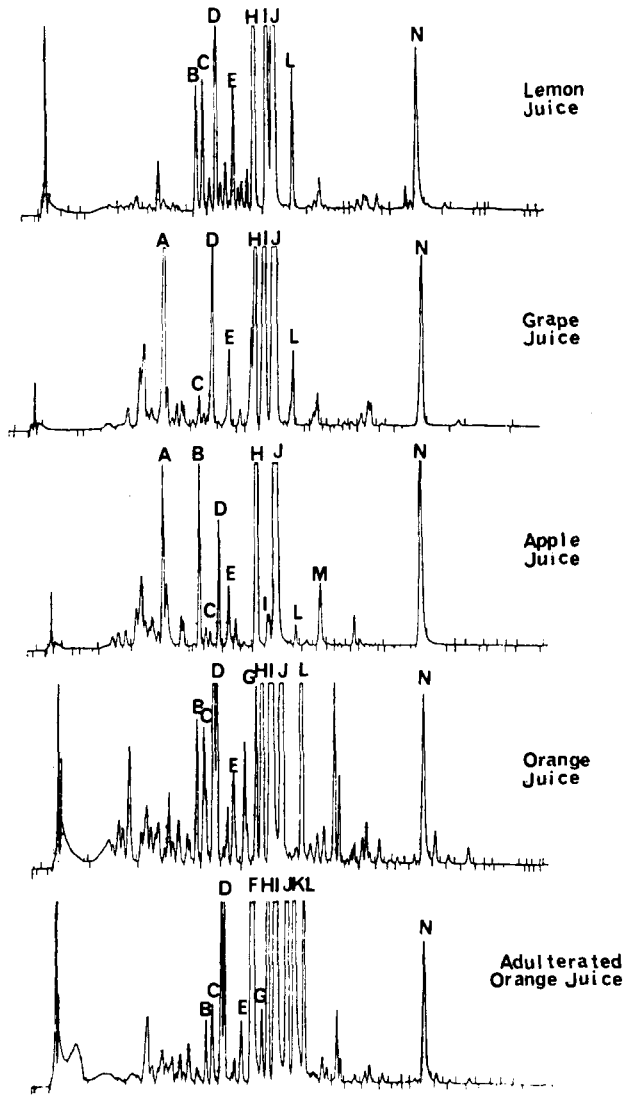

Figure 1. Key: A, unknown; B, asparagine; C, serine; D, aspartic acid plus arginine; E, glutamic acid; F, glycine; G, alanine; H, Marfey hydroxide; I, proline; J, Marfey fluoride; K, D-proline; L, GABA, M, valine, N, D-isoleucine. Run time, 105 min.

were then mixed in a 4-mL glass vial with a Teflon-lined screw cap and heated to 55 °C for 1 h. At the end of the derivatization, 30 μL of 2 N HCl was added to each vial. The derivatives are stable for at least 1 week.

A 100-μL portion of the derivative was injected into the HPLC at a constant flow of 0.7 mL/min. After 5 min of isocratic flow at 10% acetonitrile, a linear gradient was begun that reached 40% acetonitrile at 65 min. This solvent was held constant to 85 min, and then a reverse gradient was started ending at 95 min into the run. After 10 min more, the HPLC was equilibrated for the next sample.

RESULTS AND DISCUSSION

A typical chromatogram of each of the major types of juices studied is given in Figure 1. The lemon juice chromatogram did not show the separation of alanine from HO-DAA although in orange juice a clear separation was obtained. This example serves to illustrate that the resolution of alanine is not dependable. Column aging effects, temperature, and slight differences in batches of mobile phase are believed to play a role, in particular in the separation of aspartic acid and arginine. Other peaks, such as serine, in orange juice sometimes have apparent shoulders, but they do not grossly affect the data derived from such chromatograms. The other important amino acid peaks separated well despite normal chromatographic variations mentioned.

One purpose of this study was to develop a method that could easily spot the addition of racemic amino acids or glycine to raise the formol titre of adulterated juices. It is common practice in many laboratories concerned with the authenticity of juices to determine the proline values and the formol index, which is an analytical titration determining the total amino acid content. An orange juice was diluted to 50% strength and enough D,L-proline added to conceal the detection of adulteration by a colorimetric assay. Then enough glycine was added to adjust the formol titre back to within an acceptable range. Since the other adjustments an adulterator would do to conceal the act, such as sugar and acid adjustments, will not affect the results of the method, they were not done. A visual inspection of the chromatogram in Figure 1 is all that is needed to determine that this sample has been tampered with. Such adulteration would have as easily been spotted in lemon or grape juice, and in no juice tested has any naturally occurring amino acid ever been found that would be mistaken for D-proline or glycine in the amounts an adulterator would add.

The basis of identification of the labeled peaks in the chromatograms is by retention index comparison to known amino acid standards. Thus, it was determined that the important amino acids in the four juice types are asparagine, serine, arginine, aspartic acid, glutamic acid, alanine, proline, and GABA in that elution order. Ooghe and Waele (1982) used the concentration of these same eight amino acids as a criteria to confirm the authenticity of orange, apple, grape, and grapefruit juices. Therefore, it was decided to quantitate the peaks in an effort to develop a similar database, which was accomplished by comparing the peak areas to known standards carried through the same procedure. All data were corrected for the internal standard response. The possible presence of non-amino acid coeluting interferences was eliminated by treatment of samples of the juices with L-amino acid oxidase and observing that, after destruction of the amino acids, the resulting chromatograms are free of peaks.

The data collected during this study are given in Table I and the values compared, with the exception of grape, to range values found in the literature. Unfortunately, no data showing ranges for grape could be obtained for comparison, so the averages reported by Peynaud and Ribereau-Gayon (1971) were used. For the most part, the Marfey data compare well with the ranges found in the literature. Considering seasonal and cultivar variations, the data are reasonable. In addition, since most samples were from concentrates, losses of amino acids due to precipitation and participation in Mallaird-type reactions are possible.

Interpreting chromatograms is straightforward. A sample of juice is expected to fit within the established range. If it does not, sometimes key amino acids can be examined to determine what other juice was used for adulteration. For example, if lemon juice has a typical asparagine value, but a low proline value, it was probably adulterated with apple juice. If the asparagine is low, but the proline is typical, it was probably adulterated with grape. If everything is low, sugar acid water could have been used. Although not shown, glycine data could have been accumulated. It is low for every juice encountered in this study, but protein hydrolysates have a relative abundance of glycine, and thus high glycine could suggest adulteration with them. Other amino acids are also useful indicators in the chromatogram. Aspartic acid plus arginine would

Table I[a]

juice type	no. of samples	Asn	Ser	Asp + Arg[c]	Glu	Pro	GABA
orange							
Marfey value	25	1.22–4.05	0.83–2.01	2.32–7.31	0.27–0.82	8.46–27.8	1.35–4.08
lit.[d]		1.21–3.79	0.86–2.09	2.98–7.22	0.48–1.22	5.83–20.8	1.36–4.85
lemon							
Marfey value	15	1.54–6.62	1.10–4.20	0.82–7.07	0.39–2.09	2.16–8.82	0.55–1.89
lit.[d]		1.21–2.12	1.71–4.48	2.56–4.89[b]	1.09–2.11	2.52–7.30	0.68–1.55
apple							
Marfey value	16	1.85–4.30	<0.10–0.26	0.30–1.23	0.15–0.42	0.08–0.32	<0.05–0.09
lit.[e]		2.08–10.4	0.10–0.52	0.58–1.47[b]	0.18–1.63	0.01–0.09	0.02–0.19
grape							
Marfey value	13	tr	0.29–0.87	2.46–7.75	0.08–0.55	2.60–7.12	0.37–0.73
lit.[f]			0.66	1.88[g]	1.18	2.31	

[a] All data are millimolar. [b] Lemon and apple contain essentially no arginine. [c] Literature values converted to millimolar and summed. [d] Petrus and Vandercook (1980). [e] Sproer (1985). [f] Peynaud and Ribereau-Gayon (1971). [g] Grape contains little aspartic acid.

Table II.[d] **Adulteration Experiment**

sample[a]	result 1,[b] %	result 2,[c] %	Asn	Ser	Asp + Arg	Glu	Pro	GABA
10% orange	22	9	1.55	0.30	1.56	0.51	3.36	0.60
20% orange	40	22	1.49	0.54	2.31	0.55	6.19	1.08
40% orange	60	38	1.45	0.71	3.31	0.64	10.5	1.75
60% orange	83	63	1.26	0.94	4.69	0.71	16.4	2.84
80% orange	107	84	1.19	1.16	5.70	0.81	22.1	3.95
orange used			1.15	1.36	6.90	0.88	26.5	4.87
apple used			1.61	0.31	0.99	0.47	0.62	0.15
av orange of database			0.77	0.84	4.25	0.94	28.9	3.56
av apple of database			0.74	0.09	0.53	0.33	0.33	0.96

[a] Lists actual percent orange added by colleague to unknown blend. [b] Result 1 is calculated from average values of database. [c] Result 2 is calculated from actual analytical results of juices used. [d] All amino acid data are area counts multiplied by 10^7 as reported by the HP 3390A.

confirm the addition of apple juice to lemon juice, and low serine would, at least, be a probable indicator that grape or apple had been used.

The same line of logic would be applied to the study of orange juice because its' amino acid pattern is similar to that of lemon, except that the range tends to run higher. This fact, unfortunately, makes adulteration of orange with lemon hard to detect, but no adulteration scheme is universal. Carotenoid and bioflavanoid profiles would probably be more helpful. This method is best at detecting adulteration of citrus juices with the cheap juices, such as apple and grape, and protein hydrosylates or sugar acid water.

A study was undertaken to determine how well the method predicts adulteration once the component juices in a sample have been identified. Thus, a colleague submitted blind blends of apple and orange juice for testing, and pure samples of the apple and orange juice used, so that specific reference data could be obtained. After analysis, all the peak areas of the amino acids of primary interest were corrected for deviations of the internal standard response (D-isoleucine) from 1.70×10^7 area counts using direct proportioning. Alanine coeluted with Marfey hydroxide and was not determined. The peak area responses of all the other apple and orange juice data accumulated to date were also corrected for their internal standard responses and averaged to obtain general reference data. Then, from the data of each individual amino acid, the percent orange in each unknown blend was calculated, based on that amino acid response, by the simultaneous equations

$$Ax + By = C \text{ and } X = 100\% - Y$$

where x = percent apple, y = percent orange, A = reference data for the amino acid in apple, B = reference data for the amino acid in orange, and C = the area response of the amino acid in the sample. The y values arrived at using the data of the individual major amino acids are then averaged to obtain an overall y value reported in Table II.

When the general reference data are used in the calculation, the results (see result 1, Table II) never deviated more than 30% from the actual values of the blends made by our colleague. When the specific reference data were used, the data do not vary more than 5% (see result 2, Table II). This latter result could not occur if the amino acid area responses were generally deviating more than 5% from linearity within the ranges of apple and orange data, which indicates this technique holds much promise. Potential interferences were present.

Table II also includes area responses corrected for the internal standard response. This is to illustrate the strengths and weaknesses of the method. As it happened, when compared to the general reference data, the specific reference data had higher than usual values for four of the amino acids in orange and five in apple. This explains the relatively high error when the general reference data are used to calculate the results. Fortunately, the orange used was a little lower in proline and glutamic acid, which partially neutralized the effect. It should be pointed out that natural variability of this magnitude is by no means limited to amino acid data in natural products. Many adulteration indicators used to establish the authenticity of juices vary this much. Therefore, many adulteration indicators need be examined prior to concluding whether or not a juice is pure, and we feel that this method is only one of the determinations that should be done, unless of course a large amount of a D-amino acid is found.

It should further be pointed out that it was not necessary to identify the peaks before using them to calculate the results in this experiment, corresponding retention indices would have done as well, nor was it necessary to calculate the concentrations of the amino acids, the peak areas were used. This reaffirms that the method is useful as a way to derive patterns that can be used to recognize juice samples as pure, or impure.

Due to other priorities in the workplace, this research cannot be continued, and not enough data have been collected to allow proper computer-assisted processing of

Table III[a]

juice type	no. of samples	Asn	Ser	Asp + Arg	Glu	Pro	GABA
pear	7	4.16–11.8	0.15–0.56	0.45–1.20	0.33–0.63	0.42–1.54	0.05–0.21
grapefruit	4	3.98–5.28	1.62–1.99	5.94–6.87	0.53–0.93	4.35–8.93	2.28–2.58
cherry	19	1.96–23.8	tr	0.11–0.68	tr–0.27	1.29–13.8	0.36–1.45
plum	1	15.4	tr	0.32	0.28	5.27	0.56
apricot	1	18.4	tr	0.58	0.38	1.51	0.79
peach	1	12.8	tr	0.41	0.47	0.47	0.26
nectarine	1	26.3	1.43	1.12	0.98	0.83	0.16
strawberry	2	4.33–9.21	0.29–1.20	0.54–1.36	0.38–0.51	0.24–0.38	1.05–1.39
red currant	2	0.98–1.22	0.66–1.02	1.04–1.05	0.35–0.40	0.45–0.98	1.79–2.27
raspberry	1	1.68	tr	tr	0.33	0.77	0.18
cranberry	1	tr	tr	tr	0.42	0.10	0.11
blackberry	1	2.80	0.85	0.57	0.31	1.54	0.73

[a] All data are millimolar.

the database. It might, for instance, be found that some amino acids occur in a tighter range and thus should be assigned a higher dependability factor in the calculations, to provide a more reliable result. Therefore, it is impossible at this stage of research to determine specific detection limits of the various kinds of adulteration that can occur. However, we believe we could have spotted the adulteration of the 40% orange in our example because of the very low proline value, supported by the falling of most of the other amino acid values at the low end of the expected ranges. Surely, with a larger database, coupled with sophisticated statistical analysis, the detection could be improved. It is only the endeavor of this paper to evaluate the data thus far collected.

For the benefit of interested readers, limited data on various other juices are presented in Table III. As in the example of orange and lemon, amino acid profiles of other similar fruit juices do little to distinguish them from each other. Thus, the pome fruit juices, apple and pear, are similar. The stone fruit juices, cherry, plum, apricot, peach, and nectarine, generally follow the same pattern. Of the soft fruit juices, strawberry might be distinguishable from red currant by examining asparagine and GABA values, but a larger database is needed to increase the degree of certainty. Finally, grapefruit data closely parallel that of lemon and orange.

The main objective of this laboratory project was to devise a method that could detect the adulteration of lemon and orange with the cheap juices such as apple, pear, and grape. Fortunately, the juices most often used for adulteration are grossly deficient in the amino acids targeted in this study relative to the citrus juices. The same analytical methodology appears to be able to accumulate data on other amino acids that might be better indicators for other adulteration problems. For example, high valine can help spot apple in pear juice. High threonine and glutamine values may be good indicators for cherry and the soft fruits. Finally, the large peak A shown in Figure 1 seems to be present in high concentrations in apple, grape, cherry, cranberry, and plum; in medium concentrations in pear, grapefruit, red currant, raspberry, strawberry, peach, nectarine, blackberry, and apricot; and in low concentrations in lemon and orange. The peak is not hydroxyproline, pipecolic acid, nor thought to be any of the other essential amino acids.

In conclusion, amino acid profiles are like any other adulteration indicators ever experienced in this laboratory. They can address specific issues, particularly when dissimilar juices are blended. The method discussed in this paper is as accurate as any modern method but, in addition, is stereospecific, allowing the detection of sophisticated adulteration with racemic amino acids, even when the correct levels are added. The potential of this technique goes beyond the objective of this paper. Data could be collected on other useful amino acids, and adulteration of juices not analyzed extensively in this study might be detectable.

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Registry No. Asn, 70-47-3; Ser, 56-45-1; Asp, 56-84-8; Glu, 56-86-0; Pro, 147-85-3; GABA, 56-12-2; Gly, 56-40-6; Ala, 56-41-7; D-Pro, 344-25-2; Val, 72-18-4; D-Ile, 319-78-8.

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