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Adulteration of Apple with Pear Juice: Emphasis on Major Carbohydrates, Proline, and Arbutin

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Detection of juice-to-juice adulteration based on chemical composition studies is a common method used by government regulatory agencies and food companies. This study investigated the use of major carbohydrate (fructose, glucose and sucrose), polyol (sorbitol), proline, and phenolic profiles as indicators of pear adulteration of apple juice (PAAJ). For this work, a total of 105 authentic apple juice samples from 13 countries and 27 authentic pear juice samples from 5 countries were analyzed. Because the major carbohydrate ranges for these juices showed significant overlap their use as markers for PAAJ detection would be very limited. It was found that sorbitol and proline means for apple and pear were significantly different; however, their broad natural ranges would afford PAAJ at levels up to 30% without detection. In addition, careful selection of the pear juice used as the adulterant would further limit the usefulness of these markers for PAAJ detection. Arbutin was conclusively identified as a marker for pear juice on the basis of its presence in all 27 authentic pear samples and its absence (<0.5 μg/mL) in all 105 apple juice samples analyzed in this study. The application of the developed HPLC-PDA method for arbutin analysis to detect PAAJ at levels as low as 2% (v/v) was demonstrated. A confirmation method for the presence of arbutin in pure pear juice and apple adulterated with pear juice was introduced on the basis of the hydrolysis of arbutin to hydroquinone employing β-glucosidase, with reactant and product monitoring by HPLC-PDA.

KEYWORDS: Apple juice; pear juice; adulteration; carbohydrates; proline; arbutin; hydroquinone

INTRODUCTION

The adulteration of foods is not a new problem. In the early 1800s unscrupulous tea traders would treat used tea leaves with black lead to restore their color in order to resell this material as fresh (*1*). Currently, food adulteration takes many forms, including, but not limited to, intentional mislabeling of product species, geographical and botanical origins, and debasing. A common form of fruit juice adulteration is by juice substitution. This debasing method is dependent on similarities in the chemical composition, availability, and price of the adulterant. Pear juice is an ideal adulterant for apple juice because of their similarities in major chemical composition, which is a result of their botanical relationship (*2*, *3*).

The "matrix" approach was the first comprehensive method developed for the detection of apple juice authenticity (*4*). This method is based upon the analysis of a number of chemical and physical parameters of authentic juice and juice suspected of being adulterated. The adulterated samples may be identified if their chemical composition deviates significantly from the mean or lies outside the ranges of pure juice.

Carbohydrates are the main components in apple and pear juices and account for >95% of total soluble solids (*5*). Fructose and glucose are the two major carbohydrates in apple, with ranges of 3.20−10.5 and 0.17−4.10% (*6*), respectively, with a fructose-to-glucose ratio of >2.0 (*4*, *7*). The levels of these two monosaccharides in pear juices are 5.10−8.89% for fructose and 0.76−3.90% for glucose (*8*), with a fructose-to-glucose ratio of >2.7 (*5*). In addition to these monosaccharides, apple juice contains appreciable levels of sucrose and sorbitol (polyol) at levels ranging from 0.2 to 5.62% and from 0.16 to 1.20%, respectively (*6*). The concentrations of these two compounds in pear juices were found to be 0.54−3.70% for sucrose and 1.21−2.80% for sorbitol (*8*). Because of the wide natural variation of carbohydrates in these two juices, the detection of low levels of apple adulterated with pear juice based on carbohydrate concentrations and ratios appears to have limited applicability.

Proline (amino acid) levels have been suggested as a possible marker for the detection of pear adulteration of apple juices. Apple contains 1.2−13.8 mg/L levels of proline, and the value for pear ranges from 30.0 to 250.0 mg/L (*5*, *6*, *8*). Therefore, detection of higher levels (>13.8 mg/L) of proline in apple juice has been proposed as an indicator for pear adulteration (*9*).

Phenolic compounds are an important chemical and nutritional component in apple and pear juices (*5*, *10*, *11*). They are secondary plant metabolites and are found in all fruits (*12*). According to the literature, the type and levels of phenolics are

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characteristic for each fruit and can be used as indicators of fruit juice adulteration (*13*−*16*). For example, low levels of chlorogenic acid (<50 μg/mL) were observed in apple juice because of adulteration with beet sugar (*4*). However, the addition of pear juice to apple juice can restore chlorogenic acid to normal levels because it contains 30−70 μg/mL of this compound (*14*).

It has been reported that the presence of unique phenolic fingerprint marker(s) in juices can be used for identifying juice-to-juice adulteration (*16*). Phloridzin has been reported to be a characteristic apple juice phenolic (*4*, *16*), and arbutin and isorhamnetin-3-glucoside have been reported as unique phenolics present in pear juices (*13*, *16*−*18*). In a published 3-year study on commercial apple juice concentrates conducted by the National Food Processors Association, arbutin was shown to be present in 50% (13/26) of samples analyzed, with a mean concentration of 7.8 mg/L (at 11.5 °Brix) and a maximum of 28.6 mg/L (*19*, *20*). Alonso-Scales et al. (*21*) have reported the presence of five isorhamnetin glycosides, including isorhamnetin 3-glucoside in apple peel.

The objective of this study was to investigate the previously described authenticity methods of carbohydrate profiling, proline levels, and phenolic markers, specifically arbutin and isorhamnetin 3-glucoside, for the detection of pear adulteration of apple juice. For this work, the most comprehensive authentic pear juice database (27 samples from 5 countries) and a large authentic apple juice database (105 samples from 13 countries) was assembled and analyzed.

MATERIALS AND METHODS

Chemcial Standards. Arbutin, catechin, chlorogenic acid, *p*-coumaric acid, hydroquinone, D-fructose (fructose), D-glucose (glucose), phloridzin, and D-sucrose (sucrose) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sorbitol was obtained from Pfizer (Montreal, QC, Canada), and isorhamnetin 3-glucoside and isorhamnetin were obtained from Indofine Chemical Co. (Somerville, NJ).

Apple and Pear Juices. In total, 105 authentic commercial apple juice samples (5 cloudy) from 13 countries and 27 authentic commercial pear juice concentrates (2 cloudy) from 5 countries were analyzed in this study. All juice samples were collected by Dr. N. H. Low or obtained by him from reputable primary producers including Enzafoods (New Zealand), Fisher Sucos (Brazil), Jugos del Sur (Argentina), Motts (United States), Patagonia (Chile), Sun Rype (Canada), and Tree Top (United States). All samples were stored at −20 °C until required for analysis.

Chemical Analysis. *Total Solids.* °Brix values were determined using a refractometer (Leica Inc., Buffalo, NY) at 23 ± 1 °C. All samples were analyzed in duplicate.

Determination of Proline. All juice samples were diluted to 11.5 (± 0.1) °Brix with distilled deionized water (ddH_2O; Milli-Q Water System, Millipore, Milford, MA), and a 1.0 mL aliquot of each sample was placed in a stoppered test tube (1 × 15 cm). To each tube were added 1.0 mL of formic acid (Sigma-Aldrich Canada Ltd.) and 2.0 mL of a 3% (w/v) solution of ninhydrin (Sigma-Aldrich Canada Ltd.) in ethyleneglycol monoethyl ether (Sigma-Aldrich Canada Ltd.). The resulting solution was mixed, and the test tubes were placed in a water bath maintained at 98 ± 1 °C for 15 min. The test tubes were then placed in cold water (4 ± 1 °C) to bring to room temperature (23 ± 1 °C). To this solution was added 10.0 mL of *n*-butyl acetate (Sigma-Aldrich Canada Ltd.) followed by thorough shaking in order to extract color into the organic phase. This solution was then passed through fluted filter paper (Whatman no. 41; 90 mm diameter; VWR Scientific, Mississauga, ON, Canada) containing ≈1.0 g of anhydrous sodium sulfate (BDH, Toronto, ON, Canada). The absorbance of the organic phase (filtrate) was measured at 509 nm (Spectronic 1201; Thermo Spectronic US, Madison, WI). A sample blank was prepared by using 1.0 mL of ddH_2O, 1.0 mL of formic acid, and 2.0 mL of ethylene glycol monoethyl ether, under the same reaction and extraction conditions (*5*).

A standard curve was established using proline (Aldrich Chemical Co., Inc., Milwaukee, WI) in ddH_2O at a concentration range of 5.0−50.0 μg/mL. Standard curves had correlation coefficients of ≥0.994. All samples were analyzed in quadruplicate.

Determination of Glucose, Fructose, Sorbitol, and Sucrose. Samples were analyzed by high-performance liquid chromatography (HPLC) with refractive index (RI) detection (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Samples were initially diluted with ddH_2O to 11.5 ± 0.1 °Brix and then further diluted 1:1 (v/v) with ddH_2O and filtered (13 mm diameter, 0.2 μm pore size) prior to HPLC-RI analysis. Carbohydrate separation was accomplished on a 250 × 4 mm i.d. Capcell-Pak-NH_2 column (5 μm; Phenomenex, Torrance, CA) with an isocratic mobile phase of acetonitrile/water (80:20, v/v) maintained at a flow rate of 1.0 mL/min. Sample injection volume was 20 μL, and analysis was carried out at ambient temperature. Data acquisition and processing were carried out using HP Chem Station rev. A.06.0X software (Agilent Technologies Canada Inc.). Standard curves for fructose, glucose, sorbitol, and sucrose were prepared in ddH_2O at concentrations ranging from 0.5 to 5.0 g/100 mL, from 2.0 to 10.0 g/100 mL, from 0.1 to 5.0 g/100 mL, and from 0.2 to 5.0 g/100 mL, respectively, and were filtered prior to HPLC-RI analysis. All samples and standards were analyzed in triplicate.

Determination of Arbutin and Hydroquinone. The arbutin (4-hydroxyphenyl-β-D-glucopyranoside) and hydroquinone (1,4-dihydroxybenzene) contents of juice samples were determined by HPLC with photodiode array detection (PDA) (Agilent Technologies Canada Inc.). The separation of these compounds was accomplished on a 250 × 4.6 mm i.d. end-capped C_{18} column (5 μm, 100 Å; Prodigy, Phenomenex, Torrance, CA) in series with a 4 × 2 mm guard column of the same stationary phase at ambient temperature. This chromatographic method affords the separation of a wide spectrum of apple and pear polyphenols, including, but not limited to, arbutin, chlorogenic acid, hydroquinone, isorhamentin 3-glucoside, isorhamnetin, phloridzin, phloretin, and quercitin. Arbutin and hydroquinone detection was achieved by employing PDA at wavelengths of 280 and 290 nm, respectively. The mobile phase included ddH_2O containing 50 mM phosphate (KH_2PO_4) at pH 3.0 (adjusted with 1 M phosphoric acid; solvent A) and 70% acetonitrile/30% A (solvent B) in the following gradient system: initial, 100% A for 3 min, followed by a linear gradient to 4% B at 6 min, followed by a linear gradient to 10% B at 25 min, followed by a linear gradient to 15% B at 30 min, followed by a linear gradient to 80% B at 31 min, a hold at 80% B for 10 min, followed by a linear gradient to 100% B at 42 min, and a hold at 100% B for 13 min. The mobile phase flow rate was 0.8 mL/min, and the injection volume was 20 μL. All samples were diluted to 11.5 (±0.1) °Brix and further diluted 1:1 with ddH_2O and then filtered prior to HPLC-PDA analysis. All samples and standards were analyzed in triplicate.

If sample analysis of only arbutin and hydroquinone was required, 100% B could be added at 25 min and held for 15 min.

Standard curves for arbutin were prepared by the addition of this compound to apple juice samples AJ27, AJ69, and AJ16 at levels ranging from 0.5 to 110.0 μg/mL. Standard curves for hydroquinone were prepared in ddH_2O at concentrations ranging from 0.1 to 50.0 μg/mL. Standard curves for arbutin and hydroquinone had correlation coefficients of ≥0.995.

Preparation of Apple Juice Intentionally Adulterated with Pear Juice for Arbutin/Hydroquinone Content. Three apple juice samples were chosen for adulteration on the basis of their hydroquinone content, representing low (<2.0 μg/mL; AJ27), medium (2.0−5.0 μg/mL; AJ69), and high (>5.0 μg/mL; AJ16). A pear juice sample (PJ17) containing the lowest arbutin (40.5 μg/mL) level observed in the 27 authentic samples analyzed was used to adulterate the aforementioned apple juice samples. All samples were diluted to 11.5 ± 0.1 °Brix with ddH_2O, and adulteration was accomplished at levels of 2.0, 10.0, and 30.0% (v/v) using PJ17.

Preparation of Apple Juice Intentionally Adulterated with Pear Juice for Sorbitol Content. Three apple juice samples, AJ27, AJ69, and AJ24, were selected for adulteration on the basis of their sorbitol content, representing low (0.26%), medium (0.56%), and high con-

Table 1. Mean, Range, and Standard Deviation (SD) Results for Glucose, Fructose, Sucrose, Sorbitol, Proline, and Arbutin Concentrations in 105 Apple and 27 Pear Juice Samples

		glucose (g/100 mL)	fructose (g/100 mL)	sucrose (g/100 mL)	sorbitol (g/100 mL)	proline (μg/mL)	arbutin (μg/mL)
apple juice	mean	2.41	5.96	1.63	0.42	6.3	ND[a]
	SD	0.46	0.52	0.65	0.12	1.6	
	range	1.16–3.68	4.65–7.40	0.22–3.50	0.20–0.84	3.4–10.9	
pear juice	mean	1.68	5.64	0.77	2.23	16.5	72.4
	SD	0.20	0.50	0.30	0.28	8.5	23.9
	range	1.17–2.00	4.80–6.78	0.17–1.48	1.54–2.84	8.1–50.8	40.5–151.1

[a] Not detected (detection limit of 0.5 μg/mL; 3 × s/n).

centrations (0.84%). Pear juice sample PJ17 was used as the adulterant in this work because it had a sorbitol concentration that matched the mean (2.23%) for the 27 authentic pure pear samples analyzed. All samples were diluted to 11.5 ± 0.1 °Brix with ddH_2O, and adulteration was accomplished at levels of 2.0, 10.0, and 30.0% (v/v) using PJ17.

Arbutin Hydrolysis with β-Glucosidase. Three apple juice samples (AJ16, AJ27, and AJ69) were individually diluted to 11.5 ± 0.1 °Brix with ddH_2O, and arbutin was added at concentrations of 2.0, 10.0, and 30.0 μg/mL. The resulting solutions were placed in 5 mL volumetric flasks, and β-glucosidase was added at levels corresponding to 85, 140, and 170 units for 2.0, 10.0, and 30.0 μg/mL arbutin, respectively. The resulting solutions were stoppered and stirred at ambient temperature for 16 h. Enzymatic hydrolysis was quenched by heating the reaction mixture in boiling water (98 ± 2 °C) for 2 min. Negative controls consisting of the same juices without enzyme addition followed by immediate heating in boiling water were also run. The accuracy and reproducibility of arbutin hydrolysis by β-glucosidase for positive controls were determined using 10 replicates for each concentration. All samples were filtered, and each replicate was analyzed in duplicate by HPLC-PDA.

The aforementioned apple juice samples were intentionally adulterated with pear juice sample PJ17 at levels of 2.0, 10.0, and 30.0% (v/v). The resulting solutions were placed in 5 mL volumetric flasks, and β-glucosidase addition levels, hydrolysis conditions, and sample workup were identical to those previously outlined. All experiments and their analysis were performed in triplicate.

Extraction, Analysis, and Quantitation of Isorhamnetin 3-Glucoside (I3G) and Isorhamnetin in Selected Apple and Pear Juice Samples. Two apple (AJ23 and AJ29) and two pear juice (PJ13 and PJ14) samples were individually diluted to 11.5 ± 0.1 °Brix with ddH_2O. A 50 mL aliquot of each sample was adjusted to pH 1.5 (with 8 M phosphoric acid) and extracted with 2 × 50 mL of ethyl acetate. The combined extracts were evaporated to dryness using a rotary evaporator at 35 ± 2 °C, and the residue was dissolved in 1.5 mL of 95% ethanol and filtered (*17*).

Pear juice samples (PJ13 and PJ14) were individually spiked with 5.0 ± 0.1 μg/mL of I3G and isorhamnetin. Extraction of these compounds was afforded by employing the aforementioned protocol.

Storage stability studies were performed using a 200 ± 1 μg/mL solution of I3G in ethanol and a pear juice sample (PJ13) at 11.5 ± 0.1 °Brix intentionally spiked with 5.0 ± 0.1 μg/mL I3G. The 200 μg/mL sample was stored at room temperature, and the spiked pear juice sample was stored at 4 ± 1 °C. Sample aliquots were analyzed in duplicate at 1 week intervals for a 10 week period.

All samples were analyzed by HPLC-PDA (see Determination of Arbutin and Hydroquinone) with the aforementioned mobile phases with I3G and isorhamnetin detection at 354 nm. The following gradient system was employed for compound separation: initial, 100% A for 3 min, followed by a linear gradient to 4% B at 6 min, followed by a linear gradient to 10% B at 15 min, followed by a linear gradient to 15% B at 25 min, followed by a linear gradient to 20% B at 35 min, followed by a linear gradient to 23% B at 50 min, followed by a linear gradient to 25% B at 60 min, followed by a linear gradient to 30% B at 66 min, followed by a linear gradient to 50% B at 80 min, followed by a linear gradient to 80% B at 83 min, followed by a linear gradient to 100% B at 85 min. The mobile phase flow rate was 1.0 mL/min, and the injection volume was 20 μL. All samples were diluted to 11.5 (± 0.1) °Brix and further diluted 1:1 with ddH_2O and filtered prior to HPLC-PDA analysis. All samples were prepared and analyzed in triplicate.

RESULTS AND DISCUSSION

Apple and pear are closely related botanically (*22*) and are grown in many parts of the world under diverse agro−climate conditions, and these two juices have many qualitative similarities with respect to their chemical composition (*3*−*5*, *8*). Therefore, apple and pear juices are vulnerable to juice-to-juice addition when this form of adulteration does not substantially change the major chemical composition of the resulting juice.

Analysis and Quantitation of Carbohydrates and Sorbitol. Fructose, glucose, sucrose, and sorbitol were the major carbohydrates and polyol identified in all apple and pear juice samples analyzed in this study, and their concentrations were determined by HPLC-RI. The mean, range, and standard deviation results for 105 apple and 27 pear juice samples are shown in **Table 1**.

Published levels of the major carbohydrates, fructose (F), glucose (G) and sucrose (S), in apple juice ranged from 3.20 to 10.50%, from 0.17 to 4.10%, and from 0.20 to 5.60% (g/100 mL), respectively (*4*, *6*, *7*). The fructose, glucose, and sucrose levels of the 105 apple juice samples analyzed in this study ranged from 4.65 to 7.40%, from 1.16 to 3.68%, and from 0.22 to 3.50% (g/100 mL), respectively (**Table 1**). The fructose, glucose, and sucrose levels of all apple juice samples in the current study were within the ranges reported in the literature for these carbohydrates. The fructose and glucose mean values were similar to those reported (adjusted to 11.5 °Brix) by Mattick and Moyer (*7*) of 5.23 and 1.87% (g/100 mL), respectively. However, the mean sucrose level for apple juice reported (adjusted to 11.5 °Brix) by these authors was 2.42 (± 1.01)%, and in this work the mean sucrose level was much lower at 1.63 (± 0.65)%. This mean value was in agreement with other literature values (*4*, *6*) and that reported by Ryan (*23*) of 1.75%.

A fructose-to-glucose ratio of >2.0 with a lower limit of 1.6 has been suggested as an indicator for apple juice authenticity (*4*). The fructose-to-glucose ratio of all but two apple juice samples in this study had F/G ratios of >2.0. The F/G ratios of the other two samples, AJ5 and AJ92, were 1.64 and 1.88, respectively, still above the suggested lower limit.

Reported fructose, glucose, and sucrose levels in pear juice range from 5.10 to 8.89%, from 0.76 to 3.90%, and from 0.54 to 3.70%, respectively (*8*). The ranges of fructose, glucose, and sucrose of the 27 pear juice samples analyzed in this study were 4.80−6.78, 1.17−2.00, and 0.17−1.48%, respectively (**Table 1**). Individual fructose and glucose values of the 27 pear juice samples were within literature values. A fructose-to-glucose ratio of >2.7 has been suggested as an indicator for pear juice authenticity (*5*), and all but one of the pear juice samples analyzed met this requirement. For sample PJ22, the observed

F/G ratio was 2.51. On the basis of this result and the experimental standard deviation for the HPLC-RI method, it is suggested that the lower limit for F/G in authentic pear juice be set at 2.40.

In addition to these carbohydrates, apple and pear juices contain appreciable levels of the polyol sorbitol. Literature ranges for sorbitol levels in apple and pear juices are 0.16−1.20 and 1.21−2.80%, respectively (*5*, *6*). The concentration ranges for sorbitol in the 105 apple and 27 pear juice samples analyzed in this study were 0.20−0.84 and 1.54−2.84%, respectively. With the exception of the published versus observed maximum levels of sorbitol for commercial apple juice of 1.20 and 0.84%, respectively, these values were in good agreement. Apple juice samples were categorized on the basis of their sorbtol concentration as low (<0.40%), medium (0.41−0.60%), and high (>0.61%). On the basis of these sorbitol concentrations, 52 samples (49.5% of all samples) were in the low category, 47 in the medium category (44.8% of all samples), and 6 in the high category (5.7% of all samples). The literature mean for sorbitol in pear is 2.16, and the 2.23% observed in this study was in close agreement. The highest level of sorbitol observed in this study for pear juice was 2.84% (PJ13), and this sample originated from China. On the basis of these results, authentic pear juice could contain sorbitol levels as high as 2.84 ± 0.08%.

The relationship between major carbohydrates and/or sorbitol in authentic apple juices and country of origin was investigated. The only correlation in chemical composition found was between sorbitol level and New Zealand, with 9 of the 10 samples (AJ22 was the exception) from this country having a sorbitol concentration of less than the mean of 0.42%. The mean and standard deviation for sorbitol in the 10 New Zealand apple juice samples were 0.30 and ±0.10%, respectively.

Sorbitol to sucrose and sorbitol to total carbohydrates plus sorbitol ratios have been suggested as a method to detect the dilution of apple juice with water, carbohydrate, and/or pear juice (*6*, *24*). According to the literature, authentic apple and pear juices have sorbitol-to-sucrose ratios ranging from 0.112 to 0.432 and from 0.52 to 3.10, respectively (*24*). The sorbitol-to-sucrose ratios for all apple and pear samples analyzed in this study were within the literature reported ratios with the exception of one sample (AJ5) that had a ratio of 0.473. This sample had a sorbitol concentration (0.44%) that matched the mean of the 27 authentic samples, but a low sucrose concentration (0.93%); this combination resulted in the observed high ratio. Therefore, authentic samples that have undergone sucrose hydrolysis via pear storage, pear processing to fruit storage, fruit processing, or concentrate storage (*25*) may have sorbitol/sucrose ratios of >0.432. The sorbitol to total carbohydrate plus sorbitol ratios for all 105 apple juice samples were determined, and each was <0.1.

Analysis and Quantitation of Proline. The proline concentrations for apple and pear juice samples in this study were determined according to the RSK procedure (*5*), which is based upon the reaction between proline and ninhydrin; the resulting complex is extracted into *n*-butyl acetate and the absorbance of the organic phase measured at 509 nm. The mean proline content of the 105 apple juice samples was 6.3 μg/mL, with a range of 3.4−10.9 μg/mL (**Table 1**). The mean and range values observed in this study are in agreement with those reported in the literature of 5.5 and 1.3−13.8 μg/mL (*7*), 8 and 0−15 μg/mL (*5*), and 5.2 and 3.9−6.5 μg/mL (*26*). However, the revised matrix method recommended that the proline content of authentic apple juice fall within a narrow range of 2−5 μg/mL (*27*). On the basis of this narrow range, 82 (78%) of the authentic samples in this study would be considered to be adulterated. The results of this study do not support the use of this proline concentration range for authentic apple juice.

The mean proline concentration of the 27 pear juice samples analyzed was 15.5 μg/mL with a range of 8.1−50.8 μg/mL (**Table 1**). The observed range for these samples was considerably lower than the 30−250 μg/mL reported in the literature (*5*). In this study only one sample (PJ18; 50.8 μg/mL) had a proline concentration greater than the 30 μg/mL value commonly used in Europe as a minimum for proline. On the basis of this minimum, 26 of the 27 samples or 96.2% would be considered to be adulterated on the basis of proline concentration. RSK values were based mainly on European pear juice samples, which could explain the observed differences. The pear juice samples analyzed in this study represent the major world producing regions for pear juice, whereas those used to establish the RSK values (*5*) were based mainly on European samples. Therefore, the reported mean and range values from this study should be used to establish the proline content of commercial pear juices.

On the basis of these results, proline concentration as a marker for pear adulteration of apple juice would have limited usefulness. For example, an apple juice containing the mean proline value of 6.3 μg/mL intentionally adulterated with a pear juice containing the mean proline value of 16.5 μg/mL would require debasing at a level of 47% before the maximum level (as determined in this study) of proline for pure apple juice of 10.9 μg/mL was reached.

Analysis and Quantitation of Arbutin. 4-Hydroxyphenyl-β-D-glucopyranoside (arbutin) is a phenolic glucoside believed to be involved in plant defense mechanisms, on the basis of experimental results showing that pears containing arbutin oxidation products exhibit resistance to diseases (*28*). In pear juice, arbutin was found to be stable to processing as its concentration was not changed significantly by the action of enzymes, pH, and thermal processing (*12*, *14*).

Arbutin analysis of all commercial juices samples was afforded by the developed HPLC-PDA procedure. The retention time of arbutin was 14.0 ± 0.2 min, and its detection limit was 0.5 μg/mL based on a 3× signal-to-noise (s/n) ratio. The concentration of arbutin in the 27 pear juice samples ranged from 40.5 to 151.1 μg/mL with a mean value of 72.4 μg/mL (**Table 1**). Arbutin was not detected (1+1 dilution of 11.5 ± 0.1 °Brix solution) in any of the 105 apple juice samples analyzed (**Table 1**).

To ensure that the lack of arbutin detection was not due to the sample concentration (1+1 dilution of 11.5 ± 0.1 °Brix solution) used, 88 of the 105 (due to low initial amounts for 17 of these samples) samples were individually diluted to 11.5 (± 0.1) °Brix with ddH_2O, and a 50 mL aliquot of each sample was extracted with ethyl acetate and diethyl ether (*13*). The extracts were combined and evaporated, and this procedure resulted in a ≈50-fold concentration of apple phenolics. Extracts were analyzed for the presence of arbutin by HPLC-PDA, and this compound was not detected (<0.5 μg/mL) in any of these 88 samples.

These results confirm the presence of arbutin in commercial pear juice and its absence in commercial apple juice and conclusively prove its validity as a pear juice marker.

Analysis and Quantitiation of Hydroquinone. 1,4-Dihydroxybenzene (hydroquinone) is a phenolic compound that acts as a plant growth factor by accelerating seed germination and is also a substrate for enzymatic browning (*29*, *30*).

Table 2. Glucose, Fructose, Sucrose, and Sorbitol Concentrations and Standard Deviations in Pure Apple and Pear Juice Samples and in Intentionally Adulterated Apple Juice Samples

	g/100 mL			
	glucose	fructose	sucrose	sorbitol
PJ17	1.61 (0.02)[a]	6.64 (0.01)	1.00 (0.01)	2.17 (0.01)
AJ27	2.27 (0.01)	6.25 (0.03)	1.88 (0.01)	0.26 (0.00)
AJ69	2.19 (0.01)	5.39 (0.02)	2.49 (0.03)	0.56 (0.01)
AJ 24	2.71 (0.02)	5.36 (0.01)	2.28 (0.03)	0.84 (0.02)
AJ27 + 2.0%[b] PJ17	2.25 (0.01)	6.24 (0.02)	1.84 (0.02)	0.28 (0.00)
AJ27 + 10.0% PJ17	2.20 (0.01)	6.27 (0.03)	1.80 (0.02)	0.44 (0.01)
AJ27 + 30.0% PJ17	2.08 (0.01)	6.37 (0.03)	1.91 (0.02)	0.84 (0.01)
AJ69 + 2.0% PJ17	2.17 (0.02)	5.40 (0.02)	2.46 (0.02)	0.58 (0.01)
AJ69 + 10.0% PJ17	2.13 (0.02)	5.50 (0.01)	2.31 (0.02)	0.70 (0.02)
AJ69 + 30.0% PJ17	2.02 (0.02)	5.76 (0.02)	2.03 (0.02)	1.03 (0.02)
AJ24 + 2.0% PJ17	2.68 (0.02)	5.36 (0.02)	2.24 (0.02)	0.85 (0.01)
AJ24 + 10.0% PJ17	2.59 (0.01)	5.48 (0.01)	2.14 (0.01)	0.96 (0.01)
AJ24 + 30.0% PJ17	2.38 (0.02)	5.72 (0.02)	1.87 (0.03)	1.23 (0.01)

[a] Standard deviation; triplicate analysis. [b] v/v basis.

The hydroquinone content of the 105 commercial apple concentrate samples was afforded by the developed HPLC-PDA procedure. The retention time for hydroquinone was 16.5 ± 0.1 min, and its detection limit (1+1 dilution of 11.5 ± 0.1 °Brix solution) was 0.1 μg/mL based on a 3× s/n ratio. Hydroquinone was detected in all commercial apple juices analyzed in this study with a range of 0.4−21.4 μg/mL. Apple juice samples were categorized on the basis of their hydroquinone concentration as high (>5 μg/mL), medium (2−5 μg/mL), and low (<2 μg/mL). On the basis of these hydroquinone concentrations, 13 samples were in the high category, 72 in the medium category, and 20 in the low category.

Analysis and Quantitation of Carbohydrates and Sorbitol in Selected Apple Juice Samples Intentionally Adulterated with Pear Juice. Three apple juice samples were selected for adulteration with pear juice on the basis of their sorbitol content as low (AJ27; 0.26%), medium (AJ69; 0.56%), and high (AJ24; 0.84%). Pear juice sample PJ17 was used as the adulterant in this experiment because of its sorbitol concentration (2.17%), which was just below the mean (2.23%) of the 27 authentic pure pear juice samples analyzed in this study. Adulteration of the three aforementioned apple juices at levels of 2.0, 10.0, and 30.0% was made on a volume/volume basis with PJ17. As determined by HPLC-RI, the fructose, glucose, and sucrose concentrations in the adulterated samples were 5.36−6.37, 2.02−2.68, and 1.80−2.46%, respectively, whereas the pure samples ranged from 5.36 to 6.25%, from 2.19 to 2.71%, and from 1.88 to 2.49%, respectively (**Table 2**). On the basis of literature values for carbohydrates and those found for the authentic apple juice samples used in this study, all intentionally adulterated samples had glucose, fructose, and sucrose concentrations within the published ranges.

The sorbitol concentration range for intentionally adulterated samples ranged from 0.28 to 1.23% (**Table 2**). On the basis of the literature (*5*) maximum value of 1.20% for sorbitol in pure apple juice samples, only AJ24 intentionally adulterated with 30% PJ17 would exceed this value. However, even the concentration observed for this sample would be too close to be deemed adulterated on the basis of the standard deviation results observed for sorbitol measurement by HPLC-RI (**Table 2**). In addition, it has been reported that the sorbitol concentration of authentic apple samples from some geographical regions can reach 1.4−1.8% (*31*).

A sorbitol to total sugars plus sorbitol ratio of <0.1 has been suggested as the baseline value for pure apple juice (*2*, *6*). Of the nine intentionally adulterated samples, only AJ24 with 30% PJ17 had a sorbitol to total sugars plus sorbitol ratio of >0.1 (0.109), and on the basis of standard deviation results, even this sample could not conclusively be called adulterated on the suggested baseline value.

It has been proposed (*2*) that a sorbitol-to-sucrose ratio upper limit of 0.432 be used for authentic apple juice. Four of the nine samples in this study, AJ27 with 30% PJ17, AJ69 with 30% PJ17, AJ24 with 10% PJ17, and AJ24 with 30% PJ17, had sorbitol-to-sucrose ratios of 0.439, 0.507, 0.448, and 0.657, respectively, each greater than the proposed upper limit. However, the standard deviation results of 0.01 for AJ27 with 30% PJ17 would lead to ambiguity in calling this sample adulterated. It is clear that this method of adulteration detection could be readily circumvented by selecting apple and pear juices with appropriate sorbitol and sucrose concentrations.

On the basis of these results, pear adulteration of apple juice at levels up to and including 30% could not be accurately detected using major carbohydrates, sorbitol, and/or their ratios.

Analysis and Quantitation of Arbutin in Selected Apple Juice Samples Intentionally Adulterated with Pear Juice. Three apple juice samples (AJ16, AJ27, and AJ69) were intentionally adulterated with PJ17 at levels of 2.0, 10.0, and 30.0% (v/v). Pear juice sample PJ17 was used as the adulterant in this experiment due to its arbutin concentration (40.5 μg/mL), which was the lowest of the 27 authentic pure pear juice samples analyzed in this study (**Table 1**). Analysis of these samples by HPLC-PDA showed arbutin concentrations of 0.9, 4.4, and 13.1 ± 0.1 μg/mL, for AJ16, AJ27, and AJ69, respectively. On the basis of the determined detection limit for arbutin of 0.5 ± 0.1 μg/mL, the addition of as little as 2.0% pear to apple juice could be readily detected when the adulterant contained only 40.5 μg/mL of this compound. If the pear juice used as the adulterant contained the mean arbutin value (72.4 μg/mL) of the 27 authentic samples analyzed in this study and it was added at a level of 2.0% (v/v), then the arbutin concentration in the adulterated apple juice would be ≈1.4 μg/mL, well above the experimentally determined detection limit for this compound.

Confirmation of the Presence of Arbutin in Selected Apple Juice Samples Intentionally Spiked with Arbutin and Adulterated with Pear Juice. The presence of arbutin in pear samples, apple samples intentionally adulterated with pear juice, and apple samples to which arbutin was intentionally added was confirmed by treatment with β-glucosidase. Three apple juice samples were selected for arbutin addition and intentional adulteration with pear juice on the basis of their hydroquinone content as low (AJ27; 1.5 μg/mL), medium (AJ69; 4.1 μg/mL), and high (AJ16; 13.0 μg/mL). This enzyme can hydrolyze arbutin to hydroquinone, with HPLC-PDA detection of starting material and product. Three pure apple juice samples (AJ27, AJ69, and AJ16) were intentionally spiked with arbutin at concentrations of 2.0, 10.0, and 30.0 μg/mL and with pear juice AJ17 at levels of 2.0, 10.0, and 30.0% (v/v). Individual samples were treated with β-glucosidase at levels corresponding to 85, 140, and 170 units for 0.8−2.0, 4.4−10.0, and 13.1−30.0 μg/mL arbutin, respectively. The resulting solutions were stoppered and stirred at room temperature for 16 h, and enzymatic hydrolysis was quenched by heating the reaction mixture in boiling water for 2 min. Following hydrolysis, the decrease in arbutin area and corresponding increase in hydroquinone area were monitored by HPLC-PDA.

Table 3. Arbutin Peak Area and Percent Hydrolysis of Intentionally Spiked Apple Juice Samples before and after β-Glucosidase Treatment

	arbutin peak area (mAU × s)		
arbutin concn (μg/mL)	before hydrolysis	after hydrolysis	% hydrolysis
2.0	21.8 (0.5)[a]	ND[b]	>75
10.0	94.9 (0.7)	ND	>95
30.0	278.3 (1.9)	11.7 (2.3)	95.7 (0.8)

[a] Standard deviation; 10 replicates. [b] Not detected (detection limit of 0.5 μg/mL; 3 × s/n).

Table 4. Hydroquinone Peak Area and Percent Hydrolysis of Apple Juice Samples Intentionally Spiked with Arbutin after β-Glucosidase Treatment

	hydroquinone peak area (mAU × s)		
arbutin concn (μg/mL)	before hydrolysis	after hydrolysis	% hydrolysis[a]
2.0	20.1 (1.0)[b]	37.0 (1.4)	86.0 (7.3)
10.0	105.5 (1.8)	206.3 (3.6)	95.5 (3.4)
30.0	321.0 (9.6)	628.7 (3.8)	95.9 (1.2)

[a] Detection limit of 0.1 μg/mL (3 × s/n). [b] Standard deviation; 10 replicates.

For apple juice samples to which arbutin was intentionally added, hydrolysis based on arbutin peak area reduction was found to be 75.0, 95.0, and 95.7% for 2.0, 10.0, and 30 μg/mL, respectively (**Table 3**). Hydrolysis percentages of 75.0 and 95.0 for arbutin concentrations of 2.0 and 10.0 μg/mL, respectively, were based on the detection limit for this compound of 0.5 μg/mL. Arbutin hydrolysis based on the peak area increase of hydroquinone was 86.0, 95.5, and 95.9% for 2.0, 10.0, and 30.0 μg/mL, respectively (**Table 4**).

For apple juice samples intentionally adulterated with PJ17, arbutin hydrolysis based on peak area reduction was 44.4, 88.6, and 96.2% for 2.0, 10.0, and 30.0% PJ17 in AJ27, AJ69, and AJ16, respectively. Hydrolysis percentages of 44.4 and 88.6 for arbutin concentrations of 0.9 and 4.4 μg/mL, respectively, were based on the detection limit for this compound of 0.5 μg/mL, whereas the amount of arbutin hydrolyzed based on the increase in peak area of hydroquinone was 91.1, 93.6, and 89.4% for 2.0, 10.0, and 30.0% PJ17 in AJ27, AJ69, and AJ16, respectively. By comparison of HPLC-PDA analyses before and after β-glucosidase treatment, the observed increase in peak area of hydroquinone was proportional to arbutin concentration with a response of ≈11 mAU × s per μg/mL. Representative chromatograms showing arbutin and hydroquinone peaks before and after β-glucosidase addition to AJ69 intentionally adulterated with 10% PJ17 are shown in **Figure 1**.

These experimental results show that the presence of arbutin in apple juice can be confirmed by sample treatment with β-glucosidase to produce hydroquinone, with the decrease in arbutin and concomitant increase in the hydroquinone peak areas monitored by HPLC-PDA.

I3G as a Marker for Pear Juice. The 27 pear samples used in this study were analyzed for the presence of I3G (retention time of 72. 0 ± 0.1 min) according to the developed HPLC-PDA method. Experimental results showed that none of the 27 samples (at 11.5 °Brix) contained I3G at a detection limit of 1.0 μg/mL. To investigate the possibility that I3G was converted to isorhamnetin during processing and/or storage, all pear samples were analyzed for the presence of isorhamnetin (retention time of 72.8 ± 0.1 min) according to the developed

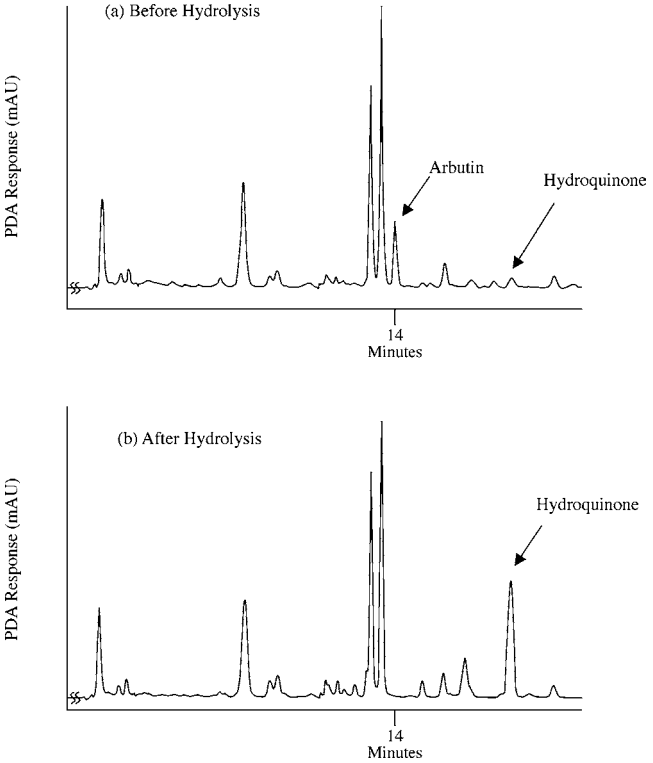

Figure 1. HPLC-PDA chromatogram of AJ69 intentionally adulterated with 10% PJ17 (**a**) before and (**b**) after β-glucosidase treatment.

HPLC-PDA method. No isorhamnetin was found in any of these samples (at 11.5 °Brix) at a detection limit of 1.0 μg/mL. To investigate the possibility that I3G and/or isorhamnetin was present at lower levels in processed pear juice, two samples (PJ13 and PJ14) were extracted (*17*) and concentrated (≈30×). HPLC-PDA analysis of the resulting solutions again showed no I3G or isorhamentin. To ensure that I3G and isorhamentin were extracted using this protocol, PJ13 and PJ14 were spiked separately with 5.0 μg/mL each of I3G and isorhamnetin, extracted, and analyzed by HPLC-PDA. Recoveries of >80% were observed for each compound. Finally, to ensure that I3-G was not hydrolyzed (at juice pH) to isorhamnetin and/or that this compound did not decompose during storage, a pear juice (at 11.5 °Brix) and a pear juice concentrate (at 71 °Brix) were intentionally spiked with 5.0 ± 0.1 μg/mL I3G. Samples were stored at 4 ± 1 °C, and aliquots were removed and analyzed weekly over a 10 week period. No significant loss (peak heights >90% of a 5.0 ± 0.1 μg/mL I3G standard) was observed for I3G over the 10 week storage period.

The absence of I3G in the 27 pure pear juice samples analyzed in this research contradicts those reported in the literature (*17*, *18*), where I3G was suggested as a distinct marker for pear. The possibility of I3G conversion to isorhamnetin during pear processing to concentrate and storage was not supported by this work as no detectable levels of this compound were observed in the 27 pear concentrates. This fact coupled with the literature (*21*) report of I3G in apple peel appears to preclude the use of this compound as a marker for pear juice adulteration of apple.

In this work 105 apple and 27 pear concentrates were analyzed for their major carbohydrate (fructose, glucose, and sucrose), sorbitol, proline, and polyphenol contents. With respect to major carbohydrates, it was observed that the natural composition and range overlap between apple and pear obviate the use of these compounds as markers for juice-to-juice adulteration. The results from this work show that the mean sorbitol concentrations in apple and pear are significantly

different, at 0.42 and 2.23%, respectively. These results indicate that sorbitol levels in apple of >0.84% could be an indicator of pear addition; however, caution must be shown when a sample is identified as adulterated on the basis of sorbitol content as there is literature evidence that authentic apple juice can contain up to 1.4% of this polyol. In this work the mean proline contents of apple and pear were shown to be quite different, with mean values of 6.3 and 16.5 μg/mL, respectively. On the basis of these results and natural range values of the samples analyzed, apple juice containing a proline concentration of >10.9 μg/mL would be considered to be adulterated, with pear juice the most likely candidate if the major carbohydrate profile was within the natural range. The most significant finding of this work was the fact that arbutin was found to be present in all 27 authentic pear samples, with a mean concentration of 72.4 μg/mL, and was not detectable in any of the 105 pure apple samples. The identification of a unique marker for pear affords the accurate (no false-positive results) identification of pear adulteration of apple juice. Because of the complexity of the polyphenolic profile of apple, the in situ hydrolysis of arbutin to hydroquinone by β-glucosidase affords an additional identification protocol for this compound in a suspected adulterated apple juice, thus eliminating the need for a more expensive and time-consuming mass spectroscopy confirmation.

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