Fluorometric Analysis of Phenolics in Persimmons

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Bruised and substandard persimmons were processed to produce persimmon extracts. Optimal fluorometric conditions (wavelength of excitation, pH range, and concentration) for measurement of the content of phenolic acids were developed in this study. The main component found in persimmon extract was p-coumaric acid.

Persimmons constitute one of the more important fruit crops grown in Israel. Since the fruit is delicate and is subject to bruising in shipment and handling, it is important to develop new uses for damaged products.

Flavonoids, including condensed tannins that are found in persimmon juice, have various biological characteristics, such as free radical scavengers, flavor astringent compounds, food additives, and absorbents.1,7-79 Phenolic compounds, which are distributed widely in plants and particularly prominent in fruits, are important in determining color and flavor. Studies of proteins in fruits have been impeded to some extent by their inherently low concentration and interference from other constituents, principally phenolic compounds and amino acids.8-10

A recent report indicates persimmon phenolics which were produced from substandard and damaged fruits. Optimal conditions (wavelength of excitation, pH range, and concentration) for measuring protocatechuic and gallic acids were found in this study. Comparison of the composition of purified phenolic extract and fruit liqueur based on natural fruit-ethanol extracts from persimmons was done.

Materials and Methods

Persimmon (Diospyros kaki L.) of two varieties: Fuio with seeds and Triumph, seedless, were used throughout all experiments.

The crude phenolics were extracted from all parts of persimmon fruit with ethyl acetate during 21 days at room temperature in ratios (w/v) of material:solvent (1:1.5). To compare the results obtained with ethyl acetate other solvents, methanol, ethanol, acetone, petroleum ether, and ethyl acetate, were used. All extracts were made under the same conditions (overnight with agitation at room temperature).

The extracted phenolic fraction was obtained by the following procedure.11-13 Ten g of persimmon tissue was macerated twice in 30 ml of methanol using a magnetic stirrer for 2 h. Debris were removed by filtration, and the methanolic solution was extracted three times with 50 ml of light petroleum (bp 60-80°C), and the remaining methanolic solutions were evaporated at 30°C to about 10 ml. The residue was diluted with 40 ml of water and extracted 8 times with 30 ml of ethyl acetate. Then the final phenolic fraction was obtained by evaporation of combined ethyl acetate extracts at 30°C. This extract was used for further spectroscopic and NMR measurements. For HPLC application the same material was dissolved in 2 M Na2CO3 solution and filtered. The pH was adjusted to 3 (2 M HCl). This acidified sample was extracted with petroleum ether, which was dried with 10 g Na2SO4, filtered, and evaporated to dryness, and the recovery of phenolic acids was measured. The remains were used for HPLC.

The 1H-NMR spectra of the compounds extracted with methanol and dissolved in deuteriomethanol were obtained with a Varian VXR 3009.

Measurement of total polyphenols of the resulting extracts using Folin-Ciocalteu reagent was done by UV spectrophotometry at 765 nm. Gallic acid was used as a standard.12-14 The absorption of standard solutions, as well as of extracted material was measured on a UVicron 930 Kautorn UV spectrophotometer. The IR spectra of lyophilized persimmon samples were measured by Fourier Transformation Infrared Spectroscopy (FTIR) as a film between KBr plates with a Fourier Transformation (FT) IR Analcet instrument. The recording was done from 4000 to 2500 cm−1 wave number.

Polyphenols and amino acids were also evaluated by fluorometry, as one of the simplest and reproducible methods. Fluorescence was measured by a Model FP-770 Jasco-Spectrofluorometer. Phenolic acids were measured by Garcia-Sanchez procedure5,16 with our modifications. p-Coumaric, gallic, and protocatechuic acids were chosen as standards for fluorometric analysis. Calibration graphs were prepared with the 0.01 m ethanolic solutions of standards. Fluorescence emission spectra were measured. For each of the phenolic acids the following conditions were used:

a) p-coumaric acid at wavelengths of excitation 330 nm and emission 443 nm; pH of the sample was adjusted to 10.7
b) gallic acid at wavelengths of excitation 260 nm and of emission 357 nm; pH of the sample was 4.63
c) protocatechuic acid at wavelengths of excitation 290 nm and of emission 363 nm; pH of the sample was 10.7

The isolated material was analyzed by high-pressure liquid chromatography (HPLC).17-18

The protein in the samples was measured by the method of Lowry et al.19 using a UVicron 930 spectrophotometer.

Measurement of proline, as described by Yokotsuka,20 included its oxidation with sodium hypochlorite and hydrogen peroxide before the reaction with o-phenhaldialdehyde in the presence of 2-mercaptoethanol and a high concentration of Brj 35 for fluorophore formation. Then this mixture was centrifuged at 1000 × g for 5 min, and the fluorescence of the supernatant was measured at wavelengths of excitation 360 nm and emission 450 nm. Proline was used as the standard.

Deionized and distilled water was used throughout. All chemicals used were purchased from Sigma Chemical Co., except for o-phenhaldialdehyde.
and 2-mercaptoethanol which were purchased from Fluka.

For the preparation of liqueurs, mashing, fermentation, distillation, extraction, and aging were used. Fruit liqueur based on natural fruit-ethanol extracts was produced by three different processes. The first two processes used ethanol extraction of fresh or dry fruits, and the third one used extraction of dry fruit with distilled alcohol from an extract of persimmon. According to the first method, fruit was crushed and pectinase\textsuperscript{7}—macerating enzyme (Novo-Industrias, Denmark) was added. For preparation of alcohol-fruit extracts the following parameters were varied: concentration of alcohol (absolute 95% and 50%), temperature (20–25°C and 36°C); ratios (w/v) of material:solvent (fruit:alcohol) 1:1; 1:1.5; 1:2; 1:3 and duration of extraction (10 days, 20 days, and 30 days). The third variation dealt with persimmon fruit crushed and treated with pectinase, fermented by Saccharomyces cerevisiae var. ellipsoideus. Dried fruit was soaked with distillate which was obtained from the fermented mash for 20 days and this double extract was the final product.\textsuperscript{21} The analyses of extracts, liqueur musts, and liqueurs were done with conventional methods,\textsuperscript{18–24} including taste panel analysis\textsuperscript{21} and statistical data.\textsuperscript{21}

Results and Discussion

Few data were found in respect to phenolic and other organic acids in persimmon.\textsuperscript{14,26,27} The NMR investigations (not shown) demonstrated two triplets around 7.5 ppm in the spectrum of a mixture of weakly acidic components. Triplets were equal in shape and slightly different in intensity. These signals correspond to the aromatic ring of phenolic compounds.\textsuperscript{28,29} Also a singlet between 5 and 6 ppm can be attributed to the vinyl group of p-coumaric acid or its derivative.

Figure 1 presents UV spectra of purified phenolic material in comparison with the p-coumaric spectra. Their absorption maxima were very close.

Figure 2 demonstrates FTIR spectra of persimmon extract. Peak A corresponds to O–H stretching vibrations of phenols, which absorb in a range 3550–3230 cm\textsuperscript{-1}. Carboxylic acids O–H stretching absorbs at 3300–2500 cm\textsuperscript{-1} (peak B). Between these broad intense bands = C–H stretching vibrations appear. The position of the C=O absorption band in the 1700–1680 cm\textsuperscript{-1} region makes it possible to assign a carbonyl band (peak C) conjugated to a double bond. The FTIR spectra of the p-coumaric acid standard and this persimmon extract were consistent with one another. These measurements are in agreement with other reports.\textsuperscript{30} Consequently, a large part of the phenolic extract is represented by benzoic and hydroxyxinnamic acids (p-coumaric, o-coumaric, and others).

The HPLC profiles of persimmon extracts in Fig. 3 show three peaks. According to the retention time of standard phenolic acids, the peaks were attributed to gallic (peak 1), p-coumaric (peak 2), and ferulic (peak 3) acids. The amount of gallic acid was slightly higher in peak (14.0%) than in pulp (9.3%)—peak 1 in Fig. 3, A, B. The amount of p-coumaric acid was higher in pulp than in pulp in 1.5 times (peak 2, Fig. 3, A, B), but the amount of ferulic acid was higher in persimmon pulp than in pulp (peak 3, Fig. 3, A, B). The recovery of the sample for HPLC application extracted with petroleum ether in comparison with ethyl acetate showed only 1.86% using Folin–Ciocalteu's reagent.

Little research has been done on the amino acids of fruits, probably because fruits are low in nitrogen and of little nutritional significance as a protein source. However, the amino acid content influences the processing of certain fruits (fermentation of grapes to form wine, browning reactions of citrus products and dehydrated fruits, extraction and preparation of phenolic extracts).

Proline, protein, and phenolic acids were measured in samples corresponding to different stages of the process and with different procedures of extraction. Total proteins (2.875 mg/g) and proline (1.023 mg/g) were measured in water extracts of persimmons. The amount of proline in persimmon extract after fermentation was as much as twice that in fresh persimmon fruit. These data correspond to information from the literature that the amount of proline increases during the process of fermentation.\textsuperscript{20}

Figure 4 shows the proline content in aqueous persimmon extracts. There is no data available for proline content in persimmons. Therefore these values of proline concentration could be compared with values found in wine, which are one order less than the reported ones.\textsuperscript{20} Proline is one of the most important amino acids during fermentation and an indicator of normal fermentation. Proline residues are responsible for the affinity towards proantocyanidins, and take part in the production of aromatic compounds and in this way influence the quality of beverages.\textsuperscript{20,23} Mostly the
amount of procyanidin is mentioned in literature, but no data are shown for the amount of antocyanidines in persimmons. Hydrolysis of tannin fraction gives cyanidin.

Colloidal turbidity of beverages is the result of interaction between polyphenols and proteins, and can be regulated by the treatment of beverages with different stabilizing agents and a filtration stage with PVPP. The protein concentration in persimmon was similar to the other known data. The small quantity of protein in the presence of a small quantity of polyphenols may give a stable product.

The following data in Table 1 show relative amounts of polyphenols in whole persimmon fruit, persimmon fruit without skin, and its skin. Total polyphenols extracted with

![Image](image_url)

**Fig. 3.** HPLC Elution Profiles of Persimmon Extracts.

Phenolic extracts from: (A) persimmon peel and (B) persimmon pulp. (1), (2), and (3) respectively gallic, p-coumaric, and ferulic acids. HPLC conditions: column, ODS (150 x 4.6 mm); mobile phase a linear gradient from 60% A:40% B (v/v) to 30% A:70% B during 20 min and then to 60% A:40% B during 10 min. Solvent A was water and solvent B was acetonitrile; flow rate, 1 ml/min; detector, UV monitor at 210 nm; temperature, 40°C. The HPLC equipment included at LKB 2150 HPLC pump connected to an LKB controller, LKB Producer AB, Bromma, Sweden.

![Image](image_url)

**Fig. 4.** Proline Content in Persimmons, Measured by Fluorometric Method.

a, corresponds to the proline concentration in extract, diluted 40 times; b, curve of standard proline, corresponding to its concentration of 45 μg/ml. Insert: calibration curve of proline. Proline was extracted in all samples with water.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Total weight</th>
<th>Total polyphenols</th>
<th>Phenolic fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH</td>
</tr>
<tr>
<td>Whole fruit</td>
<td>99.2±0.97</td>
<td>0.139±0.05</td>
<td>91.80±0.57</td>
</tr>
<tr>
<td>Fruit/no skin</td>
<td>77.2±0.76</td>
<td>0.115±0.01</td>
<td>90.00±0.51</td>
</tr>
<tr>
<td>Skin</td>
<td>22.0±0.37</td>
<td>0.214±0.05</td>
<td>85.93±0.58</td>
</tr>
<tr>
<td>Whole fruit</td>
<td>105.3±0.99</td>
<td>0.148±0.02</td>
<td>93.40±0.90</td>
</tr>
<tr>
<td>Fruit/no skin</td>
<td>80.7±0.81</td>
<td>0.121±0.02</td>
<td>91.70±0.79</td>
</tr>
<tr>
<td>Skin</td>
<td>24.1±0.29</td>
<td>0.186±0.03</td>
<td>83.52±0.57</td>
</tr>
</tbody>
</table>

* Results show mean values ± SE for 8 measurements.
* Triumph variety.
* Fuio variety (seeds are 5% of whole fruit weight).
* EtOH, ethanol soluble; MeOH, methanol soluble; EtOH + MeOH, soluble in a mixture of ethanol (1) + methanol (1); residue, alcohol nonsoluble phenolics.
ethyl acetate were in the range of 0.139% and were concentrated mostly in the skin (Table I). The percentage of total polyphenols and phenolic fractions, which are shown in Table I (86–92%, 2.4–4%; 1–4%), were received after extraction of total polyphenols with ethyl acetate and then reextracted with ethanol and methanol. Ethanol-soluble polyphenols in fruit were slightly higher than in skin, but methanol-soluble ones were as much as twice higher in skin than in fruit. Phenolics soluble in the two alcohols were in similar amounts in all parts of persimmons. The equation for calculation of total polyphenols is the following: amount of polyphenols in whole fruit is equal to per cent of the weight of the pulp multiplied by the polyphenol concentration in pulp per cent of the weight of the skin multiplied by the polyphenol concentration in the skin. The results of extraction of polyphenols with some solvents and measurement by Folin–Ciocalteau’s reagent showed the following order (µg/g): methanol (1215.09)> acetone (6210)> ethanol (4851.6)> ethyl acetate (920)> petroleum ether (17.08). The amount of total polyphenols extracted with the same solvents and weighed showed the following order (% dry matter): methanol (15.618%)> ethanol (15.322%)> acetone (14.559%)> petroleum ether (0.351%)> ethyl acetate (0.139%). A slight difference between the Folin–Ciocalteau’s measurement and total weight of polyphenols can be explained by interference with other substances, such as sugars, fibers, pectins, and proteins. Amounts of polyphenols in methanol and ethanol, which are shown as the first members in this order can be explained by high solubility of tannins in these solvents.\textsuperscript{13,14}

\textit{p}-Coumaric, protocatechuic, and gallic acids were chosen as the most representative phenolic acids in the phenol composition of persimmon fruits. Optimal conditions (wavelength of excitation, pH range, and concentration) for measuring the content of protocatechuic acid and gallic acids were found in this study. Emission spectrum for each of these benzoic acids was run at different pH’s and wavelengths of excitation. Optimal conditions were found in respect to these parameters. Then selectivity of the method was tested in mixture of all these acids at appropriate concentrations. Interference was not observed. Also emission spectra of fenolic, vanillic, syringic, and tannic acids were measured for the same purpose. But conditions for the measurement of these acids were not found.

The reviewed data showed that the highest yield extraction in first experiment was done with 50% ethanol, during 20 days at room temperature with a 1:1 (w/v) ratio of fruit to ethanol.

Chemical composition of the second experiment showed that more effective extraction was done with 50% ethanol, during 20 days, at room temperature and also during incubation at 36°C and a ratio of fruit to ethanol of 1:2.5 (w/v).

Data of chemical composition of the third experiment showed that the best extraction was obtained with 50% distillate, during 20 days, at room temperature and a ratio of fruit to distillate of 1:2.5 (w/v). The taste (8.95) and aroma (9.00) scores were the highest among all the data of the three different experiments.\textsuperscript{21}

To improve the taste of liqueur, sugar was added. In addition treatment of liqueur with clarifying agents and filtration was done.

### Table II. Characteristics of Persimmon Liqueurs

<table>
<thead>
<tr>
<th>Sample number \footnote{Respectively group data of ( n = 13 \times 2 ) replications.}</th>
<th>Preparation of extract \footnote{Experiment 1 = fresh persimmon (1) to 50% alcohol (1). Experiment 2 = dry persimmon (1) to 50% alcohol (2.5). Experiment 3 = dry persimmon (1) to 50% persimmon distillate (2.5).}</th>
<th>Composition (%)</th>
<th>Taste, scores</th>
<th>Aroma, scores</th>
<th>Taste &amp; aroma, scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Experiment 1</td>
<td>30</td>
<td>27</td>
<td>20</td>
<td>5.25</td>
</tr>
<tr>
<td>2</td>
<td>Experiment 1</td>
<td>30</td>
<td>27</td>
<td>30</td>
<td>6.75</td>
</tr>
<tr>
<td>3</td>
<td>Experiment 2</td>
<td>30</td>
<td>27</td>
<td>20</td>
<td>5.75</td>
</tr>
<tr>
<td>4</td>
<td>Experiment 2</td>
<td>30</td>
<td>27</td>
<td>30</td>
<td>6.50</td>
</tr>
<tr>
<td>5</td>
<td>Experiment 3</td>
<td>30</td>
<td>27</td>
<td>20</td>
<td>6.75</td>
</tr>
<tr>
<td>6</td>
<td>Experiment 3</td>
<td>30</td>
<td>27</td>
<td>20</td>
<td>6.00</td>
</tr>
<tr>
<td>7</td>
<td>Experiment 3</td>
<td>30</td>
<td>27</td>
<td>30</td>
<td>5.25</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>30</td>
<td>24</td>
<td>20</td>
<td>6.15</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>30</td>
<td>24</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

The scores, given in Table II are the average of three taste panels. According to the data of Table II the best results were achieved in samples 6 and 2 which have 20% and 30% fruit extract respectively and 27% alcohol.

According to data of Table II samples of liqueur prepared from persimmon extracts had high aroma and taste scores. The results of taste and aroma scores in regards to sample 6 are in agreement with the literature.\textsuperscript{8,32} A similar chemical composition was obtained in sample 4.

Extract from the persimmon Fuio was lower in taste score than from the persimmon Triumph. The bitter taste in extract Fuio can be explained on the basis of the nature and behavior of phenolic deposits and tannin solubility in persimmon fruit.\textsuperscript{10,33–36} Therefore our research focused on the preparation of liqueurs from persimmon (Triumph) ethanol and phenolic extracts.

The process of preparing fruit extracts without previous drying, fermentation, and distillation must be faster and more effective than using all the stages of technology. All these data were in the range of standard fruit and corresponded with data of references 18, 23, and 27.

Phenolic acids (\textit{p}-coumaric, protocatechuic, and gallic), which were measured in the extracts of persimmons and liqueurs are shown in Table III. These compositional data are difficult to compare because different researchers use a variety of sampling techniques.\textsuperscript{23,26} The amounts of \textit{p}-coumaric acid found in skin were 826 μg/g and in fruit itself 480 μg/g.

The calculations gave the following: 826 × 0.22 + 480 × 0.78 = 614. This number exactly corresponds to the amount of \textit{p}-coumaric acid for whole fresh fruit in Table I. As can be seen from Table III, the persimmons Triumph and Fuio differ in polyphenol content. The amount of all three phenolic acids was the highest in the liqueur made on the basis of an extract from Fuio. Persimmon Fuio has 3.84 times more protocatechuic acid than persimmon Triumph.
Table III. Polyphenols* in Persimmon Extracts and Liquors

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Phenolic acids (µg/g)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-Coumaric</td>
<td>Protocatechuic</td>
<td>Gallic</td>
</tr>
<tr>
<td>Persimmonb</td>
<td>614.8 ± 1.07</td>
<td>62.6 ± 0.72</td>
<td>221.2 ± 2.58</td>
</tr>
<tr>
<td>Persimmonc</td>
<td>425.2 ± 1.29</td>
<td>240.6 ± 2.49</td>
<td>158.5 ± 1.98</td>
</tr>
<tr>
<td>Liqueursd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 2e</td>
<td>45.3 ± 0.36</td>
<td>18.9 ± 0.27</td>
<td>55.7 ± 0.70</td>
</tr>
<tr>
<td>Sample 6f</td>
<td>188.0 ± 0.84</td>
<td>28.5 ± 0.78</td>
<td>115.4 ± 1.98</td>
</tr>
<tr>
<td>Sample 7g</td>
<td>135.2 ± 1.72</td>
<td>114.5 ± 2.38</td>
<td>92.1 ± 0.58</td>
</tr>
<tr>
<td>Sample 20i</td>
<td>52.1 ± 0.74</td>
<td>27.7 ± 0.52</td>
<td>46.3 ± 0.83</td>
</tr>
<tr>
<td>Sample 6i</td>
<td>184.7 ± 1.53</td>
<td>31.6 ± 0.48</td>
<td>122.7 ± 1.82</td>
</tr>
<tr>
<td>Sample 2j</td>
<td>123.3 ± 0.75</td>
<td>120.7 ± 1.77</td>
<td>84.9 ± 0.75</td>
</tr>
</tbody>
</table>

* Results show mean values ± SE for 8 measurements.
| Persimmon Triumph. |
| Persimmon Fuio. |
\* See Table II for samples composition.
\* Samples 2, 6, and 7 prepared in 1986.
\* Samples 2, 6, and 7, after 6 years storage.

However, the content of p-coumaric and gallic acids in Triumph variety is larger by 1.4 times than Fuio. Six years of storage did not drastically influence the polyphenol quantity. Some of the phenolic compounds mostly in Fuio persimmon fruit cause undesirable taste and color changes in food products and decreases in protein nutritive value.4 The ratio of phenolic acids content of liqueur 2 to that of persimmon Triumph extract, is respectively 7.4%, 30.0%, and 25.0% for p-coumaric, protocatechuic, and gallic acids. In liqueur 6 this ratio of phenolic acids increased to p-coumaric 30.6%, protocatechuic 45.5%, and gallic acid 52.0%. Liqueur 7 from Fuio persimmon includes a fermentation stage. All maximum emission positions of spectra shown in Fig. 5 correspond to one another, because all samples were prepared under the same extraction conditions. Ethanol as a solvent was used for polyphenol extraction. Ratio of phenolics to that of persimmon Fuio extract was: p-coumaric 31.8%, protocatechuic 47.6%, and gallic 58.0%.

Figure 5 demonstrates changes in the contents of p-coumaric acid.

Persimmon extracts were treated with bentonite to decrease the level of phenolics, which can cause turbidity; therefore the content of phenolics was measured before and after treatment. The amount of phenols was similar to data shown by different authors.18,32 Absorbance of liqueur clarified with bentonite was lower than in untreated samples, indicating the removal of phenols.31,33 The samples prepared from extracts with previous fermentation (Table III, sample 6) were higher in these phenols than without fermentation (Table III, sample 2), as well as samples prepared from persimmon Fuio (Table III, sample Persimmonb). The numbers of peaks in liqueur samples were smaller than in pure standards, suggesting that the extracted compounds were a mixture of phenols. As it is seen from Tables I–III, our data agreed in levels of protein and amino acids content, and in total polyphenols.15,18,23

This paper presents:
1. Optimal fluorometric conditions (wavelength of excitation, pH range, and concentration) for measurement of the content of phenolic acids were developed in this study.
2. The results of the effects of process variables on the amount of phenolic acids in persimmon extracts measured by different analytical methods (UV, NMR, FTIR, HPLC, and fluorometry);
3. Suitability of two varieties of persimmons for extract preparation with a preference for the Triumph seedless type. Improvement of the mode of extraction, purification, and preservation of persimmon phenolics, in the form of extracts;
4. The change of some parameters such as the ratios of raw and dry materials to a solvent, conditions of fermentation, and degree of distillation based on our
previous investigation gave the possibility to make a beverage with high aroma and taste and low amounts of polyphenols and proteins;

5. Comparison of the composition of purified phenolic extract and fruit liqueur based on natural fruit-ethanol extracts from persimmons was done.

The main component found in persimmon extract was 3-m-coumaric acid, and 3-m-coumaric, protocatechuic, and gallic acids were measured in fruit-ethanol extracts.

References
6) K. Sakaguchi, Japan Kokai Tokkyo Koho, 90134746 (May 24, 1990).

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