TECHNICAL COMMUNICATIONS

Improved Liquid Chromatographic Method for Determination of Organic Acids in Leaves, Pulp, Fruits, and Rinds of Garcinia

GUDDADARAVYANAHALLY K. JAYAPRAKASHA, BHARANI S. JENA, and KUNNUMPURATH K. SAKARIAH
Central Food Technological Research Institute, Human Resource Development, Mysore-570 013, India

An improved liquid chromatographic (LC) method was developed for determination of organic acids in leaves, pulp, fruits, and rinds of Garcinia. At present, the commonly used LC method for analysis of organic acids in Garcinia extracts uses direct application of the extracts on the column. This practice gradually reduces efficiency of the column and shortens its life. In the improved method, the interfering substances such as pigments and xanthones were effectively removed by passing the aqueous extract through an ODS cartridge. With subsequent injection on a C18 reversed-phase column, using 6.0mM phosphoric acid as the mobile phase with a flow rate of 1.0 mL/min and UV detection at 210 nm, the organic acids were determined in the extracts. The major organic acid was (−)-hydroxycitric acid at the level of 2.5, 0.8, 3.0, and 20.1% in leaf, pulp, fresh fruit, and dried rinds, respectively. Minor quantities of hydroxycitric acid lactone, oxalic acid, and citric acid were also identified. Limits of detection and recoveries were 0.9–1.5 μg and 93.9–99.8%, respectively. This is the first report on the composition of organic acids from Garcinia pedunculata.

In recent years, (−)-hydroxycitric acid (HCA) has gained considerable attention as a promising weight-controlling agent. In extensive animal studies, HCA has been proven to effectively curb appetite, suppress food intake, increase the rates of hepatic glycogen synthesis, reduce fatty acid synthesis and lipogenesis, and decrease body-weight gain. Derivatives of HCA are incorporated into many pharmaceutical preparations. In extensive animal studies, HCA has been proven to effectively curb appetite, suppress food intake, increase the rates of hepatic glycogen synthesis, reduce fatty acid synthesis and lipogenesis, and decrease body-weight gain. Derivatives of HCA are incorporated into many pharmaceutical preparations. HCA is the principal organic acid in the fruits of certain species of Garcinia. There are approximately 180 different species of Garcinia (3), but only a few contain the HCA substantially, such as G. cambogia, G. indica, G. atroviridis (4–6) and, as we more recently reported, in the rinds of G. cowa (7). G. cowa and G. pedunculata are found in the northeastern parts of India and the Andaman Islands. G. pedunculata is a tall, stately tree with short spreading branches. It has fleshy pericarp used in place of lime or lemon; however, there are no reports on the composition of organic acids from G. pedunculata.

Small and complex molecules have been isolated from the various species of Garcinia, which include xanthones and xanthone derivatives (8–10). Besides HCA and its lactone (Figure 1), the other major solute components in aqueous extracts of Garcinia are pectins and polyphenols (11). The existing method of assay of organic acids consists of titration of extract against standard sodium hydroxide (12). Gas chromatography (GC) requires derivatization of the acid before analysis (6). Some authors (13–15) have described methods for the determination of organic acids by liquid chromatography (LC). In these methods, reversed-phase LC required a lengthy ion-exchange column cleanup. Also, in most of the methods (7, 16, 17) the extract was directly injected into the LC system without removing the pigments. This resulted in shortening of column life and inaccuracy in quantification due to interfering substances.

In the present study, we report a method for the determination of organic acids in leaves, pulp, fruits, and dried rinds of G. pedunculata by LC, in which attempts were made to overcome the shortcomings of earlier methods. This is the first report on the composition of organic acids from G. pedunculata.

Experimental

Apparatus

(a) LC series instrument.—HP 1100 (Hewlett-Packard, La Jolla, CA).

(b) Nuclear magnetic resonance (NMR) spectrometer.—1H and 13C NMR spectra (D2O) were recorded at 400 and 100 MHz, respectively, on a Bruker AMX-400 FT instrument using tetramethyl silane as the internal standard (Bruker, Rheinstetten, Germany).

(c) Mass spectrometer.—Shimadzu QP-5000 Quadrupole (Shimadzu, Tokyo, Japan).

(d) Swinnex-type filter.—Pore size 0.45 μm (Millipore, Bangalore, India).

(e) Glass filter.—Borosil (Bangalore, India).

Chemicals

(a) Hydrochloric acid GR (Cat. No. 61012505001046), phosphoric acid (Cat. No. 61805700251046), methanol LC
Isolation of (–)-Hydroxycitric Acid Lactone

Pure HCA lactone is not commercially available. Hence, HCA lactone was isolated from the fruits of *G. indica* by the method reported earlier and its purity was analyzed by gas liquid chromatography (GLC) and optical rotation (18). Five different concentrations (100, 250, 500, 750, and 1000 μg/mL) HCA lactone solutions were prepared in triple-distilled water, pH 6.8, and stored at 5°C until further use.

Preparation of Free (–)-Hydroxycitric Acid

As free HCA is not available commercially, it was prepared by deionizing calcium hydroxycitrate as described earlier (7). Calcium hydroxycitrate (80 mg) was suspended with 5.0 mL triple-distilled water and treated with 1 g Dowex 50 (H⁺). The supernatant was collected and the resin was washed to neutral pH. The washes and supernatant from the resin were pooled, diluted to 20 mL, and filtered through a glass filter. Standard HCA solutions of various concentrations (100, 250, 500, 750, and 1000 μg/mL) were prepared and stored at 5°C until further use. The resin was then activated as follows: Five resin volumes of 5% HCl were percolated in the Dowex 50 for 50 min, and then displaced with 5 resin volumes of distilled water for 50 min. Finally, the resin was rinsed with distilled water to get a neutral pH and Dowex 50 in the activated form.

Preparation of Standard Oxalic Acid and Citric Acid

Stock solutions of oxalic and citric acids were prepared by dissolving 10 mg from each acid in triple-distilled water and diluted to 10 mL with triple-distilled water. Standard solutions of oxalic and citric acids were prepared to obtain a concentration ranging from 100 to 1000 μg/mL.

Sample Preparation

(a) Fresh leaves, pulp, and fruits.—From each sample, 5 g was extracted with 50 mL water at 10 psi for 20 min and filtered through muslin cloth. The extraction and filtration were repeated once again for complete extraction of the organic acids. The extracts were pooled and concentrated to 20 mL under vacuum, treated with 100 mL ethanol to remove pectinaceous material, and centrifuged at 1200 × g for 15 min. The supernatant was concentrated under reduced pressure to 25 mL and stored at 4°C until further use. The acid content of leaves, pulp, and fruits was determined by acid–base titration using phenolphthalein indicator.

(b) Dried rinds.—(1) First method.—Rinds (5 g) of *G. pedunculata* were extracted with 100 mL water at 15 psi for 30 min and filtered through a glass filter. The extraction and filtration was repeated twice for complete extraction of the organic acids. The extract was concentrated to 30 mL under vacuum, treated with 120 mL ethanol to remove pectinaceous material, and centrifuged at 1200 × g for 15 min. The supernatant was concentrated under reduced pressure to 25 mL and stored at 4°C until further use. The acid content was determined by acid–base titration. (2) Second method.—Rinds (10 g) of *G. pedunculata* were extracted in a Soxhlet extractor with 100 mL acetone and methanol separately for 8 h each. The extracts were filtered using Whatman No. 1 filter paper and concentrated to 10 mL under vacuum. An acetone and MeOH concentrate was suspended in 20 mL water separately, heated in a boiling water bath for 30 min, and filtered through a glass filter. The filtrate was diluted to

Figure 1. Structures of organic acids identified in *G. pedunculata*.

Figure 2. Electron-impact mass spectrum of (–)-hydroxycitric acid lactone.
50 mL with water. The acid content of acetone and MeOH extracts were determined by acid–base titration.

**Cleanup Procedure**

The ODS cartridge was preconditioned by first passing 10 mL MeOH and water successively for 10 min. An empty syringe was then used to pass air through the cartridge to expel remaining water; 100 μL extract was passed through the cartridge for 60 s and the organic acids were eluted with 1.0 mL triple-distilled water for 60 s and diluted to 1.5 mL. The eluate was filtered through a 0.45 μm membrane filter before injection into the LC column. After use, the cartridge was washed with 10 mL 60% (v/v) methanol and with 4 mL methanol and water successively for further use.

**LC Analysis**

The LC system consisted of a Hewlett-Packard LC Model HP 1100 Series equipped with a quaternary LC pump, fitted with a Zorbax C18 analytical column, 25 cm × 4.6 mm id, 5 μm particle size, Part No. 880967-902. The injection system (Rheodyne) used contained a 20 μL sample loop. Detection was performed on an HP 1100 Series variable wavelength detector at wavelength of 210 nm. The mobile phase used was 6.0 mM phosphoric acid with a flow rate of 1.0 mL/min. The compounds were quantified on HP CHEMSTATIONS software.

**Quantification of Organic Acids in Samples**

Samples (20 μL) prepared as above were injected into the LC system and the concentration of HCA, HCA lactone, oxalic acid, and citric acid were obtained from the calibration graph and by application of the dilution factor. The concentrations of organic acid in the samples were expressed as g/100 g of sample.

**Recovery Studies**

To verify the accuracy of the sample cleaning procedure, the recovery studies were performed for free HCA and HCA lactone. Aliquots of 100 μL free HCA stock solution (2 mg/mL) and 50 μL sample (leaves, pulp, fresh fruit, and rinds extract) were mixed and passed through a preconditioned ODS cartridge. The organic acids and HCA lactone were eluted with 0.7 mL triple-distilled water, the final volume was adjusted to 1.0 mL with water, and 20 μL was injected into the LC column. Similarly, 50 μL samples (leaves, fruit, and rinds extract) were passed through a preconditioned ODS cartridge; it was eluted as described above, and 20 μL was injected into the LC column. The concentration of free HCA present in 20 μL was calculated by using a regression equation. Aliquots of 200 μL HCA lactone stock solution (1 mg/mL) and 50 μL sample (leaves, pulp, fresh fruit, and rinds extract) were mixed and passed through a preconditioned ODS cartridge. The organic acids and HCA lactone were eluted with 0.7 mL triple-distilled water, the final volume was adjusted to 1.0 mL with water, and 20 μL was injected into the LC column. Similarly, 50 μL samples (leaves,

---

**Table 1. Composition of organic acids in leaves, pulp, fruits, and rinds of G. pedunculata determined by LC and titration methods**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Oxalic acid (g/100 g)</th>
<th>(–)-Hydroxycitric acid lactone (g/100 g)</th>
<th>(–)-Hydroxycitric acid (g/100 g)</th>
<th>Citric acid (g/100 g)</th>
<th>Total (g/100 g)</th>
<th>Titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinds</td>
<td>0.783 ± 0.021</td>
<td>3.298 ± 0.180</td>
<td>20.113 ± 0.87</td>
<td>2.374 ± 0.449</td>
<td>26.567</td>
<td>28.66</td>
</tr>
<tr>
<td>Pulp</td>
<td>0.090 ± 0.039</td>
<td>0.201 ± 0.040</td>
<td>0.821 ± 0.080</td>
<td>1.056 ± 0.030</td>
<td>2.168</td>
<td>3.93</td>
</tr>
<tr>
<td>Fruit</td>
<td>0.231 ± 0.002</td>
<td>0.242 ± 0.003</td>
<td>3.019 ± 0.002</td>
<td>0.232 ± 0.017</td>
<td>3.493</td>
<td>4.32</td>
</tr>
<tr>
<td>Leaf</td>
<td>0.110 ± 0.003</td>
<td>1.450 ± 0.150</td>
<td>2.450 ± 0.099</td>
<td>0.309 ± 0.020</td>
<td>4.319</td>
<td>5.11</td>
</tr>
</tbody>
</table>

---

**Figure 3. Liquid chromatograms of (A) standard oxalic acid, HCA lactone, free HCA, and citric acid; (B) dried rinds, MeOH extract; (C) dried rinds, acetone extract; (D) dried rinds, water extract; (E) fresh fruit, water extract; (F) leaf, water extract; (G) pulp, water extract.**
pulp, fresh fruit, and rinds extract) were passed through a pre-conditioned ODS cartridge; it was eluted as described above and 20 μL was injected into an LC column. The concentration of HCA lactone present in 20 μL sample was calculated by using a regression equation.

Validation of LC Method

(a) Calibration and linearity.—The linearity of the method was evaluated by analyzing a series of organic acids. A 20 μL aliquot of each acid of the 5 working standard solutions containing 1–20 μg oxalic acid, HCA lactone, free HCA, and citric acids was injected into the LC system to obtain peak area responses. The calibration curves for oxalic acid, HCA lactone, free HCA, and citric acids were prepared by plotting concentration of organic acids vs peak area.

(b) Range.—The calibration range was established by considering the practical range necessary according to the HCA concentration present in the samples. This range includes concentrations from lower limit of quantitation (LOQ) to the upper LOQ (7).

(c) Determination of LOQ.—The LOQ for organic acids such as oxalic acid, HCA, HCA lactone, and citric acid was determined as described earlier (7). The LOQ was defined as the lowest amount of oxalic acid, HCA, HCA lactone, and citric acid concentration that can be determined with an accuracy and precision of <20%.

Results and Discussion

As the standard HCA lactone is not commercially available, it was isolated from the fruits of *G. indica* by the method reported earlier, and its purity was analyzed by GLC and optical rotation (18). HCA lactone had a melting point of 182°C and optical rotation [α]_D^20_ = +99.7°C (c = 1.0; H_2O); these values matched well with those of HCA lactone reported values (18). HCA lactone is stable at humid-free and neutral pH conditions. The HCA lactone ring opened when it was heated with alkali (pH > 10.0) for approximately 30 min. The purity of isolated HCA lactone was 99%. The structure of HCA lactone was further established by _1^H_ and _13^C NMR spectra. _1^H_ NMR spectra of HCA lactone signals matched reported values (18). _13^C_ NMR of HCA lactone showed signals at 174.1, 171.1, 168.2, 84.1, 79.2, and 39.8. Further, the structure of HCA lactone was confirmed by mass spectra. The molecular ion peak for HCA lactone can be seen at 191, which confirms identification of HCA lactone (Figure 2). Figure 2 also shows fragments for 191 (M + 1; 5.1%), 162 (2%), 145 (35%), 127 (10%), 116 (48%), 99 (70%), 88 (100%), 69 (45%), 60 (40%), and 55 (20%).

Table 1 shows the composition of organic acids in leaves, pulp, fruits, and rinds of *G. pedunculata* as determined by LC and acid–base titration methods. Acid–base titration gives higher values due to the presence of other organic acids. The values obtained by LC accounted only for HCA, because the values correspond to the areas of HCA peak. Major organic acid found in leaves, fruits, and rinds of *G. pedunculata* by LC is HCA, as shown in liquid chromatograms in Figure 3 and Tables 1 and 2, whereas in pulp, citric acid was the major acid. Three of the minor peaks were identified as HCA lactone, oxalic, and citric acids by co-injection of standard acids. All 4 organic acids were resolved as a single peak in all samples analyzed (Figure 3). The identity of oxalic acid, HCA lactone,

<table>
<thead>
<tr>
<th>Solvents used</th>
<th>Oxalic acid</th>
<th>(–)-Hydroxycitric acid lactone</th>
<th>(–)-Hydroxycitric acid</th>
<th>Citric acid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.391 ± 0.009</td>
<td>1.061 ± 0.020</td>
<td>13.437 ± 0.400</td>
<td>1.24 ± 0.444</td>
<td>16.13</td>
</tr>
<tr>
<td>MeOH</td>
<td>0.429 ± 0.002</td>
<td>1.685 ± 0.014</td>
<td>13.367 ± 0.139</td>
<td>1.184 ± 0.179</td>
<td>16.67</td>
</tr>
<tr>
<td>Water</td>
<td>0.783 ± 0.020</td>
<td>3.298 ± 0.18</td>
<td>20.113 ± 0.867</td>
<td>2.374 ± 0.449</td>
<td>26.567</td>
</tr>
</tbody>
</table>

Table 3. Recovery study of (–)-hydroxycitric acid lactone added to selected samples of *G. pedunculata*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Initial</th>
<th>Added</th>
<th>Found</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinds</td>
<td>109.00</td>
<td>200</td>
<td>67.62</td>
<td>97.47</td>
</tr>
<tr>
<td>Fruits</td>
<td>65.38</td>
<td>200</td>
<td>111.58</td>
<td>98.74</td>
</tr>
<tr>
<td>Pulp</td>
<td>8.58</td>
<td>200</td>
<td>12.58</td>
<td>95.02</td>
</tr>
<tr>
<td>Leaves</td>
<td>14.17</td>
<td>200</td>
<td>18.17</td>
<td>97.79</td>
</tr>
</tbody>
</table>

*Present in 20 μL.*

*Added for recovery study.*
HCA, and citric acid peak were confirmed by determination of relative retention time and by spiking with authentic samples. The retention times of the oxalic acid, HCA lactone, HCA, and citric acid in all samples were 3.71 ± 0.07, 4.53 ± 0.03, 4.82 ± 0.04, and 7.93 ± 0.06 min, respectively. The run time of LC (Figure 3) was kept for 25 min to see whether some other organic acids may elute beyond 15 min.

To study the extractability of organic acids from the rinds of *G. pedunculata*, we used different polar solvents. Dried rinds were extracted with acetone, MeOH, and water, and the organic acids were determined by LC (Table 2). Among the 3 solvents, water extracted maximum organic acids, which may be due to higher polarity of water than other solvents. The quantitation of organic acids by standard addition to untreated sample before extraction from *G. cambogia*, *G. indica*, and *G. cowa*, and commercial samples was discussed earlier (2, 7, 16–18). In those studies, the recovery of organic acids was satisfactory, and therefore was not conducted in the present study.

The acid–base titration method was used to determine organic acids in the extracts, which gives the total acidity of extracts (12). But in this method, the concentration of HCA, HCA lactone, citric and oxalic acids cannot be estimated separately. Generally, GC estimation involves the conversion of acid to volatile silyl derivatives. For silylation, the sample should be dried completely, but HCA has the tendency for lactonization during drying (4) and because of its highly hygroscopic nature, it is rather difficult to dry the sample. Recently, Jayaprakasha and Sakariah (2, 16, 17) developed LC methods for determination of HCA as well as other organic acids in commercial samples of *G. cambogia* extracts, leaves, and rinds of *G. indica* and fruits of *G. cambogia*. However, in those methods, extracts were injected directly into the LC system without removing the interfering compounds from the extract before the injection. In the present study, the samples were passed through the ODS cartridge to remove the interfering pigments/xanthones and injected into the LC column for determination of organic acids from *G. pedunculata*.

Calibration curves were derived from 3 injections of 5 concentrations of oxalic acid, HCA lactone, HCA, and citric acid. Linearity was in the 1.5–20 μg concentration range, with good reproducibility and accuracy. The regression equations for oxalic acid, HCA lactone, HCA, and citric acid was $y = 803x - 125$, $y = 73x + 63$, $y = 125x - 18$, and $y = 79x + 163$, respectively, where $y$ is the peak area and $x$ is the concentration of organic acid. The correlation coefficients ($r^2$) of the oxalic acid, HCA lactone, HCA, and citric acid were $0.99$, $0.99$, $0.99$, and $0.96$, respectively. The estimated LOQs in this study for oxalic acid, HCA lactone, HCA, and citric acid were $0.9$, $1.4$, $1.5$, and $1.3$ μg, respectively. Similar organic acid composition was found in leaves, fresh fruits, and dried rinds of *G. pedunculata* in variable amounts. Water was the most preferable solvent for extraction of organic acids (Table 2).

The data in Tables 3 and 4 show that recovery of HCA lactone and HCA was almost complete in the experiments. The recovery of HCA lactone and HCA by LC after passing through an ODS cartridge was in the range of 95.0–98.7% and 93.9–99.8%, respectively, which emphasizes the accuracy of the method. The recovery rate of HCA lactone and HCA from extracts of leaves, pulp, fruit, and rinds was calculated by the ratio of mean peak area obtained from injection of known concentration of extract passed through ODS cartridge and that of extract and standard HCA passed through the LC column. The present study explored the presence of bioactive constituent of *Garcinia*, i.e., HCA and its lactone, and found that these were the major compounds in *G. pedunculata* (Tables 1 and 2); hence, we conducted recovery studies for HCA and its lactone.

### Conclusions

The present method for determination of organic acids by LC from fruits and plant materials may provide longer life to the column and better resolution of the peaks by passing the extracts and samples through the ODS cartridge before injection into the LC column. This study demonstrates the potential of using an ODS cartridge for purification of *Garcinia* extracts before injection into an LC column. With this method, good repeatability of results is established. A recovery of 95.0–98.7% and 93.9–99.8% of HCA lactone and free HCA, respectively, was obtained from the ODS cartridge. This is the first report that fruits of *G. pedunculata* contain a substantial amount of HCA.

### Table 4. Recovery study of (–)-hydroxycitric acid added to selected samples of *G. pedunculata*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initiala</th>
<th>Addedb</th>
<th>Founda</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinds</td>
<td>565.78</td>
<td>200</td>
<td>568.87</td>
<td>99.79</td>
</tr>
<tr>
<td>Fruits</td>
<td>408.73</td>
<td>200</td>
<td>411.81</td>
<td>96.73</td>
</tr>
<tr>
<td>Pulp</td>
<td>35.98</td>
<td>200</td>
<td>37.96</td>
<td>97.18</td>
</tr>
<tr>
<td>Leaves</td>
<td>40.80</td>
<td>200</td>
<td>43.88</td>
<td>93.90</td>
</tr>
</tbody>
</table>

* a Present in 20 μL.
* b Added for recovery study.
Acknowledgments

We thank V. Prakash and M.C. Varadaraj (Central Food Technological Research Institute, Mysore, India) for their keen interest in this work.

References