CHAPTER 5

METHODS DEVELOPED FOR THE ESTIMATION OF HYDROXY CITRIC ACID

5.1 INTRODUCTION

Hydroxy citric acid, a reputed natural product in dietary supplement is the active constituent of Garcinia cambogia fruit. HCA was identified by some scientists worked at CFTRI, Mysore, during 1960s [Lewis et al. 1965]. Previously HCA was mistakenly identified as citric acid and tartaric acid and titration procedures were used for estimations. The other methods available like, Paper chromatography, HPLC by comparative method etc. provide inconsistent results. The need for a reliable analytical procedures for estimation of HCA is essential at this moment, since lot of products incorporating HCA are being marketed worldwide. In this chapter new analytical procedures developed for the estimation of hydroxy citric acid are described, which are;
1. Spectrophotometric determination of HCA

2. Estimation of HCA using HPTLC

3. Estimation of HCA by HPLC using C\textsubscript{18} RP amide HPLC column

5.2 MATERIALS

The dried fruit rinds of *Garcinia cambogia* grown in Sri Lanka (commercial imported variety) were used for the studies. The chemicals used were of AR grade. HPLC grade solvents were used for HPLC and HPTLC analyses.

5.3 METHODS

5.3.1 Spectrophotometric determination of HCA

Background of this investigation

While reviewing the literature no method has been seen using spectrophotometer for the estimation of HCA. According to Lewis *et al.* (1964), a colour spot was developed when sodium meta vanadate solution was sprayed on paper which is unique to HCA. So a standardized procedure was developed based on this color complex formation.
Preparation of sample for Spectrophotometric analysis

Water extract of leaves, bark, rind and fruit of G. cambogia were used for the experiment. About 100g-chopped dried material (moisture content–15%) was boiled with 300mL water (3 times), filtered, concentrated under vacuum to 50% moisture level. The thick concentrated liquid was filtered, washed the residue with small portion of water and combined all washes with the mother liquid. It was neutralized with 4N NaOH solution maintained at pH 7.5. Fifty percent (50%) solution of CaCl₂ was added and stirred well. Precipitated residue was filtered through a Buchner funnel and dried (Moisture – 4%). Twenty-five gram material was obtained. Accurately weighed 0.2g of this material dissolved in 5mL of 1N H₂SO₄ and diluted to 25mL with distilled water. This was decolorized using 10% activated carbon. The solution was filtered into a 50mL standard flask, washed the residue with small portion of distilled water and made up to the volume.

Preparation of standard solution

Ethylene diamine salt of HCA was used as the standard. The standard solutions were prepared as follows.

Working standards were prepared by ethylene diamine salt of HCA (98% ED – HCA). The salt equivalent to 0.0429g of the free acid was weighed accurately and dissolved in 5mL of 1N H₂SO₄ and approximately 25mL of
distilled water was added. It was filtered and transferred into a 50mL volumetric flask and made up to the volume using distilled water [Conc. 828 µg/mL].

**Procedure**

One milliliter of the above standard solution was pipetted out into a 100mL volumetric flask and made up to volume using distilled water. Exactly 0.9mL 5% sodium meta vanadate in water was added and noted the time. The color of the solution became yellow. As time advanced, the yellow color slightly changed to orange red. Absorbance was noted at 467nm after 20 minutes. A blank solution was prepared. Repeated the same experiment using 1.5mL, 2mL, 2.5mL and 3mL of standard solution. A calibration graph was plotted against the concentration of HCA and absorbance. Dilute the sample solution so that final concentration should be 0.5mg/mL. Repeated the same experiment using sample solution and the amount of HCA was calculated from the calibration graph.

**5.3.2 Estimation of HCA using HPTLC**

Thin Layer Chromatography (TLC) is one of the most flexible methods of chromatographic analysis for the separation and identification of chemical substances. It excels whenever large numbers of samples must be analyzed by an economical method that is ready for immediate use and does not require complicated sample preparation.
HPTLC is a modern version of TLC with accurate spotting and quantification, which plays an important role in the quality control of food stuffs, usually with emphasis on the investigation of ingredients like lipids, carbohydrates, vitamins and organic acids and the detection of harmful impurities (e.g. aflatoxins, pesticides). HPTLC is a suitable technique for checking the purity and testing the stability of raw materials and finished preparations.

The flexibility of HPTLC is a consequence of the absence of restrictions on the choice of mobile and stationary phases. It is also convenient for data processing and, therefore, for quantification / calibration reference and test samples can be chromatographed on the same HPTLC plates and hence in the same chromatographic system. *Garcinia cambogia* extracts were analyzed by HPTLC for HCA content.

**Sample preparation**

Three hundred (300mg) milligram weight of standardized calcium hydroxy citrate was dissolved in 10mL 0.1N HCl. Filtererd and the filterate was used for analysis.

<table>
<thead>
<tr>
<th>Applicator</th>
<th>DESAGA AS 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Densitometer</td>
<td>DESAGA CD 60</td>
</tr>
</tbody>
</table>

**Chromatographic conditions**

<table>
<thead>
<tr>
<th>Method</th>
<th>Ascending, one – dimensional development in the HPTLC developing chamber, without chamber saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application</td>
<td>1-2 and 3μL with the applicator AS 30 as 5 mm bands</td>
</tr>
</tbody>
</table>
Methods developed for the estimation of HCA

Stationary phase: Precoated HPTLC plates, NH$_2$F$_{254}$ S Merck No:13192, pre-washed with methanol

Mobile phase Methanol + water (6+2, v/v)

Distance run : 7cm

Running time : 30 min.

Detection : After development the plate was heated 10 min. at 150°C and the resultant fluorescent zones were examined under the UV – lamp at 366 nm.

**In situ quantitation**

The fluorimetric analysis was carried out at 366 nm. and 420 nm with the densitometer CD60.

**DESAGA CD 60 Method**

DESAGA CD 60 method is shown below

Chromatogram No. : 0000230 printed 02/02/1999 10:59
Name of method : Garcinia cambogia extract

Start – coordinate X : 12.0 [mm] Width of slit : 0.2 [mm]
Start – coordinate Y : 5.0 [mm] Height of slit : 3.0 [mm]
End – coordinate Y : 70.0 [mm] Filterposition : open 420 nm
Chapter 5

Methods developed for the estimation of HCA

Meanderwidth : 0.0 [mm]

Number of lanes : 4 Wave length Measurement: 366 nm

Distance of Lanes : 10.0 [mm] Wavelength Reference : 0 [nm]

Mode : Remission Fluorescence Signal positive Lamp Hg

Resolution at Measurement : 0.100 [mm]
Number of Measurements / Position : 4

Smoothing factor : 0

Linearizing acc. to kubelka / Munk. : 0

Scale for signal in graphics : 500

Backgroundcorr. (Meander only) : No

Automatic Zeroing at start : Yes

Point after Measurement : No printing

Data saving : No

Call user program : Start no Used Program

Spot Optimization : No Optimization

Resolution for Optimization : 0.025 [mm]

Number of Measurements / Position : 4

Evaluation during Measurement : Yes

Width of window (mm) : 1.000
Threshold for Peak detection : 0.500
Maximum slope of Base line : 10.000
Minimum Peak height : 5.000
Minimum Peak area : 0.000
Evaluation – Interval from to (mm) : 5.000 70.000

Number of components : 1
Number of standard – concentrations: 3
Number of different samples : 0
Unit of standard : ng
Unit of results : ng
Factor of conversion : 1.00000
Number of Digits of standard : 2
Number of Digits of Results : 2

Type of Calibration Function : Straight Line \( y = a \cdot x + b \) without \((0,0)\)
Calibration on Peak – Height / Area : Height

Correlation of Lanes
Standard 1 : 1
Standard 2 : 3
Standard 3 : 4
Concentration of standards:

<table>
<thead>
<tr>
<th>Standard</th>
<th>K1</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1</td>
<td>K1</td>
<td>1.00</td>
</tr>
<tr>
<td>Standard 2</td>
<td>K1</td>
<td>2.00</td>
</tr>
<tr>
<td>Standard 3</td>
<td>K1</td>
<td>3.00</td>
</tr>
</tbody>
</table>

Automatic Peak identification:

<table>
<thead>
<tr>
<th>Component</th>
<th>Name of component</th>
<th>Position [mm]</th>
<th>Tolerance [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydroxy citric acid</td>
<td>55.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
5.3.3 Estimation of HCA by HPLC using C\textsubscript{16} RP amide column

**Preparation of standard**

Fifty milligram of ethylene diamine salt of HCA was accurately weighed out into a 50mL volumetric flask. Dissolved the material by adding 5mL 50\% \text{H}_2\text{SO}_4 and made up to the volume by HPLC grade water. The resulting stock solution contained 500\mu g/mL HCA.

An internal standard solution was prepared by dissolving 50mg L– aspartic acid in 5mL 50\% \text{H}_2\text{SO}_4 in water taken in a 100mL volumetric flask. Made up to the mark using HPLC grade water. The concentration of the solution was found to be 500 \mu g/mL. Different strengths of standard solutions were prepared by taking different volumes of the stock solution. 3mL of the internal standard solution was added and made up to 10mL using HPLC grade water.

**Preparation of samples**

Different salts of HCA and HCA lactone were prepared as described in Chapter 4. Accurately weighed out 250mg sample into a 100mL standard flask. Then added 5mL water and 5mL concentrated sulphuric acid. Dissolved the material and made up to the volume using HPLC grade water. The pH was adjusted to 2.1.
Sample solutions of other organic acids were prepared by taking 50 – 70mg sample in 100mL HPLC grade water.

All the solutions including standard, and samples were sonicated for half an hour and filtered in 0.45 micron filter.

**Procedure**

10 – 20μL of this solution was injected into the HPLC column and the elution profile was noted.

Using standard solutions, regression graph was drawn.

**Instrument conditions**

Instrument : HPLC class VP system supplied by Shimadzu, Japan

Column : Supelco RP amide, 2.5cm x 4.6cm x 0.5μm C16 guard column

Mobile phase: 0.1M sodium sulphate in HPLC water, pH adjusted to 2.1 with sulphuric acid, filtered and sonicated.

Flow rate : 0.5mL/ minute
Detection wave length : 203nm
Injection volume : 10 – 20μL
The percentage of HCA in the sample solution was calculated as

\[
\frac{\text{Area of HCA peak of sample}}{\text{Concentration of standard}} \times \frac{\text{Area of HCA peak of std.}}{\text{Concentration of sample}}
\]

5.4 RESULTS

5.4.1 Color reaction between HCA and meta vanadate

When HCA reacted with sodium meta vanadate, an orange red coloured complex was formed. In order to find out the absorption maxima of this product, the solution was scanned between 200 – 800nm in UV Spectrophotometer. The absorbance measured was plotted against wavelength. The spectrum obtained was shown in Figure 5.1.
Fig. 5.1: Spectrum of colour complex formed when HCA reacted with sodium meta vanadate

From the spectrum, it is clear that the color complex formed has absorption maxima at 467nm in the visible region.
5.4.2 Stability of color complex formed between HCA and sodium meta vanadate

When HCA mixed with sodium meta vanadate reacted, a yellow coloured solution was formed immediately, which on keeping changed into an orange colored complex. This colour remained for 10 minutes and then slowly its intensity was decreased. The stability of the color complex was studied by taking absorbance at 467 nm at different time intervals. The time scan of HCA and sodium meta vanadate was done and shown in Figure 5.2.
Fig. 5.2: Time scan of HCA and sodium meta vanadate solution immediately after mixing

![Time scan graph]

The color tends to stabilize after 15 – 20 minutes of mixing between HCA and sodium meta vanadate solutions and this color remains for 10 – 13 minutes and then started decreasing.
5.4.3 Optimization of the Spectrophotometric procedure

After finding the absorption maxima and stabilizing the time interval, the other parameters like concentration of sample solution, strength of meta vanadate reagent and sulphuric acid to be needed were optimized. These optimum parameters were shown in Table 5.1.

Table 5.1: Optimum parameters required for estimation of HCA by Spectrophotometric method

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Parameters</th>
<th>Conditions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Concentration of sample solution**</td>
<td>0.5 – 0.6mg/mL</td>
</tr>
<tr>
<td>2</td>
<td>Volume and strength of H₂SO₄ needed</td>
<td>5 mL 1 N H₂SO₄</td>
</tr>
<tr>
<td>3</td>
<td>Vol. of meta vanadate solution</td>
<td>0.9mL 5% sodium meta vanadate</td>
</tr>
<tr>
<td>4</td>
<td>Time limit</td>
<td>Absorbance should be taken exactly 20 minutes after the addition of sodium meta vanadate</td>
</tr>
</tbody>
</table>

* The values were obtained as the average of 6 experiments

** Samples were salts of HCA
The optimum concentration of sample was 0.5 – 0.6mg/mL. The quantity of sulphuric acid was constant but volume of meta vanadate solution was critical and found out that 0.9mL of 5% solution was required for 0.5 – 0.6mg/mL of sample solution. The most important point is the time of absorbance noted. The colour remained stable from 15 – 25 minutes after the addition of sodium meta vanadate and optimum time for absorbance was found to be 20 minutes.

5.4.4. Analysis of Hydroxy citrates using UV – Spectrophotometric method

Different salts of hydroxy citric acid like calcium hydroxy citrate and potassium hydroxy citrate were analyzed by Spectrophotometric method. The samples were collected from different manufacturers. The datas are shown in the Table 5.2 and 5.3
Table 5.2: HCA Analysis of different samples of Calcium hydroxy citrate by Spectrophotometric method

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Source</th>
<th>% HCA claimed by manufacturer</th>
<th>% of HCA analyzed by spectrophotometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arjuna Natural Extracts Ltd., India</td>
<td>54.1%</td>
<td>53.5%</td>
</tr>
<tr>
<td></td>
<td>Batch No.: GCC - 033/0008/B - 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NutriScience Innovations LLC - USA</td>
<td>53.8%</td>
<td>51.3%</td>
</tr>
<tr>
<td>3</td>
<td>Transglobal Resources Inc. - USA</td>
<td>51.8%</td>
<td>51%</td>
</tr>
<tr>
<td>4</td>
<td>Quimdis S.A, France</td>
<td>60.7%</td>
<td>56.5%</td>
</tr>
<tr>
<td>5</td>
<td>Inabata Koryo Co. Ltd., Japan</td>
<td>52.2%</td>
<td>51.1%</td>
</tr>
</tbody>
</table>

* The values expressed are the average of three-consecutive analysis

The HCA analysis showed that, the content of HCA was almost same as that claimed by manufacturer but with slight deviation in Quimdis product.
Table 5.3: HCA Analysis of different samples of Potassium hydroxy citrate by Spectrophotometric method

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Source</th>
<th>% HCA claimed by manufacturer</th>
<th>% of HCA analysed by Spectrophotometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arjuna Natural Extracts Ltd., India</td>
<td>51.1%</td>
<td>50.5%</td>
</tr>
<tr>
<td></td>
<td>Batch No.: GCP - 018/0104/B - 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NutriScience Innovations LLC – USA</td>
<td>50.5%</td>
<td>49.0%</td>
</tr>
<tr>
<td>3</td>
<td>Transglobal Resources Inc. – USA</td>
<td>54%</td>
<td>52.2%</td>
</tr>
<tr>
<td>4</td>
<td>Quimdis S.A, France</td>
<td>50.4%</td>
<td>50%</td>
</tr>
</tbody>
</table>

* The values obtained are the average of three analyses

HCA data showed that values were almost same as that claimed by the manufacturer.
5.4.5 Interference of HCA lactones on sodium meta vanadate

Since HCA is easily converted into lactones, the interference of lactones on meta vanadate reaction was checked. Lactones were prepared by the method described in chapter 2. Different quantities of this lactones were mixed with known and standardized quantity of HCA and were analysed. The results are shown in Table 5.4.

Table 5.4: Interference of HCA Lactones with sodium meta vanadate in presence of known HCA

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>% of lactones added</th>
<th>% of HCA in the sample</th>
<th>HCA analysed by Spectrophotometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>60%</td>
<td>17%</td>
<td>17.4%</td>
</tr>
<tr>
<td>2.</td>
<td>50%</td>
<td>22%</td>
<td>23.1%</td>
</tr>
<tr>
<td>3.</td>
<td>40%</td>
<td>26.1%</td>
<td>25.6%</td>
</tr>
<tr>
<td>4.</td>
<td>30%</td>
<td>31.5%</td>
<td>31%</td>
</tr>
</tbody>
</table>

* Known quantity of calcium hydroxy citrate (50% HCA) was added into the lactone. All results are expressed on dry weight basis.
Table 5.4 shows the non-interference of HCA lactone in color complex formation with sodium meta vanadate and Hydroxy citric acid. We have seen that colour complex formed between lactone and sodium meta vanadate was yellow color but for HCA, color was orange reddish. This is a reliable method to distinguish between HCA lactone and HCA.

5.4.6 Comparison of HCA by Spectrophotometric method and HPLC methods

The HCA content obtained by Spectrophotometric method was compared with HPLC method. HPLC analyses were carried out at Industrial Laboratories, USA and Shiva Analytical Lab, Bangalore. These values are shown in Table 5.5. From their analysis reports, it is clear that HPLC method is comparable with that of UV – Spectrophotometric method.
Table 5.5: Comparison of HCA by Spectrophotometric method and HPLC method (for different samples)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Sample *</th>
<th>HCA content by Spectrophotometric method</th>
<th>HCA content by HPLC method (outside analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Industrial Lab, USA</td>
</tr>
<tr>
<td>1.</td>
<td>Calcium hydroxy citrate&lt;br&gt;Batch No.: GCC-023/0010/B-34&lt;br&gt;(Arjuna Natural Extracts Ltd.)</td>
<td>54.2%</td>
<td>52.1%</td>
</tr>
<tr>
<td>2.</td>
<td>Potassium hydroxy citrate&lt;br&gt;Batch No.: GCP - 018/0007/B- 4&lt;br&gt;(Arjuna Natural Extracts Ltd.)</td>
<td>52.6%</td>
<td>51.2%</td>
</tr>
<tr>
<td>3.</td>
<td>Magnesium hydroxy citrate&lt;br&gt;Batch No.: MHC - 011/0004/B - 1&lt;br&gt;(Arjuna Natural Extracts Ltd.)</td>
<td>56.4%</td>
<td>55.7%</td>
</tr>
</tbody>
</table>

* All samples were manufactured by Arjuna Natural Extracts Ltd., India

The Spectrophotometric analysis of HCA gives comparable results with that of outside laboratories.
5.4.7 Analysis of HCA using HPTLC

Method was developed for the analysis of HCA by HPTLC. Samples were spotted on HPTLC plate, developed and scanned using Densitometer CD 60. The fluorescent chromatogram obtained was digitized by the frame grabber. This is shown in Figure 5.3.
Method developed for the estimation of hydroxycitric acid

20 - 100 µg sample was loaded in precoated HPTLC plate, NH₄F₂54S (Merck). The plate was developed up to 7 cm in the mobile phase (Methanol: Water 6:2). The plate was heated for 10' at 150⁰C. Cooled and scanned at 366 nm (Fluorescence). Lanes 1, 3 and 4 represent Garcinia Cambogia Extract samples and lane 2 represents pure citric acid.
**DESAGA CD 60 Peak list**

DESAGA CD 60 peak list is shown below.

<table>
<thead>
<tr>
<th>Chromatogram No.</th>
<th>0000230 printed 02/02/1999 11:01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of object</td>
<td>Hydroxy citric acid</td>
</tr>
<tr>
<td>Name of method</td>
<td>Garcinia cambogia extract</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peak Comp. No.</th>
<th>Name</th>
<th>Y - Pos. [mm]</th>
<th>Area [mm²]</th>
<th>Area height [%]</th>
<th>PM ng</th>
<th>Std.Conc. ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane number : 1 (Standard No. 1) X - coordinate : 12.0 [mm]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13.4</td>
<td>347.2</td>
<td>70.8</td>
<td>134.93 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>37.5</td>
<td>56.9</td>
<td>11.6</td>
<td>25.06 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1 Hydroxy citric acid</td>
<td>55.0</td>
<td>86.3</td>
<td>17.6</td>
<td>24.97 b</td>
<td>1.000</td>
</tr>
</tbody>
</table>

| Lane number : 2 X - coordinate : 22.0 [mm] |
| 1              | 13.1          | 350.0         | 100.0      | 267.32 b       |       |              |

| Lane number : 3 (Standard No. 2) X - coordinate : 32.0 [mm] |
| 1              | 13.2          | 11.7          | 1.5        | 150.62 b       |       |              |
| 2              | 13.8          | 539.7         | 69.3       | 192.26 f       |       |              |
| 3              | 1 Hydroxy citric acid | 55.1 | 227.7 | 29.2 | 56.20 b | 2.000 |

| Lane number : 4 (Standard No. 3) X - coordinate : 42.0 [mm] |
| 1              | 8.6           | 4.1           | 0.3        | 5.31 b         |       |              |
| 2              | 12.3          | 123.9         | 9.8        | 146.53 b       |       |              |
| 3              | 13.4          | 711.7         | 56.1       | 165.86 f       |       |              |
| 4              | 1 Hydroxy citric acid | 55.5 | 428.8 | 33.8 | 88.25 b | 3.000 |
Regression graph of hydroxy citric acid by HPTLC is shown in Figure 5.4. The detection level was found to be in between 20μg-100μg. Citric acid in the line 2 has different Rf value than HCA. Figure 5.5 shows the chromatogram of the all-4 lanes.

5.4.8 Estimation of HCA by HPLC using C₁₆ RP amide column

HCA and other organic acids had clear elution profile on C₁₆ RP amide column. The lactones also had separate peak.

Here the results obtained for the estimation of HCA present in different hydroxy citrates, lactones and other organic acids were discussed. The elution profile of HCA present in calcium hydroxy citrate, potassium hydroxy citrate and sodium hydroxy citrate are shown in Figure 5.6, 5.7 and 5.8. The regression graph obtained when different strengths of standard ethylene diamine salt of HCA were injected is shown in Figure 5.9.

5.4.9 Elution profile of other organic acids using C₁₆ RP amide column

The elution profile of HCA lactone is shown in Figure 5.10. The elution profiles of other organic acids like oxalic acid, tartaric acid, citric acid and aspartic acid are shown in Figures 5.11, 5.12, 5.13 and 5.14.
Fig. 5.4: Regression graph of HCA by HPTLC method

Component No. : 1 hydroxy citric acid
Calibration : Straight line $y = a\times x + b$ without (0,0)
Calib. function : $y = 3.16e+01\times x - 6.81e+00$
Residual dev. : 0.3348

(Std. x Shp. x) without conv. factor.)
Chapter 5

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Fig. 5.5: HPTLC profile of HCA
Fig. 5.6: Elution profile of Calcium hydroxy citrate by HPLC (C$_{18}$ RP amide column) method

Calcium hydroxy citrate solution was prepared as described in materials and methods. 30–40 μg material was injected into C$_{18}$ RP–amide column. Retention time and area percentage were noted.
Chapter 5 Methods developed for the estimation of HCA

Fig. 5.7: Elution profile of Potassium hydroxy citrate by HPLC ($C_{16}$ RP amide column) method

Potassium hydroxy citrate solution was prepared as described in materials and methods. 50-60 µg material was injected into $C_{16}$ RP–amide column. Retention time and area percentage were noted.
Sodium hydroxy citrate solution was prepared as described in materials and methods. 75-85µg material was injected into C16 RP–amide column.

Retention time and area percentage were noted.
Fig. 5.9: Regression graph of HCA by HPLC (C\textsubscript{16} RP amide column) method

This is the regression graph obtained when different concentrations of ethylene diamine salt solution of HCA was injected into C\textsubscript{16} RP-amide column. Ratio in Y-axis indicates the ratio of HCA against ratio of internal standard.
Fig. 5.10: Elution profile of Lactones by HPLC ($C_{16}$ RP amide column) method

Hydroxy citric acid lactone was prepared as described in materials and method. 160-170μg sample was injected into $C_{16}$RP-amide column. Retention time and area percentage were noted.
Fig. 5.11: Elution profile of Oxalic acid by HPLC ($C_{16}$ RP amide column) method

Elution profile of oxalic acid using C16 RP – amide column.

60 - 65µg sample was injected.
Fig. 5.12: Elution profile of Tartaric acid by HPLC 
(C_{16} RP amide column) method

Elution profile of tartaric acid using C16 RP – amide column.  
60 - 65µg sample was injected.
Fig. 5.13: Elution profile of Citric acid by HPLC (C_{18} RP amide column) method

Elution profile of citric acid using C16 RP – amide column.
90 - 100 μg sample was injected
Fig. 5.14: Elution profile of Aspartic acid by HPLC (C$_{16}$ RP amide column) method.

Elution profile of aspartic acid using C16 RP - amide column.

10 - 15µg sample was injected.
HCA content in calcium hydroxy citrate was compared by spectrophotometric method and HPLC method using C₁₆ RP amide column. These results are shown in Table 5.6

### Table 5.6 Comparison of HCA by Spectrophotometric method and HPLC (C₁₆ RP amide column) Method

<table>
<thead>
<tr>
<th>SI. No.</th>
<th>Sample</th>
<th>HCA content by Spectrophotometric method* (%)</th>
<th>HCA content by HPLC method* (C₁₆RP amide column)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Garcinia cambogia (Calcium salt)</td>
<td>53</td>
<td>52.8</td>
</tr>
<tr>
<td></td>
<td>Provided by Arjuna Natural Extracts Ltd.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Garcinia cambogia (Calcium salt)</td>
<td>59</td>
<td>60.1</td>
</tr>
<tr>
<td></td>
<td>NutriScience Inc. USA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Garcinia cambogia (Calcium salt)</td>
<td>52</td>
<td>51.6</td>
</tr>
<tr>
<td></td>
<td>Fabrichem Inc., USA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* percentage on dry weight basis

The result of HCA content analysis by UV and HPLC (C₁₆ RP amide column) were almost same without interference of lactone.
Table 5.7: Analysis of HCA in different branded products by Spectrophotometric and HPLC methods

<table>
<thead>
<tr>
<th>Brand name</th>
<th>*Medication per capsule</th>
<th>HPLC method (C₁₈RP amide column)</th>
<th>Spectrophotometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td>LivSlim</td>
<td>490 mg</td>
<td>41.3%</td>
<td>40.6%</td>
</tr>
<tr>
<td>M/s LivLong Neutaceutical Ltd.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioslim</td>
<td>497 mg</td>
<td>21.5%</td>
<td>22.1%</td>
</tr>
<tr>
<td>M/s Dabur</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ayurslim</td>
<td>495 mg</td>
<td>31.1%</td>
<td>30.9%</td>
</tr>
<tr>
<td>M/s Himalaya Drug Company</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Medication is a mixture of different ingredients including *Garcinia cambogia* extract (calcium salt)

Different branded products of *G.cambogia* extract were tested and showed in Table 5.7. Content of *G.cambogia* extract was different in the products. So the HCA content also varied from product to product.
5.5 Discussion

Some analytical procedures were developed in order to estimate HCA content in hydroxy citrates, extracts and derivatives. Titration methods to find out acidity or acid value provide only a crude or rough idea about the ingredient. Advanced technology or elaborate analytical procedures are necessary for estimation of HCA. The procedures developed using UV, HPLC and HPTLC were described in this chapter provide an accurate estimation of HCA.

The analytical procedure for Spectrophotometric method is very simple. The color complex formed when HCA reacted with sodium meta vanadate is unique and specific. No color complex was formed when citric acid was taken as sample. This indicates that color complex formed is directly proportional to quantity of HCA present in *Garcinia cambogia* fruit. Citric acid is present in *Garcinia cambogia* at a level of 3-5%. This was estimated by pentabromoacetone method. Color complex formed has absorption maxima of 467nm i.e., in the visible region. Lactone does not interfere with color formation.
The reaction is time dependant. It takes about 15 – 20 minutes to stabilize the color. This incubation time is quite satisfactory to carry out the analysis. Hydroxy citrates were hydrolyzed using H$_2$SO$_4$. The optimum conditions were also studied.

We have analyzed different samples collected from different manufacturers of HCA. There was not much difference in HCA level based on the claims.

In the second method, HCA was separated by HPTLC. Here HCA is compared with citric acid. Since the Rf values obtained for HCA and Citric acid are very different, these can be detected very easily. One major advantage of method is that, analysis is possible for a large number of samples simultaneously. This will provide a comparative study for many samples. The detection level of HCA was found to be in between 20µg - 100µg.

The third method described is HPLC using C$_{18}$ RP amide column. α-hydroxy organic acids are easily separated and identified using this C$_{18}$ RP amide column and it is specific for α-hydroxy acids. The HPLC analysis done by JIX Antony et al. described a method for HCA analysis. But, the results are obtained by a comparative method by calculating the total acids and by deducting the percentage of citric acid from the total acids. Direct estimation of HCA is not included in that procedure. The method described here has provided separate
elution profile for organic acids, lactone and hydroxy citrates and can compare the results. But analysis-using HPLC is more expensive than any of the other methods. The only draw back of spectrophotometric method is its time dependence. The color complex formed is unstable and change its color on keeping. Though the HPLC analysis is time consuming and expensive, it is more accurate and specific. For larger number of samples, HPTLC method is the best and cheapest in terms of recurring cost.