1. Purpose

To determine the contents of polyphenols, catechin, epicatechin, catechin gallate, epicatechin gallate, gallocatechin gallate, epigallocatechin gallate, epigallocatechin and gallic acid in green tea and grape products.

2. Materials

The following materials are needed to carry out the analysis:

2.1 Standards:

- (+)-Catechin hydrate, Sigma Chemical Co.
- (+)-Epicatechin, Sigma Chemical Co.
- (-)-Catechin gallate, Sigma Chemical Co.
- (-)-Epicatechin gallate, Sigma Chemical Co.
- (-)-Gallocatechin gallate, Sigma Chemical Co.
- (-)-Epigallocatechin gallate, Sigma Chemical Co.
- (-)-Epigallocatechin, Sigma Chemical Co.
- (-)-Gallocatechin, Sigma Chemical Co.
- Gallic Acid, Sigma Chemical Co.

2.2 Apparatus:
• HPLC instrument: Hewlett-Packard HP 1100 Series HPLC equipped with autosampler, DAD detector, and HP ChemStation Software or equivalent.
• HPLC column: MetaChem PolarisTM Amide C18, 5µm, 4.6 x 250 mm
• Calibrated analytical balance accurate to ±0.01 mg
• Sonicator, with temperature control
• Volumetric flasks, appropriate sizes
• Syringes, 3-cc disposable with Luer-lok tip
• Filters, 0.45-µm, glass PVDF or Cellulose Acetate
• Pipettes, class A, assorted sizes

2.3 Reagents:

• Water, deionized, HPLC grade
• Phosphoric acid, 85%, reagent grade
• Acetonitrile, HPLC grade

3. HPLC Condition

_The following HPLC condition should be used when carrying out this analysis:_

3.1 Project and location: Polyphenol HPLC program (pp01.m) for separating and checking and quantifying isoflavones is in the C:\HPCHEM\1\Methods\ directory of HP ChemStation software system. Sequence: pp01.s.
3.2 Column: MetaChem PolarisTM Amide C18, 5µm, 4.6 x 250 mm
3.3 Column Temperature: 35° C
3.4 Mobile Phase: 0.1% phosphoric acid/acetonitrile gradient (see table)

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>0.1% H3PO4</th>
<th>% Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>85.0</td>
<td>15.0</td>
</tr>
<tr>
<td>12.00</td>
<td>75.0</td>
<td>25.0</td>
</tr>
<tr>
<td>20.00</td>
<td>75.0</td>
<td>25.0</td>
</tr>
<tr>
<td>22.00</td>
<td>85.0</td>
<td>15.0</td>
</tr>
<tr>
<td>30.00</td>
<td>85.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

3.5 Flow Rate: 1.0 mL/min
3.6 Detection Wavelength: 280 nm
3.7 Injection volume: 10 µL
Alternative HPLC Column

- Column: Waters SymmetryShieldTM, RP18, 5 µm, 4.6 x 250 mm
- Mobile Phase: 0.1% phosphoric acid/acetonitrile gradient (see table)

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>% 0.1% H3PO4</th>
<th>% Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>92.0</td>
<td>8.0</td>
</tr>
<tr>
<td>35.00</td>
<td>22.0</td>
<td>78.0</td>
</tr>
<tr>
<td>36.00</td>
<td>22.0</td>
<td>78.0</td>
</tr>
<tr>
<td>30.00</td>
<td>92.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

- Flow rate: 1.0 mL/min
- Detection Wavelength: 280 nm
- Injection volume: 10 µL

3.8 Retention Times (approximate):

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MetaChem RP C18</th>
<th>Waters RP C18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic Acid</td>
<td>2.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>4.4</td>
<td>12.0</td>
</tr>
<tr>
<td>Catechin</td>
<td>5.8</td>
<td>14.6</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>6.8</td>
<td>20.0</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>9.4</td>
<td>21.0</td>
</tr>
<tr>
<td>Gallocatechin gallate</td>
<td>11.2</td>
<td>23.2</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>13.3</td>
<td>32.2</td>
</tr>
<tr>
<td>Catechin gallate</td>
<td>15.2</td>
<td>31.6</td>
</tr>
</tbody>
</table>

Note:
ECG and CG reverse elution order between columns. Run reference standards to verify order of elution.

4. Preparation of Standards:

4.1 (+)-Catechin Stock Standard Solution
• Accurately weigh approximately 5 mg (± 0.1 mg) of (+)-catechin into a 10-mL volumetric flask. Record the weight to the nearest 0.01 mg.
• Add approximately 7 mL extraction solution and sonicate for 10 minutes or until all solids have dissolved. Allow flask to cool to room temperature and bring to volume with extraction solution. Mix well.
• Store standard sealed with parafilm in the refrigerator when not in use.

4.2 (+)-Catechin Working Standards

• Prepare working standards as dilutions of the stock to cover the expected range of concentration of catechins in the samples. Suggested dilutions are 1:10, 1:50 and 1:100.
• Store standard sealed with parafilm in the refrigerator when not in use.

4.3 Gallic Acid Stock Standard

• Accurately weigh 7.5 mg (± 0.1 mg) of gallic acid into a 100-mL volumetric flask. Record the weight to the nearest 0.01 mg.
• Add approximately 70 mL of extraction solution and sonicate for 15 minutes, or until all solids have dissolved. Allow flask to cool to room temperature and bring to volume with extraction solution. Mix well.
• Store standard sealed with parafilm in the refrigerator when not in use.

4.4 Gallic Acid Working Standards

• Prepare working standards as dilutions of the stock to cover the expected range of concentration of gallic acid in the samples. Suggested dilutions are 1:10, 1:50 and 1:100.
• Store standard sealed with parafilm in the refrigerator when not in use.

4.5 Extraction Solution Preparation-Phosphoric Acid, 0.1%

• Prepare by carefully pipetting 1.2 mL phosphoric acid into about 950 mL water in a 1-L volumetric flask. Mix, and bring to volume with water. Perform operation in a fume hood.

5. Sample Preparation
5.1 Principal

Samples are dissolved and extracted in 0.1% phosphoric acid in water with the aid of sonication. The samples are analyzed by HPLC and the seven catechins quantified against (+)-catechin as an external standard. Gallic acid is quantified against the pure standard. The HPLC column is a MetaChem PolarisTM Amide C18, 5µm, 4.6 x 250 mm. The mobile phase is a gradient, consisting of 0.1% phosphoric acid in water and acetonitrile, with a flow rate of 1 mL/min. Detection is at 280 nm.

5.2 Accurately weigh the appropriate amount of material (see table) into a volumetric flask.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Sample Weight</th>
<th>Sonication Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf Powder</td>
<td>100 mg (± 10 mg)/100 mL</td>
<td>60 minutes</td>
</tr>
<tr>
<td>Extract Powder (50 - 75%)</td>
<td>50 mg (± 5 mg)/50 mL</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>

- Add approximately three-fourths of extraction solution and sonicate for the specified amount of time at 60°C.
- Allow solution to cool to room temperature and bring to volume with extraction solution. Mix well.
- Using a 3-cc disposable syringe, filter the solution through a 0.45-µm filter into an HPLC vial and cap.

6. Procedure

6.1 Prepare reference standard solutions and sample preparations as directed.
6.2 Prepare an extraction solvent blank.
6.3 Make a single injection of the blank. Make single injections of the reference standard preparations.
6.4 Prepare a linearity curve for (+)-catechin and gallic acid, with the origin ignored, using peak area vs. concentration (mg/mL). Perform linear regression analysis on the data.
6.5 Make single injections of the sample preparations. Calculate the percent catechins in the sample.
7. Quality Assurance:

7.1 A duplicate sample preparation and standard preparation should be analyzed with each set of 20 or less samples.
7.2 To monitor method variance, a green tea sample of known composition should be assayed with each batch and the assay results control charted.
7.3 The correlation coefficient of a linear regression curve for both catechin and gallic acid should be >0.999.

8. Calculations

8.1 Quantify each catechin against (+)-catechin using the following equation and factors.

\[
\% \text{ w/w analyte} = \frac{C \times FV \times d \times F \times 100}{W}
\]

*Where:*

\(C\) = The concentration of the individual catechin determined by linear regression analysis of (+)-catechin, mg/mL (corrected for hydration)

\(FV\) = The final volume of the sample preparation, mL

\(d\) = Dilution of the sample, if needed

\(w\) = The weight of the sample in mg

\(F\) = The correction factor for the individual catechin response vs. (+)-catechin

\(F=1.000\) for (+)-Catechin hydrate
\(F=1.020\) for (+)-Epicatechin
\(F=0.327\) for (-)-Catechin gallate
\(F=0.382\) for (-)-Epicatechin gallate
\(F=0.482\) for (-)-Gallocatechin gallate
\(F=0.543\) for (-)-Epigallocatechin gallate
\(F=3.450\) for (-)-Epigallocatechin.

8.2 Quantify gallic acid against the pure standard using the following equation:
% Gallic acid = \( \frac{C \times FV \times d}{W} \times 100\% \)

Where:

C = The sample's gallic acid concentration (mg/mL) from linear regression analysis
FV = The final volume of the sample preparation (mL)
d = The dilution factor of the sample preparation
w = The sample weight in mg

References:

**APPENDIX:**

*Structures of the Standard Compounds:*

(+) Catechin ($C_{15}H_{14}O_{6}$, MW: 290)

(2R, 3S)

(-) Epicatechin ($C_{15}H_{14}O_{6}$, MW: 290)

(2R, 3R)

(-) Catechin gallate ($C_{22}H_{18}O_{10}$, MW: 442)

(2S, 3R)

(-) Epicatechin gallate ($C_{22}H_{18}O_{10}$, MW: 442)

(2R, 3R)
(-)-Gallocatechin gallate (C_{22}H_{18}O_{11}, MW: 458)

(-)-Epigallocatechin gallate (C_{22}H_{18}O_{11}, MW: 458)

(-)-Epigallocatechin (C_{15}H_{14}O_{7}, MW: 306)

Gallic acid (C_{7}H_{6}O_{5}, MW: 170)