CHAPTER-6

ESTIMATION OF GALLIC ACID USING VANADIUM (V)–ERIOCHROME CYANINE-R SYSTEM AND APPLICATION TO SOME HERBAL PLANTS, FRUIT JUICES AND WINE SAMPLES
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6 Introduction

6.1 Eriochrome cyanine R (ECR)

The IUPAC name of Eriochrome cyanine R is Tri-sodium5-[(Z)-(3-carboxylato-5-methyl-4-oxo-2, 5-cyclohexadien-1-ylidene) (2-sulfonatophenyl)methyl] -2-hydroxy-3-methyl benzoate. (Molecular formula= C_{23}H_{15}O_{9}SNa_{3}, Molecular mass =536.40). It is a red to reddish brown colour solid and soluble in water. It finds use as a redox indicator in analytical chemistry, and as a microscopic stain in biology [1]. It has many analytical applications such as in the extractive-spectrophotometric determination of Chlorpromazine [2], estimation of beryllium [3], determination of Fluoride with Zirconium [4], Photometric [5], and Solid-Phase Spectrophotometry determination of trace amounts of aluminium in water [6], and in rapid modified method for determination of aluminum in water [7]. Eriochrome cyanine R film modified glassy carbon electrode is used for selective oxidation of serotonin and norepinephrine [8]. ECR finds application also in the column pre-concentration of Aluminum [9], Fluorimetric determination of human serum albumin, in speciation of vanadium (IV) and vanadium (V) in natural waters by solid phase spectrophotometry [10]. The structural formula of ECR is as shown below.

![Structural formula of Eriochrome cyanine R](image)

In the present study an attempt was made to develop a simple and inexpensive spectrophotometric method for the estimation of gallic acid using ECR and vanadium (V) as a chromogenic system. In the present study ECR forms purple complex with vanadium (IV) after reduction of vanadium (V) with gallic acid. This reaction is exploited for the estimation of gallic acid in fruit, plant and wine samples.
6.2 Experimental

6.2.1 Instrumentation

A JASCO model UVIDEC-610 ultraviolet (UV-Vis) spectrophotometer with 1.0-cm matched cells was used for all absorbance measurements. A water bath shaker (NSW 133, New Delhi, India) was used to maintain constant temperature while incubation. CM 101 cyclo mixer and REMI centrifuge (Bombay, INDIA) was used to homogenize and centrifuge the extracts prepared.

6.3 Reagents and solutions

All the chemicals used in the assay were of analytical grade and double distilled water was used throughout the assay procedure.

6.3.1 Sodium metavanadate

Sodium metavanadate was purchased from E. Merck (Germany) and 50 µg/mL solution of vanadium was prepared by dissolving 12 mg in 0.5 mL HCl (0.2M) and diluting the solution to 100 mL with water.

6.3.2 Eriochrome cyanine R (ECR)

Eriochrome cyanine R (ECR) was obtained from E. Merck (Germany) and 500 µg/mL solution of ECR was prepared by dissolving 50 mg in 100 mL of water.

6.3.4 Cetyl trimethyl ammonium bromide (CTAB)

Cationic surfactant cetyl trimethyl ammonium bromide was from Sigma Aldrich and 200 µg/mL solution of CTAB was prepared by dissolving 20 mg in 100 mL of acetate buffer of pH 5.5.

6.3.5 Acetic acid/sodium acetate buffer

An acetic acid/sodium acetate buffer (0.2 M) of pH 5.5 was prepared by dissolving suitable quantity of sodium acetate in water and diluting the glacial acetic acid in water.

6.3.6 Gallic acid solution

Gallic acid was purchased from Sigma Aldrich and a stock solution of 200 µg/mL was prepared by dissolving 2 mg in 10 mL of standard flask using double distilled water.
6.4 General assay procedure for estimation of gallic acid

In a 10 mL standard flask 7.5 μg/mL vanadium (V) solution, 100 μL of various concentrations of standard gallic acid (0.4-9.0 μg/mL) solution, 100 μg/mL ECR solution and 40 μg/mL of CTAB were added. The solutions were swirled and allowed to stand for 30 min. The absorbance of the reaction mixture was measured at 580 nm with reference to the blank containing all reagents except gallic acid. Similarly 100 μL aqueous extract of medicinal plant, fruit and wine sample were subjected to gallic acid content test as described above. The absorbance of each was recorded at the wavelength of 580 nm.

6.5 Results and Discussion

6.5.1 Spectral characteristics

The absorption spectrum of colored product was measured in a spectrophotometer containing the reaction mixture of 7.5 μg/mL vanadium (V) solution, 100 μL of various concentrations of standard gallic acid solution (4, 6.5, and 9.0 μg/mL) and 100 μg/mL ECR solution and 40 μg/mL of CTAB in the wavelength region 400–700 nm against the reagent blank at room temperature. The optimum wavelength with maximum absorption was found to be 580 nm. The absorption spectra of the colored products are shown in Figure 6.1 for various concentrations of gallic acid. The intensity of the coloured species at 580 nm increased proportionately with increase in gallic acid concentration. This occurred as a result of the redox reaction involved. Hence 580 nm wavelength was selected for all further studies.
Figure 6.1 Absorption spectra of V(V) + ECR + CTAB in buffer + various concentrations of gallic acid. (▲) 4.0 µg/mL (■) 6.5 µg/mL (♦) 9.0 µg/mL

6.5 Optimum reaction condition

In designing the proposed spectroscopic procedure, all the reaction conditions were optimized in order to attain a rapid formation of the complex with maximum stability and sensitivity. This was done by conducting controlled experiments by measuring the absorbance at 580 nm for concentration of the reagents used and other parameters by varying one factor at a time and fixing the others constant.

6.5.1 Effect of reagent concentration

To enhance the performance of the proposed method for the determination of gallic acid, effects of concentration of vanadium (V), ECR and CTAB were studied by using a fixed concentration of GA in 10-mL standard flasks. It was found that 7.5 µg/mL of V (V) were needed for getting maximum color intensity. Hence, 7.5 µg/mL of V (V) was selected for all further studies. ECR (500 µg/mL) was tested in volumes 0.5 to 2.5mL and optimum result was obtained with 100 µg/mL of ECR. Hence, this was used for further experiments. The effect of concentration of CTAB on the formation, sensitivity and stability of the colored species was studied. It was observed that 40 µg/mL of CTAB was found optimum with respect to sensitivity, linearity, and stability of the purple colored species. These optimized concentrations and conditions were adopted for all further studies in 10 mL of the reaction mixture.
6.5.2 Effect of time and temperature on color stability of colored product

The absorbance of the violet colored species was studied from 0 to 60 minutes at every 5 minutes intervals. The species was found stable for 50 min. Maximum and constant absorbance was obtained between 20 to 50 min. Therefore, 30 min of incubation time was selected for studies.

Temperature sensitivity was determined by pre-incubating 7.5 µg/mL of vanadium (V) solutions, 20 µg/mL of standard gallic acid solution and 100 µg/mL of ECR and 40 µg/mL of CTAB in 10 mL for 30 minute at temperature ranges of 0–60°C. The temperature was registered as a function of absorbance of the colored species at 580 nm. No significant changes were observed in the absorbance value between 20 and 40°C. Hence the reaction was carried out at room temperature (28±5°C).

6.5.3 Study of the reaction route

The reaction route was investigated by studying products of the reaction. Vanadium (V) and gallic acid are colorless, while the product of the reaction Vanadium (IV) with ECR is purple in colour, i.e., gallic acid reduces vanadium (V) to vanadium (IV), thus forming V(IV)-ECR complex, which was stabilized by adding cationic surfactant CTAB in buffer to form ternary species. The probable reaction path way for the proposed reaction is given in the Scheme 6.1.
Scheme 6.1  Proposed reaction pathway for formation of vanadium (IV)-ECR complex for estimation of gallic acid.
6.6 Analytical appraisal of the method

6.6.1 Calibration curve of gallic acid solution alone and in herbal plant, fruit and wine sample

Calibration graphs were constructed using standard solutions under optimum experimental condition. A linear relationship was observed between the absorbance and concentration of gallic acid from 0.4-9.0 µg/mL. The molar absorptivity and sandell’s sensitivity for gallic acid were calculated from beer’s law. The ringbom plots demonstrated the optimum range of concentration of 0.6-9.0 µg/mL for gallic acid, by the proposed method. The graph showed an intercept, which was calculated by the least square methods regression equations. \( y = a + bc \), at 580 nm. Where \( c \) is the concentration of gallic acid in µg/mL, ‘a’ is the intercept and \( y \) is the absorbance of the colored solution. The value obtained gave a linear plot in the concentration range from 0.4 to 9 µg/mL with a slope of 7.89 x10^4 and a correlation coefficient 0.995 \((n = 6)\). Regression analysis of the results shown in Table 6.1 revealed that absorbance verses concentration relationships with the proposed procedure were perfectly linear \((R^2 = 0.995)\) for the gallic acid and also shown in Figure 6.2. Two-way analysis showed that the calibration lines of gallic acid in the aqueous solution and in complex (plants, fruits and wine) samples were essentially parallel and there was no difference at 95% confidence level between the slopes of these lines as shown in Figures. 6.3, 6.3a and 6.3b. This means there was no interaction between the tested gallic acid and the gallic acid constituents of complex matrices ((plants, fruits and wine) that would otherwise cause chemical deviations from Beer’s law.
Table 6.1  Optical parameters in the estimation of Gallic acid

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>580</td>
</tr>
<tr>
<td>Beer’s Law range ($\mu$g/mL)</td>
<td>0.4-9.0</td>
</tr>
<tr>
<td>$\varepsilon$ (L mol$^{-1}$ cm$^{-1}$ x 10$^4$)</td>
<td>8.12</td>
</tr>
<tr>
<td>Sandell’s sensitivity ($\mu$g/cm$^2$) x 10$^{-3}$</td>
<td>0.56</td>
</tr>
<tr>
<td>Limit of detection ($\mu$g/mL) x 10$^{-3}$</td>
<td>4.4</td>
</tr>
<tr>
<td>Limit of quantification ($\mu$g/mL)</td>
<td>0.0146</td>
</tr>
<tr>
<td>Slope (b) ± tS$_b^a$</td>
<td>0.079±0.0042</td>
</tr>
<tr>
<td>Intercept (a) ± tS$_a^b$</td>
<td>-0.040±0.013</td>
</tr>
<tr>
<td>Correlation coefficient (R)</td>
<td>0.997</td>
</tr>
<tr>
<td>RSD$^c$</td>
<td>1.48</td>
</tr>
</tbody>
</table>

$^a$Confidence interval for slope at 95% confidence limit for 6 degree of freedom.

$^b$Confidence interval for intercept at 95% confidence limit 6 degree of freedom.

$^c$Average of 6 determinations (concentrations of 1.5 $\mu$g/mL of pure gallic acid)
Figure 6.2 The calibration curve of gallic acid

Figure 6.3 The calibration curve of gallic acid alone and in wine sample. (●) For gallic acid solutions of varying concentrations. (○) For 1 mL wine sample + gallic acid solutions of varying concentration
**Figure 6.3a** The calibration curve of gallic acid alone and in Amla (*Phyllanthus emblica*) leaves extract. (▲) Gallic acid solutions of varying concentration (●) 0.2 mL Amla (*Phyllanthus emblica*) leaves extract + gallic acid solution of varying concentration

**Figure 6.3b** The calibration curve of gallic acid alone and in orange juice (●) Gallic acid solutions of varying concentration (-) 0.4 mL range (*Citrus aurantium Risso*) juice + gallic acid solutions of varying concentration
6.6.2 Interference studies

The effect of common excipients present in the herbal, fruit, and wine sample were studied by analyzing sample solutions containing the 4.5 µg/mL of gallic acid as mentioned in Table 6.2. The tolerance limit was defined as the concentration which gave an error of ± 3.0% in the determination of gallic acid. The common excipients such as sucrose, glucose, lactose, salicylic acid, benzoic acid, BHT, BHA, ferulic acid, p-coumaric acid, catechin, glycine, Ca²⁺, Cl⁻, sorbinose and glycol had no effect on the analysis of gallic acid as the tolerance ratio for these were found to nearly 1000. The other excipients interference was as summarized in Table 6.2 for the determination of gallic acid such as hydrogen peroxide, Fe(III), Cl⁻, Al³⁺, citric acid, tartaric acid, sulfosalicylic acid, phenol, resorcinol, Mn²⁺, ascorbic acid, phloroglucinol and hydrogen peroxide. Among them Fe (II), hydrogen peroxide, ascorbic acid, phloroglucinol and tannic acid interferes seriously as the tolerance ratio for these species is very less 0.20.
Table 6.2  Interference study

<table>
<thead>
<tr>
<th>Inhibiting species</th>
<th>Tolerance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch, glucose, sucrose, lactose salicylic acid, benzoic acid, ( p )-coumaric acid, ferulic acid, glycol, chloride, glycine, sorbinose, catechin, ( \text{Ca}^{2+} ), BHT, BHA, ( \text{Al}^{3+} ), Citric acid,</td>
<td>1000</td>
</tr>
<tr>
<td>Nitrophenol, 2,4 dinitrophenol, Tartaric acid</td>
<td>500</td>
</tr>
<tr>
<td>Sulfosalicylic acid, oxalic acid, Phenol</td>
<td>200</td>
</tr>
<tr>
<td>Resorcinol, ( \text{Mn}^{2+} ), Fe(^{3+}), Fe(^{2+}), Hydrogen peroxide, phluroglucinol, ascorbic acid tannic acid</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^{a}\)Tolerance ratio corresponds to the ratio of inhibiting species concentration to that of concentration of gallic acid (5µg/mL)
6.7 Linearity, precision and accuracy

The proposed method was validated for linearity, precision and accuracy. Method linearity was established by measuring at least four solutions of gallic acid compound at known concentrations. Each measurement was repeated six times and the mean value was used for calculation of the regression lines. The precision of the method was evaluated by estimating the method repeatability. The method repeatability was investigated by measuring gallic acid for six times at different concentrations. The mean value of the relative standard deviation of the tested solutions covering all the working range was 1.48%. Similarly, the assay for daily precision (inter day precision) at the same concentration level was repeated for 6 consecutive days as presented in Table 6.3. The reliability and accuracy of the proposed method were further ascertained through recovery studies using the standard addition method by adding different amount of gallic acid to the preanalyzed fruit juice samples such that the cumulative amount after adding the gallic acid did not exceed their linearity range. The mean recovery was estimated to be equal to 100.3±5.5%.
### Table 6.3 Intra day and inter day precision data

<table>
<thead>
<tr>
<th>Amount of gallic acid taken (µg/mL)</th>
<th>Intra day, Recovery ±RSD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inter day, Recovery ±RSD&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>0.79±0.1</td>
<td>0.81±0.3</td>
</tr>
<tr>
<td>1.2</td>
<td>1.19±0.2</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>2.8</td>
<td>2.78±0.3</td>
<td>2.81±0.5</td>
</tr>
<tr>
<td>3.5</td>
<td>3.4±0.1</td>
<td>3.51±0.1</td>
</tr>
<tr>
<td>6.0</td>
<td>5.82±0.1</td>
<td>5.9±0.3</td>
</tr>
<tr>
<td>7.5</td>
<td>7.43±0.1</td>
<td>7.5±0.2</td>
</tr>
<tr>
<td>8.0</td>
<td>8.1±0.7</td>
<td>7.96±0.14</td>
</tr>
<tr>
<td>8.5</td>
<td>8.6±0.1</td>
<td>8.4±0.19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean of 6 determinations,

<sup>b</sup> Mean of 6 determinations performed over a period of 6 days
6.8 Application of the method to natural samples

Medicinal plants Shankapushpi (*Convolvulus pluricaulis*), Amla (*Phyllanthus emblica*), Shallika (*Boswellia serrata*), Fenugreek (*Trigonella foenum-graecum*) were collected from Ayurvedic centre in Mysore. Red grape (*Citrus paradise*), orange (*Citrus aurantium Risso*), green grape (*Citrus paradise*) and also wine samples were purchased from super market in Mysore. Gallic acid is a natural phenolic antioxidant compound widely distributed in herbal plants, fruits, wine and vegetables. Structurally gallic acid has phenolic groups that serve as a source of readily available hydrogen atoms such that the subsequent radicals produced can be delocalized over the phenolic structure [11, 12]. The interest in gallic acid compound is due to its pharmacological activities such as radical scavenging [13, 14], antimutagenic, anticarcinogenic and antioxidant property [15-17]. Gallic acid is incorporated into the alcoholic beverages as a result of hydrolysis of tannins and is related to the aging/maturation process.

6.8.1 Preparation of herbal extracts

The plant materials collected were dried for 2 h in an oven at 60°C and ground to fine powder form. Powder samples (1g each) were refluxed in 50 mL double distilled water (using air cooled condenser and round bottomed flask) for 1 h using magnetic stirrer and heater apparatus. The refluxed solution was filtered using Whatmann no. 41 filter paper. The aqueous plant extracts were prepared freshly for reliability of the results. However, the assay results did not change even after 48 h storage of the medicinal plant extracts at 4°C.

6.8.2 Preparation of fruit extracts

Fruits collected were cut into half and blended by using a food processor and strained to remove pip, pulp and sacs. The freshly prepared extract was then filtered using Whatmann no. 41 filter paper so that no turbidity was present in the extract. The clear solution of the extract was accurately diluted for proposed method and reference NBD [18] assay for determining gallic acid.

Gallic acid content of fruit, some medicinal plants and wine samples by the proposed spectrophotometric method was evaluated. The absorbance was plotted in the calibration curve of gallic acid from which gallic acid content of the sample can be calculated. The results obtained by the developed method were compared with those obtained by NBD assay and the results are tabulated in Table 6.4. Gallic acid determination can be done by various methods including liquid chromatography [19-26] gas chromatography with mass spectrometric detection [23], capillary
electrophoresis [27], spectrometry [28], chemiluminescence with flow injection analysis [29] and voltammetry [30]. Chromatographic methods are time consuming, expensive and involve complicated procedure for the assay to perform. HPLC [31, 32] and capillary electrophoresis are powerful separation tools. But they have disadvantages in analyzing different samples. In most cases analysis follows sample pretreatment which includes liquid–liquid [20, 21, 33] or solid-phase extraction [22].

The reliability and accuracy of the proposed method were further ascertained through recovery studies using the standard addition method by adding different amounts of gallic acid to the pre-analyzed samples. The $F$-test and Student $t$ test values at 95% confidence level confirmed that there were no significant differences between the precisions (variances) of the proposed and reference methods.
### Table 6.4 Application of the proposed method for the determination of gallic acid in various samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gallic acid found (µg/mL)</th>
<th>Gallic acid found (µg/mL)</th>
<th>Recovery %</th>
<th>RSD</th>
<th>t-test</th>
<th>F-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proposed method</td>
<td>Reference method</td>
<td>Gallic acid added (µg/mL)</td>
<td>Proposed method</td>
<td>Reference method [27]</td>
<td></td>
</tr>
<tr>
<td>Red Grape</td>
<td>4.3</td>
<td>4.1</td>
<td>2.0</td>
<td>5.9</td>
<td>5.1</td>
<td>93.65</td>
</tr>
<tr>
<td>Green grape</td>
<td>6.9</td>
<td>----</td>
<td>2.0</td>
<td>7.76</td>
<td>1.77</td>
<td>85.39</td>
</tr>
<tr>
<td>Wine-1</td>
<td>2.9</td>
<td>2.95</td>
<td>3.0</td>
<td>6.1</td>
<td>5.8</td>
<td>103.3</td>
</tr>
<tr>
<td>Wine-2</td>
<td>1.96</td>
<td>1.8</td>
<td>1.5</td>
<td>3.6</td>
<td>3.5</td>
<td>95.74</td>
</tr>
<tr>
<td>Amla</td>
<td>4.8</td>
<td>4.2</td>
<td>4.5</td>
<td>8.9</td>
<td>8.2</td>
<td>95.69</td>
</tr>
<tr>
<td>Shankapushpi</td>
<td>1.6</td>
<td>----</td>
<td>4.5</td>
<td>6.0</td>
<td>4.42</td>
<td>98.36</td>
</tr>
<tr>
<td>Shallaki</td>
<td>2.4</td>
<td>2.7</td>
<td>3.5</td>
<td>6.1</td>
<td>6.0</td>
<td>103.3</td>
</tr>
<tr>
<td>Fenugreek</td>
<td>6.9</td>
<td>6.1</td>
<td>1.5</td>
<td>8.5</td>
<td>8.0</td>
<td>100.1</td>
</tr>
</tbody>
</table>

*a* Calculated for average of six determinations  
*b* Tabulated $F$ value for (5,5) $df$ at $P$ (0.05) is 5.05  
*c* Tabulated $t$ value for 5 $df$ at $P$ (0.05) is 2.28.  
Wine 1 and 2 collected from different supermarkets
6.9 Conclusion

The proposed method has distinct advantages over other assay methods with respect to simplicity, availability and stability of reagents, reproducibility over a wide concentration ranges and the analysis requires only room temperature condition. Only aqueous extraction is needed and the method is applicable to medicinal plants as well as wine samples. The reagent is fast enough to oxidize gallic acid. It can be used by conventional laboratories using standard equipment like a colorimeter. The total gallic acid content obtained by the proposed method is comparable with the reference method. In this system no sample pretreatment is needed, linearity is maintained over a wide range of concentrations and sensitivity is high. For these reasons, the proposed vanadium (V)-ECR system appears to be a viable method over the reference method.
Literature cited:


