A Simple Spectrophotometric Method for Determination of Glyoxylic Acid in its Synthesis Mixture

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ABSTRACT

A new simple and reliable spectrophotometric method is described to determine glyoxylic acid in its synthesis reaction mixture containing oxalic acid, glycolic acid, acetic acid, glyoxal and ethylene glycol by means of a modified Hopkins-Cole reaction between glyoxylic acid and tryptophan in presence of ferric chloride and concentrated sulphuric acid. The linear range of glyoxylic acid concentration is 0 - 0.028 M. The limits of detection LOD and quantitation LOQ are 0.0019 M and 0.00577 M, respectively. The LOD, LOQ, standard deviation, relative standard deviation and recovery ratio of the proposed method are comparable with a selected HPLC reference method. Both methods displayed same precision and credibility. Reaction stoichiometry between tryptophan and glyoxylic acid is assumed to be 2:3. Reaction mechanism has been postulated based on identified molar ratios of reactants. Glyoxal gave a negative test with tryptophan although it is a di-aldehyde.
**Key words:** glyoxylic acid, oxoacetic acid, spectrophotometric, determination, tryptophan

1- Introduction

Glyoxylic acid (GA) is an important organic acid in the chemical, cosmetic, pharmaceutical, and food industries. It is found in plants and involved in the metabolic cycle of animals. Glyoxylic acid is produced in several ways; by nitric acid oxidation of glyoxal, by catalytic oxidation of ethylene or acetaldehyde, and by electrochemical reduction of oxalic acid [1].

The determination of glyoxylic acid in its electrochemical synthesis reaction mixture is a difficult process because this mixture contains carboxylic acids with convergent acidic dissociation constants and other compounds carrying organic groups that can undergo oxidation or reduction such as ethylene glycol, glyoxal and glycolic acid. Thus, it is expected that glyoxylic acid will be very difficult to determine quantitatively using traditional analytical methods such as acid-base or redox titrations, separation, or precipitation [2, 3].

This paper provides a simple, reliable, and inexpensive spectrophotometric method for determination of glyoxylic acid in such reaction mixtures directly without the need of separation. The method depends on a colored chromogen product formation
between glyoxylic acid and tryptophan when they react according to a reaction discovered in 1901 by Hopkins and Cole [4], see eq. (1). They suggested that one molecule of glyoxylic acid can react with two molecules of tryptophan to give a crimson-violet color that absorbs at 540-545 nm. This reaction ideally occurs in an anhydrous medium pledged by concentrated sulphuric acid [5-8].

\[ \text{Tryptophan} + 2 \text{Glyoxylic acid} \xrightarrow{\text{H}_2\text{SO}_4} \text{Crimson colored product} \]  

Since its discovery, Hopkins-Cole test has been neither easy nor reliable for tryptophan quantitative measurements due to slow rate of product formation, various color evolutions and low stability of the product. This pushed copious researchers to suggest many improvements as in [9-17]. In [9,10] auxiliary materials such as copper sulphate were added that gave dark crimson-violet color product. Cary 1928 [9] was the pioneer in the description of the crimson-violet color absorption at 560 nm. Other researchers [11-14] studied the compounds and stoichiometry that can form between organic aldehydes and tryptophan. Quantitative determination of tryptophan in proteins succeeded by addition of mild oxidants such as persulfate and Fe$^{3+}$ ions, which accelerated chromogen appearance and increased its stability [15-17].
However, the test wasn’t satisfactory for quantitative glyoxylic acid determination so far, eventually due to the factors affecting its accuracy as to be elucidated in the context of this article.

Glyoxylic acid spectrophotometric analysis has been also used to determine enzyme activity by a color reaction with 2-aminobenzaldehyde and glycine [18] or by forming a color product with phenylhydrazine, but this method can be affected by the presence of other aldehydes in the mixture [19].

Determination of glyoxylic acid in similar organic mixtures using HPLC technique has been successful in [20, 21] using refractive index and photo diode array detectors, respectively. Another method required the conversion of glyoxylic acid into one of its derivatives with 2,4-dinitrophenylhydrazine, [22,23]. Glyoxylic acid in other mixtures was also determined by this technique however using a more sensitive detector; the fluorescence detector, after converting glyoxylic acid into a fluorocarbon derivative with detection limit reaching 5 nanomol/L, [24].

Other methods such as differential pulse polarography technique [25] and ion chromatography technology [26] have been likewise used to analyze similar reaction mixture with diverse degrees of success.
The aim of this work is to provide a new rapid, reliable, and inexpensive spectrophotometric method for determination of glyoxylic acid in electrochemical synthesis mixture directly without the need of separation.

**Materials:**

Oxalic acid (BDH) 99%, Glyoxylic acid (Merck) water solution 50 %, Glycolic acid (Sigma Aldrich) 99%, Glyoxal (Merck) water solution 40%, Ethylene glycol (BDH) 99.5%, Glacial Acetic acid (SDFCL) 99.5%, L-Tryptophan (BDH) 93%, Sulphuric Acid (Panreac) 95-98 %, Ferric chloride hexahydrate (Avonchem) 98% fresh packaging.

**Instruments:**

Spectrophotometer (721-2000) - Caihong Corporation Limited.
HPLC – Column: Knauer Eurokat columns H Form, RI detector (Knauer).

**Experimental work:**

The reaction of Hopkins and Cole (eq. 1) was applied on each component of the reaction mixture consisting of oxalic acid, glyoxylic acid, glycolic acid, glyoxal, ethylene glycol and acetic acid solution in water at 0.028 M concentration in the following procedure:

To each individual test tube place 0.25 mL of the reactant solution, 0.6 mL of tryptophan 0.016 M solution and 2 mL of fresh ferric chloride 0.025 M solution. Mix
thoroughly, then pour 5 mL of concentrated sulphuric acid divided in around 1 mL portions over 30 minutes under continuous stirring in a cold bath of running tap water to prevent any increase in temperature beyond 50°C. Then read absorbance at 560 nm within 10-20 minutes. If to allow samples to stand elongated time, secondary transformations involving Fe²⁺ ion might cause a color change and test failure.

**Results and discussion:**

1. **Applicability of the method for quantitative determination of glyoxylic Acid in mixture**

Figure (1) shows the spectra obtained using proposed test procedure on glyoxylic acid and other mixture constituents in addition to a blank sample. Water replaced the organic compound in the blank sample.
Three absorption maxima can be observed; at wavelengths 440, 500, and 560 nm. The maximum at 560 nm is characteristic for glyoxylic acid. The other two maxima are common for all compounds, including the blank sample. Thus they can be attributed to the interaction with ferric chloride, see paragraph 2.2. Accordingly, the Hopkins-Cole reaction test by means of proposed method is selective only to glyoxylic acid without any interference of other compounds of the studied mixture including glyoxal containing aldehyde group. The baseline, however, has to be zero set using the blank sample in order to compensate the minor effect of ferric chloride interaction with tryptophan.

**Figure (1):** Optical absorption spectrum of reaction product between each component of the studied mixture and tryptophan.

**Figure (2):** Optical absorption spectrum of a mixture containing oxalic acid, glyoxylic acid, glycolic acid, glyoxal, ethylene glycol and acetic acid in comparison with pure glyoxylic acid, each at a concentration of 0.028 M, after a reaction with tryptophan in presence of ferric chloride and concentrated sulphuric acid.
Furthermore, Figure (2) depicts the spectra obtained for 0.028 M glyoxylic acid solution alone and 0.028 M glyoxylic acid in mixture solution using the proposed test procedure. It can be clearly seen that both pure glyoxylic acid and the glyoxylic acid in mixture exhibit practically the same absorbance at 560 nm.

2. Optimization of the method variables

The process of the optimization aims to make the Hopkins-Cole reaction only related to the concentration of glyoxylic acid in the reaction mixture.

2.1 Influence of concentrated sulphuric acid

Added volume of concentrated sulphuric acid was varied; 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 mL, while amounts of reactants were kept constant at: 0.25 mL glyoxylic acid 0.028 M solution, 0.6 mL tryptophan 0.016 M solution and 2 mL ferric chloride 0.025M solution. The final volume of liquid in tubes was corrected with distilled water. Test procedure was described in the experimental work section. Figure (3) shows an increase of optical absorbance up to 5 mL sulphuric acid addition. Which means that sufficient amount of concentrated sulphuric acid must be present in order to absorb reaction- and hydration water. Otherwise the aldehyde group of glyoxylic
acid may be hydrated to germinal diol (K=300 at 25 °C), and eventually further dimerized to hemiacetal, thus making it not be available for reaction with tryptophan.

**Figure (3):** Optical absorption versus volume of concentrated sulfuric acid in the reagent with 0.25 mL glyoxylic acid 0.028 M, 0.6 mL tryptophan 0.016 M and 2 mL of ferric chloride 0.025 M

2.2 *Influence of ferric chloride:*

The added volume of ferric chloride 0.025 M solution was varied; 0.25, 0.5, 1, 1.5, and 2 mL, while the other mixture constituents and test procedure remained unchanged, see paragraph 2.1. Figure (4) shows a proportional increase of absorbance with increasing ferric chloride solution volume up to 1 mL, followed by a slight further increase up to 2 mL ferric chloride addition. Thus, oxidizing agent
need to be present in reaction mixture in sufficient amount for satisfactorily performance.

**Figure (4):** Optical absorption versus volume of ferric chloride in the reagent at 0.25 mL glyoxylic acid 0.028 M and 0.6 ml tryptophan 0.016 M.

To confirm its responsibility for absorption maxima at 440 and 500 nm in Figures (1) and (2), a separate test has been run without addition of ferric chloride solution. Figure (5) displays the disappearance of these maxima. However the maximum at 560 nm, which is representative for glyoxylic acid, showed far less intensity than that shown in Figure (1). It hence demonstrates that ferric chloride, in agreement with [16, 17], increases the yield of pigment product.
The mechanism of the iron III ion effect can be ascribed to oxidation reaction in the leuco dye molecule, which results from condensation of two tryptophan molecules with one glyoxylic acid molecule, see Figure (6) and mechanism discussion paragraph.

**Figure (5):** Optical absorption spectrum for reaction product of glyoxylic acid and tryptophan 0.016 M in presence of concentrated sulfuric acid and absence of ferric chloride.

The mechanism of the iron III ion effect can be ascribed to oxidation reaction in the leuco dye molecule, which results from condensation of two tryptophan molecules with one glyoxylic acid molecule, see Figure (6) and mechanism discussion paragraph.

**Figure (6):** Proposed reaction from leuco- to pigment compound under effect of iron ion as of an oxidizing agent, concurring Fearon [13]

2.3 **Influence of Tryptophan:**

The volume of tryptophan 0.016 M solution was varied; 0.1, 0.2, 0.25, 0.3, 0.4, 0.6, 0.8, 1.0 mL, while other mixture constituents and test procedure remained unchanged, see paragraph 2.1. As can be seen in Figure (7), there is a proportional
Increase in absorbance with increased added volume of tryptophan solution until reaching 0.3 mL. At this point, tryptophan amount \((0.48 \times 10^{-5}\) moles\) should have reacted with the complete amount of glyoxylic acid present in the sample (amounting to \(0.7 \times 10^{-5}\) moles). Afterwards, absorbance increased sluggishly nearing a plateau at 0.6 mL (or \(0.6 \times 0.016=0.96 \times 10^{-5}\) mol). The later increase in absorbance with larger tryptophan solution volume can be ascribed to the interaction between tryptophan and iron II ion produced during reaction. We decided to keep the amount of tryptophan in this excess in all after coming tests to compensate for its loss in reaction with iron II ion.

**Figure (7):** Optical absorption versus volume of tryptophan 0.016M solution in the reagent at 0.25 mL glyoxylic acid 0.028 M and 2 mL ferric chloride 0.025 M and 5 mL concentrated sulfuric acid

**2.4 Influence of temperature:**
Numerous tests were conducted (not shown here) at different temperatures ranging from ambient to 90°C. At ambient temperature, it was practically difficult to conduct a successful test due to rapid and intense heat generation by the reaction itself and by sulphuric acid hydration. Whereas, tests carried out above 50°C produced differing colors from crimson-violet and lacked reproducibility. This is not astonishing, because tryptophan-glyoxylic acid reds are known to be greatest unstable pigments. They readily lose CO₂ and soon oxidize on little warming [13]. Fortunately, tests conducted at temperatures 48-50°C exhibited very good reproducibility and formed stable color.

3. The standard curve:

The standard calibration curve was prepared for glyoxylic acid concentration according to the optimized procedure conditions. The spectrophotometer was zero sat using the blank sample that comprised water instead of glyoxylic acid in test mixture. Figure (8) shows the relation between absorbance and glyoxylic acid concentration at 560 nm. A linear relation between color intensity and glyoxylic acid concentration was obtained in the range 0–0.028 M representative for the calibration curve for glyoxylic acid. At the point 0.028 M, it is supposed that glyoxylic acid amount \((0.7 \times 10^{-5}\) moles\)) reacts with the corresponding stoichiometric amount of tryptophan \((0.48 \times 10^{-5}\) moles from Figure (7)). The further slight increase in
absorbance afterwards can be ascribed to interaction of the excess of glyoxylic acid with tryptophan’s secondary products with iron II ion.

**Figure (8):** Standard curve for glyoxylic acid and optical absorption in relation to glyoxylic acid concentration at optimized conditions, 0.6 mL tryptophan 0.016 M and 2 mL ferric chloride 0.025 M and 5 mL concentrated sulfuric acid.

4. **Mechanism Discussion**

In the early stage of Hopkins-Cole test discovery it was confirmed that tryptophan is capable to give two distinct colored products when reacted with glyoxylic acid [13]: With the minimum amount of the aldehyde and temperature kept below 15 °C, the first colored product is carmine. The mechanism of its formation is assumed to follow the scheme postulated in Figure (9). Here, the aldehyde unites the carbon atoms of the benzene rings of two tryptophan molecules to form the leuco compound, this upon oxidation forms the quinonoid configuration in the molecule responsible of the color (pigment compound (1)); the same product that appears in
eq. (1). Such suggested pathway requires the presence of a strong condensing agent like concentrated H$_2$SO$_4$ or pure acetic acid, and a mild oxidant like Fe$^{+3}_{aq}$.

![Proposed reaction mechanism of condensation reaction](image)

**Figure (9):** Proposed reaction mechanism of condensation reaction involving two tryptophan molecules and one molecule of glyoxylic acid

By allowing the temperature to rise to around 50 °C, sufficient activation energy would be available to trigger further condensation steps leading to the second colored product (pigment compound (2)) that appears then violet to blue; depending on water content; blue only in its solid form. Thus, the firstly formed pigment compound (1) condenses further with aldehydes of two more glyoxylic acids. This condensation, nevertheless, occurs between the nitrogen of side-chain and the carbon atom of the indole nucleus in same tryptophan molecule. It leads consequently to closure of the tryptophan side-chain and formation of a pyridine nucleus, [13]. Such colored product might be formed according to the pathway speculated in Figure (10).
It also absorbs at the same wave length; 560 nm due to alike quinonoid configuration in the molecule responsible of the color.

Friedman & Finley, 1971 [27] proposed another reaction mechanism between glyoxylic acid and tryptophan in presence of a reducing agent, NaNO₂, in which glyoxylic acid unites the carbon atoms of the indole nucleus instead of benzene rings of two tryptophan molecules to form the leuco compound.

In our work we calculated the reaction stoichiometry based on Figures (7) & (8). It is found that the reaction stoichiometry of tryptophan to glyoxylic acid is 2:3, thus confirming the proposed mechanism.
Figure (10): Proposed reaction mechanism of condensation involving two molecules of tryptophan and three molecules of glyoxylic acid

Not all aldehydes seem to react with tryptophan, because glyoxal gave a negative test result. It appears that only aldehydes that react readily with the benzene ring, indole nucleus or amino-group are capable of condensing with tryptophan.

5. Validation of the method:

To validate our method we measured glyoxylic acid in two organic mixtures comprised the studied compounds with known concentrations using this method and a reference HPLC method after [21]. The results are shown in Tables (1-3). Spectrophotometric method achieved comparable results with the selected HPLC method in terms of lower standard deviation, relative standard deviation and better recovery. Glyoxylic acid can be determined by the proposed spectrophotometric method in more diluted solutions as noticed from LOD and LOQ values. Both methods however ensure same precision and same credibility based on F- and t-statistical tests respectively, because both exhibit calculated test values smaller than critical one.
Table (1): Comparison between spectrophotometric and HPLC methods in terms of standard deviation, recovery % and RSD%

| Method       | Glyoxylate acid concentration in the mixture M | Determined glyoxylate acid in the mixture M | Standard deviation ± M | Recovery % | ¹RSD % |
|--------------|-----------------------------------------------|--------------------------------------------|------------------------|------------|-------|
| Spectrophotometric | 0.35 | 0.354 | 0.00132 | 101 | 0.37 |
| HPLC         | 0.50 | 0.504 | 0.00110 | 109 | 0.22 |
| HPLC         | 0.50 | 0.508 | 0.00457 | 102 | 0.90 |

*Samples diluted 25 times in the spectrophotometric determination only.

Table (2): Statistical comparison between spectrophotometric and HPLC methods

| Method       | Linear Regression Equation | Correlation Coefficient $R^2$ | $^2$LOD M | $^3$LOQ M |
|--------------|----------------------------|-------------------------------|-----------|-----------|
| Spectrophotometric | $y = 56.737x + 0.016$ | 0.998 | 0.001905 | 0.005773 |
| HPLC         | $y = 24337025.58x + 219631.2716$ | 0.997 | 0.052893 | 0.160282 |

Table (3): Statistical comparison between spectrophotometric and HPLC methods-continued

| Method       | F – test * Calculated | F – test Critical** | t – test * Calculated | t – test Critical** |
|--------------|-----------------------|---------------------|-----------------------|---------------------|
| Spectrophotometric | 12                   | 19                  | 1.35                  | 4.3                 |
| HPLC         |                       |                     |                       |                     |

* F- and t- tests were calculated using measurement data of 0.35 M GA concentration, with 2 degrees of freedom and 95% confidence level for both series.

** According to statistical tables given in [28]

**Conclusions**

Glyoxylate acid has been determined in an organic mixture consisting of oxalic acid, glyoxylic acid, glycolic acid, acetic acid, glyoxal and ethylene glycol by a new spectrophotometric method based on its reaction with tryptophan in presence of

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¹ RSD : Relative standard deviation
² LOD: limit of detection
³ LOQ: limit of quantitation
ferric chloride and concentrated sulphuric acid. The new method doesn’t require prior separation of glyoxylic acid from the mixture. The reaction between tryptophan and glyoxylic acid was found to be affected by reaction conditions. Good control of temperature and heat removal and correct reagent’s amounts are critical for success of the new method. The method was found to be effective within the range 0.00557-0.028M of glyoxylic acid concentration. The limit of detection, limit of quantitation, standard deviation, relative standard deviation and recovery of the proposed method were found comparable with a selected HPLC reference method. Both methods displayed same precision and credibility. Reaction stoichiometry of tryptophan to glyoxylic acid was found to be 2:3, and the product is tryptophan blue dye. Although it is a dialdehyde, glyoxal didn’t react with tryptophan according to Hopkins-Cole test.

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