

Assessment of Phenolic Compounds by Spectrophotometry: Exploring the Technique's Limitations - A Mini-Review

Farida Benmezziane

Department of Agronomic Sciences, Faculty of Sciences of Nature and Life, Chadli Bendjedid University of El-Tarf, BP 73, 36000 El Tarf, Algeria
Laboratory of Biomathematics, Biophysics, Biochemistry and Scientometry, Faculty of Sciences of Nature and Life, Bejaia University, Bejaia (06000), Algeria
benmezziane-farida@univ-eltarf.dz

Abstract. Several techniques are dedicated to the determination of phenolic compounds in plants. Spectrophotometry coupled with the Folin-Ciocalteu method is by far the most used technique for rapid quantification of these compounds. This review aims to bring together the most recent information on the UV-VIS spectrophotometry technique and its application in the determination of phenolic compounds. Despite its simplicity, this technique is limited by its non-specificity. However, and to remedy this obstacle, an optimization prior to the actual assay, in terms of extraction and quantification conditions, is necessary in order to obtain a content as close as possible to reality.

Keywords: Spectrophotometry; Folin-Ciocalteu; Quantification; Advantages and Disadvantages.

I. Introduction

Bioactive molecules, particularly polyphenols and carotenoids, are natural secondary metabolites found abundantly in fruits and vegetables. These compounds have been broadly investigated and their manifold health benefits are widely recognized. Through numerous years of epidemiological researches, these substances have revealed a numberless of protective effects and biological activities. Among their prominent properties are antiviral, anti-allergenic, neuroprotective, analgesic, cardioprotective, antioxidant, anti-inflammatory, anti-diabetic, antimicrobial, anti-carcinogenic, hepatoprotective and many others [1-9]. These effects arise from their property to interact with various free radical species through a reducing action (action to capture free radicals), so inhibiting or stopping the oxidative mechanisms that could potentially result in various diseases or disorders in organism. Polyphenols, or phenolic compounds are the products of secondary plant metabolism. Natural polyphenols include a wide group of compounds characterized by the presence of at least one

aromatic nucleus and one or more hydroxyl groups, along with other constituents. All classes of phenolic compounds have a large number of structures differing in number and position of hydroxyl and methoxyl groups on the basic backbone. Polyphenols are found throughout all parts (roots, stems, leaves, flowers, pollens, fruits, seeds and wood) of plants including fruits, vegetables, plants and herbs, with a particular abundance in medicinal plants. The polyphenols from foods can be quantified using a range of techniques, including spectrophotometry, high-performance liquid chromatography (HPLC), near infrared spectrometry, and mass spectrometry. Nevertheless, the spectrophotometric technique stands out as the most commonly employed approach to assess the total polyphenol content in foods, beverages, herbs, and other plant extracts. There are several variants of the spectrophotometric assay of polyphenols [10-11], but this technique is as far, the most widely used method to quantify these substances, as evident in numerous research papers [12-15]. The principle of the spectrophotometric assay of bioactive molecules and their antioxidant activities is based on color changes resulting from chemical reactions between the assay reagents and the functional groups, especially hydroxyl groups, found in the bioactive substances present in the extract. Nevertheless, it's important to note that the spectrophotometric assay of phenolic compounds does have limitations, in particular in terms of specificity. As such, the objective of this literature review is to provide a brief overview of the spectrophotometric technique, with a specific focus on its application in the analysis of phenolic compounds. This review will also highlight both advantages and disadvantages of thi method compared to other assay techniques.

II. What is Spectrophotometric Technique?

UV-Vis spectrophotometry is a versatile analytical technique employed for the quantitative determination of various substances or groups of substances, including organic substrates like amino acids and sugars [16], antibiotics [17], and bioactive molecules [18]. This method is based on measuring the absorbance or transmittance of a sample as a function of concentration. The substance of interest exhibits its maximum absorbance at a specific wavelength, generating a distinctive peak within its characteristic spectrum. The absorption spectrum in the ultraviolet and visible light regions encompass one or more absorption bands. Compose. The absorbance occurs over a wide spectrum range from 200 to 800 nm [19]. This technique finds applications in various fields, such as clinical, pharmacological, and food analysis.

III. Principle of Spectrophotometry

According to the findings of **Di Benedetto and Breuil [20]**, the absorption spectrometry in the ultraviolet and the visible (UV-Vis) is based on the fundamental concept of molecules absorbing radiation within a specific wavelength range, spanning from 190 to 800 nm. This range include both the ultraviolet (UV) extending from 190 to 400 nm, and the visible which spans from 400 to 800 nm. Advanced spectrophotometers have extended their cover into the near infrared (NIR) spectrum, reaching wavelength up to 2500 nm (Fig.1).

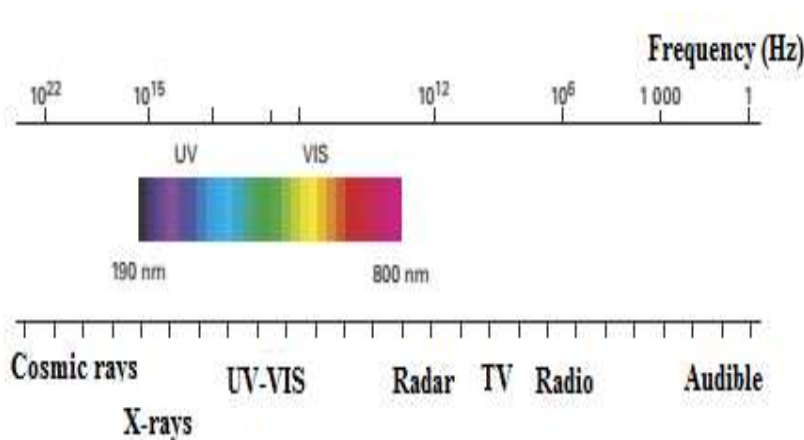


Fig. 1. Spectral range of electromagnetic radiation [20]

The absorption of UV-VIS radiation by these molecules serves as a means to determine the quantity, or more specifically, the concentration of molecules present in the path of the radiation. The concentration values are not obtained through direct measurement; instead, it necessitates the preparation of a calibration curve. This involves the preparation of standard solutions with known concentrations of the molecules to be assayed.

Standard solutions are prepared following the same procedure as the sample itself and are placed into absorption cells crossed by UV-VIS radiation at a specific wavelength corresponding to the target molecules. At this wavelength, a fundamental simple law links the transmitted radiation's intensity through the medium with the concentration of absorbing molecules (assuming only the species being analyzed absorbs at this wavelength). This principle governs quantitative spectrophotometric analysis and is known as the Beer-Lambert law, which establishes the relationship between absorption at a given wavelength (λ) and the concentration (C) of absorbing molecules. If the initial intensity of radiation at wavelength λ before passing through the cell is denoted as $I_{\lambda 0}$, the intensity after traversing the cell, denoted as I_{λ} , can be expressed in relation to $I_{\lambda 0}$ by the following formula:

$$I_{\lambda} = I_{\lambda 0} \exp(-\epsilon_{\lambda} l c) \text{ or } A_{\lambda} = \log \frac{I_{\lambda 0}}{I_{\lambda}} = \epsilon_{\lambda} l c$$

Where :

A_{λ} : absorbance of the medium at wavelength λ (nm) in the UV-VIS;

ϵ_{λ} : specific coefficient of molar absorbance (molecular extinction coefficient) in $L \cdot \text{mole}^{-1} \cdot \text{cm}^{-1}$;

l : optical path of the cell in cm;

c : concentration in mole $\cdot L^{-1}$ of molecules which absorb at wavelength λ .

The principle of spectrophotometry and the Beer-Lambert law are presented in Figure 2.

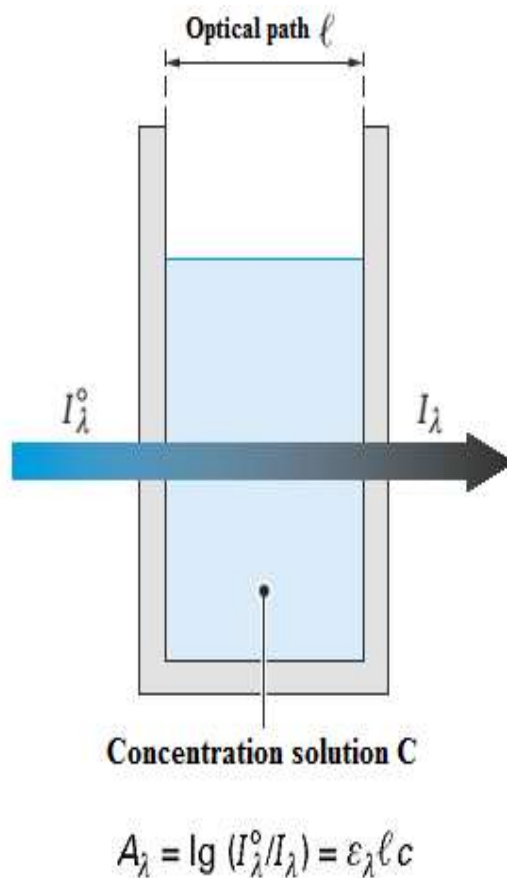


Fig. 2. Absorption cell and Beer-Lambert's law [20]

IV. The Calibration Curve in Spectrophotometry

The calibration line is necessary in a spectrophotometric analysis. The method involves the preparation of a series of dilutions with known concentrations, followed by the measurement of the absorbance of each of these standard solutions. The collected results make it possible to plot the calibration curve, represented as $A = f(C)$, where "A" represents absorbance, and "C" signifies concentration.

When analyzing an unknown concentration solution, its absorbance is measured under the same conditions as the standard solutions. The obtained absorbance value is then plotted on the previously established calibration curve.

The concentration of the solution to be assayed can be determined from the calibration curve, by ensuring that the range of standard solutions covers the expected concentration of the unknown sample. Figure 3 provides an illustrative example of a calibration curve for reference.

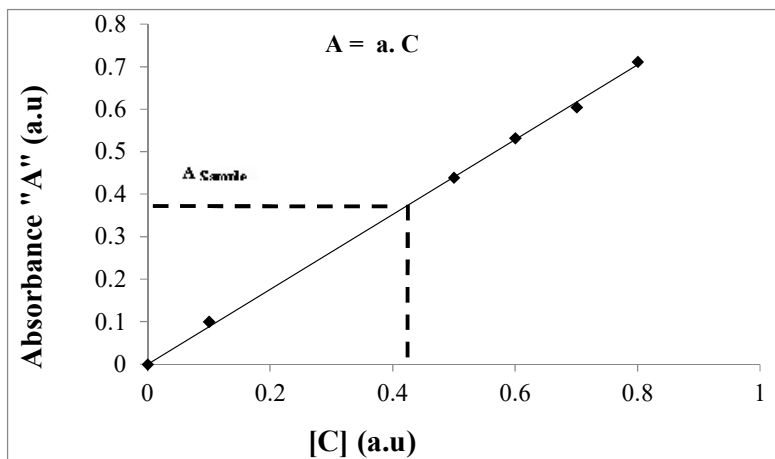
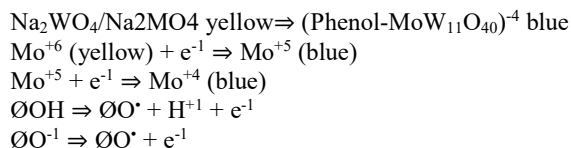


Fig. 3. Plotting the calibration curve and the determination of the unknown concentrations

The graph exhibits a linear relationship represented by a straight line, passing through the origin. This relationship follows the Beer-Lambert's law, suggesting that absorbance is directly proportional to the concentration of the target species. This proportional relationship is represented by the equation of the straight line: $A = a \cdot C$, where "a" represents the slope of the line, symbolizing the molar extinction coefficient "ε".

V. Spectrophotometry UV-VIS Applications in Polyphenols Quantification

Broadly established classic spectrophotometric tests are simple and fast screening methods for quantifying various classes of phenolic compounds in crude plants extract. Several scientific studies have employed spectrophotometry as a pivotal tool to quantify the Total Phenolic Content (TPC) based on Folin-Ciocalteu reagent use, a fundamental oxidation-reduction reaction. The color changes observed during this reaction indicates the concentration of the TPC in plant extracts. This concentration can be determined using visible-light spectrophotometry, and the results are commonly expressed as gallic acid equivalents. The reaction between polyphenols in plants extracts and the Folin-Ciocalteu reagent gives rise to a blue chromophore, composed of a phosphotungstic-phosphomolybdenum complex. The maximum absorption of these chromophores is contingent upon the alkaline solution and the concentration of TPC in the extracts. As elucidated by Agbor et al. [21], the Folin-Ciocalteu phenol reagent is constated by a mixture of heteropoly acids, specifically, phosphomolybdic and phosphotungstic acids. Both molybdenum and the tungsten are in the 6+ oxidation state. During the reaction with a reducing agent, they form molybdenum blue and a tungsten blue complex, with the mean oxidation state of the metals oscillating between 5 and 6. According to the same authors, the interaction between polyphenols and the Folin-Ciocalteu reagent unfolds as follows:



In a study conducted by **Dini et al. [22]**, polyphenols of extra virgin olive oil were quantified using the Folin-Ciocalteu method, after a solid phase extraction. Their findings revealed the robustness and precision of this procedure with an excellent recovery rate ($\geq 95\%$) within a measurement range of 100 to 500 ppm. Consequently, the authors concluded that this technique can be used for the official routine method for polyphenols quantification in extra virgin olive oil. **Blainski et al. [23]** conducted an optimization and validation of the spectrophotometric assay of polyphenol compounds extracted from *Limonium brasiliense* roots. The study determined the optimal conditions for analysis, including time of 30 min, a wavelength of 760 nm, and the use of pyrogallol as a standard substance. Under these conditions, the method demonstrated excellent performance characteristics such as linearity, specificity, precision, reproducibility, robustness and ease of perform.

Rover and Brown [24] reported that the bio-oil TPC (wt% gallic acid equivalent) using the Folin-Ciocalteu method, was comparative to the TPC obtained by liquid-liquid extraction. All the results fell within the margin of error and the uncertainty of the Folin-Ciocalteu method measurement. This supposes that there were no significant differences in the results between the two methods. The uncertainty of measurement for the Folin-Ciocalteu method was $\pm 1.1\%$ at the 95% confidence level.

In their study on red grape juice and tea, **Margraf et al. [25]** identified a high and statistically significant correlation between the amount of the total phenolics determined using the Folin-Ciocalteu method and that evaluated using the Prussian blue method. However, the authors favored the Prussian blue method due to its simplicity, rapidity, reduced reagents consumption, and higher selectivity compared to the Folin-Ciocalteu method. Under optimized conditions (60–80 min of interaction between the extract and the Folin-Ciocalteu reagent, measurements at wavelength of 760 nm, and the use of gallic acid and rutin as reference substances), the analytical procedure for polyphenols quantification in *Salvia sclarea* growing in Ukraine was found to be robust and easy for performing within pharmaceutical development and routine control laboratories [26]. Similarly, **Galvão et al. [27]**, whom assessed the performance of the Folin-Ciocalteu method, after optimization of the experimental conditions (30 min of reaction; wavelength of 760 nm; 1.0 mL of Folin-Ciocalteu; 29% of Na_2CO_3 (w/v)), concluded that this simple procedure is suitable and can be adopted as an appropriate and effective analytical tool for quality control of herbal drugs derived from *Libidibia ferrea*. It's commonly understood that the content of total polyphenols varies not only depending on the origin of the raw material used and the agro-ecological cultivation conditions, but also within the same variety, as illustrated in Table 1, contingent upon the extraction conditions and / or sensitivity of the analytical method chosen.

Table 1. Source, extraction conditions and content of polyphenols quantified by Folin-Ciocalteu method.

| Polyphenol sources | Extraction conditions | Content | Ref. |
|--|---|---|------|
| Algerian fresh eggplant | Maceration in aqueous acetone (70%; v/v) at 25°C temperature for 40 min using a 0.5g/50 mL solid to solvent ratio. | 4838.34 mg GAE/100 g DW | [13] |
| Algerian fresh eggplant | Maceration in aqueous acetone (70%; v/v) at 25°C for 60 min using a 1g/50 mL solid to solvent ratio and three successive extractions | 794.94 mg GAE/100 g FM | [14] |
| Algerian fresh eggplant | Maceration of 5 g of sample powder in 100 mL of ethanol for 2 hours using a Soxhlet extractor | 91.42 ± 15.48 - 50.98 ± 5.93 mg GAE/100g DE | [28] |
| Algerian fresh eggplant | Maceration of 100g of crushed material in 625 mL of aqueous methanol (80%, v/v) for 3 days at 4 °C | 292.96 ± 4.29 µg GAE/ mg DE | [29] |
| Algerian fresh eggplant byproduct (peel) | Maceration in aqueous acetone (70%; v/v) at ambient temperature for 40min using a 0.5g/50 mL solid to solvent ratio. | 29.3 ± 1.23 mg GAE/100 g DE | [30] |
| Algerian fresh Eggplant byproduct (peel) | Maceration in 130 mL ethanol at ambient temperature for 2hours using Soxhlet apparatus and 0.1 mL of the extract for TPP determination. | 23.78 mg GAE/ g DE | [31] |
| French dried prune | Maceration of 2g of prune in 20 mL of aqueous ethanol (50%; v/v) at room temperature for 40 min | 0.74 g GAE/100 g DW | [32] |
| French dried prune | Maceration of 2g of prune in 20 mL of aqueous methanol (50%; v/v) at room temperature for 40 min | 0.76 g GAE/100 g DW | [32] |
| Mexican tomato | Maceration of 1 g of lyophilized fruit tissue | 37.1 - 85.6 mg GAE 100 g FW | [33] |
| Mexican tomato | Sequential extraction with 75% aqueous methanol (v/v) and dichloromethane (1: 1 v/v) | 10.2 ± 2.1 - 64.1 ± 1.9 µg/g GAE FW | [34] |
| Indian tomato | Maceration of homogenized sample in aqueous ethanol (80%,v/v) | 41.10 - 139.59 mg GAE/100 g FW | [35] |
| Indian tomato | Maceration of 5 g of homogenized samples in 30 mL of ethanol (80%, v/v) by stirring and sonicating for 30 min in dark | 26.34 - 66.08 mg GAE/100 g FW | [36] |
| Indian tomato | Maceration of 500 mg of Grounded dry food material in 10 mL of acetone (80%, v/v) by stirring for 1 min. | 4.71 ± 0.32 mg GAE/g DW | [37] |

VI. Limits of the Technique

The UV-Vis spectrophotometry is still widely used in contemporary scientific practices. It offers several advantages, including ease of operation, simplicity, cost-

effectiveness, and widespread popularity, particularly when compared to more intricate techniques such as High Performance Liquid Chromatography (HPLC). Nevertheless, the problem that arises with spectrophotometric techniques is their lack of specificity. Indeed, during quantification of the bioactive molecules through spectrophotometry, any substances possessing a chromophore similar to that of the analyte will also be quantified indistinguishably from the analyte itself. Furthermore, **Ratnayake and Ranaweera [38]** pointed out that the exact chemical nature of the Folin's reagent is not known, and the reagent lacks specificity for phenolic compounds, as it can be reduced by numerous non-phenolic substances such as vitamin C and minerals. Therefore, this can lead to the overestimation of analyte content. This observation aligns with the findings of **Akowuah et al. [39]**, who reported that HPLC detection proved to be more efficient for quantifying berberine, the main bioactive component in *Coscinium fenestratum* extract, compared to spectrophotometric determinations. In fact, the authors recorded significantly elevated berberine levels using the UV spectrophotometric method compared to the HPLC method across all selected λ_{max} values for compound detection. Consequently, the authors concluded that despite its affordability and simplicity, spectrophotometric measurement for berberine in botanical extracts is not suitable due to the interference from other plant constituents. When employing the conventional method for the determination of TPC, it's important to note that the Folin-Ciocalteu reagent reacts not only with the hydroxyl (-OH) groups of polyphenols but also with other non-phenolic reducing substances. This dual reactivity can lead to an overestimation of the TPC as reported by **Mojzer et al. [40]**. For instance, the polyphenols content was found to be lower in the acetone extract of fresh eggplant compared to baked, grilled or fried eggplant. This difference was attributed, in part, to the various cooking methods applied, which can induce degradation and the formation of new compounds such as the Maillard reaction products. These newly formed compounds also react with the Folin-Ciocalteu reagent, causing an apparent increase in the polyphenol content [13]. Furthermore, **Wekre et al. [41]**, conducted a study in which they explored the quantification of total polyphenols in a green macroalga, *Ulva intestinalis*, using three different analytical methods: ¹H-NMR (qNMR), HPLC-DAD, and the Folin-Ciocalteu assay. The authors discovered that the same samples yielded varying TPC values when analyzed with these different methods. This highlights the challenges associated with accurately quantifying polyphenols in extracts, emphasizing the need for careful consideration of the chosen analytical method

VII. Conclusion

The objective of this review article was to provide a general overview of UV-Vis spectrophotometry as technique for the determination of polyphenols using the Folin-Ciocalteu reagent. While the spectrophotometric assay of polyphenols presents inherent limitations due to its lack of specificity, it can be effectively employed with optimization of extraction and assay conditions. This optimization coupled with rigorous validation through statistical methodologies, ensures accurate quantification of polyphenols. This not only highlights the versatility of the method but also underscores its adaptability to various research as analytical contexts. Despite the specificity challenges encountered in spectrophotometric analysis of polyphenols, spectrophotometry remains a resilient, easy-to-handle, and cost-effective tool for routine polyphenol analysis. As we continue our efforts to unravel the complexities of

polyphenolic compounds, it is evident that spectrophotometry will continue to play a key role in our pursuit of knowledge and discovery.

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