IMPROVEMENT OF SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF PHENOLIC COMPOUNDS BY STATISTICAL INVESTIGATIONS

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The determination of phenolic compounds by the spectrophotometric method has many advantages: it is very easy to implement, requires less resources and provides a global response concerning the content of phenolic compounds. This spectrophotometric method is known as the Folin-Ciocalteu index determination. In the literature are proposed several wavelengths at which can be made Folin-Ciocalteu index determination: 725 nm, 750 nm, 760 nm, 765 nm. In this paper, it was shown, using statistical methods, the best wavelength at which can be determined the Folin-Ciocalteu index.

Key words: Folin-Ciocalteu index, natural antioxidants, statistical method, UV-Vis spectrophotometry.

1. INTRODUCTION

The determination of Folin-Ciocalteu index is a spectrophotometric method based on the measurement of absorbance at a wavelength of about 760 nm. For several reasons, this method is much better than traditional chemical methods (e.g. Chromatography), because it is easier, requires fewer resources and provides a global response about content of phenolic compounds [1].

The Folin-Ciocalteu index is specific for phenolic compounds with reducing properties, but there may be interferences with various chemicals: proteins, ascorbic acid, etc. [2]. This index is useful for quantitative determination of phenolic compounds in different extracts.

The determination is based on the reaction of Folin-Ciocalteu reagent (consisting of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$)) with phenolic compounds from the sample, resulting in a mixture of blue oxides ($W_8O_{23}$ and $Mo_8O_{23}$) (Fig. 1) [3, 4]:

The reaction occurs in alkaline medium, the intensity of blue coloring obtained is dependent on the amount of phenols in solution. But this color is not stable and evolves over time following two phases:
- rapid phase (0–30 minutes) that leads to blue color;
- slow phase after 30 minutes, with evolution of color to dark blue.

If absorbance reading is done after 30–45 minutes, the error is very small and the obtained value is reproducible.

Regarding to working mode, determination of Folin-Ciocalteu index varies from one author to another [5–8]. The method is adapted by each author based on the desired applications and available resources. The most important wavelengths,
at which Folin-Ciocalteu index is measured, are: 725 nm [9], 750 nm [10], 760 nm [11] and 765 nm [12].

The differences of opinions in the literature about the most appropriate wavelength to determine the Folin-Ciocalteu index have led to their investigation by mathematical and statistical methods in this paper.

2. EXPERIMENTAL

2.1. REAGENTS PREPARATION

*Gallic acid calibration solutions.* For preparation of gallic acid calibration solutions it was prepared a solution of 5 g/L gallic acid from gallic acid with purity $\geq 98\%$ (Merck).

From this solution they were obtained 8 calibration solutions with these concentrations: 0, 25, 50, 75, 100, 250, 350 and 500 mg/L.

*Folin-Ciocalteu reagent.* Folin-Ciocalteu reagent preparation is made in several steps and requires a long time [13]. To avoid reagent contamination with phenolic compounds at its preparation in laboratory and for more qualitative determinations, it was used Folin-Ciocalteu 2.0 N produced by Merck.

*Na$_2$CO$_3$ 20 % solution.* They were dissolved 200 g anhydrous Na$_2$CO$_3$ (Merck) in 800 mL deionized water and heated until complete dissolution of the reagent. After cooling, a few crystals of Na$_2$CO$_3$ was added in solution and left for 24 hours at room temperature. After this step, the solution was filtered into a 1 L flask and brought to volume with distilled water.

2.2. SAMPLE PREPARATION

For extraction of phenolic compounds from grape skin it has been used ethanol-water solvent (70:30 v/v). The skin of 6 grape samples (Căpșunică 2014, Cardinal, Tâmâioasă Neagră, Moldova, Coarne Moldovenești, Căpșunică 2013) was removed from the seeds and pulp, after which it was inserted in the solvent.

Each grape skin (100 g) was processed using 50 mL C$_2$H$_5$OH and 20 mL of ultrapure water (18.2 MΩcm). The mixture is kept for 5 days at room temperature in the dark. To remove any impurities, the grape skin extracts were filtered through Macherey-Nagel Chromafil membrane filters with a pore diameter of 0.45 µm.

For preparation of samples and reagents, it was used ultrapure water (18.2 MΩcm at 25 °C).

2.3. WORKING MODE WITH FOLIN-CIOCALTEU REAGENT

Folin-Ciocalteu index determination occurs in several stages [14]:
- *For blank:* In a 25 mL flask it is transferred 0.25 mL distilled (deionized) water. *For calibration solutions:* For each calibration standard (25, 50, 75, 100, 250, 350, 500 mg/L gallic acid) it is transferred approximately 0.25 ml of each solution in 25 mL-flasks. *For grape extracts:* In 25 mL flasks it is transferred approximately 0.25 mL sample. If the absorbance is higher than the absorbance of the most concentrated calibration standard (500 mg/L), it is necessary to apply a sample dilution.

- Add 17.5 mL of distilled or deionized water, followed by 1.25 mL of Folin-Ciocalteu reagent. The mixture is stirred and allowed to incubate 1-8 minutes at room temperature;
- Add 3.75 mL Na₂CO₃ 20 % solution;
- It is made up to volume with distilled water, the mixture was stirred and allowed to incubate 2 hours at room temperature;
- 2 mL of solution is transferred to a quartz cuvette. Absorbance at 765 nm is measured using a UV-Vis spectrophotometer (to identify the best wavelength for Folin-Ciocalteu index determinations have been made measurements, also, at other wavelengths: 725, 750 and 765 nm);
- Subtract blank absorbance from the absorbance values of the other calibration standards.

### 2.4. THE SPECTROPHOTOMETRIC MEASUREMENTS

Calibration standards after preparation were processed according to the working mode for Folin-Ciocalteu index. They were read at the 4 wavelengths of interest: 725, 750, 760 and 765 nm using Analytik Jena Specord 250 Plus UV-Vis spectrophotometer.

The resulting calibration curves are used to determine the concentration of phenolic compounds (expressed in mg/L gallic acid). Thus, all results are reported in mg/L gallic acid equivalents.

### 2.5. STATISTICAL ANALYSIS

Interpretation of the results was performed using statistical analysis software Microsoft Excel and IBM SPSS Statistics. The statistical techniques used in this paper are:

- Linear regression [15];
- Pearson correlations [16];
- Principal component analysis (PCA) [17];
- Student t-test [18].
Using linear regression, Pearson correlations and principal component analysis (PCA), it could identify the best wavelength for determining the Folin-Ciocalteu index, but by Student t-test they were able to see the differences between the results of Folin-Ciocalteu index recorded at different wavelengths.

3. RESULTS AND DISCUSSIONS

The spectrophotometric measurements have included not only investigation of calibration standards at 4 wavelengths (Table 1), but, also, quantitative determination of phenolic compounds in six grape extracts using calibration curves at these wavelengths (Table 2).

All samples (both calibration standards and grape extracts) were processed according to the procedure of working with Folin-Ciocalteu reagent.

**Table 1**
Absorbance values for calibration standards of gallic acid (0, 25, 50, 75, 100, 250, 350, 500 mg/L) at investigated wavelengths (725, 750, 760, 765 nm)

<table>
<thead>
<tr>
<th>Gallic acid concentration (mg/L)</th>
<th>Absorbance</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A_{725\text{nm}})</td>
<td>(A_{750\text{nm}})</td>
<td>(A_{760\text{nm}})</td>
<td>(A_{765\text{nm}})</td>
</tr>
<tr>
<td>0</td>
<td>0.0000 ± 0.0000</td>
<td>0.0000 ± 0.0000</td>
<td>0.0000 ± 0.0000</td>
<td>0.0000 ± 0.0000</td>
</tr>
<tr>
<td>25</td>
<td>0.0275 ± 0.0004</td>
<td>0.0318 ± 0.0006</td>
<td>0.0319 ± 0.0005</td>
<td>0.0310 ± 0.0004</td>
</tr>
<tr>
<td>50</td>
<td>0.0578 ± 0.0008</td>
<td>0.0658 ± 0.0010</td>
<td>0.0676 ± 0.0010</td>
<td>0.0668 ± 0.0010</td>
</tr>
<tr>
<td>75</td>
<td>0.1060 ± 0.0010</td>
<td>0.1182 ± 0.0015</td>
<td>0.1204 ± 0.0017</td>
<td>0.1191 ± 0.0016</td>
</tr>
<tr>
<td>100</td>
<td>0.1251 ± 0.0012</td>
<td>0.1362 ± 0.0017</td>
<td>0.1395 ± 0.0016</td>
<td>0.1385 ± 0.0015</td>
</tr>
<tr>
<td>250</td>
<td>0.3206 ± 0.0020</td>
<td>0.3463 ± 0.0024</td>
<td>0.3519 ± 0.0026</td>
<td>0.3517 ± 0.0024</td>
</tr>
<tr>
<td>350</td>
<td>0.4398 ± 0.0032</td>
<td>0.4688 ± 0.0036</td>
<td>0.4749 ± 0.0034</td>
<td>0.4733 ± 0.0036</td>
</tr>
<tr>
<td>500</td>
<td>0.6311 ± 0.0041</td>
<td>0.6651 ± 0.0048</td>
<td>0.6712 ± 0.0046</td>
<td>0.6712 ± 0.0047</td>
</tr>
</tbody>
</table>

Folin-Ciocalteu index is very useful to characterize the antioxidant activity of grape extracts, the measurements being performed at 4 wavelengths: 725, 750, 760 and 765 nm. They were used grape extracts samples of several varieties: Capsunica 2014 (P1), Cardinal (P2), Târnăioasă Neagră (P3), Moldova (P4), Coarne Moldovenesti (P5) and Capsunica 2013 (P6).

To identify the most correct wavelength for determination of Folin-Ciocalteu index, the results were processed by mathematical and statistical methods: linear regression, Pearson correlations, principal component analysis (PCA).
Table 2
Folin-Ciocalteu index determination of grape extracts (P1, P2, P3, P4, P5, P6) at wavelengths (725, 750, 760, 765 nm)

<table>
<thead>
<tr>
<th>Dilution factor (sample volume: deionized water volume)</th>
<th>Folin-Ciocalteu index (in mg/L gallic acid equivalents)</th>
<th>Measured at 725 nm</th>
<th>Measured at 750 nm</th>
<th>Measured at 760 nm</th>
<th>Measured at 765 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 1:4</td>
<td>1402.29 ± 7.12</td>
<td>1391.13 ± 8.78</td>
<td>1295.07 ± 8.03</td>
<td>1297.40 ± 9.55</td>
<td></td>
</tr>
<tr>
<td>P2 1:4</td>
<td>1458.25 ± 8.07</td>
<td>1444.19 ± 8.99</td>
<td>1350.11 ± 7.56</td>
<td>1343.60 ± 9.45</td>
<td></td>
</tr>
<tr>
<td>P3 1:4</td>
<td>2160.60 ± 13.70</td>
<td>2117.45 ± 12.73</td>
<td>2008.32 ± 11.82</td>
<td>2008.60 ± 10.73</td>
<td></td>
</tr>
<tr>
<td>P4 1:9</td>
<td>3233.96 ± 20.89</td>
<td>3190.21 ± 19.54</td>
<td>3169.54 ± 20.07</td>
<td>3167.11 ± 21.36</td>
<td></td>
</tr>
<tr>
<td>P5 1:9</td>
<td>3230.64 ± 19.99</td>
<td>3176.69 ± 21.29</td>
<td>3167.88 ± 20.33</td>
<td>3155.47 ± 20.84</td>
<td></td>
</tr>
<tr>
<td>P6 1:9</td>
<td>4328.15 ± 28.44</td>
<td>4239.61 ± 27.50</td>
<td>4206.69 ± 29.17</td>
<td>4200.41 ± 29.56</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 – Linear regressions for calibration curves at the 4 wavelengths (725, 750, 760 and 765 nm).

Linear regression. Linear regression is one of the most widely used mathematical techniques to compare two data strings. For a good reliability between two strings, $R^2$ must be more than 0.995.

By plotting calibration curves at the investigated wavelengths (Figure 2), it was observed that the best $R^2$ is at 725 nm ($R^2 = 0.9993$). The other calibration curves can also be used to determine Folin-Ciocalteu index, because the condition of $R^2 > 0.995$ is respected, however $R^2$ is lower ($R^2 = 0.9988$).

Pearson correlations. By interpretation of the Pearson correlations applied on absorbance values of the gallic acid solutions (Table 3) at the 4 wavelengths (725, 750, 760 and 765 nm), it has been identified two groups of data: the first group of wavelengths (725 nm and 750 nm), that correlate with concentration at a rate of
1.000 (p < 0.01), and the second group of wavelengths (760 nm and 765 nm), that correlate with concentration at a rate of 0.999 (p < 0.01).

Pearson correlations between the concentration levels in grape extracts obtained at four wavelengths (725, 750, 760 and 765 nm) have been showed high similarities (1.000, p < 0.01). By this statistical analysis could not highlight differences between the levels of gallic acid equivalents in the samples.

Measurements on extracts have not identified differences related to the wavelength using the Pearson correlations. But by investigating gallic acid solutions at the 4 wavelengths through the Pearson correlations can be seen that the wavelengths 725 and 750 nm are better.

**Table 3**

Pearson correlations between gallic acid concentration and absorbance values at 4 investigated wavelengths: 725, 750, 760 and 765 nm

<table>
<thead>
<tr>
<th>Concentration</th>
<th>(A_{725\text{nm}})</th>
<th>(A_{750\text{nm}})</th>
<th>(A_{760\text{nm}})</th>
<th>(A_{765\text{nm}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>1.000**</td>
<td>1.000**</td>
<td>0.999**</td>
<td>0.999**</td>
</tr>
<tr>
<td>(A_{725\text{nm}})</td>
<td>1.000**</td>
<td>1</td>
<td>1.000**</td>
<td>1.000**</td>
</tr>
<tr>
<td>(A_{750\text{nm}})</td>
<td>1.000**</td>
<td>1.000**</td>
<td>1</td>
<td>1.000**</td>
</tr>
<tr>
<td>(A_{760\text{nm}})</td>
<td>0.999**</td>
<td>1.000**</td>
<td>1.000**</td>
<td>1</td>
</tr>
<tr>
<td>(A_{765\text{nm}})</td>
<td>0.999**</td>
<td>1.000**</td>
<td>1.000**</td>
<td>1</td>
</tr>
</tbody>
</table>

**, p < 0.01 for N=8.

**Principal Component Analysis (PCA).** Absorbance values have been investigated by principal component analysis followed by Varimax rotation method with Kaiser normalization [19] (Table 4). It was observed that absorbance values are very close together regarding the 2 resulted components. Although the differences are not significant between the values of the components, gallic acid concentration is attributed to component 2 and all absorbance values to component 1.

**Table 4**

Principal component analysis (PCA), followed by Varimax rotation method with Kaiser normalization (3 iterations), applied on absorbance values at the 4 wavelengths

<table>
<thead>
<tr>
<th>Component</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>0.695</td>
<td>0.719</td>
</tr>
<tr>
<td>(A_{725\text{nm}})</td>
<td>0.710</td>
<td>0.704</td>
</tr>
<tr>
<td>(A_{750\text{nm}})</td>
<td>0.717</td>
<td>0.697</td>
</tr>
<tr>
<td>(A_{760\text{nm}})</td>
<td>0.719</td>
<td>0.695</td>
</tr>
<tr>
<td>(A_{765\text{nm}})</td>
<td>0.718</td>
<td>0.696</td>
</tr>
</tbody>
</table>

Assessing the rapprochement between the two component values of concentration and absorbance at different wavelengths, it can be seen the following order: 725 nm (0.704), 750 nm (0.697), 765 nm (0.696), 760 nm (0.695). Also, for
the component 1, it is noted the same trend: 725 nm (0.710), 750 nm (0.717), 765 nm (0.718), 760 nm (0.719).

After applying principal component analysis followed by Varimax rotation method with Kaiser normalization (3 iterations) on Folin-Ciocalteu index values obtained on grape extracts (Table 4), two main components have resulted:

– component 1, characterized by grape extracts concentration values (in gallic acid equivalents) at the wavelengths: 725 and 750 nm;

– component 2, characterized by grape extracts concentration values (in gallic acid equivalents) at the wavelengths: 760 nm and 765 nm.

Component 1 is defined by greater concentration values, but component 2 by lower concentration values of phenolic compounds in samples.

Normally, the results should be similar, however the values obtained at 725 nm and 750 nm seem to differ a great extent from those at 760 nm and 765 nm.

Taking into consideration the Table 4, it can be concluded that 725 nm is the best wavelength that correlates the most with the concentration values. Thus, the results obtained at 765 nm 760 nm contain a high error ratio, because of this they are distant from the results obtained at 725 nm and 750 nm.

**Student t-test.** Student’s t-test was applied to the concentrations of the six grape extracts samples (Table 5) measured at the four wavelengths (725, 750, 760 and 765 nm).

It was observed that relative standard deviation at some samples is very high (± 6.89%) by comparing results obtained at different wavelengths. This proves that between calibration curves at the 4 wavelengths are big differences.

By principal component analysis applied on phenolic concentrations, obtained at 4 wavelengths, in 6 samples of grape extracts (Figure 3), followed by Varimax rotation method with Kaiser normalization, they were obtained two main components:
– component 1, characterized by the concentrations of grape extracts, which had relatively high standard deviation (Table 5);
– component 2, characterized by the concentrations of grape extracts, which have showed relatively low standard deviation (Table 5).

![Principal component analysis](image)

Fig. 3 – Principal component analysis, followed by Varimax rotation method with Kaiser normalization, applied to the concentration values of phenolic compounds in the grapes extracts obtained at four wavelengths (725, 750, 760 and 765 nm).

According to the 3 types of statistical analysis (linear regression, principal component analysis, Pearson correlations) it can concluded that the wavelength of 725 nm is most suitable.

Thus, at the wavelength of 725 nm it is observed the strongest correlation between absorbance and concentration values.

4. CONCLUSION

By using statistical methods (linear regression, Pearson correlations, principal component analysis, Student t-test), it could define the most suitable wavelength for determination of Folin-Ciocalteu index.

The importance of identifying the best wavelength is related to the quality of results. The measurement of Folin-Ciocalteu index at the four wavelengths can cause large differences between real and the result obtained at other wavelength (up to 10%).

Thus, wavelength of 725 nm according to mathematical calculations is the best to determine Folin-Ciocalteu index.
REFERENCES