PERFORMANCE CHARACTERISTIC EVALUATION OF FOLIN-CIOCALTEU MICRO-METHOD FOR TOTAL POLYPHENOLS DETERMINATION FROM PLANT EXTRACTS

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Summary

This paper aims to describe the standard operation procedure for performance characteristic evaluation of Folin-Ciocalteu micro-method for total polyphenols analysis from vegetal matrices. The key of this method consists in the use of a microplate reader due to multiple advantages: using small amounts of extract and reagent as well, assuring a good repeatability and a considerable reduction of the total analysis time. Besides that, it is an accurate and easy to accomplish method. Standard calibration curve was performed using concentrations of 0, 0.25, 0.50, 0.75, 1 mg/ml of gallic acid and a microplate reader TECAN Infinite M1000 Pro at 750 nm was used. Statistical evaluation of linear calibration function was performed through following parameters: the standard deviation and coefficient of variation with simple and repeated analysis, the repeatability and reproducibility, the limit of detection (LOD) and the limit of limit of quantification (LLOQ).

Keywords: polyphenols, micro-method, method validation, UV/VIS, Folin-Ciocalteu

Polyphenols are a category of plant compounds that offer various health benefits. They have antioxidant, anti-inflammatory and biological effects that are manifested in the prevention of pathological conditions. Polyphenols can act as antioxidants, meaning they can neutralize harmful free radicals that would otherwise damage the cells and increase the risk of conditions like cancer, diabetes, and heart disease (10).

Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. These compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators, contributors to plant pigmentation, antioxidants, and protective agents against UV light, among others (5).

Although quantitative determination of polyphenols is hampered by their structural complexity and diversity, several methods have used to determine polyphenols in plant extracts (1).

Polyphenols in plant extracts react with specific redox reagents as the Folin-Ciocalteu reagent to form a blue complex that can be quantified by visible-light

spectrophotometry (8).

The reaction forms a blue chromophore constituted by a phosphotungstic-phosphomolybdenum complex (6, 8) where the maximum absorption of the chromophores depends on the concentration of the phenolic compounds (8).

Materials and methods

For the quantification of the phenolic compounds in some dried plants, Folin-Ciocalteu method was used following the protocol described by TAMAS-KRUMPE Octavia Maria *et al*. (9). This method consisted in several steps: 25 µl of the final solution (alcoholic extract of dried plant material), 125 µl of the Folin-Ciocalteu reagent and 100 µl Sodium carbonate (Na_2CO_3) were pipetted into a 96-well plate.

The standardized method, with a reaction time of 30 min, wavelength of 760 nm and Gallic acid as the standard was used to validate the method for the determination of total polyphenols. Analyses were performed in triplicate. The absorbance of the samples was read with a microplate reader TECAN Infinite M1000 Pro.

Analytical method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use (7).

According SR ISO CEI 17025:2018 method validation analytical laboratory is „confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled".

The clause 5.4.5.2 of ISO/IEC 17025 presents that validation must be performed in the following cases: „non-standard methods; laboratory-designed/developed methods, standard methods used outside their intended scope, amplifications and modifications of standard methods''.

Verification and validation are independent procedures that are used together for checking that a product, service, or system meets requirements and specifications and that it fulfils its intended purpose (11). These are critical components of a quality management system according ISO 17025:2018.

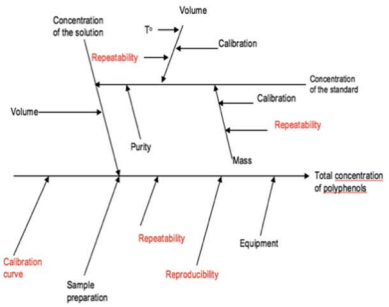

Fig. 1. Ishikawa diagram for the determination of the concentration of polyphenols

For the validation report several factors have to be taken into consideration such as: linearity, accuracy, precision, specificity, selectivity, sensitivity, stability, uncertainty matrix effect, repeatability and reproducibility, limit of detection and quantification.

To create an overview with all those factors we managed to introduce them into the Ishikawa diagram: a causal diagram

used to identify potential factors that could cause an overall effect. The factors that have been taken into consideration and analysed were: the calibration curve, the repeatability and reproducibility and the limit of detection and quantification.

Calibration curve

The calibration curve of the Gallic acid was determined from five concentration points over the range of concentrations: 3.90, 7.80, 15.62, 31.25, 62.50, 125.00, 250.00 mg/ml, following the Lambert-Beer law (2). Statistical analysis was performed according LGC Guide for calibration curve preparation. Interpretation of the statistical results was performed according ISO14502-1:2005.

Repeatability and reproducibility

According to the standard ISO 5725-1:1994, the *repeatability conditions* are „Conditions where independent test results are obtained by the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short time intervals" and *the reproducibility conditions,* according to the same standard are "Conditions where the results are obtained with the same method, on identical test items, in different laboratories, with different operators, with using different equipment".

For this paper, repeatability and reproducibility have been calculated according the Standard *ISO 14502-1:2005.*

Limit of detection and quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (3). For the validation of this method, the limit of detection was calculated with the following formula: **LOD = 3.3 × (S Y / a)** – where SY is SD of blank response "a" for the slope of a linear calibration line.

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices (3). For the determination of this parameter, the following formula was used: **LLOQ = 10 × (S Y / a)** – where SY is SD of blank response "a" for the slope of a linear calibration line.

Results and discussion

Molecular absorption spectrophotometry in ultraviolet/visible light (UV/VIS) is an analytical method based on the property of an ion or molecular species to absorb at certain wavelengths of UV/VIS radiation. Thus, the absorption can be considered as a specific process related to the structure of the absorbing species, which determines the energy involved in the electron transition. However, to make the method more selective, normally reagents are used to convert the species of interest into a form that allows the absorption of the radiation to be measured with greater sensitivity and/or selectivity (2).

The European Pharmacopoeia indicates a single general method for determination of total polyphenols in all herbal drugs: the Folin-Ciocalteu method (4) witch we managed to transform into a micro-method because of the number of advantages that is confers.

During the process of developing this method, we intended to achieve a repeated number of readings to make a correlation between them regarding *repetability* and *reproducibility.*

At the first reading the following results were obtained (Table 1, Fig. 2)

Table 1

The results obtained at the first reading

Concentration of standards	Instrument readings
3,9000	0,0358
7,8000	0,0566
15,6200	0,1121
31,2500	0,2266
62,5000	0,3782
125,0000	0,8317

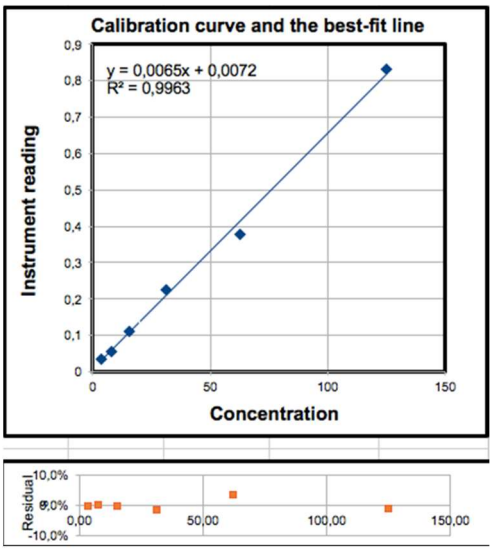

Fig. 2. The Correlation between absorbance and concentration on the first calibration curve

After a period of 5 days the same procedure was reassessed in the same laboratory conditions by a different co-worker, resulting the following data (Table 2, Fig. 3):

Table 2

The results obtained at the second reading

Concentration of standards	Instrument readings
3,9000	0,0433
7,8000	0,0913
15,6200	0,1398
31,2500	0,2595
62,5000	0,4598
125,0000	0,8233

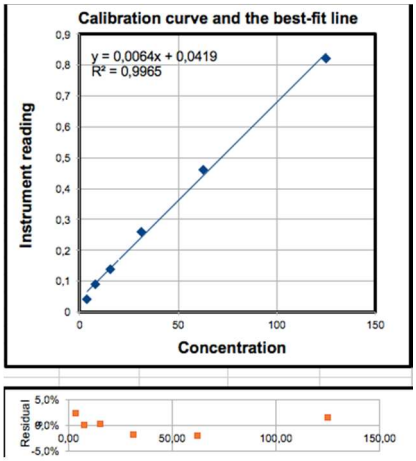

Fig. 3. The Correlation between absorbance and concentration on the second calibration curve

In order to validate the method, we determined: repeatability, reproducibility limit of detection and limit of quantification using the formulas mentioned above and compared the results.

As one can observe in the Table 3 the results are related from a determination to another.

Table 3

Correlation between the parameters calculated with the results obtained at the first and second reading

Calibration curve	Reproducibility	Repetability	LOD	LLOQ
1.	1,0081	0,0529	0,0624	0,1891
2.		0,0033	0,0039	0,0119

Conclusions

This paper's purpose was to develop and validate a micro-method for the determination of the total polyphenols in some dried plants according to Folin-Ciocalteu technique. This method grants a significant number of benefits that should be considered.

Firstly, by the use of the micro-method, the reagent consumption is reduced up to ten times in comparation with the classical macro-method. Secondly, the incertitude budget is drastically reduced by assuring homogenous sample preparation of the samples which takes only a few minutes (with the use of a multichannel pipette) and a very good quality of the reading which is done in only a few seconds.

Regarding repeatability and reproducibility, because the micro-method is so fast and easy to perform, with conditions that do not change over time, the measurement results will not be affected, proving a highly method robustness.

Concerning the evaluated parameters the method is verified and it can be used in the working conditions of the laboratory and is suitable for the proposed purpose.

Aknowledgment

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