STUDY OF THE PERFORMANCE OF THE POLYVINYLPYRROLIDONE POLYMER PHENOL CAPTURE

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***Abstract*— The Lowry Method (LM) is daily used in proteic amount determination in different biological samples, however, phenolic components presence is a limiting factor in the method. This paper investigates the LM output about protein amount, in a phenolic matrix, applying a Phenol Capture Method (PCM), from pre-treatment of the sample with polyvinylpyrrolidone (PVP). For this, a factorial experimental design was applied, with a central point and three independent variables. The best conditions suggested by the planning were 75% (w/w) of PVP, 5000 rpm, and 10°C, with a protein content of 87.80 µg.mL^{-1} and an experimental error of 22.9%. The PCM is efficient for protein amount quantification by LM, preventing the turbidity of the medium. Assessments using the PCM are still necessary to verify the matrix effect and the response as a function of protein variation (method validation), however, the results obtained allowed a reasonable estimate of the protein content under the conditions usual.**

***Keywords*— Up to five keywords should be provided. No keyword has to be in the title, they should be different words to help readers to use the search engine.**

I. INTRODUCTION

In laboratory analysis routines, the methods used to determine the protein content are selected considering sample matrix, working range, sensibility (detection limit), and possible interferences (Rekowski *et al.*, 2021; Silva *et al.*, 2020; Edelhoch, 1967). Protein quantification can be determined spectrophotometrically in the ultraviolet region 100-400 nm (Edelhoch, 1967) by groups present in the side chains of the constituent amino acids, such as aromatic residues (~280 nm) (Antosiewicz and Shugar, 2016; Cissé *et al.*, 2020) and from colored products resulting from reactions between proteins and chemical compounds, specific in the visible range (400-750 nm), (Kielkopg *et al.*, 2020; Sarkar *et al.*, 2020). Among the experimental techniques, the Lowry Method is usually the most used (Hayes, 2020; Peterson, 1979), given its high sensitivity (5 – 100 µg/mL) (Zaia *et al.*, 1998). However, due to its peculiarities and limitations, the presence of phenolic compounds in the sample can act as an interference, causing an analytical error (Cao *et al.*¸ 2020). The reagent for this test consists of a mixture of molybdate, tungstate and phosphoric acid (Folin-Ciocalteu) (Kaur *et al*., 2021), which are reduced, by side chains of amino acids (tyrosine, tryptophan, cysteine, asparagine and histidine), as part of electrons advancing the tetrapeptide units of the proteins. The addition of copper (II) accelerates the reaction, forming a chelating complex with the proteins, which favors the transfer of electrons with the Folin-Ciocalteu reagent, and finally, generates a change in the color of the medium, enabling the spectrophotometric determination in 750 nm (Contardi *et al.*, 2021).

Thus, solving the problem for protein quantification in a phenol-containing matrix, this paper proposes the application of Polyvinyl Pyrrolidone (PVP-40) polymer as a phenolic capturing agent (Park *et al.*, 2015). The phenolic compounds form strong complexes for hydrogen bonding with PVP-40, allowing the Folin-Ciocalteu reagent to be free to react with the protein-copper complex (Park *et al.*, 2015). He advantages of PVP polymer's strong interactions with phenolic compounds extend to different methods, such as controlled drug delivery, as well as a multitude of applications in pharmaceutical sciences (Contardi *et al.*, 2019).

This study aims at the elaboration of a 2^k factorial experimental design (*software Statistica 8*), in order to obtain the best conditions for determining protein amount by Lowry Method, in the phenol-containing matrix and with the application of PVP-40 as capturing agent. For experimental design, it is suggested the delineation of parameters and experimental conditions such as concentration of capturing agent (PVP-40), temperature (T °C) and rotational speed at the after centrifugation/solubilization stage. Parameters previously verified and that can favor a better performance of the method.

II. METHODS

A. Standard Solution of BSA

A standard solution of bovine serum albumin (BSA; ≥96%; Sigma-Aldric) was prepared at a concentration of 1.0 mg/mL then diluted to 71.42 µg/mL of BSA, in a phenolic (hydroxybenzene) solution of 34.79 mmol/L (99% Phenol; Sigma-Aldrich). The suggested phenol concentration was based on the constituents present in vaccines where phenol acts as a preservative/antibacterial.

This solution was used in all experiments of the experimental design to determine the protein content (Lowry Method) in the presence of phenol.

B. Determination of protein content - Lowry Method

To determinate the protein content, the Lowry method was evaluated in the presence of polymeric solution

(PVP-40; average molar weight, 40,000 Da) , acting as a phenol sequestrant, following the standard protocol (Bennett, 1992). In a test tube containing 1.0 mL of the sample were added: 1.0 mL of alkaline copper reagent and 0.50 mL of Folin-Ciocalteu reagent, previously diluted 1:5. Optical density readings were obtained at 750 nm in a UV-Visible spectrophotometer (Spectrophotometer SP 1102 – Bel photonics–Brazil).

C. Phenol capture method - PCM- Experimental design

The Factorial Experimental Design (FED) $2^{(k-p)}$, (k=3; p=0) was designed with two levels (high and low) and a central point, with three independent variables: percentage concentration of polyvinylpyrrolidone (PVP-40; % (w/w)), centrifugation temperature and rotation, setting a centrifugation time of 5 min (Universal 320R, Benchtop centrifuge). The parameters were established according to Table 1. For the FED, the *software Statistica 8* generated nine compositions, in triplicates with a central point, totaling 27 experiments (Table 1). The experiments were carried out randomly, for each run of experiments, a blank solution was prepared with the percentage by mass of PVP-40 indicated in the absence of protein. The rotation was based on a rotor with a diameter of 6 cm, but the rotation values can be converted to Relative Centrifugal Force (RCF) or G-force using the equation RCF = 1.12 x Radius x (rotation/1000)^2 (Legrand and Bouazza, 1991).

III. RESULTS

The data presented in Table 2 were applied for statistical verification between the variables PVP-40% (w/w), solubilization temperature (T°C), and rotation speed. The statistical significance (*p-value* ≤ 0.05) for all analyzed factors is presented in the Pareto chart, as shown in Fig. 1. The overlap of the reference line, over the bars in the chart, indicates that the effects, concentration of PVP- 40; interactions between PVP-40 and T°C; rotation speed; and the interactions between PVP-40, rotation and temperature have a statistically significant association with the response variable (protein content), (*p-value* ≤ 0.05). That is, they are process factors that influence the execution of the phenol capture method.

It is observed that the concentration factor of PVP-40 and its interaction with temperature favored the increase in protein content. This is confirmed by some studies that show the efficiency of this polymer as a protector of proteins, such as oxidase enzymes (peroxidase), in the presence of natural phenolic compounds, with the capture of phenol by hydrogen bonding between hydroxyl groups (-OH) and the polymer. Removal of natural phenolic compounds from raw eggplant pulp and peel extracts was also observed using PVP (Legrand and Bouazza, 1991).

In the Pareto chart, the statistical value of the *Student's t-test* (negative) indicates that the rotation factor is inversely proportional to the achievement of the protein content, but it cannot be considered in isolation for the method, as it does not consider the influence of the other factors in the process. However, for positive values, it indicates a proportionality between the factors, thus, the greater the interaction between factors 1 and 3 (PVP and temperature) and the increase in PVP mass, the greater the protein content obtained.

Table 1. Factors and factor levels used for the PCM method to determine the protein content by the Lowry method.

Factors	Low level	Center point	High level
PVP-40 %(w/w)	25	25	75
Cooling T(°C)	0	5	10
Rotation (rpm)	5000	7000	9000
PVP-40 %(w/w)	25	25	75

[a] PVP: polyvinylpyrrolidone; r.p.m: rotation per minute; T: temperature (°C). %PVP-40 added in medium containing phenol 31.87 mmol (3 mg/mL) and 71.40 µg/mL BSA.

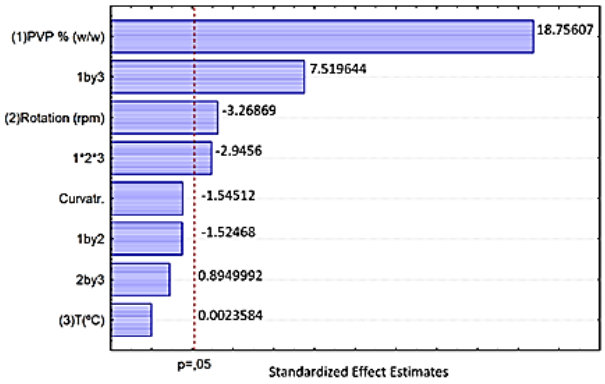

Figure 1: Pareto plot for protein content determination (Lowry method) in phenolic solution using PCM. Data as a function of Student t-test statistic values

The statistical model for the 2^k experiment can be presented here, as the curvature of the plane was not very significant (*p-level* ≥ 0.05), according to Eq. 1.

$$\text{Protein content (µg/mL)} = 64.4 + 13.25P - 2.31\,R + 5.03\,P.T - 2.08\,P.R.T, \quad (1)$$

where: P: PVP-40; R: rotation and T: temperature (°C); with the coefficient of determination, R^2 =0.960 and R^2adj= 0.942.

Figure 2a, presents the means plot, for the interaction of rotation, temperature, and PVP-40 content factors. The graph shows that more moderate rotations are enough to guarantee the solubilization of the polymer in the medium, not being necessary a greater increase in this factor for a better result. Also, it is observed that the solubility of PVP-40 in an aqueous solution was facilitated when the system was kept refrigerated under agitation at 10°C and 5000 rpm. What favored the polymer solubility, allowing lower viscosity, better interaction between polymer and phenol. Other studies, however, have successfully applied PVP to remove phenolic compounds at lower temperatures (Mäntele and Deniz, 2017).

Figure 2b, shows the response surface indicating that under conditions of low centrifugation speed (5000 rpm), percentage by mass of 75% of PVP-40 and temperature of 10 °C, indicated a set of conditions for phenol capture in the medium and therefore the possibility of determining the protein content by the Lowry method. When compared with the protein content obtained by the Lowry Method, in the absence of interfering phenol (theoretical protein content of 71.40 µg/mL), the experimental error using PCM and subsequent application of the Lowry

Table 2. Protein amount (Lowry method) obtained for factorial experimental design 2(k), (k=3), using software *Statistica 8*.

PVP % (w/w)	(rpm)	T (°C)	Protein content (µg/mL)*SD
50	7000	5	61.80 (±0.99)
75	9000	10	74.73 (±2.39)
75	9000	10	80.17 (±2.47)
50	7000	5	61.80 (±1.98)
75	5000	0	66.55 (±2.13)
75	5000	0	73.37 (±2.35)
25	9000	0	51.60 (±1.65)
75	5000	0	82.89 (±2.65)
75	9000	10	79.49 (±2.54)
25	9000	0	55,00 (±0.88)
50	7000	5	59.00 (±0.96)
25	5000	10	45.48 (±0.73)
25	5000	10	43.44 (±0.69)
25	9000	0	50.92 (±0.81)
25	5000	10	44.12 (±0.71)
25	5000	0	55.46 (±0.89)
25	5000	0	62.87 (±1,01)
25	5000	0	62.87 (±1,01)
75	9000	0	70.64 (±1,13)
75	9000	0	68.79 (±1,10)
75	9000	0	71.76 (±1,15)
75	5000	10	87.31 (±1,40)
75	5000	10	88.05 (±1,41)
75	5000	10	88.05 (±1.98)
25	9000	10	48.05 (±0,77)
25	9000	10	45.09 (±0,72)
25	9000	10	48.79 (±0,78)

PVP: Polyvinylpyrrolidone; rpm: rotation per minute, T: temperature (°C). *SD: standard deviation. Data considering Lowry Method's error (±20 %).

Method was 22.90% (protein content obtained 87.80 µg/mL). This error can be overcome by applying a calibration curve that contains the presence of polymer and phenol, which can be investigated by observing the matrix effect, based on validation tests. However, the experimental error of the classical Lowry method of 20% (Lowry *et al.*, 1951) must be considered, which then allows a reasonable estimate of the protein content used in PCM. The variation in the molecular weight of the polymer is an important factor for future studies applied to the conditions optimized in this work.

Wintersan and Minchin (2005) tried to determine the protein content bound to ortho-diphenols by the Lowry Method, assuming that there is no inherent presence of this compound in the presence of copper. The authors observed a variation of 20 to 50%, concerning the reference values estimated by the Lowry Method.

In the test tube, it is observed that the turbidity of the protein solution in a medium containing phenol and Folin-Ciocalteu reagent, used in the Lowry Method, makes it impossible to obtain O.D. This is because the Folin-Ciocalteu reagent (acid phosphotungstic and phosphomolybdic acid), used for detection of phenolic compounds, in an alkaline medium, also reduces the Mo $^{6+}$ ion to Mo $^{+5}$, leading to the formation of the phenolate anion and, therefore, forming tungsten molybdenum oxide (Magalhães *et al.*, 2010).

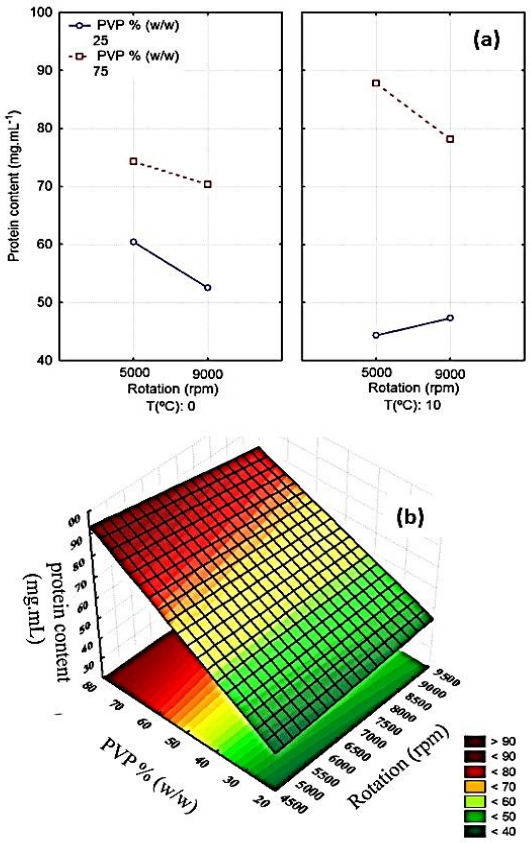

Figure 2. (a) graphs of maximum trends for protein content (µg/mL) obtained by the Lowry Method after PCM (phenol capture method), at working temperatures of 0 and 10 °C; % PVP-40 of 25 and 75% and rotation of 5000 and 9000.

V. CONCLUSIONS

From the results obtained, it is possible to reasonably estimate the protein content in phenolic solutions, in the established tests, from the capture of phenol by PVP. However, it is necessary to investigate the existence of the matrix effect (PVP and phenol) for better comparison with the control sample (absence of phenol) and also to evaluate the limitation of PCM at low protein concentrations, since the experiments carried out here kept the concentrations fixed of BSA and phenol. It is possible, however, to imprint its efficiency to the PCM, since it promoted interactions of the Folin-Ciocalteu reagent primarily with the side groups of the BSA protein, by sequestering the phenol molecules by the PVP-40 polymer. Please insert a continuous break at the end of the manuscript so both columns end at approximately the same height.

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