Advances in Science and Technology Research Journal

Advances in Science and Technology Research Journal, 2027, 21(1), 623–635
https://doi.org/10.12913/22998624/241621
ISSN 2299-8624, License CC-BY 4.0

Received: 2026.09.02
Accepted: 2026.11.16
Published: 2026.12.01

Statistical evaluation and validation of Folin-Ciocalteu assay protocols for reliable determination of total polyphenols in onion skin bio-waste extracts

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ABSTRACT

A systematic statistical evaluation of five Folin-Ciocalteu (FC) assay protocols was performed to address the lack of standardization and limited reproducibility in Total Polyphenol Content (TPC) determination. The protocols were critically compared using rigorous statistical analysis to identify the most accurate and reliable procedure. Method validation was carried out using a commercially available Vitaflavan extract and gallic acid standard solutions. The optimal protocol was found to involve a 120 min incubation with the FC reagent at room temperature, providing the highest accuracy and reproducibility among the tested approaches. The selected protocol was subsequently applied to determine TPC in the extracts from yellow onion skin bio-waste. Additionally, the effect of ultrasound-assisted extraction on TPC was investigated and compared with conventional solid–liquid extraction. The results demonstrated an increase in TPC when ultrasound treatment was applied, yielding 55.5 ± 1.3 mgGAE/g of dry onion skin compared to 32.9 ± 1.1 mgGAE/g obtained by conventional extraction. The proposed approach provides a standardized framework for improving the reliability and comparability of FC-based TPC measurements in complex matrices. Protocol P5 is recommended as a standardized, high-precision method for the reliable characterization of polyphenols in food-waste valorization and plant science.

Keywords: Folin-Ciocalteu assay; total polyphenol content; ultrasound-assisted extraction; method validation; polyphenols; onion skin; standardization

INTRODUCTION

Polyphenols are a large and diverse group of chemical compounds naturally occurring in the plant world. They are products of secondary plant metabolism and are characterized by the presence of numerous phenolic rings [1]. The main sources of polyphenols in the human diet are berries,

grapes, olive oil, cocoa, onion, nuts, potatoes, as well as other fruits and vegetables. Since ancient times, plant extracts containing polyphenols have been used in folk medicine because of their perceived health-promoting effects. Numerous scientific studies have shown that polyphenols play an important role in positively influencing human health. They have been attributed with antioxidant, anticancer, anti-inflammatory, antibacterial, antiviral, antifungal, anti-atherosclerotic, anticoagulant, immunomodulatory, vasodilatory, analgesic, antidiabetic, and hepatoprotective effects [2–5]. Their beneficial effects are due to their ability to neutralize free radicals, blocking their damaging effects on cells. Therefore, research on polyphenols is currently a hot topic in many laboratories. Thus, there is a need for polyphenol determination by many researchers. The commonly used method for assessing total polyphenol content, widely reported in the literature as a standard protocol, is the spectrophotometric assay with Folin-Ciocalteu (FC) reagent [6]. It relies on the transfer of electrons in an alkaline environment from phenolic compounds to form a chromophore constituted by a phosphotungstic/phosphomolybdenum complex, the maximum absorbance of which depends on the concentration of phenolic compounds [7]. The reduced Folin-Ciocalteu reagent can be measured spectrophotometrically in the range of 690–710 nm. Although the test with Folin-Ciocalteu reagent is commonly used, many modifications have been described, differing in reagent concentrations, incubation time, and temperature [8, 9, 6].

Onion (*Allium cepa*) is a rich source of biologically active compounds such as polyphenols and flavonoids [10–12]. Onion has strong, characteristic aromas and flavors that have made it an important food ingredient. Onion extracts have recently been reported to be effective in cardiovascular disease, because of their hypocholesterolemic, hypolipidemic, antioxidant, antiasthmatic, and antithrombotic activity [13]. Onion is one of the most important crops among all horticultural commodities, and it is second to tomato in terms of global production [14]. About three-quarters of the EU's onion production in 2022 came from the Netherlands (19.7% of the EU's onion area and 24.2% of its production), Spain (13.9% and 20.0%, respectively), France (10.2% and 11.5%, respectively), Poland (13.3% and 10.5%, respectively), and Germany (9.9% and 10.3%, respectively) [15].

Onion skins, external fleshy scales, bulb tops, pods, and roots generated during industrial peeling are the main onion residues and constitute approximately 38% of the weight of fresh onions unfit for consumption [16].

The ultrasound-assisted extraction (UAE) has proven to be the simplest and cheapest technique; it allows the use of different solvents, low temperatures, has no restrictions on the polarity of the compound of interest or the moisture content of the matrix; it also has good reproducibility and high potential for scale-up [17]. It has been demonstrated that the application of ultrasound in extracting bioactive components has great potential [18].

In this work, the authors focused on investigating different protocols, based on Folin-Ciocalteu reagent, for determination of total polyphenol content (TPC). Employing statistical studies, the most accurate one was selected, and then applied for TPC analysis in the extracts obtained from yellow skin onion. Moreover, the efficiency of UAE was compared with a traditional solid-liquid extraction.

MATERIALS AND METHODS

Materials

The chemicals used in this study were of analytical grade and obtained from commercial suppliers. Sodium carbonate (Na_2CO_3, purity ≥99.5%) was provided by Fisher Scientific (Hampton, NH, USA). This inorganic salt served as a source of carbonate ions to neutralize the Folin-Ciocalteu reagent under alkaline conditions. Folin-Ciocalteu's phenol reagent (stock concentration 2.2 mol/L, calculated as acid) was supplied by PanReac AppliChem ITW Reagents (Barcelona, Spain). Gallic acid (purity ≥98.5%) was used as a reference standard for calibration curves and quantification of polyphenols, and absolute ethanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). A polyphenol extract (Vitaflavan, batch OC-DRT-16-02900) containing around 90% of polyphenols, primarily oligomeric proanthocyanidins, was generously provided by Purextract® (Coutras, France) and served as a sample to validate the methods. All chemicals were used without further purification. The purity and source of the chemicals ensured the accuracy and reproducibility of the methods

evaluated in this study. Yellow onion skin (biowaste derived from the onion processing sector) used for polyphenol extraction was kindly supplied by Cebollas Marchite company (Fustiñana, SPAIN), which is one of the most important onion exporters in the world.

Determination of Total Polyphenols Content

Five different protocols (P1-P5) for determining total polyphenol content in samples were investigated and compared. Detailed information on each protocol, including the order and volume of reagent additions, incubation time, Na_2CO_3 stock concentration, final reaction volume, and final Na_2CO_3 concentration after all reagent additions, is provided in Table 1. The Folin-Ciocalteu reagent was used as supplied by the manufacturer for protocols P1, P3, and P5, whereas for P2 the supplied FC reagent was diluted 1:10 by mixing 1 mL of FC reagent with 9 mL of water. For P4, a 10% (v/v) FC reagent solution was prepared from the supplied FC reagent. Total polyphenol content was determined spectrophotometrically using different variations of Folin-Ciocalteu's method by means of a UV-VIS UV-1800 Shimadzu spectrophotometer (Tokio, Japan) using standard 5 mL polystyrene cuvettes (KARTEL.L S.p.A., Italy; Cat. No. 01937-00) with a 10 mm optical path length. The Folin-Ciocalteu reagent, a mixture of phosphomolybdic and phosphotungstic acids, reacts with phenolic compounds and is reduced, forming a blue chromophore detectable spectrophotometrically. Seven concentration levels of gallic acid standard were used to construct the calibration curves: L1 – 0.6 g/L, L2 – 0.4 g/L, L3 – 0.2 g/L, L4 – 0.1 g/L, L5 – 0.05 g/L, L6 – 0.025 g/L, and L7 – 0.0125 g/L. A 1 g/L gallic acid stock solution was prepared in Milli-Q water. Each concentration level was prepared independently three times and measured in triplicate according to all the investigated polyphenol determination methods presented in Table 1. Once the calibration curves for each method were prepared, two independent standard solutions, each prepared in triplicate, were used for method validation. The first one was gallic acid at a concentration of 0.2 g/L prepared in Milli-Q water. The second standard was a commercial polyphenol extract, Vitaflavan, at a concentration of 0.2 g/L. Each standard solution was measured in triplicate using all the methods investigated, as presented in Table 1.

Polyphenol extraction from yellow skin onion

Before the extraction, the sample of yellow onion skin was ground into small pieces with a Bosch coffee grinder MKM6003 180W type KM13 (Munich, Germany) for 45 s. The total solid content was analyzed by drying the sample in the oven at 105 °C until constant weight, giving the value of 87.8 ±1.1%.

Table 1. Detailed procedure for the determination of total polyphenol content in investigated Protocols 1-5

Procedure order	P1	P2	P3	P4	P5
Sample (ml)	1	2*	0.02	0.02	0.1
Water (ml)	60	0	0.1	1.58	2
FC reagent (ml)	5	10*	0.1	0.1**	0.1
Incubation (min)	0.5	5	3	10	5
Na_2C_o3					
Volume (ml)	15	8	0.2	0.3	1.6
Concentration (%)	20	7.5	20	16	7.5
Water (ml)	19	0	1.58	0	0
Incubation					
Time (min)	120	120	20	60	120
Temperature (°C)	RT	RT	40	RT	RT
Final volume (ml)	100	20	2	2	3.8
Final Na_2CO_3 (%)	3.0	3.0	2.0	2.4	3.2
Measurement					
Wavelength (nm)	765	760	750	765	765

Note: * Diluted 1:10, ** 10% (v/v).

After sample grinding, the solid-liquid extraction was carried out by mixing 1 g of biomass with 30 ml of 60% ethanol. Then, the mixture was stirred for 1 h at room temperature (22 ±2°C) under a magnetic stirrer set at 500 rpm. Upon completion of the extraction, the mixture was centrifuged at 9000 rpm for 10 min and then filtered. The supernatant was collected for total polyphenol analysis, which was performed using the validated protocol (P5). The extraction procedure was performed in triplicate, and each supernatant was analyzed three times. Prior to polyphenol analysis, the extract samples were diluted at a ratio of 1:10. A blank prepared with the extraction solvent (60% ethanol) and subjected to the same dilution factors as the corresponding samples was used to correct for any absorbance contribution arising from the solvent. The applied dilution factors ensured that all analyzed samples were within the validated calibration range.

It should be noted that no specific matrix-effect studies were performed for the onion skin extracts. Nevertheless, all samples were analyzed using the same extraction procedure and analytical conditions, allowing for a consistent comparison of the results obtained.

The total polyphenol content (TPC) in yellow onion skin was expressed as mg gallic acid equivalent (GAE) per g of biomass dry weight (DW) and calculated using the following equation:

$$TPC(mg_{GAE}/g_{DW}) = \frac{PC\left(\frac{mg_{GAE}}{L}\right)\times DF\times SE(L)}{WB\,(g)\times DW\,(\%)} \quad (1)$$

where: *TPC* – the total polyphenol content of yellow onion skin; *PC* – the polyphenol concentration determined in the extract by UV-Vis spectrophotometry; *DF* – the dilution factor applied prior to UV-Vis analysis; SE – the volume of solvent used for extraction; WB – the mass of biomass (i.e., yellow onion skin) used for extraction; DW – the dry matter content (total solids content) of the biomass.

In the case of ultrasound-assisted extraction, first the mixture was exposed to ultrasound treatment (9 cycles of 30 s treatments with 59s break between each treatment) using a BANDELIN SONOPULS HD 4200 ultrasonic homogenizer (Merk, Madrid, Spain) at a working frequency of 20 kHz and an applied power of 400 W. Then, the pre-treated mixture was used in the extraction procedures as described above.

Statistical analysis and validation

All standards and samples were independently prepared in at least three times, and each prepared solution was analyzed in triplicate. The results are expressed as mean standard deviation (*SD*). The normality of the data distribution was assessed using the Shapiro-Wilk test. Statistical comparisons between the experimental means and expected values were conducted using a one-sample t-test in Statistica 13.1 (StatSoft Inc., Tulsa, OK, USA) with a significance level of a=0.05.

Method validation parameters were evaluated in accordance with the principles of the International Council for Harmonisation (ICH Q2(R1)) and ANVISA (Resolution RE No. 899/2003) guidelines. The established acceptance criteria were set as follows: linearity coefficient of determination $R^2 \geq 0.990$, accuracy (analytical recovery) within 95.0%–105.0%, and precision expressed as relative standard deviation RSD≤2.0 for pure gallic acid standards and RSD≤5.0 for complex plant extracts. It should be noted as a study limitation that method accuracy (recovery) and precision (repeatability) were evaluated at a single mid-range concentration benchmark (0.200 g/L). This single-point assessment served to compare performance across the five protocols, rather than to constitute a full, multi-level range validation across the entire calibration curve.

The analytical performance of the Folin-Ciocalteu modifications (P1–P5) was evaluated following the National Health Surveillance Agency of Brazil (RE 899) guidelines. The validation parameters included the slope, y-intercept, and the coefficient of determination (R^2). Precision was expressed as the relative standard deviation (*RSD%*), while the accuracy of the regression models was monitored via the standard error of estimate (*SEe*). Limits of detection (*LOD*) and quantification (*LOQ*) were calculated based on the standard deviation of the intercepts (*S*) and the mean slope of three independent calibration curves as follows [19–27]:

$$LOD = 3.3S/Slope \quad (2)$$

$$LOQ = 10S/Slope \quad (3)$$

Accuracy was calculated according to the formula:

$$Accuracy\% = \left(\frac{Mean\ Measured\ Concentr.}{Expected\ Reference\ Concentr.}\right)\times 100 \quad (4)$$

Relative percent error (RPE%) calculation was performed using the formula:

$$RPE\% = |Accuracy\% - 100\%| \quad (5)$$

Precision (RSD %) was calculated following the formula:

$$Precision\ RSD\ (\%) = \left(\frac{SD}{\bar{x}}\right) \times 100 \quad (6)$$

RESULTS AND DISCUSSION

Comparative characteristics of the evaluated protocols

Five methodological variants of the Folin-Ciocalteu assay were systematically evaluated to assess their impact on polyphenol quantification (Table 1). Protocol P1 was adapted from the classic 1965 paper by Singleton and Rossi [28], where they described polyphenol determination using Folin-Ciocalteu reagent. This procedure involves a very short (30-second) incubation with the reagent, followed by a 2-hour incubation at room temperature after adding sodium carbonate. Measurement is performed at 765 nm. Protocol P2 is based on the later paper by Singleton et al. [29]. It is a micromethod adapted for smaller sample volumes. The sample is incubated with Folin-Ciocalteu reagent for 1-8 minutes; sodium carbonate is then added, and the mixture is incubated for 2 hours at room temperature. Absorbance is measured at 760 nm. Protocol P3 was taken from the procedure routinely used in a work by Cicco et al. [8]. It involves incubating the sample with Folin-Ciocalteu reagent for 3 minutes, then neutralizing with carbonate and incubating for another 20 minutes at 40 °C. Absorbance is measured at 750 nm wavelength. Protocol P4 originates from the routine protocol applied in the paper by Sotenko et al. [30]. The sample is incubated with Folin-Ciocalteu reagent for 8-10 minutes, then sodium carbonate is added and incubated for 1 hour in the dark at room temperature. Absorbance is measured at 765nm. Protocol P5 was adapted from the work of Tsibranska et al. [31] describing the extraction of bioactive compounds from plants. This procedure involves a 5-minute incubation of the sample with Folin-Ciocalteu reagent, followed by the addition of sodium carbonate and a 2-hour incubation in the dark at room temperature. Measurement is taken at 765 nm.

All presented protocols, ranging from the classical macro-methods of Singleton and Rossi (P1, P2) to modernized micro-scale adaptations (P3–P5), differ primarily in reagent stoichiometry, incubation kinetics, and thermal conditions. While the fundamental redox reaction remains consistent, the combined procedural parameters of each protocol-including reagent stoichiometry, dilution factors, incubation duration, and thermal conditions-collectively influence the reaction's endpoint and color development.

It should be noted that Protocols P1–P5 represent distinct, multi-factorial analytical procedures that differ simultaneously across several operational parameters (reagent stoichiometry, sample dilution, temperature, and incubation duration). Consequently, performance variations reflect holistic, protocol-level outcomes, rather than isolated single-factor kinetic effects.

Linearity and dynamic range

Table 1 shows how studied protocols differ primarily in reagent concentrations, incubation times, and temperatures. During the first set of experiments, gallic acid was used as a reference phenolic to estimate the total polyphenol equivalent by calibration curve, and then during the second set of experiments two independent standard solutions were used for validation of the investigated protocols. The first one was an aqueous solution of gallic acid at a concentration of 0.2 g/L, while the second standard was an aqueous solution of a commercially available polyphenol extract, Vitaflavan, at a concentration of 0.2 g/L.

Figure 1 provides calibration curves generated during the experiments carried out following the investigated protocols, while Table 2 presents statistical parameters calculated from triplicate repetitions of each experiment. The calibration curves for protocol P3 and protocol P4, as well as for pristine Singleton methods P1 and P2, exhibited linearity across the full range of standard concentrations from 0.0125 to 0.6 g/L with a coefficient of determination R^2 above 0.99. This indicates a high-quality fit of the linear regression model and the ability to accurately quantify polyphenols in this range. The maximum concentration level of 0.600 g/L in Protocol P5 yielded an absorbance exceeding 1.4. Although the calibration model remained linear across the full range (R^2=0.999), high absorbance values (A>1.0) are generally avoided in quantitative spectrophotometry due to

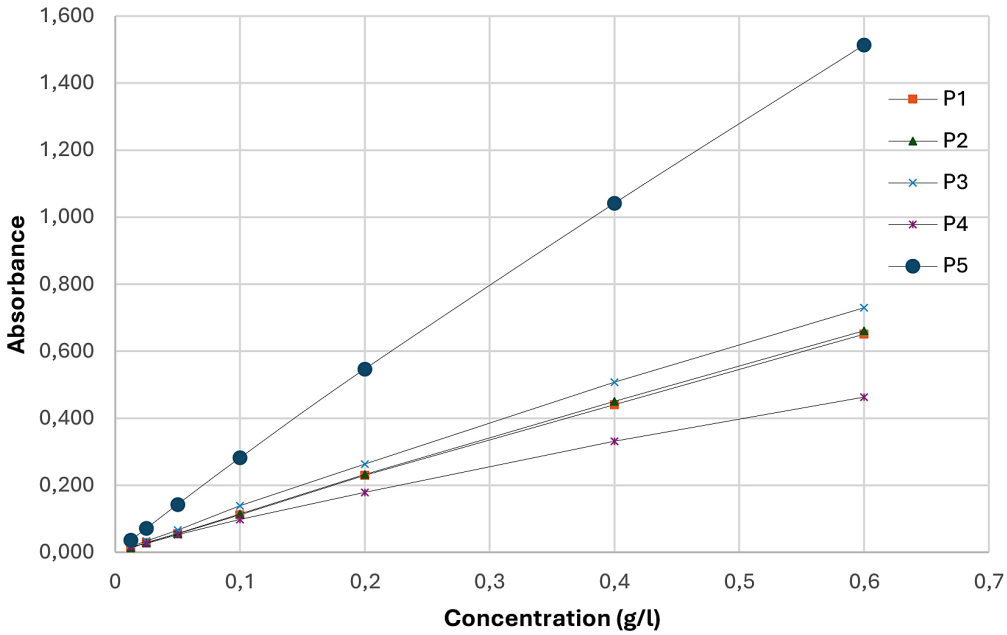


Figure 1. Calibration curves generated for each protocol of gallic acid solution

increased measurement uncertainty and potential stray light interference. Therefore, the proposed working range for Protocol P5 was capped at 0.400 g/L (absorbance A ≈ 0.98), ensuring that measurements remain within the optimal spectrophotometric response range (A ≤ 1.0) without requiring high sample dilutions.

Diluting the samples allows avoiding excessive absorbance and accurately determining polyphenol content within the linear measurement range. The calibration curve parameters should be considered when selecting the optimal technique.

Validation

Two standard solutions were applied for the validation of the investigated protocols . Table 3 presents statistical and comparative analysis of total polyphenols concentration in aqueous solutions of Vitaflavan and gallic acid (TPC, g/L) across different protocols. It is important to highlight that according to the information provided by the Vitaflavan extract provider, the total polyphenol content in the product is around 90 wt%. However, the analytical method used to

Table 2. Statistical parameters for the calibration curves – total polyphenols concentration (g/L)

Specification	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5
Slope (a)	1.087	1.106	1.218	0.765	2.519
Intercept (b)	0.003	0.003	0.010	0.015	0.020
Intercept SD (S)*	0.00500	0.00498	0.00974	0.00949	0.01562
R^2	0.999	0.999	0.999	0.997	0.999
LOD**	0.015	0.015	0.026	0.040	0.020
LOQ***	0.046	0.045	0.080	0.124	0.062
(SEe)	0.005	0.005	0.010	0.010	0.016

Note: The linear calibration range for Protocols P1–P4 is 0.0125–0.600 g/L, whereas for Protocol P5 it is 0.0125–0.400 g/L due to capping the upper calibration level at 0.400 g/L to maintain absorbance within the optimal spectrophotometric range (A≤1.0).

***** Intercept SD **(**S**)** is the standard deviation of the regression intercepts obtained from n = 3 independent calibration curves. ****** Limit of detection **(**LOD**)** was calculated as LOD = 3.3S/a, where S is the intercept standard deviation and a is the calibration slope. ******* Limit of quantification **(**LOQ**)** was calculated as LOQ = 10S/a. ******** Proposed working range defines the estimated concentration interval bounded by the statistical LOQ (lower limit) and the maximum evaluated linear calibration level (upper limit). For Protocol P5, the upper limit is capped at 0.400 g/L to maintain spectrophotometric absorbance within A≤1.0 and minimize measurement uncertainty.

determine this value was not specified, and therefore it is unclear whether it can be directly compared with results expressed as gallic acid equivalents (GAE). Therefore, for the purposes of this study, the expected total polyphenol content of the Vitaflavan solution was considered to be the average TPC value calculated from the results obtained using the five UV-Vis methods evaluated in this study, with gallic acid used as the calibration standard (Table 3). For the Vitaflavan samples prepared at a concentration of 0.2 g/L, the calculated mean TPC value was 0.18 g/L. Consequently, this value was used as the expected total polyphenol content of the Vitaflavan solution throughout this work. Average polyphenol contents with deviations and standard errors are also shown in Figure 2 for Vitaflavan extract solution (Figure 2a) and for gallic acid solution (Figure 2b).

In the case of the one-sample t-test for the Vitaflavan solution (Table 3), Protocols P2 ($p=0.647$), P4 ($p=0.924$), and P5 ($p=0.368$) showed no statistically significant differences from the expected value ($p>0.05$). In contrast, Protocols P1 ($p=0.007$) and P3 ($p=0.020$) yielded means that were statistically significantly different from 0.180 g/L ($p<0.05$). Systematic errors were evaluated directly using physical Mean Bias (g/L), 95% Confidence Intervals ($CI_{95\%}$), and Relative Percent Error (RPE%). Protocol P1 exhibited a small positive bias of +0.007 g/L (RPE=3.89%, $CI_{95\%}$=[0.185; 0.189] g/L), P3 showed a negative bias of -0.008 g/L (RPE= 4.44%, $CI_{95\%}$= [0.167; 0.177] g/L, while Protocol P5 demonstrated outstanding accuracy with a minimal bias of +0.002 g/L (RPE=1.11%, $CI_{95\%}$= [0.175; 0.189] g/L).

The results of the Folin-Ciocalteu assay are strongly influenced by the types of polyphenols present in a sample. Simple phenolic acids, such as gallic acid, typically exhibit high reactivity toward the Folin-Ciocalteu reagent, whereas more structurally complex or sterically hindered compounds generally display slower and/or lower reactivity [32]. In Vitaflavan extract, polyphenols are predominantly procyanidins (47%), epicatechin and its derivatives (28%), and catechin (22%), with gallic acid accounting for only 2% of the total [32]. Because most polyphenols in Vitaflavan are complex procyanidins and flavan-3-ol derivatives, the measured TPC using the Folin-Ciocalteu assay, depending on the protocol applied, could be lower than that obtained with gallic acid alone. This effect is particularly pronounced under protocol P3, which employs a

Table 3. Descriptive statistics and comparative analysis of total polyphenols concentration in aqueous solutions of Vitaflavan and gallic acid across different protocols.

Protocol	Analyte/standard	Mean (g/L)	SD (g/L)	Bias (g/L)*	95% confidence interval (g/L)**	Expected value (g/L)	p-value (t-test)***
P1	Aqueous solution of Vitaflavan	0.187	0.001	+0.007	[0.185;0.189]	0.180	0.007
	Aqueous solution of gallic acid	0.196	0.004	-0.004	[0.186;0.206]	0.200	0.225
P2	Aqueous solution of Vitaflavan	0.176	0.013	-0.004	[0.144;0.208]	0.180	0.647
	Aqueous solution of gallic acid	0.204	0.003	+0.004	[0.197;0.211]	0.200	0.147
P3	Aqueous solution of Vitaflavan	0.172	0.002	-0.008	[0.167;0.177]	0.180	0.020
	Aqueous solution of gallic acid	0.206	0.008	+0.006	[0.186;0.226]	0.200	0.324
P4	Aqueous solution of Vitaflavan	0.179	0.016	-0.001	[0.139;0.219]	0.180	0.924
	Aqueous solution of gallic acid	0.213	0.002	+0.013	[0.208;0.218]	0.200	0.008
P5	Aqueous solution of Vitaflavan	0.182	0.003	+0.002	[0.175;0.189]	0.180	0.368
	Aqueous solution of gallic acid	0.198	0.001	-0.002	[0.196;0.200]	0.200	0.074

Note: * Bias (g/L) = mean measured concentration – expected value**.** Positive values indicate overestimation (positive bias), while negative values indicate underestimation. ** 95% confidence intervals were calculated using Student's t-distribution for n=3 independent replicate measurements: $CI_{95\%}$ = Mean ± t (0.025, df=2) x (SD/sqrt(3)), where t (0.025, df=2) = 4.303. *** p-values were obtained from a one-sample t-test comparing the experimental mean to the respective expected value ($\alpha = 0.05$).

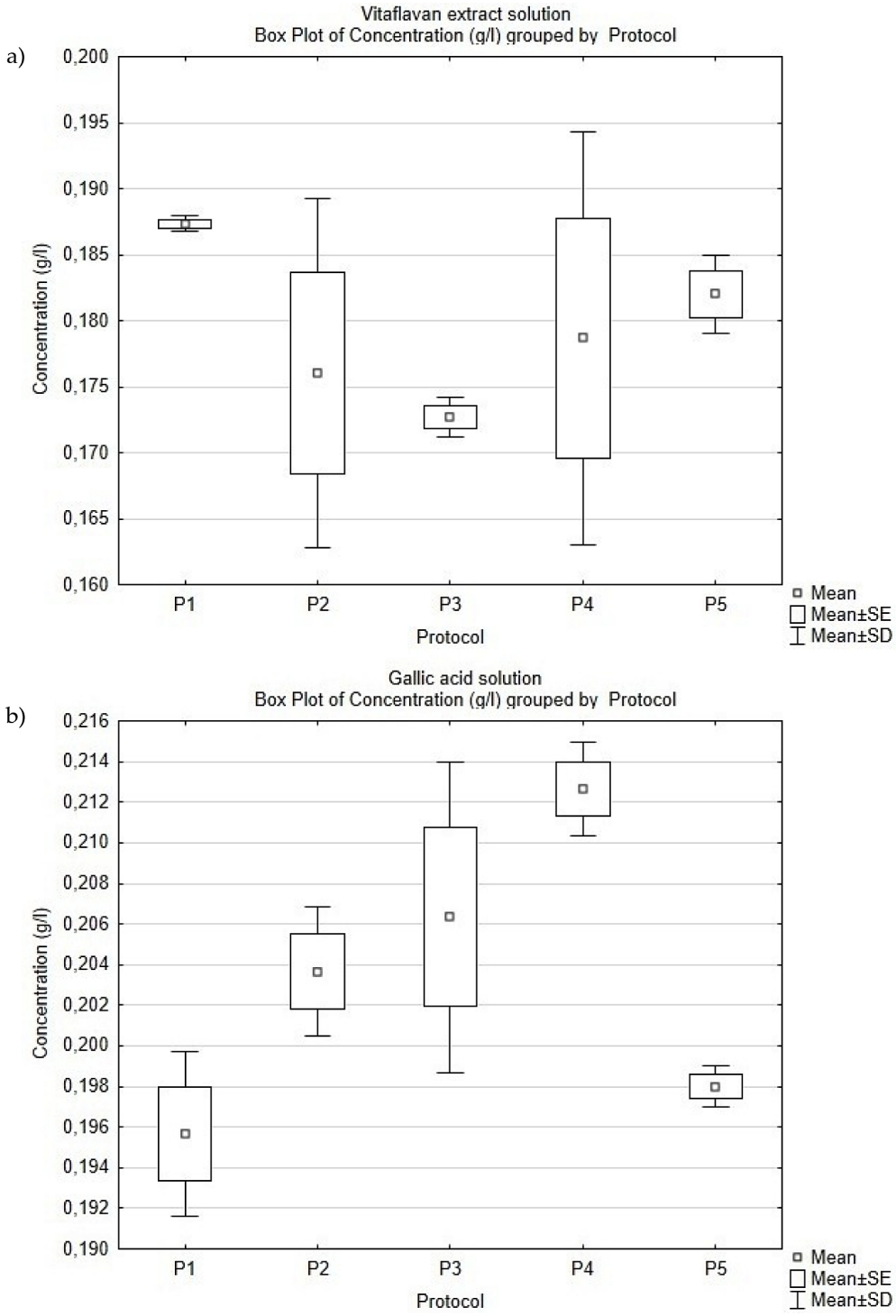


Figure 2. Average TPC with deviations for Vitaflavan extract solution (a), and for gallic acid solution (b)

shorter incubation time and the lowest final sodium carbonate concentration (2%, see Table 1), resulting in TPC values significantly below the theoretical value (Table 3, Figure 2).

In conclusion, the results show that protocols P5 and P2 provide the most reliable determination of total polyphenols in plant extracts like Vitaflavan. The selected analytical method significantly influences the measured polyphenol content, so performance parameters should guide the choice of optimal procedure.

In the case of the mean values for the gallic acid standard (0.200 g/L), no statistically significant differences from the expected theoretical benchmark were detected for Protocols P1 ($p=0.225$), P2 ($p=0.147$), P3 ($p=0.324$), and P5 ($p=0.074$) under the evaluated experimental conditions ($p>0.05$). Because a non-significant t-test

result ($p>0.05$) does not inherently prove complete accuracy or the total absence of bias, method trueness was evaluated directly using physical parameters: Mean Bias (g/L), Relative Percent Error (RPE %), and 95% Confidence Intervals (CI_{95}%). Among these, Protocol P5 exhibited minimal systematic bias (-0.002 g/L, RPE=1.00%, CI_{95}%=[0.196; 0.200] g/L) combined with outstanding repeatability (RSD=0.51%). In contrast, Protocol P4 yielded a sample mean that was statistically significantly higher than 0.200 g/L ($p=0.008$, bias=+0.013 g/L, RPE=6.50%), indicating a tendency toward overestimation.

Subsequently, the relative percent error (RPE) between the measured value and theoretical value was calculated (Table 4). The error parameter is crucial for selecting the optimal analytical procedure, as it identifies the methods with the highest reliability. All methods gave results close to the theoretical values for both analyzed standards.

The accuracy and precision metrics presented in Table 4 reveal substantial differences in performance across the five evaluated Folin-Ciocalteu assay protocols. For the complex Vitaflavan botanical extract (evaluated against the 0.180 g/L reference benchmark), Protocols P4 and P5 demonstrated the highest accuracy, yielding RPE values of 0.56% (accuracy=99.44%) and 1.11% (accuracy=101.11%), respectively, whereas Protocol P3 exhibited the largest deviation (RPE=4.44%, accuracy=95.56%). However, an evaluation of method repeatability revealed that Protocol P4 suffered from unacceptable precision variation (RSD=8.94%), exceeding the established 5.0% threshold for plant extracts, whereas Protocols P1 (RSD=0.53%), P3 (RSD=1.16%), and P5 (RSD=1.65%) maintained good repeatability.

For the pure gallic acid standard (0.200 g/L benchmark), Protocol P5 achieved superior overall performance, combining the lowest systematic error (RPE=1.00%, accuracy=99.00%) with outstanding precision (RSD=0.51%). Conversely, Protocol P4 yielded the highest systematic error (RPE=6.50%, accuracy=106.50%). Although all protocols maintained RPE values below 7% across both matrices, Protocol P5 was uniquely distinguished by consistently satisfying both accuracy (RPE≤1.11%, accuracy 99.00–101.11%) and precision criteria (RSD≤1.65%) for both simple standards and complex botanical extracts. In contrast, Protocol P4 demonstrated matrix-dependent instability, showing low systematic error for Vitaflavan but poor precision (RSD=8.94%) and elevated error for gallic acid (RPE=6.50%), thereby confirming Protocol P5 as the most robust, accurate, and universally applicable analytical procedure.

These findings suggest that protocol P5 offers the optimal balance of accuracy and reproducibility for simultaneous determination of diverse polyphenolic compounds, while protocol-specific optimization may be required when targeting individual analytes.

Finally, total polyphenol content was measured in yellow skin onion extract obtained from solid-liquid extraction as well as solid-liquid ultrasound-assisted extraction by applying protocol P5. The total polyphenol contents were 32.9 ±1.1 mgGAE/g of dry onion skin and 55.5 ±1.3 mgGAE/g of dry onion skin, from solid-liquid extraction and ultrasound-assisted extraction, respectively.

To assess accuracy and systematic bias, the protocols were validated against two distinct standards: a simple phenolic acid (gallic acid) and a complex botanical extract (Vitaflavan, 90% polyphenols). The results of this investigation provide valuable insights into optimizing the popular Folin-Ciocalteu assay for quantifying total polyphenols. The obtained findings demonstrated significant variability between common procedural modifications of this method. This underscores the importance of rigorous validation to understand how subtle differences

Table 4. Validation parameters for Vitaflavan and gallic acid

Protocol	Vitaflavan			Gallic acid		
	RPE (%)	Accuracy (%)	Precision (RSD%)	RPE (%)	Accuracy (%)	Precision (RSD%)
P1	3.89	103.89	0.53	2.00	98.0	2.04
P2	2.22	97.78	7.39	2.00	102.00	1.47
P3	4.44	95.56	1.16	3.00	103.00	3.88
P4	0.56	99.44	8.94	6.50	106.50	0.94
P5	1.11	101.11	1.65	1.00	99.00	0.51

in protocol impact analytical performance and to identify optimal parameters for accurate polyphenol analysis. In agreement with the presented study, previous works have also shown that changing reagent concentrations, incubation times, and measurement wavelengths can markedly influence Folin-Ciocalteu test results. Cicco et al. [8] reported that longer incubation times consistently increased color development and the apparent phenolic content. Moreover, the authors found that raising the incubation temperature to 40°C reduced the end-point time to 20 min. Similarly, Georgé et al. [33] demonstrated that higher temperatures, such as 50°C, further shortened the end-point time to 15 min. However, they observed that the coloration began to decline after 30 min. This suggests that while color formation occurs more rapidly at elevated temperatures, temperatures above 40°C can also cause the color to fade more quickly [34]. Several authors also reported that increasing the sodium carbonate concentration in the Folin-Ciocalteu assay generally leads to higher absorbance and, consequently, a higher measured phenolic content [35-37]. However, the magnitude of this effect depends strongly on the sample matrix, with different optimal sodium carbonate concentrations required for different types of samples [36, 37]. The conducted investigation also revealed tendencies for certain variants to over- or underestimate true polyphenol levels. In the presented studies, protocol P3 (which incorporates a 40°C heating step) maintained high calibration linearity (R^2=0.999), but yielded lower recovery for complex polyphenol extracts compared to the unheated, multi-hour Singleton protocols (P1 and P2). It was shown that protocol P5 offered the highest accuracy and reliability, while Singleton's protocols P1 and P2 showed adequate but marginally lower performance. Polyphenol analysis is highly sensitive to procedural variations, as small changes in methodology can alter redox kinetics and color formation [38,39]. The conducted work provides guidance for the researchers selecting and standardizing Folin-Ciocalteu assays for their specific aims and sample types. Further optimization is still needed, however, to develop protocols maximizing simplicity, precision, and cost-effectiveness without sacrificing accuracy. Standardizing polyphenol analysis techniques remains an ongoing challenge. This investigation serves as an important reference for choosing appropriate experimental parameters and improving validation methods. As Musci & Yao [9] emphasized, inadequate assay optimization and evaluation is a persisting issue undermining the credibility of polyphenol research. More comprehensive analytical characterizations will strengthen the reliability of findings and allow clearer comparisons between studies.

Application to real samples: onion skin valorization

The conducted studies on valorization of TPC in yellow onion skin biowaste extracts demonstrated that by performing the solid-liquid extraction (using 60% ethanol solution), 32.9 ±1.1 mgGAE/g DW of total polyphenols can be obtained from 1 g of dried yellow onion skin. Numerous studies have been conducted exploring the biological profile of the onion plant [40, 14, 41]. The works by Yardimici [42], Kim et al. [43], and Ramires et al. [44, 45] reported that onion skin extract could contain: taxifolin, gallic acid, ferulic acid, protocatechuic acid, kaempferol, free quercetin, quercetin glycosides, and oxidized quercetin derivatives. Moreover, recently published papers indicate that TPC in onion skin could range from 27.8 mgGAE/g to 97.28 mgGAE/g of dried materials, depending on the type of onion and location of its cultivation [43]. By applying the ultrasounds pre-treatment, it was possible to increase TPC up to 55.5 mgGAE/g of dried materials in the studied extract. Different studies have shown that UAE can significantly enhance TPC from fruits, vegetables, and herbs [46-49]. The effectiveness of ultrasound, compared to the simple solid-liquid extraction method, is attributed to the cavitation effect, which facilitates the release of extractable compounds and increases mass transport by diffusion and/or by disrupting plant cell walls.

This study confirms that the choice of FC protocol is not trivial. While all methods showed acceptable linearity, Protocol P5 provided the most robust performance across different chemical structures. As noted by Musci & Yao [9], the lack of standardization in polyphenol assays often leads to irreproducible data in food science. By adopting a standardized and systematically evaluated protocol like P5, researchers can improve the consistency and comparability of TPC values across different laboratories and plant matrices.

CONCLUSIONS

This study demonstrates that the inherent procedural variability of the Folin-Ciocalteu assay is a critical factor that can significantly skew the quantification of total polyphenols in in investigates material and extraction conditions. Through a systematic statistical evaluation of five distinct protocols at a mid-range concentration benchmark, Protocol P5 was identified as the most robust and precise methodology, offering superior linearity and minimal bias.

The following key findings can be indicated. Firstly, standardization is essential – subtle changes in sodium carbonate concentration and incubation kinetics, often overlooked in routine analysis, directly impact the redox stability and color development of the FC reagent. Procedural conditions also play a significant role. While protocol variants incorporating moderate heating (40 °C) shorten analysis time, multi-factorial procedures like P3 may yield lower measured TPC values when applied to complex phytochemical profiles containing oligomeric proanthocyanidins. Extraction efficiency is the subsequent key finding of this study. The practical application of the standardized P5 protocol confirmed that ultrasound-assisted extraction is a highly effective green technology for the valorization of yellow onion skin, yielding a 68.7% higher polyphenol recovery compared to conventional solid-liquid extraction.

Protocol P5 provides a reliable, high-precision framework for the standardization of polyphenol analysis. Adopting this validated approach will enhance the comparability of results across diverse research fields, from food chemistry to the development of bioactive ingredients from agricultural biowaste. Future research should continue to evaluate the impact of specific matrix interferents on these optimized parameters to further refine the global standardization of antioxidant assays.

Acknowledgments

This work was supported by the project "PHENOLEXA – Benign cascade extractive biorefinery for converting agri-food side streams into high-value polyphenolic bioactives and functional fibres for pharma, cosmeceuticals, nutraceuticals and food products" that has received funding from the Bio Based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation programme under grant agreement No. 101023225.

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