Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.35122

Determination of total phenolic compounds in plant extracts via Folin-Ciocalteu's method adapted to the usage of digital images

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Abstract

The food industry shows great interest on researching innovative and alternative methods to determine phenolics compounds'. Hence, the present study aims to develop a technique based on Folin-Ciocalteu's method to determine the total amount of phenolic compounds of a sample using a smartphone as a tool for obtaining digital images of the analysis' and evaluating them through the Colorgrab® application. In this study, color parameters S, L, V and I were evaluated, as well as experimental parameters such as distance between sample and camera, light temperature, and sample's volume. To improve analytical conditions, phenolic compounds were determined through the conventional Folin-Ciocalteu's method, with the results being compared to the data obtained from the adapted method. After optimization, the procedure was carried out using the S color parameter, cold light (6500 K), 7 cm distance between sample and camera, and a solution volume of 1.5 mL. The test showed suitable results for precision (RSD < 2.4%) and accuracy (98.4 to 103.8%) when compared to the conventional method. The proposed technique proved to be an innovative alternative to determine total phenolic compounds in routine analysis for different sectors of the food industry, showing good analytical results without requiring sophisticated equipment.

Keywords: food industry; Colorgrab®; smartphone; eco-friendly; eggplant; lemon balm.

Practical Application: This study sought to develop an analytical method through the use of digital images obtained from a smartphone as an easily available alternative to quantify a sample's total phenolic compounds. This technique aims to replace the use of a spectrophotometer, reducing the need for bulky equipment, as well as reducing environmental impacts by using less solvents, all while proposing a technique with relatively lower cost compared to those commonly used and following the recommendations of green chemistry.

1 Introduction

Bioactive compounds show great interaction capacity in live tissues, presenting functional biological properties such as antioxidant, anti-inflammatory, anticarcinogenic and antiviral activities (Ramakrishna et al., 2019). When it comes to food, examples of bioactive compounds that display these properties include non-digestible carbohydrates (soluble and insoluble fibers) and antioxidants (phenolic compounds, carotenoids, tocopherols, isoflavones and anthocyanins) (Ramakrishna et al., 2019; Subiria-Cueto et al., 2022). Thus the functional potential of many bioactive compounds is usually closely related to phenolic compounds, which is one of the most common groups of chemical compounds found in food (Quideau et al., 2011). These compounds are also able to improve the sensorial properties of a given product, such as color and smell (Costa et al., 2015). Thus, these compounds are used as natural food preservatives, coloring agents, and can even be used in the cosmetic industry (Silva et al., 2010). Their major use, however, is related to food preservation, considering that such compounds promote oxidative stability due to their high antioxidant potential. Many studies have been performed on bioactive compounds, introducing them as functional dietary ingredients, in order to reduce pathologies such as obesity, diabetes and cardiovascular diseases (Vargas-Madriz et al., 2022; Siriwardhana et al., 2013). The antioxidant potential of phenolic compounds plays a major role in the food industry if one considers the current trend of researching natural antioxidants (Banwo et al., 2021).

Phenolic compounds' capacity to eliminate free radicals can be measured through many assays. The most commonly applied test to determine phenolic compounds concentration is the Folin-Ciocalteu colorimetric technique (Vuolo et al., 2019). This method is based on molecular absorption spectrophotometry, which is an efficient technique that shows great accuracy and precision, however, it also requires specific equipment, adequate facilities and trained personnel to perform the analysis (Bhawani et al., 2015). Currently, industries have been searching for alternative equipment and eco-friendly technologies that allow tests to be quickly and easily performed while also being portable, reducing costs by minimizing the amount of solvents required, and implementing versatile equipment that can be used in both laboratory and sampling sites (Armenta et al., 2015; Vieira et al., 2020). In this context, the use of mobile devices

Received 26 Feb., 2022
Accepted 12 May, 2022
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become a promising alternative to perform chemical analyses, especially colorimetric tests (Helfer et al., 2017). The use of digital images in colorimetric analyses is based on image acquisition, where the reaction's color intensity is captured and evaluated using smartphones, cameras, webcams, and scanners in order to measure the sample's chemical parameters (Hong & Chang, 2014).

Smartphones are in the forefront when it comes to image acquisition via mobile devices, especially due to their portability, accessibility and high resolution, allowing for the acquisition of high quality digital images that can be used to determine the concentration of specific analytes (Gallegos et al., 2013). The use of such equipment is recognized as an innovative technology with numerous applications. Recently, many studies have shown the usage of smartphones as a promising and innovative tool in the food industry, among which stands out the determination of ascorbic acid in natural juice (Porto et al., 2019); the bioactive compounds' potential in freeze dried açaí (Caramês et al., 2021); determination of bioactive compounds in grape juice (Porto et al., 2019); and to quantify furfural in lager beer samples (Rico-Yuste et al., 2016). Considering the great potential of smartphone's usage in colorimetric tests and the lack of studies on determining the amount of phenolic compounds of different food samples without the use of expensive equipment, the present study seeks to propose a rapid, easily applied, eco-friendly and low-cost methodology to determine phenolic compounds through digital images, using smartphones and the Colorgrab® application.

2 Material and methods

2.1 Samples

The chosen food samples for this study were whole eggplant (*Solanum melongena*), containing peel, pulp and seeds, and lemon balm (*Melissa officinalis*) leaves, obtained at the local market in Santa Maria city (Rio Grande do Sul, Brazil). Samples were oven-dried (MA 033/100, Marconi, Brasil) at 45 ± 5 °C for 48 h, and then stored in polyethylene bags at -18 °C until analysis.

2.2 Hydroalcoholic extraction

The extracts' preparation was performed accordingly to Boeira et al. (2018), by using 5 g of sample, 50 mL of ethyl alcohol in a 1 : 10 proportion (m/v) (99.5% purity, Sigma-Aldrich, USA). The extraction lasted for 20 min, using a heated plate at 60 °C under constant stirring. Finally, extracts were filtered using paper filters (0.17 mm) and the final volume was adjusted to 50 mL by adding distilled water, and stored at -18 °C until analysis.

2.3 Phenolic compounds' determination using UV-VIS spectrophotometry

Phenolic compounds' content was determined in triplicate by the Folin-Ciocalteu spectrophotometric method, described by Singleton & Rossi (1965), with some modifications. A 0.5 mL aliquot from each extract was added to 2.5 mL Folin-Ciocalteu's reagent solution (0.2 M) (Alphatec, USA). The solution was left to rest for 5 min before being added with 2 mL of 7.5% p/v sodium carbonate (Impex, Brazil). The resulting color absorbance was measured at 760 nm in a UV-Vis spectrophotometer after 15 min reaction time at 40 °C. To obtain the phenolic compounds' concentration data, a calibration curve was constructed using different concentrations of gallic acid (10 to 70 $mg{\cdot}L^{-1}$). Total phenolic compounds' content was expressed in mg of gallic acid equivalents per L of sample ($EAG{\cdot}L^{-1}$ of sample).

2.4 Determination of phenolic compounds using smartphone

Sampling and preparation of standard solutions were performed using the conventional Folin-Ciocalteu method, with the resulting liquid being placed in a 24 well Elisa microplate. To obtain the digital images, a Samsung Galaxy A5 smartphone equipped with a 13 MP resolution camera (Samsung Eletronics, Suwon, Gyeonggi, South Korea) was used. Images were captured directly via the Colorgrab® app (Loomatix, Haifa, Israel, 2021).

In order to guarantee a standardization of the acquired images, a lightbox (Figure1) was constructed to allow for adequate plate positioning and image capturing. The box was internally coated with 15 cm x 15 cm white ethylene vinyl acetate (EVA) sheets. A led light stripe (10 W/m, Stella) was fixed on the lid borders and a 7 cm x 4 cm clipping was made in the upper part of the box in order to accommodate the smartphone.

The obtained images contained the sample's color information in RGB, thus, color intensity was determined in only three colors: red (R), green (G) and blue (B), considering a 0 to 255 numeric range. Conditions were optimized and it was decided to use the saturation analytical signal (S) to determine phenolic compounds' content in plant extracts, which was calculated through Equation 1.

$$S = \frac{C}{M} \tag{1}$$

Where, $M = max(R,G,B)$ and $C = M - min(R,G,B)$.

2.5 Optimization and experimental conditions

In order to optimize the proposed method, the determination of phenolic compounds from lemon balm leaves was performed using the UC-Vis spectrophotometric technique. The same extract was used to evaluate the distance between camera and sample

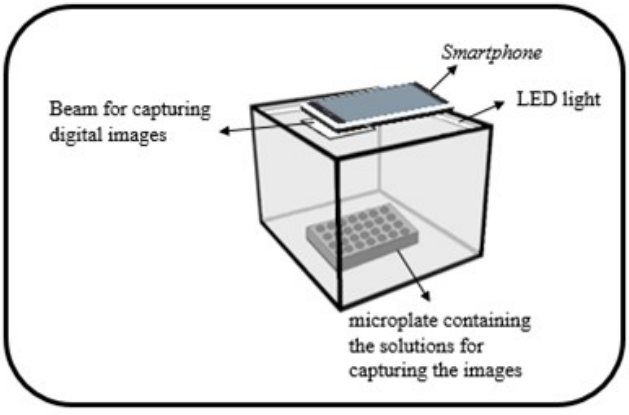

Figure 1. Lightbox for colorimetric reaction's digital image obtainment.

(5; 7; 9; and 11 cm); the sample's final volume in the microplate well (1.5; 2; 2.5; and 3 mL); and light's color/temperature, which ranged between cold (6500 K), warm (3000 K) and natural light temperature. The proposed method (n = 5) results' precision and accuracy were compared to those obtained from the conventional analysis (n = 5). For each evaluated condition, the calibration curve and five replicates (quintuplicates) from each sample were also evaluated.

All improvements were performed using the lightbox (Figure 1), in order to insert the microplate and standardize the sample's reading. In addition to the S value, color glow representation (Hanbury, 2008), such as intensity (I), illumination (V) and luminance (L) were also reviewed. In order to do so, the RGB data was handled according to Equations 2-4 (Hanbury, 2008).

$$V = M \tag{2}$$

$$I = \frac{(R+G+B)}{3} \tag{3}$$

$$L = \frac{M+m}{2} \tag{4}$$

Where $M = max(R,G,B)$ and $C = M - min(R,G,B)$.

To evaluate the accuracy of the proposed method, two vegetable extract samples were submitted to determination of phenolic compounds using the conventional technique and the proposed method. To compare them, the concordance between both methods was calculated, observing average results obtained in triplicate.

2.6 Statistical analysis

All experiments were statistically analyzed using the T-student test to compare two means with a 95% significance level, using the R software (RStudio, 4.1.0).

3 Results and discussion

3.1 Improvement of experimental conditions and data acquisition using smartphones

Two evaluations of experimental conditions were conducted in order to obtain greater accuracy and precision results when compared to the conventional method. The first one is related to color representation parameters (S, I, V and L), and the second one sought to optimize the distance between sample and camera, the sample's volume in the microplate wells, and illumination conditions. Thus, lemon balm leaves' extract was used to compare the total phenolic compounds' data found using the proposed and the conventional method.

Evaluation of the color representation parameter

Preliminary tests were conducted using natural light in order to choose the best color parameter to perform the analysis. Quintuplicate readings of phenolic compounds' concentration on lemon balm leaves' extract were performed using 1.5 to 3 mL samples with distances between 5 and 11 cm, resulting in 80 different concentration results. V, S, L and I parameters were then calculated from the obtained RGB data. The mean values and standard deviation of each color parameter evaluated are shown in Figure 2.

The columns represent phenolic compounds concentration; the error bars represent standard deviations obtained from the proposed method; yellow and dashed lines represent lemon balm extract's phenolic compounds concentration and standard deviation obtained from the conventional method, respectively. No significant difference was observed between color parameters (T- student test, $p > 0.05$).

As shown in Figure 2, the yellow line represents the average concentration of phenolic compounds, while the dashed lines represent the standard deviation obtained from the conventional analysis. The mean values of the evaluated color parameters (bars) was different from S, V, L and I, due to the fact that each color parameter is distinctly calculated, thus, different values can be obtained from the same sample. Regarding the method's precision, the S parameter displayed less variation between measurements when compared to others. Table 1 shows the concentration data of phenolic compounds, standard deviation and results' concordance when compared to the conventional method.

The results show that the S color parameter provided greater concordance rates when compared to the conventional method (99.73%), and greater precision was observed (RSD = 5.02%) when compared to V, L and I parameters (Table 1). Furthermore, the S parameter showed proportional positive relations to concentration, while all other parameters had inversely proportional results. Nonetheless, the best determination

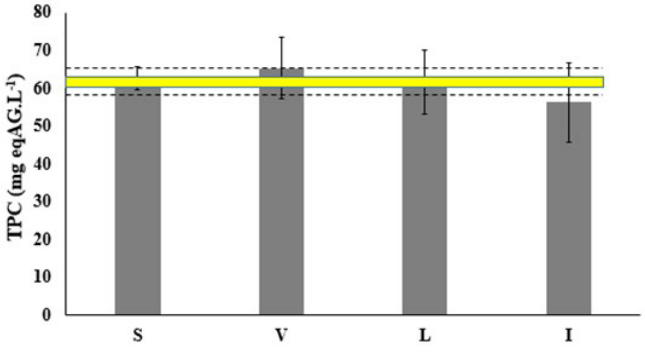

Figure 2. Evaluation of S, V, L and I color parameters.

Table 1. Data for each evaluated color parameter under natural light.

	Conventional	Conversion parameters RGB			
		S	V	L	I
TPC (mg·L^{-1} AG)	62.53	62.70	65.40	61.80	56.35
SD	1.33	3.15	8.06	8.39	10.57
RSD	2.13	5.02	12.33	13.57	18.76
Agreement (%)		99.73	95.87	105.82	109.67
R^2	0.9999	0.9774	0.9611	0.9069	0.9400

TPC = phenolic compounds concentration; SD = standard deviation; RSD = relative standard deviation; R^2 = determination coefficient of the calibration curves considering 1.5 mL and 7 cm distance.

coefficient between the different color parameters was linked to the S parameter, suggesting greater reliability. Thus, the S parameter was then chosen to improve experimental variables.

Experimental variables evaluation

After the S parameter was chosen, the sample volume, light temperature and distance between sample and camera were evaluated. Figure 3 shows results related to the evaluated variables (bars), comparing them to the conventional method (yellow line). As observed in Figure 3, the sample's volume was one of the parameters that generated significant variations in analyte concentration, considering that, the greater the sample's volume, the smaller the concentration of phenolic compounds. Such parameter was determined to simplify the method, reduce reagent costs, and contribute to an eco-friendly practice.

The results obtained when using a sample volume of 1.5 mL (5 cm, cold, natural and warm light; 7 cm under cold and warm light; 9 cm under natural light and 11 cm under ambient light) did not show significant differences ($p > 0.05$) when compared to the conventional method. Furthermore, this condition produced more accurate results considering light and distance variables.

When using 2 mL sample volume at a distance of 9 cm, as well as 2.5 mL sample volume at a distance of 9 and 11 cm under natural light, the detector's saturation point was reached, not allowing for the accurate measurement of phenolic compounds' concentration. Moreover, it was also observed that the greater the sample's volume in the microplate well, the standard deviation would increase, while concordance between samples would be reduced when compared to the conventional method. Thus, 1.5 mL sample volume was chosen, since it shows a mean standard deviation of 1.09 (RSD = 1.74%) and 100.5% mean concordance.

Furthermore, imaging conditions directly impact the analysis quality, as well as its results. Variables such as light source, the roughness of the object's surface, and color intensity can influence the quality of digital images obtained using smartphones, greatly impacting the image's precision and results (Zamora-Garcia et al., 2021). In order to reduce image acquisition problems, sharpness and light conditions (cold, warm and natural light) were evaluated in relation to the distance. According to the obtained results (Figure 3), it can be observed that the light temperature plays an important role. Warm light sources have shown a greater disparity in the average total phenolic compounds values when compared to cold and natural light. When cold light was applied, the obtained results were more precise and exact when compared to those found in the conventional method, presenting similar TPC means and lower SDs. The optimization of these conditions indicates that the evaluated variables presented significant differences when compared to the conventional method. However, the best analytical signal was obtained at a 7 cm distance, using a 1.5 mL sample volume and cold light settings, for it didn't show significant differences at 95% significance level, while also displaying a 101.8% concordance rate and a 0.59 (RSD = 0.92%) standard deviation when compared to the conventional Folin-Ciocalteu method.

Smartphone digital image-capture method's accuracy evaluation

To evaluate the accuracy of smartphone digital image-capture, the determination of total phenolic compounds was carried out through a spectrophotometric technique, performed on two different plant extracts (eggplant and lemon balm). Figure 4 shows the calibration curves obtained from both techniques, demonstrating that both methods show significant linearity between analytic signal and concentration, considering that the obtained determination coefficients (r^2) were very similar (0.9945 and 0.9973) between the smartphone digital image-capture and the conventional method, respectively. Calibration curves displayed high linearity, allowing for a reliable determination of a sample's total phenolic compounds. Figure 5 displays the standard deviation and concentration values for both samples, expressed in mg of gallic acid equivalent.

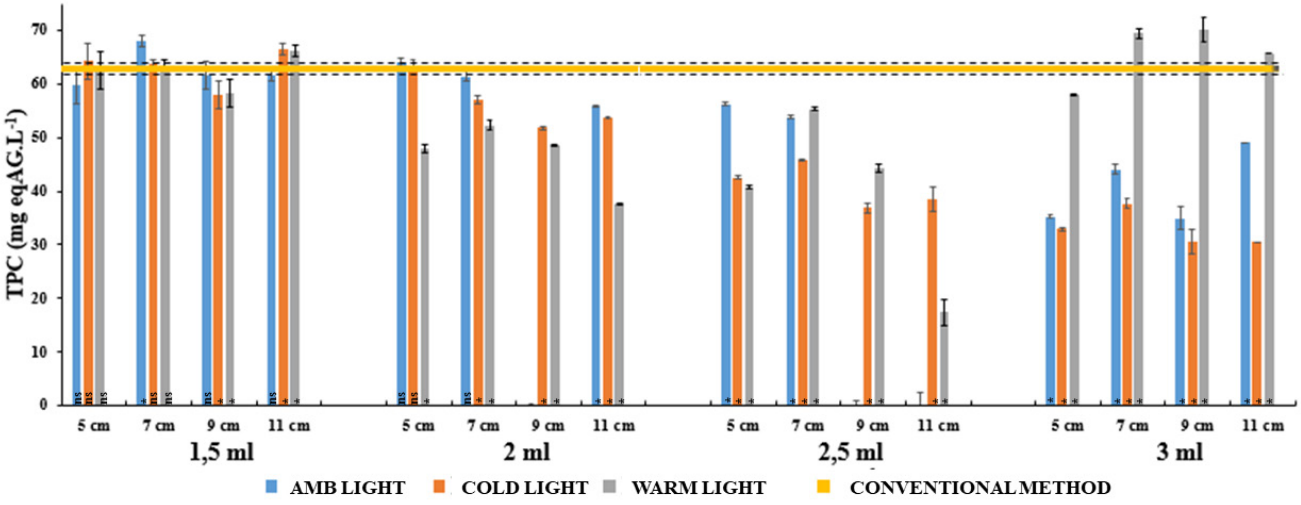

Figure 3. Light, volume and distance parameters on phenolic compounds' determination, compared to the conventional method. Ns: non-significant (T-student test, $p > 0.05$); *: significant (T-student test, $p > 0.05$).

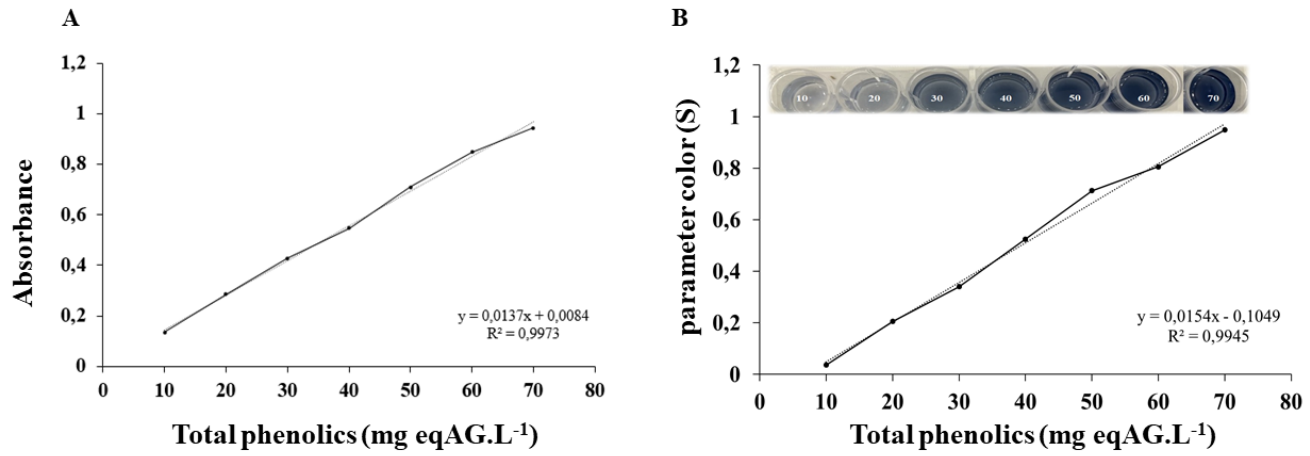

Figure 4. (A) conventional method; (B) smartphone digital image-capture method calibration curves for determination of total phenolic compounds.

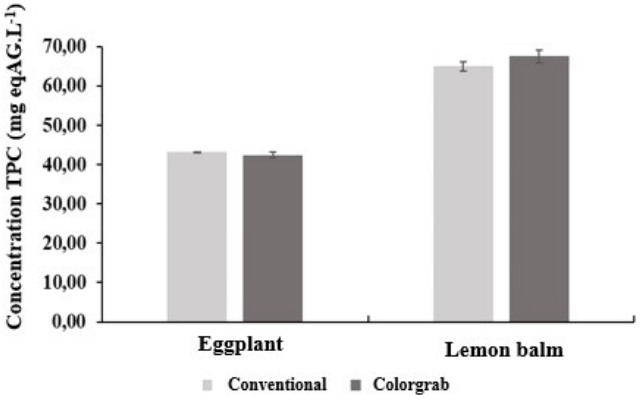

Figure 5. Smartphone digital image-capture method's accuracy assessment.

Columns represent phenolic compounds concentration, and error bars represent standard deviations. No significant difference was observed between both techniques and samples (T-student test, $p > 0.05$).

Eggplant samples displayed a phenolic compound concentration of 143.11 ± 0.19 mgEAG·L^{-1} and 42.42 ± 0.72 mgEAG·L^{-1} for conventional and proposed methods, respectively, while lemon balm samples showed a concentration of 65.03 ± 1.15 mgGAeq·L^{-1} for the conventional method and 67.53 ± 1.59 mgGAeq·L^{-1} for the smartphone digital image-capture method. Both evaluated extracts showed no significant difference between the methods ($p > 0.05$). The test's accuracy was similar, with concordance rates ranging from 98.4 to 103.8%, indicating that the smartphone digital image-capture method is precise.

Many studies have been conducted using digital images, such as tetracycline determination in milk samples (Masawat et al., 2015). The authors compared their proposed method to official analysis techniques, evidencing that no significant statistical difference was shown between the assessments. The authors also found that the concordance rate remained between 93.1 and 101% when compared to the conventional method (Masawat et al., 2015). Beltrame et al. (2019) have studied the use of digital imaging to quantify grape juice's total anthocyanin content and antioxidant capacities, finding rates of 0.9258 and 0.8479, respectively, which are significantly lower than those found in the present study, especially when considering that concentration rates remained above 0.99 (Beltrame et al., 2019). Other studies have also found great concordance rates (99%) in comparison to conventional methods when determining ascorbic acid content in fruit juices (Aguirre et al., 2019). Therefore, it is possible to assume that the use of smartphones when performing day-to-day analyses is an easily accessible, advantageous, and innovative alternative. Moreover, the technique provides fast, accurate and precise results, alongside a relatively low cost, which allows for the determination of total phenolic compounds in different vegetable extracts. The smartphone digital image-capture method also represents reduced energy consumption and no need for sophisticated equipment or solvents, making it a less aggressive alternative to the environment.

4 Conclusion

The present study allowed us to obtain of invaluable analytic data on the determination of total phenolic compounds found in eggplant and lemon balm extracts, via the use of digital images obtained by smartphones. The smartphone digital image-capture method showed great precision and accuracy when compared to the conventional Folin Ciocalteau analysis, also displaying great linearity between analytic signal and concentration. Thus, the smartphone digital image-capture method demonstrates a promising analytical capacity in determining total phenolic compounds of different plant extracts, while also pushing for the use of smartphones in quality control analysis for the food industry, as it is a portable, easily applied and economically viable piece of equipment, allowing for an innovative and eco-friendly approach by reducing the amount of required chemicals and solvents in order to evaluate total phenolic compound concentration.

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