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Adaptation of the Folin-Ciocalteu and Fast Blue BB spectrophotometric methods to digital image analysis for the determination of total phenolic content: Reduction of reaction time, interferences and sample analysis

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ABSTRACT

The Folin-Ciocalteu (FC) method is the most common method used for total phenolic content (TPC) determination, but in recent years, a more specific method that does not detect non-phenolic reducing compounds has been developed, the Fast Blue BB (FBBB) assay. In this study, the reference spectrophotometric methods have been adapted to a rising, simple, fast and low-cost technique (Digital Image Analysis, (DIA)) based assay while studying if the reaction time of both methods could be reduced and applied for the determination of TPC in food samples. Moreover, the interaction of ascorbic acid (AA) and fructose (F), with both reagents, has been observed.

Two linear methods were obtained by DIA with R^2 values of 0.997 and 0.995, low detection limits (LOD), a precision and accuracy below 10%, and no statistically significant differences ($p > 0.05$) with the spectrophotometer. The reaction times were 60 and 30 min for FC and FBBB, respectively. The FC method required lower concentrations of AA and F to observe interactions in contrast to the FBBB, which did not exhibit any interactions with F. DIA has successfully been applied with both methods on a variety of drink samples, with results ranging from 10 to 600 mg GAE/g sample.

1. Introduction

Polyphenols are water-soluble plant-derived secondary metabolites, containing at least two phenol rings and one or more hydroxyl substituents (Singla et al., 2019). They are a very diverse group of compounds, with a wide variety of structures and widely distributed in plants. Due to this large diversity, there are different ways to classify them, being the most frequent classification the one that divides polyphenols into 5 different groups: phenolic acids, flavonoids, lignans, stilbenes and others (Belščak-Cvitanović et al., 2018, pp. 3–44).

They are present in many foods (vegetables, cereals, legumes, fruits, nuts, etc.) and drinks (wine, cider, tea, etc.) in variable amounts even among cultivars of the same species, due to genetic factors, environmental conditions, the level of maturation, variety, processing, etc. (Silva & Pogačnik, 2020).

Over the years, polyphenols have been extensively studied due to their interesting functional properties that include metal chelating activity, ability to form polyphenol-protein complexes and antioxidant activity thanks to their capacity to neutralize free radicals by donating an electron or a hydrogen atom. These properties make polyphenols beneficial for the human health, as they are involved in the prevention of diseases such as cancer or cardiovascular diseases, among others (Belščak-Cvitanović et al., 2018, pp. 3–44; Bravo, 1998).

There are several assays for the determination of antioxidant activity of food components. One of them is the total phenolic content (TPC) determination assay, which is directly related to food antioxidant capacity. This is a simple and highly relevant technique for the identification of functional properties in food.

The most common method used for this determination has always been the Folin-Ciocalteu (FC) method, but in recent years, a more

Abbreviations: FC, Folin-Ciocalteu; FBBB, Fast Blue BB; DIA, Digital image analysis; TPC, Total phenolic content; GA, Gallic acid; AA, Ascorbic acid; F, Fructose; GAE, Gallic Acid Equivalents; EJCR, Elliptical Joint Confidence Region.

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specific method, which does not detect non-phenolic reducing compounds, has been developed, the Fast Blue BB (FBBB) assay. FC method has been reported to interact with a number of substances (particularly sugars like fructose or sucrose, aromatic amines, sulfur dioxide, organic acids like ascorbic acid and other enodiols) (Prior et al., 2005).

Both assays are spectrophotometric methods used to determine phenolic compounds in plant origin hydrophilic solutions. In the FC method, phenolic compounds react with a reagent at basic pH (pH 10). Under basic conditions, the phenolate ion formed reduces FC through a redox reaction and generates the formation of a Mo (V) complex that presents a blue color with a maximum absorbance at a wavelength of 765 nm (Singleton & Rossi, 1965).

On the other hand, FBBB is based on a coupling reaction of phenolic compounds with the diazonium salt, which, at basic pH, results in the formation of azo complexes. The FBBB salt contains an electrophilic diazonium group ($-N_2^+$) where the nitrogen is retained in coupling with the reactive activating group (–OH) of the phenolic group. Coupling occurs mostly *para* to the phenolic activating group, unless the position is already occupied, then substitution occurs *ortho* to the activating group. The yellowish coloration presents an absorption maximum at 420 nm, and it is quantified by spectrophotometry based on a calibration curve made with gallic acid. Gallic acid is usually used as the standard polyphenol because it is a representative natural phenolic found in many plants, including tea (Medina, 2011a; Medina, 2011b).

Nowadays, techniques that allow obtaining data from images are rising due to its simplicity, low-cost and speed. These techniques are used for several analytical chemistry applications such as determination of bioactive compounds from grape juice (Beltrame et al., 2021), determination of TPC in beer based on the Folin-Ciocalteu method (Ledesma et al., 2019) or determination of ascorbic acid (Coutinho et al., 2017) among others. Images are obtained using either smartphones, scanners or digital cameras.

A digital photo divides the image into pixels and the numerical value of each pixel is described by color spaces, which are composed of some channels. Several color models have been used in different studies, including the RGB model, which is decomposed into three primary color channels: red (R), green (G) and blue (B). These channels will have value ranges from 0 to 255 (Fan et al., 2021). The channel used to calculate the absorbance will be the complementary to the maximum absorption. Therefore, with an image, the light transmitted by a solution can be measured and hence, Lambert Beer's law can be applied to determine an analyte concentration just as in spectrophotometry.

In this work, both, spectrophotometry and digital image analysis (DIA) have been used for the determination of TPC by FC and FBBB methods.

The aim of this work was to adapt the Folin-Ciocalteu and Fast Blue BB spectrophotometric methods to Digital Image Analysis based assays using microplates to reduce wastes and sample and reagents volumes, and a smartphone, an instrument that is cheaper and more accessible than a UV–Vis spectrophotometer. In addition, beverage samples, rich in polyphenols and known for their beneficial properties, were used to compare the two methods and determine whether DIA was a suitable replacement for UV–Vis.

2. Material and methods

2.1. Reagents and equipment

Fast Blue BB (4-benzoylamino-2, 5-dimethoxybenzenediazonium chloride hemi-[zinc chloride]) salt, gallic acid and D (−) fructose were purchased from Sigma-Aldrich (Missouri, USA). Sodium hydroxide, sodium carbonate, ethanol, ascorbic acid and Folin-Ciocalteu's reagent were obtained from PanReac (Barcelona, Spain). All reagents used were of analytical grade.

Absorbance measurements were carried out with an Agilent 8453 UV–Visible spectrophotometer and the Agilent ChemStation software.

A 96 well microplate was use for DIA and digital images were taken with a Samsung Galaxy A6 smartphone with ISO 200, white balance in Auto mode and room lights on. R, G, B values were calculated using MATLAB R2021a.

2.2. Samples

On one hand, seven types of infusions were purchased from different supermarkets. Two flavored plant and fruit infusions (ingredients are expressed in relation to 100 g of sample): I_1, with 57 g rooibos and 19 g hibiscus as main ingredients; and I_2, which contains dehydrated apple (25 g), mint (23 g), fennel (17 g), elderberry (10 g), mallow (10 g), and chicory (6 g); a green tea (T_1); a black tea (T_2); a red tea (T_3); two pennyroyals (MP_1 and MP_2); and a linden (L_1).

For each sample, the content of five different bags was mixed, 1 g was weighed and infused in 200 mL of heated (98–100 °C) doubly distilled water for 5 min as it is advised in most commercial teas. After that time, samples were filtered using a strainer. Each sample was prepared in triplicate. Further dilutions were made for each sample depending on the method.

On the other hand, five commercial juices and drinks were also purchased from a local supermarket. The samples were grape and apple juice (J_1 and J_2, respectively), orange and pineapple nectars (N_1 and N_2) and a lemon juice refreshing beverage (B_1), as well as lemons and oranges for the extraction of manually pressed juice (J_3 and J_4).

2.3. Total phenolic content (TPC)

The total phenolic content (TPC) of the drinks was measured with two different methods and procedures, Folin-Ciocalteu (FC) and Fast Blue BB (FBBB), and two different techniques, UV–Vis spectrophotometry and Digital Image Analysis.

In the first procedure, FC method was followed with some modifications (Singleton et al., 1999). A standard calibration curve with gallic acid (n = 8) was prepared from 1 mg/L to 8 mg/L 0.25 mL of FC reagent was added to 4 mL of gallic acid solutions, and after 5 min, 0.75 mL of Na_2CO_3 (7.5 g/100 mL) was added to complete the reaction. When analyzing samples, the standard solution volume was replaced with the desirable sample solution. A part of the prepared reaction solution was transferred into a microplate for the Digital Image Analysis (DIA), where three wells were filled with 400 μL each. The rest of the solution was used for the spectrophotometric determination. The reaction was left in the dark at room temperature for 2 h during which, measurements were carried out every half an hour using both a UV–Vis spectrophotometer and DIA.

For the second procedure, Fast Blue BB (FBBB) method was followed with some modifications (Medina, 2011a; Medina, 2011b). Gallic acid standard solutions were prepared, and a calibration curve (n = 7) was obtained within 3–25 mg/L concentration range. Following the method adaptation, 4 mL of standard solution or sample were mixed with 0.4 mL of FBBB (0.1 g/100 mL, dissolved in 35 mL/100 mL ethanol) and after 1 min, 0.4 mL of NaOH (5 g/100 mL) were added and mixed well (Maieves et al., 2015). Afterwards, the same process as in the FC method was followed.

To study if the reaction time of the method proposed for the two reagents could be reduced, the absorbance of the calibration curve was measured at different times. For the spectrophotometric measurement, absorbance was read after 30, 60, 90 and 120 min at a wavelength of 765 nm for the FC method, and after 30, 60 and 90 min at 450 nm for the FBBB assay. Photos were also taken at the same reaction times and the processing was made using MATLAB R2021a. In this case, to calculate the absorbance, the channel of the RGB color space complementary to the color of the reaction solution of each method was chosen, R channel for the bluish color of the FC reaction and B channel for the yellow-orange color of the FBBB assay.

Total phenolic content was expressed in mg Gallic Acid Equivalents

(GAE)/g $_{sample}$ for tea and infusion samples and in mg GAE/L for juice samples. Determinations for each sample were performed in triplicate.

2.4. Data analysis

A *t*-test, to compare the slopes of two regression lines (D.L. Massart et al., 1997), was used to study if reaction times could be reduced.

Other statistical analyses were carried out using the statistical package IBM SPSS Statistics v.27.0.1.0. A *t*-test for paired samples was performed to compare the two different techniques. Moreover, a one-way ANOVA and a post-hoc test were conducted using Tukey's HSD to assess differences in TPC between samples and to make an inter-drinks comparison. Finally, MATLAB R2021a was used to perform the Elliptical Joint Confidence Region (EJCR) test.

3. Results and discussion

3.1. Reaction times (Folin-Ciocalteu and Fast Blue BB methods)

The absorbance of a standard curve for gallic acid (GA) was measured at different reaction times, using both methods, to determine the total phenolic content by UV–Vis spectrophotometry (Fig. 1). The range of each calibration curve was chosen according to its linearity (0–8.16 mg/L for FC and 3–25 mg/L for FBBB).

In the FC method, both, absorbance values and the slopes of the calibration curves obtained increased with time. However, in the FBBB method, absorbance values and slopes decreased with time.

To check if the values showed significant differences, the slopes obtained at different times were compared using a *t*-test following the method of comparing the slopes of two regression lines (D.L. Massart et al., 1997). The calculated t (t_{cal}) was lower than the tabulated t (t_{tab}) for both methods and techniques (1.78 (DIA) and 1.34 (UV–Vis) < 2.18 for the FC method and 1.36 (DIA) and 0.63 (UV–Vis) < 2.23 for the FBBB assay). The comparison was made with the slopes of the times established in the reference methods, 120 min for FC method (Singleton & Rossi, 1965) and 60 min for FBBB assay using NaOH (Medina, 2011b). In both cases, those reaction times could be reduced, to 60 min in the first method and to 30 min in the second.

Furthermore, calibration curves obtained spectrophotometrically were compared to those obtained with DIA (Fig. 2) comparing the slopes using the same *t*-test than before, and the results did not show significant statistical differences ($p > 0.05$) between both techniques in neither of the methods ($t_{cal} = 1.04 < t_{tab} = 2.18$ for the FC method and $t_{cal} = 1.21 < t_{tab} = 2.23$ for the FBBB assay).

3.2. Validation parameters

Validation parameters of both methods for DIA technique are shown in Table 1. The coefficient of determination (R^2) of the calibration curves obtained at the chosen reaction times (60 min for FC and 30 min for FBBB) present no differences between UV–Vis spectrophotometry (0.999) and DIA (0.997) in the FC method, and the opposite in the FBBB assay, 0.992 and 0.995 for the respective techniques, although the difference is minimum.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated as three and ten times, respectively, the standard deviation of the response divided by the slope of the calibration curve. LOD for the FC method was 0.46 mg GAE/L, and for the FBBB assay 1.81 mg GAE/L. In the case of LOQ, for the FC method was 1.52 mg GAE/L and for the FBBB assay, 6.03 mg GAE/L.

Intra- and inter-day precision were obtained with results of the same day and different days, respectively, and expressed as the percentage of relative standard deviation (RSD_r and RSD_R%). The precision of the method for small concentrations was lower than for higher concentrations, but in anyway below the expected RSD_r% (7.3–11%) and predicted RSD_R% (11–16%) listed in appendix F of the guideline for standard method performance requirements (AOAC International, 2016). Accuracy was calculated by comparing DIA technique with the reference spectrophotometric technique and in both methods, the average was under a 10% of relative error. The precision and accuracy of this technique can be significantly impacted by factors like ambient light or illumination; when accuracy and precision are studied, small differences between images can result in slightly high errors.

Selectivity of the methods was determined by measuring the interferences of non-phenolic reducing compounds, such as ascorbic acid (AA) and fructose (F), with DIA for the chosen reaction time for each method. These two compounds were added in the same concentration (mol/L) as GA at different points of the calibration curve. In the FC method, F did not show any interaction adding it at the same concentration as GA, therefore higher concentrations (x1000) were added at three points to observe possible interferences. The same process was followed for the FBBB assay. No interactions were detected with neither of the compounds, hence, ten times GA concentration of AA and F were added at two points of the calibration curve.

Results are shown in Fig. 3, where the line of GA is represented in blue, and in orange, the line of GA, to which AA or F have been added at three points for the FC method and at two points for the FBBB assay.

On the FC method, AA showed a positive interaction with FC reagent as the absorbance obtained increased around 50% in the two higher concentrations, while a thousand times higher concentration of F (0.025 mol/L and 0.045 mol/L to $2.4{\cdot}10^{-5}$ mol/L and $4.8{\cdot}10^{-5}$ mol/L of GA, respectively) had to be added to observe an increment. This increment was of a 15% in the lowest concentration added, 56% in the second point and 119% in the highest concentration.

On the FBBB assay, F did not show interferences at any concentration used, while AA had to be added at a concentration ten times higher than GA to see an interaction with the reagent, and contrary to the previous method, the interference was negative, as it showed a 50% decrease in

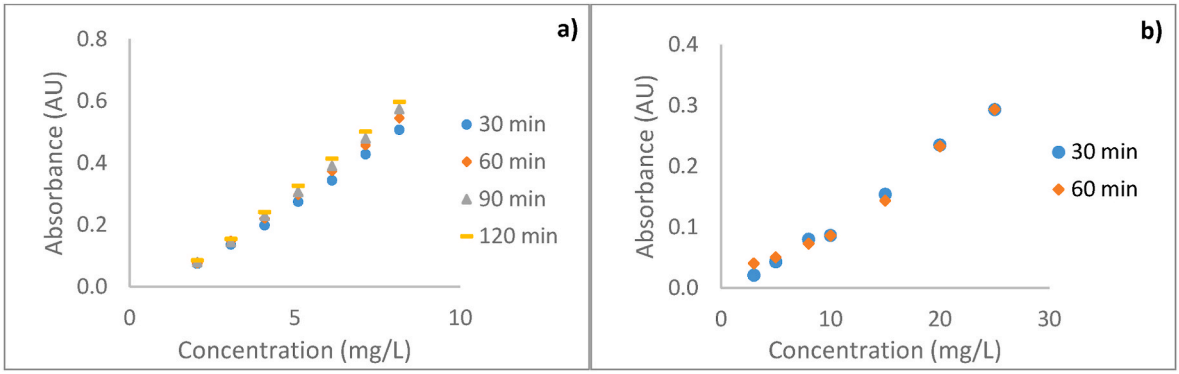

Fig. 1. GA calibration curves for FC (a) and FBBB (b) spectrophotometric methods at different reaction times.

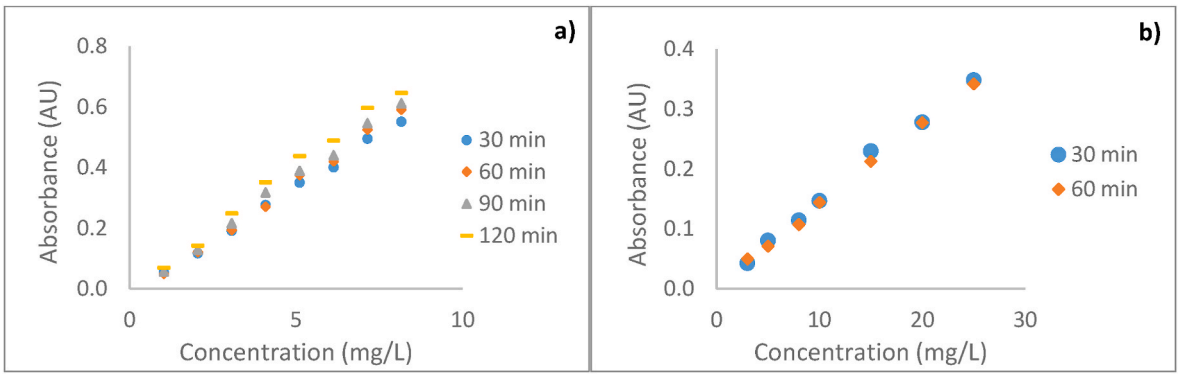

Fig. 2. Gallic acid calibration curves for FC (a) and FBBB (b) methods at different reaction times by DIA.

Table 1
Validation parameters for Digital Image Analysis.

Parameters	FC method	FBBB assay
Linearity	$R^2 = 0.997$ (1–8.16 mg/L)	$R^2 = 0.995$ (3–25 mg/L)
LOD	0.46 mg GAE/L	1.81 mg GAE/L
LOQ	1.52 mg GAE/L	6.03 mg GAE/L
Intraday precision (RSD_r, n = 3)	3.8%	6.7%
Interday precision (RSD_R, n = 3)	6.0%	8.5%
Accuracy (RE)	8.9%	9.4%

the absorbance of both concentrations added.

These results agree with those presented in literature where interferences of FC (Bridi et al., 2014; Muñoz-Bernal et al., 2017) and FBBB reagents (Lester et al., 2012; Roslan et al., 2019) with AA and F are determined by spectrophotometry.

3.3. Sample analysis

3.3.1. Comparison of techniques

There is great interest in the phenolic content of food, and there are many studies on the determination of them in different drink samples. Most of those researches use the FC spectrophotometric method and a few of them DIA and the FBBB assay.

In this study, the determination of the TPC of 15 different samples was made using both methods and techniques in order to evaluate the suitability of the less used ones. Among the samples, eight were tea/infusion samples including green (T_1), black (T_2) and red (T_3) tea, pennyroyal (MP_1 and MP_2), linden (L_1) and two aromatized plant and fruit infusions (I_1 and I_2). The other seven samples were different types of fruit beverages, including orange and pineapple nectars (N_1 and N_2), grape and apple juice (J_1 and J_2), manually pressed lemon and orange juices (J_3 and J_4) and a lemon juice refreshing beverage (B_1). The results are presented in Table 2, Table 3 and Fig. 4.

The results obtained by UV–Vis spectrophotometry were higher for all tea samples in the FC method and all but red (T_3) and black (T_2) tea in

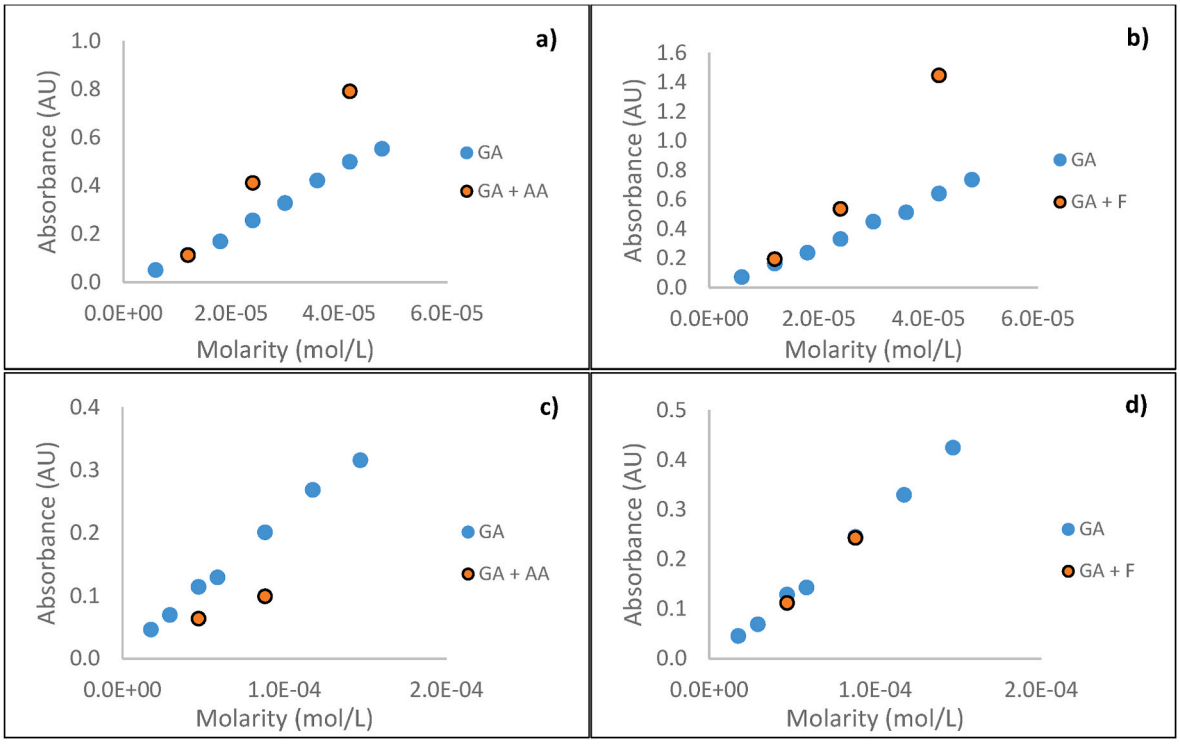

Fig. 3. Influence of non-phenolic compounds (ascorbic acid and fructose) on the standard curve of gallic acid. a) Ascorbic acid interaction with FC. b) Fructose interaction with FC. c) Ascorbic acid interaction with FBBB. d) Fructose interaction with FBBB.

Table 2
Total phenolic content (mg GAE/g $_{sample}$) of eight tea samples by FC method and FBBB assay, spectrophotometrically and with DIA. Ratio FBBB/FC refers to DIA.

Sample	FC method		FBBB assay		Ratio FBBB/FC
	UV–Vis	DIA	UV–Vis	DIA	
I_1	24.6 ± 2.4^{b}	24.0 ± 1.3^{b}	100.7 ± 30^{a}	82.4 ± 20^{a}	3.4
I_2	18.4 ± 2.9^{a}	17.8 ± 1.9^{ab}	53.8 ± 13^{a}	35.8 ± 1.7^{a}	2.0
T_1	69.7 ± 1.5^{e}	58.1 ± 7.1^{d}	598.8 ± 77^{c}	577.4 ± 83^{c}	9.9
T_2	70.8 ± 1.5^{e}	64.4 ± 3.8^{d}	507.9 ± 34^{c}	556.1 ± 31^{c}	8.6
T_3	43.8 ± 3.0^{d}	37.7 ± 4.8^{c}	218.7 ± 48^{b}	242.2 ± 22^{b}	6.4
MP_1	40.0 ± 1.1^{cd}	37.7 ± 3.7^{c}	74.7 ± 12^{a}	53.3 ± 11^{a}	1.4
MP_2	37.7 ± 2.2^{c}	37.0 ± 1.2^{c}	73.5 ± 7.3^{a}	74.9 ± 3.6^{a}	2.0
L_1	15.3 ± 0.6^{a}	11.8 ± 2.6^{a}	79.1 ± 9.4^{a}	63.3 ± 10^{a}	5.4

Results are expressed as mean ± standard deviation (n = 3). In each column, samples are grouped within same letters and different letters mean groups with statistically significant differences ($p < 0.05$) compared by Tukey HSD test.

Table 3
Total phenolic content (mg GAE/L) of seven different juices, nectars and drinks samples by FC method and FBBB assay, spectrophotometrically and with DIA. Ratio FBBB/FC makes reference to DIA.

Sample	FC method		FBBB assay		Ratio FBBB/FC
	UV–Vis	DIA	UV–Vis	DIA	
N_1	452.0 ± 30^{cd}	388.2 ± 7.5^{c}	345.2 ± 57^{ab}	236.4 ± 12^{a}	0.6
N_2	406.3 ± 23^{c}	364.2 ± 25^{c}	223.1 ± 72^{a}	203.0 ± 27^{a}	0.6
J_1	233.7 ± 23^{ab}	259.2 ± 19^{b}	468.4 ± 33^{b}	581.4 ± 26^{d}	2.2
J_2	443.4 ± 33^{c}	460.8 ± 14^{d}	305.7 ± 44^{ab}	305.7 ± 0.2^{bc}	0.7
J_3	266.9 ± 18^{b}	227.7 ± 8.8^{b}	264.2 ± 39^{a}	226.8 ± 32^{a}	1.0
J_4	514.9 ± 16^{d}	465.4 ± 15^{d}	453.5 ± 120^{b}	316.8 ± 7.3^{c}	0.7
B_1	189.5 ± 19^{a}	142.6 ± 21^{a}	307.2 ± 39^{ab}	243.9 ± 31^{ab}	1.7

Results are expressed as mean ± standard deviation (n = 3). In each column, samples are grouped within same letters and different letters mean groups with statistically significant differences ($p < 0.05$) compared by Tukey HSD test.

the FBBB assay, compared to DIA. In the case of fruit juice drinks, the TPC concentration determined by DIA was only higher in apple (J_2) and grape (J_1) juices, the latter also showing higher values by DIA in the FBBB assay. Nonetheless, regarding the techniques used for the determination of TPC in the samples, after performing a paired sample *t*-test, only two of the samples showed statistically significant differences ($p < 0.05$) between the two techniques in the FC method, T_3 and B_1. The results of the rest of the samples were in close agreement with those obtained using the reference method, at a confidence level of 95% in both methods. This indicates that, in general, DIA could be successfully applied to estimate the TPC of tea and fruit juice drinks with either of the methods analyzed. This is also in agreement with the results obtained by other authors about the use of the FC method by DIA to evaluate the reducing capacity of samples that required high values of dilution factor, colorless samples (Abderrahim et al., 2016), or acerola extracts (Martins et al., 2021).

An EJCR test was also performed to determine the accuracy and precision of FC and FBBB methods by DIA in predicting TPC in different beverages compared to UV–Vis spectrophotometry. This test is based on ordinary least squares (OLS) analysis of predicted concentrations against nominal concentrations to calculate intercept and slope, and comparison with the expected theoretical values (Almasvandi et al., 2020). The results are presented in Fig. 5 and showed that there are not significant difference at 95% confidence level for TPC of samples analyzed with the FBBB assay, as the ideal point (*) is inside the black line ellipse. On the contrary, there are significant differences with the FC method although the ideal point is very close to the line of the ellipse (dash red line).

3.3.2. Total phenolic content in teas and fruit beverages

As for the comparison of the two methods analyzed by DIA in tea samples, higher concentrations were obtained with the FBBB method and the FBBB/FC ratios were greater than 1, indicating larger amounts of phenolic compounds (Medina, 2011b). In fruit juice drink samples, there is more diversity in the results. Orange juice (J_4), orange and pineapple nectars (N_1 and N_2) and apple juice (J_2) samples had a higher concentration with the FC method and, therefore, FBBB/FC ratios were below 1, which indicates that they have a greater amount of reducing non-phenolic compounds. These samples are indeed the ones that have vitamin C among its ingredients. These results could confirm the interferences mentioned in section 3.2 since in the presence of other antioxidants, the FC method shows a positive interaction. The grape juice (J_1) and the lemon refreshing drink (B_1) have a higher concentration of polyphenols with the FBBB method, even if the latter also has vitamin C in its composition. In the case of the manually press lemon juice (J_3), the concentration is similar for the two methods and does not show significant differences between the concentrations obtained by both methods.

Among the infusion samples, green (T_1) and black (T_2) tea were the ones with higher TPC by far, followed by red tea (T_3), and at a long distance regarding the remaining infusions which turn out to be associated to other plant species as *Mentha piperita* sp. (MP_1, MP_2), *Tilia* sp. (L_1) and fruit and plant infusions (I_1 and I_2). All the teas analyzed are made from *Camellia Sinensis* sp., but the plant leaves in each of them follow a different process to achieve the final product. Green tea undergoes less oxidation during its processing, so polyphenols do not suffer as much degradation as in black tea, which undergoes a complete oxidation, and that makes its TPC lower than in green tea (Cleverdon et al., 2018). At the same time, the manufacture of herbal infusions made with a mixture of fruit and plants (I_1 and I_2) could involve processing procedures (such as drying or grinding) that may degrade the phenolic compounds and, hence, there may be a lower TPC in these samples (Horžić et al., 2009).

As the post-hoc Tukey HSD test showed, the infusions and teas analyzed could be classified into three different groups based on the TPC determined using both FBBB method and DIA technique. In the first group, the green (T_1) and black (T_2) teas with the highest concentration, followed by the red tea (T_3) in another group and, finally, the flavored infusions of plants and fruits, the pennyroyal samples and linden. In the case of fruit juice drinks, the sample with the highest TPC was the grape juice, followed by manually pressed orange juice and apple juice. The samples with the lowest TPC were the manually pressed lemon juice and the two nectars.

TPC could vary significantly depending on the environmental conditions, the varieties or the processing of the products used in the different studies, which makes comparison difficult. For example, the results obtained by DIA for green tea using the FC method (58.1 ± 7.1 mg GAE/g $_{sample}$) are quite consistent with those obtained by some other authors, 62.9 ± 11.9 mg GAE/g $_{sample}$ (Musci & Yao, 2017) or 64.7 ± 1.3 mg GAE/g $_{sample}$ (Abdel Azeem et al., 2020). This last author also analyzed black tea (60.3 ± 5.1 mg GAE/g $_{sample}$), and the results are similar to those obtained in this study (64.4 ± 3.8 mg GAE/g $_{sample}$). However, other authors obtain similar results for green tea (49.6 mg GAE/g $_{sample}$) but not for black tea (37.3 mg GAE/g $_{sample}$) (Gómez Ordoñez et al., 2021). Regarding juice samples, the data obtained in this study are similar to those obtained with the FC spectrophotometric method in literature for pineapple juice (357.4 mg GAE/L), apple juice

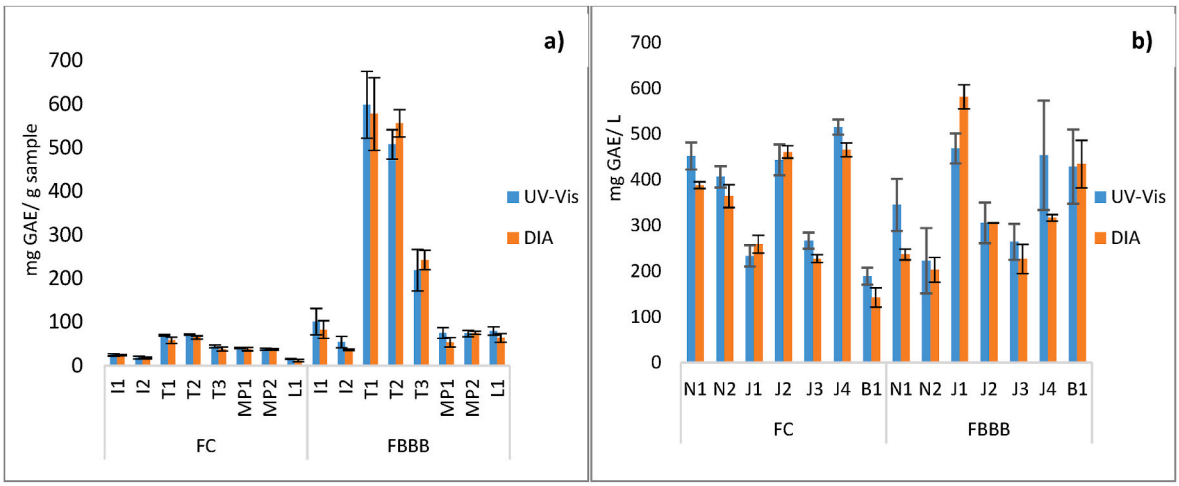

Fig. 4. a) Total phenolic content (mg GAE/g sample) of eight tea samples by FC method and FBBB assay, spectrophotometrically and with DIA. b) Total phenolic content (mg GAE/L) of seven different juices, nectars and drinks samples by FC method and FBBB assay, spectrophotometrically and with DIA. Results are expressed as mean ± standard deviation (n = 3).

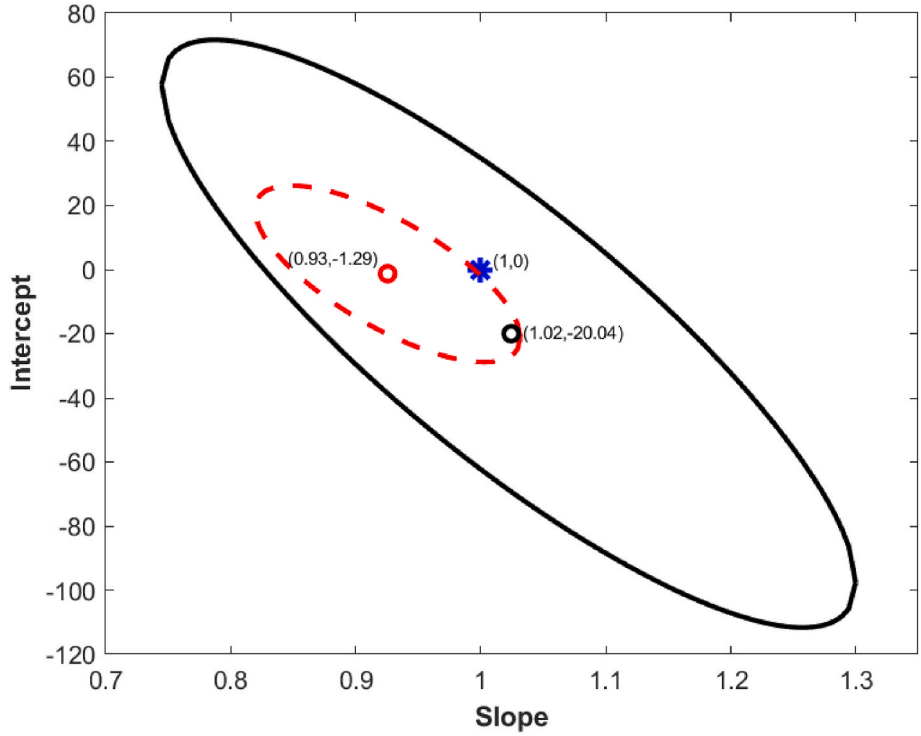

Fig. 5. EJCR test for the analysis of samples with FC (dash red line) and FBBB (black line) methods by DIA in comparison with UV–Vis spectrophotometry.

(428.1 mg GAE/L), grape juice (337.1 mg GAE/L) and fresh orange juice (542.8 mg GAE/L) (Mahdavi et al., 2010).

The studies done using the FBBB assay are scarce and therefore more researchers are needed to compare the results and reach an adequate conclusion. The range of results obtained for different juices and nectars using both methods (500–4000 mg/L with FBBB and 500–1500 mg/L with FC) are superior to those obtained in this study despite not being comparable since the juices are of different fruits (Li et al., 2021). The results obtained in other works (Medina, 2011b) using FBBB spectrophotometric assay for natural orange juice (200 mg GAE/L) are slightly lower than those obtained in this paper, and those obtained for green tea (1520 mg GAE/L) and black tea (1850 mg GAE/L) are much higher.

4. Conclusions

This study showed that it is possible to reduce the reaction time of FC and FBBB methods with the reference technique, and it is possible their adaptation to DIA. Moreover, the results obtained in this research indicated that DIA could be an alternative to the traditional spectrophotometric TPC determination methods that further reduces the total time needed due to its capacity to analyze up to 96 samples at the same time. The characteristics of this technique make it cheaper, more manageable and more environmentally friendly thanks to the reduction of waste volumes. Although it also has its limitations due to variables like ambient light or the camera's parameter settings that can affect and slightly worsen its precision and accuracy.

When analyzing the possible interferences of both methods with

ascorbic acid and fructose, it was observed that FBBB assay is the best method for the determination of TPC on teas and fruit juice drinks since non-phenolic reducing compounds present in the samples do not interfere as much with it. However, the combination of both methods could be useful to obtain a better perspective of the antioxidant capacity since each method reacts with different components. Regarding the samples analyzed in this study, overall, juices showed a higher TPC content, although the samples with the highest TPC were green and black teas along with grape juice. Red tea could be grouped with the rest of the fruit juices in terms of TPC. The remaining infusions showed a lower TPC and similar between them.

It would be interesting to use these methods on a wider variety of food samples to have a larger database, be able to make more comparisons, and relate in this way the antioxidant capacity between foods.

CRediT authorship contribution statement

Maider Zugazua-Ganado: Investigation, Methodology, Validation, Writing – original draft. **Ane Bordagaray:** Conceptualization, Supervision, Writing – review & editing. **Jokin Ezenarro:** Investigation, Methodology. **Rosa Garcia-Arrona:** Writing – review & editing. **Miren Ostra:** Conceptualization, Supervision, Writing – review & editing. **Maider Vidal:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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