

Development of a new microfluidic paper-based device (μ PAD) for total phenolic content determination of beverages by the fast blue BB method

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ABSTRACT

Phenolic compounds constitute a broad family of secondary metabolites synthesized by plants responsible for their color, flavor, and astringency, as well as being very important in the human diet as the main source of antioxidants. This work describes a simple and disposable microfluidic paper-based analytical device (μ PAD) for the determination of total phenolic content in different beverages. The detection was based on the colorimetric reaction between the Fast Blue BB reagent and the active hydroxyl groups of the phenolic compounds in an alkaline medium. The developed μ PAD enabled TPC determination for gallic acid equivalents in the range of 5–200 mg/L and catechin equivalents of 25–125 mg/L, with a detection limit below 5.5 mg/L. Tea and wine samples were analyzed with the developed μ PAD and compared to the conventional spectrophotometric method, with no significant differences ($p > 0.05$). The device remained stable for 7 days under a vacuum atmosphere.

1. Introduction

Phenolic compounds, also commonly known as polyphenols, are a varied group of plant compounds that are abundant in fruits, vegetables, and beverages, including wine and herbal infusions. These compounds have gained significant attention for their potential health benefits, primarily attributed to their antioxidant properties. They have been associated with anti-cancer and anti-diabetic effects, as well as cardiovascular protection, anti-inflammatory effects, and neuroprotective activity (El Rayess et al., 2024; Studzińska-Sroka et al., 2021).

Each beverage has a unique polyphenol profile, including phenolic acids, catechins, and flavonols. Teas and infusions are a rich source of natural antioxidants. In tea, approximately 20% of the dry matter of a leaf is polyphenols (International Organization for Standardization, 2024). The abundant polyphenol will depend on the treatment suffered by the leaves of the plant to be infused. In general, gallic acid and epicatechin 3-O-gallate, followed by catechin and epicatechin, were the most abundant polyphenols found by Pinto et al. (Pinto et al., 2020). However, due to the fermentation process, phenolic compounds will be converted into other compounds, so depending on the type of infusion, the most abundant polyphenol will vary. For this reason, thanks to its

good stability and its presence in most foods, gallic acid is one of the most used polyphenols as a standard for the determination of total phenolic compounds (Abdel Azeem et al., 2020; Baldelli et al., 2025).

Wine, mainly red wine, is also renowned for its high polyphenol content, with resveratrol and proanthocyanidins being well-known examples. Wine polyphenols also influence organoleptic properties like taste, color, and astringency (Gutiérrez-Escobar et al., 2021). Different phenolic compounds can be used as markers for the identification of grape varieties and regions based on their concentration, and the degree of antioxidant activity could also be used as an indicator of the age of wine, as the chemical structure of phenolics changes during storage and reduces their antioxidant effect (Chen et al., 2022; Eder et al., 2023). Gallic acid is the most common phenolic compound used as a reference for the determination of TPC by Folin-Ciocalteu, but catechin has also been used in some studies (Eder et al., 2023; Stevanato et al., 2004). According to the results obtained by Chen et al., catechin proved to be more stable during bottle storage than gallic acid. Several methods are used to quantify total phenolic content in foods and beverages (Medina, 2011a; Singleton et al., 1999). In this study, the Fast Blue BB method has been chosen as it provides a more specific measurement compared to the more often used traditional Folin-Ciocalteu method.

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This method is based on the reaction of the fast blue diazonium salt with the active hydroxyl groups of the phenolic compounds, forming colored azo complexes in an alkaline medium that enhances the reactivity of the salt (Medina, 2011b). After the reaction, the intensity of the color produced can be measured by UV–Vis spectrophotometry or by digital image analysis (DIA), whether using microplates or μ PADs. When using DIA, the intensity values obtained from the pixels of interest of the image are converted into absorbance values using the formula $A = \log(I_0/I_s)$, where I_0 is the intensity of the blank signal (water) and I_s is the intensity of the standards or sample signal (Kohl et al., 2006).

Microfluidics is known as a technology that uses small volumes of reagents and is being applied in diagnostics, drug delivery, and chemical analysis (Whitesides, 2006). The integration of microfluidics with paper-based devices offers advantages such as low cost, ease of fabrication, and portability. Microfluidic paper-based analytical devices (μ PADs) are developed using different techniques to precisely define hydrophilic and hydrophobic regions, and they use the capillary of filter paper to transport fluids without the need for external help (Almeida et al., 2018). The most used patterning techniques to create the hydrophobic barriers include wax printing, screen printing, inkjet printing, and photolithography, which use different materials (wax, polymers, inks...) for that purpose (Carrilho et al., 2009; Kamali et al., 2018; Sittiwong & Unob, 2016). Once the hydrophobic region is obtained, the paper might need the integration of detection elements, reagent immobilization, or surface modifications so the reaction can occur, and proper analytical detection can be achieved. μ PADs can have 2D structures, which only need a layer and are simpler to create, or they can be 3D, with more than a layer allowing lateral and vertical flow and giving the advantage of more complex analysis, as the reaction order and the flow speed can be controlled. When using 3D structures, papers need to be aligned before the final assembly, which can be complex and lead to lower reproducibility. Different detection methods can be used with μ PADs, among which are amperometric detection when introducing electrodes, colorimetric detection, or fluorescence detection (Ozer et al., 2020; Pradela-Filho et al., 2023).

To address the environmental and operational disadvantages of traditional UV–Vis spectrophotometry, miniaturized analytical devices aligned with Green Analytical Chemistry (GAC) principles and the WHO REASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid/Robust, Equipment-free, and Deliverable) have attracted growing interest. To quantify the eco-friendliness of analytical procedures, standardized evaluation metrics such as the Analytical GREENness (AGREE) index have become essential tools for assessing method sustainability across all 12 GAC principles (Pena-Pereira et al., 2020). In this context, paper-based analytical devices (μ PADs) combined with Digital Image Analysis (DIA) via flatbed scanners offer a highly sustainable, portable, and low-cost alternative for point-of-need (PON) quality control.

To contextualize the analytical performance of the proposed device within the current state of the art, different optical, electrochemical, and microfluidic sensing protocols developed for Total Phenolic Content or specific phenolic compounds are summarized in Table 1. Reported sensing platforms exhibit a wide variety of analytical strategies and detection methods. Traditional paper devices (μ PADs) predominantly rely on colorimetric DIA coupled with Single Electron Transfer (SET) redox chemistry, such as the Folin-Ciocalteu assay. Although these paper formats provide low cost and operational simplicity, their redox mechanism is non-specific, leading to overestimations in real samples containing non-phenolic reducing species (e.g., ascorbic acid, reducing sugars, or organic acids). To mitigate matrix interferences, specialized analytical strategies have been developed, including functionalized polymer chemosensors, or silver nanoparticle (AgNP) aggregation protocols (Gutorova et al., 2021; Puangbanlang et al., 2019). At the same time, advanced sensing protocols using electrochemical platforms (such as unmodified boron-doped diamond electrodes (BDDE), reduced graphene oxide nanoarrays (PtNPs/3DNRGO), and 2D MXene

functionalized surfaces) as well as advanced LC-MS/MS techniques, have been introduced for targeted phenolic compounds and contaminants such as bisphenol A (Indirakumari et al., 2025; Jayakumar et al., 2024; Puangbanlang et al., 2019). Although these electrochemical and chromatographic protocols achieve very low limits of detection, their in situ availability is still not possible due to the need of complex multi-step electrode fabrication, surface fouling, specialized benchtop instrumentation, and high operational costs.

As shown in Table 1, there are a few studies combining μ PADs and colorimetric detection for the determination of total phenols in different food samples using the FC method (Ferraz et al., 2021; Hao et al., 2021; Martínez-Pérez-Cejuela et al., 2022), but according to the available literature, there is no research using μ PADs to determine TPC by the FBBB method. In this work, a novel and simple spot test μ PAD has been designed for quantification of TPC based on the FBBB method, following the assembly of the devices developed by the UCP research group (Aguilar et al., 2022; Ferreira, Mesquita and Rangel, 2021; Ferreira, Catalão et al., 2021; Martínez-Pérez-Cejuela et al., 2022; Melo et al., 2023) that uses lamination. Different parameters such as paper type, thickness, pore size, etc. were optimized, and the developed devices proved good sensitivity, stability, and accuracy for teas, infusion and wine samples. The 24 determinations of a μ PAD (four repetitions of five samples and a blank) consume 2.16 mg of FBBB reagent, 24 mg of NaOH, and 60 μ L of each sample, in line with the GAC goals of using small amounts of reagents.

2. Materials and methods

2.1. Reagents and solutions

All reagents used were of analytical grade, and solutions were prepared with Milli-Q® Water (resistivity >18.2 MW.cm, Millipore, Bedford, MA, USA).

Fast blue BB (4-benzoylamino-2, 5-dimethoxybenzenediazonium chloride hemi-[zinc chloride]) salt and all phenolic standards (gallic acid, catechin, epicatechin, trans-ferulic acid, caffeic acid and syringic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and sodium hydroxide and ethanol from PanReac (Barcelona, Spain).

A stock solution of 500 mg/L of each phenolic compound was used to prepare the working standards in the range of 5 to 200 mg/L or 25–125 mg/L. The Fast blue BB reagent was prepared by dissolving the salt in 35% (v/v) ethanol to obtain a 0.9% (w/v) stock solution.

2.2. μ PAD assembly

The microfluidic paper-based devices (μ PADs) developed in this work were assembled using previously perforated laminating pouches with 3 mm holes for sample insertion (75 × 110 × 0.125 mm, Q-connect) as the hydrophobic layer of the μ PADs. Hydrophilic units were created by aligning two filter paper discs as one unit, placed in 4 × 6 rows and columns aligned with the previously mentioned 3 mm holes, creating 24 hydrophilic detection units in every μ PAD (Fig. 1.a).

Every unit had two different types of paper where different reagents were immobilized: sodium hydroxide (5%) in the bottom layer with Whatman™, grade 3 (W3) and FBBB (0.9%, dissolved in 35% ethanol) in the top layer with Whatman™, grade 54 (W54). The bottom layer consisted of a 9.5 mm diameter (puncher 3/8" EK tools, Lindon, USA) qualitative filter paper disc (W3), loaded with 20 μ L of NaOH (5%) and dried for 20 min at 50 °C in a stove. The top layer consisted of a hardened low-ash filter paper disc (W54) of a 9.5 mm diameter with 10 μ L of FBBB (0.9%) and dried for 10 min at 50 °C. Both paper discs were aligned in the plastic pouches for lamination (Rexel Style A4 laminator, ACCO Brands Europe, Style/CBT12698) obtaining a device with proper hydrophobic and hydrophilic zones ready to use. Once the device is prepared, the standard/sample (15 μ L) is loaded through the 4 mm holes.

Table 1

Comparative performance of paper-based, optical, and electrochemical sensing protocols for polyphenolic compound determination.

Measured parameter	Sensing Protocol	Analytical Strategy	Matrix	Reference standard	Linear Range	LOD	Selectivity (Interferents)	Ref.
TPC	Colorimetric solution assay - DIA	Chelation between phenolic compounds and ferric chloride (FeCl ₃) forming a yellow complex	Tomato Strawberry Coffee extracts	Quercetin (Phenols)	0–31.8 mg/L	0.03 mg/L	Non-specific; measures total reducing capacity and chelating phenolics across roasting stages	(Bazani et al., 2021)
TPC	μPAD - DIA	Microfluidic chip matching reaction kinetics via FC method	Green, black and Puerh tea	Gallic acid	10–100 mg/L	< 10 mg/L	Tea pigments / anthocyanins in black / Puerh tea cause minor optical grayscale interference No significant interference observed from ethanol, glycerol, sodium sulfite, D-glucose, and tartaric acid	(Hao et al., 2021)
TPC	μPAD - DIA	FC method in a vertical flow two-layer μPAD	Sparkling and table wines	Gallic acid Taniraisin	4.1–50 mg/L	1.2 mg/L		(Martínez-Pérez-Cejuela et al., 2022)
TPC	Polymer chemosensor films - DIA	Hydrophilic colorimetric polymer film disks with diazonium groups forming colored azo groups Direct irreversible electro-oxidation of epicatechin at +1.05 V (vs. Ag/AgCl) in 0.1 M HNO ₃ using voltammetry	Multifloral and honeydew honeys	Gallic acid	50–260 mg/L	ND	Free from interference from highly concentrated fructose and glucose matrices	(González-Ceballos et al., 2021)
TPC	Voltammetric Sensing		Black and green teas	Epicatechin	1.0–50.0 mg/L	0.28 mg/L	Studies against epigallocatechin gallate, catechin, tannic acid, and gallic acid	(Ali et al., 2023)
TPC	Diazotized colorimetric spots - DIA	Spot reactions on a porcelain plaque using three diazotized aminobenzenes	Tea Fruits	Gallic acid	20–400 mg/L	5.5 mg/L	High tolerance to ascorbic acid and reducing sugars; Affected by high Fe (II) levels	(Abdel Azeem et al., 2020)
TPC AC	Symmetric quad-channel μPAD - DIA	Wax-printed symmetric quad-channel design matching ABTS, CUPRAC, and FC assays	Teas Wines Juices	Gallic acid	0.5–6 mM (FC and CUPRAC)	4.5 mg/L (FC)	Uses APTES and PEG to prevent dye migration; Affected by reducing sugars and vitamin C	(Puangbanlang et al., 2019)
Flavonoids	μPAD - Spectrophotometry (DRS)	Reduction of silver nitrate to silver nanoparticles (AgNPs) in a composable modular paper device	Hawthorn tincture Onion peel	Quercetin Morin	7–100 μg/mL (Quercetin) 16–100 μg/mL (Morin)	2.3 μg/mL (Quercetin) 5.2 μg/mL (Morin)	Tested against ions (Na ⁺ , K ⁺ , Cl ⁻), glucose, and starch. Affected by ascorbic acid at a 1:1 ratio.	(Gutorova et al., 2021)
	LC-ESI-MS/MS	LLPE followed by advanced tandem MS with negative multiple reaction monitoring (MRM). Single-step electrodeposition of platinum nanoparticles and reduced graphene oxide (PtNPs/3DNRGO) on a glassy carbon electrode	Mineral, river, and tap water.	Bisphenol A	10 ng/mL to 1 pg/mL	0.037 ng/mL	High structural selectivity via MRM; rapid 2.35 min runtime without SPE cleanup	(Indirakumari et al., 2025)
Bisphenol A (Phenolic endocrine disruptor)	Electrochemical 3D nanoarray		Plastic bottle leaching studies	Bisphenol A	0.7 to 20 μM	6 nM	High selectivity against phenolic interferents	(Jayakumar et al., 2024)
	MXene electrochemical platform (REVIEW)	2D MXene (Ti ₃ C ₂ T _x) surface functionalization; Tunable surface group electro-oxidation	Environmental waters and food packaging	Bisphenol A	Variable (pM to mM ranges evaluated)	Variable (e.g. 0.001–100 μg/mL in aptasensors)	Tunable surface terminations (-F, -OH, -O) accelerate electron transfer for phenolic groups. Matrix interference study with a control μPAD without reagent	(Jayakumar et al., 2026)
TPC	μPAD - DIA	FBBB assay in a two layer μPAD	Teas Infusions Wines	Gallic acid Catechin	5–200 mg/L 25–125 mg/L	4.2 mg/L 5.3 mg/L		This work

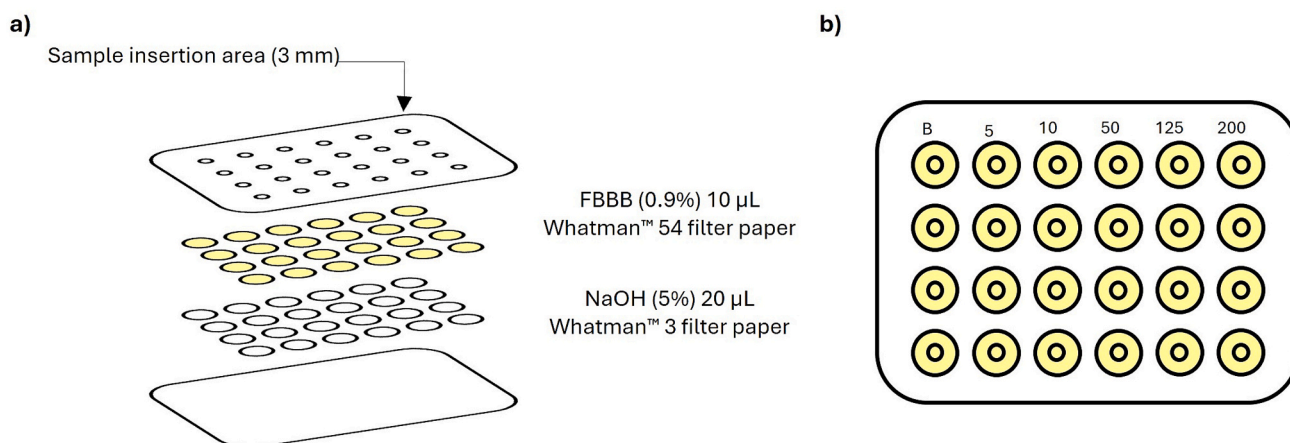


Fig. 1. Illustration of the μ PAD assembly; a) alignment of the μ PAD's 3D structure. b) μ PAD top view indicating the loaded solutions for a calibration curve.

2.3. Total phenolic content determination

To perform the total phenolic content determination, 15 μ L of standard/sample was loaded in the assembled device. After adding the standard/sample, the device is left, avoiding light, to dry and react for 10 min before scanning it on the side of the top layer and proceeding to the image analysis. Digital images were acquired with a digital scanner (Epson Stylus SX100, Los Alamitos, CA, USA) and processed with free software (ImageJ) using an RGB filter for intensity counts measurement. The chosen filter was the G channel as the complementary color of the formed orange-reddish reaction product.

The color intensity of the reaction was obtained using the software ImageJ, which allows processing the image to a RGB stack and obtaining the values of each of the channels. The green filter was chosen as the complementary color of the formed product. To measure the intensity of the pixels, a circular area of 32×32 pixels was selected in the filter paper units, coinciding with the sample insertion area where the color was concentrated and excluding edge gradients, ensuring low repeatability error. The intensity values were then converted into absorbance values using the intensity of the blank signal and the intensity for the G channel of the standards/sample ($A = \log(I_0/I_s)$). For each reading of the blank or standard/sample, 4 measurements were made (one per row).

A gallic acid calibration curve from 5 to 200 mg/L or a catechin calibration curve from 25 to 125 mg/L was performed using one device and first column for the blank signal (Fig. 1.b). Total phenolic content was expressed in mg Gallic Acid Equivalents (GAE)/g sample for tea and infusion samples and in mg Catechin Equivalents (CAE)/L for wine samples. Determinations for each sample were performed in triplicate, and all samples were diluted before analysis (Table S1).

2.4. Samples

2.4.1. Wines

Total phenolic content of eighteen red wines and one white wine was determined using the developed μ PAD. All wine samples were diluted before analysis, as shown in the supplementary material (Table S1).

2.4.2. Teas and infusions

Six different teas and infusions (Table S2) were used in the developed μ PAD to determine total phenolic content. For teas and infusions, about 1 g of sample was weighed to 200 mL of heated (98–100 $^{\circ}$ C) distilled water for 5 min. For the bael fruit infusion, package instructions were followed, and 1 g of the sample was infused in 20 mL. After that time, samples were filtered, and further dilutions were made when necessary for determination.

2.5. Validation - Spectrophotometric assay

The results obtained with the μ PAD devices were compared to results obtained with the conventional UV-Vis spectrophotometric batchwise procedure, based upon a previously reported method (Zugazua-Ganado et al., 2024). The procedure was carried out by mixing 44.4 μ L of a 0.9% FBBB solution with 4 mL of the sample, followed by the addition of 400 μ L of NaOH (5%) one minute later. Absorbance was then measured after 30 min at 450 nm (Hitachi, 100–40 UV-vis, Tokyo, Japan).

A *t*-test for paired samples was performed to compare μ PAD and UV-Vis results using the statistical package IBM SPSS Statistics v.27.0.1.0. For accuracy assessment, recovery percentages were calculated based on the addition of 50 and 100 mg/L of GA to tea and infusion samples. Small volumes of standard solution were added to prevent significant alteration of the sample matrix.

3. Results and discussion

3.1. μ PAD design

The engineering of the μ PADs uses a vertical-flow capillary transport model governed by the Lucas-Washburn equation, where pore radius, surface tension, and viscosity dictate fluid penetration through the stacked paper discs (Jafry et al., 2020). A two-layer μ PAD was established to ensure a vertical flow approach, and set to have one layer with FBBB reagent and one layer with NaOH. A preliminary test was made aiming to set the layers' order, and it was performed using grade 1 Whatman™ paper discs in both layers.

Having the FBBB at the top layer and the NaOH at the bottom layer resulted in higher sensitivity and linearity (Fig. S1a). This assembly ensured that the sample was loaded through the reagent layer.

Better sensitivity was also obtained when scanning the FBBB layer facing the scanner (detection side). As visually expected (Fig. S1c), scanning the reverse side (NaOH layer) resulted in unsatisfactory correlation coefficients.

3.2. Filter paper type

Different filter paper pore sizes and manufacturing treatment were tested for both layers establishing calibration curves for each tested condition and choosing the one that resulted in a higher calibration curve slope, higher sensitivity.

The first studies focused on the first layer, the color reagent (FBBB) and detection layer, and the NaOH bottom layer was kept with Whatman™ grade 1 filter paper (W1) loaded with 20 μ L NaOH (5%). The

calibration curves for each paper grade tested were obtained loading 10 μL of FBBB (0.9%) in the top layer paper disc and inserting 15 μL of the standard. Four different pore sizes of qualitative WhatmanTM filter papers were studied: grade 1 (W1, 11 μm), grade 2 (W2, 8 μm), grade 4 (W4, 20–25 μm), and grade 5 (W5, 2.5 μm). The highest sensitivity, calibration curve slope, was obtained after 20 min with W4 (Fig. 2.a), corresponding to 20–25 μm pore size so it was the pore size chosen. These filter papers were also the ones with lower reaction times, opposed to the 30, 27 and 25 min needed when using W1, W5, and W2, respectively. This difference is attributed to the significantly bigger pore size of W4, which minimizes flow resistance compared to the other grades.

Afterwards, filter papers with similar pore sizes but different manufacturing treatments were studied. Three different treatments were tested: a hardened low-ash filter paper with a pore size of 22 μm (W41), an ashless 20 μm pore size paper (W54), a hardened ashless 22 μm paper (W541) and the W4 as the qualitative paper. Hardened low-ash (W41) and ashless filter (W54) papers showed the highest sensitivity (Fig. 2.b).

As the reaction and detection occur at the top layer, the bottom layer with the NaOH only buffers the reaction and serves as a reservoir.

So, the next step was to try a thicker filter paper in the bottom layer, testing the qualitative WhatmanTM grade 3 (W3) as it has the same manufacturing treatment of W1 and a similar pore size (W3 pore size 6 μm and W1 pore size 11 μm) but it is thicker: 180 μm for W1 and 340 μm for W3. Because the results obtained with W41 and W54 in the top layer had been very similar (Fig. 2.b), they were both tested with W3 in the bottom layer (Fig. 2.c). Using the W3 filter paper, the drying time was reduced to 10 min while achieving better sensitivity, especially with the W54 filter on the top layer. W3 filter paper for the bottom layer and W54

filter paper for the top layer were established as the μPAD design for the determination of TPC by the FBBB method.

3.3. Filter paper size and sample volume

After establishing appropriate filter paper for both layers, to distinguish the individual effects of standard volume and paper diameter, pairwise comparisons were performed. Different combinations of three different paper sizes were tested: (i) both layers of 9.5 mm, (ii) a bottom layer of 9.5 mm and a top layer of 6.35 mm, (iii) a bottom layer of 12.7 mm and a top layer of 9.5 mm, and (iv) a bottom layer of 12.7 mm and a top layer of 6.35 mm. The 6.35 mm top layers were loaded with 6 μL of FBBB to ensure that all the volume stayed in the filter. All configurations were loaded with 15 μL of standard, except for the (iv), which had 20 μL . Along with these configurations, another device, with both layers of 9.5 mm (v), was loaded with 20 μL of standard to test if a larger volume of sample gave better sensitivity (Fig. 3).

The best sensitivity was achieved when using the same size filters for both layers (9.5 mm) and loading less volume of standard (15 μL). This arrangement was chosen for the next studies.

3.4. Phenolic compounds standards selection

Although the optimization studies were performed with gallic acid as a model phenolic compound, other phenolic compounds, potentially present in foods, were tested on the developed device.

The color development of the FBBB assay relies on electrophilic diazo coupling, which requires an aromatic ring with activating hydroxyl groups. Consequently, the measured response originates from active

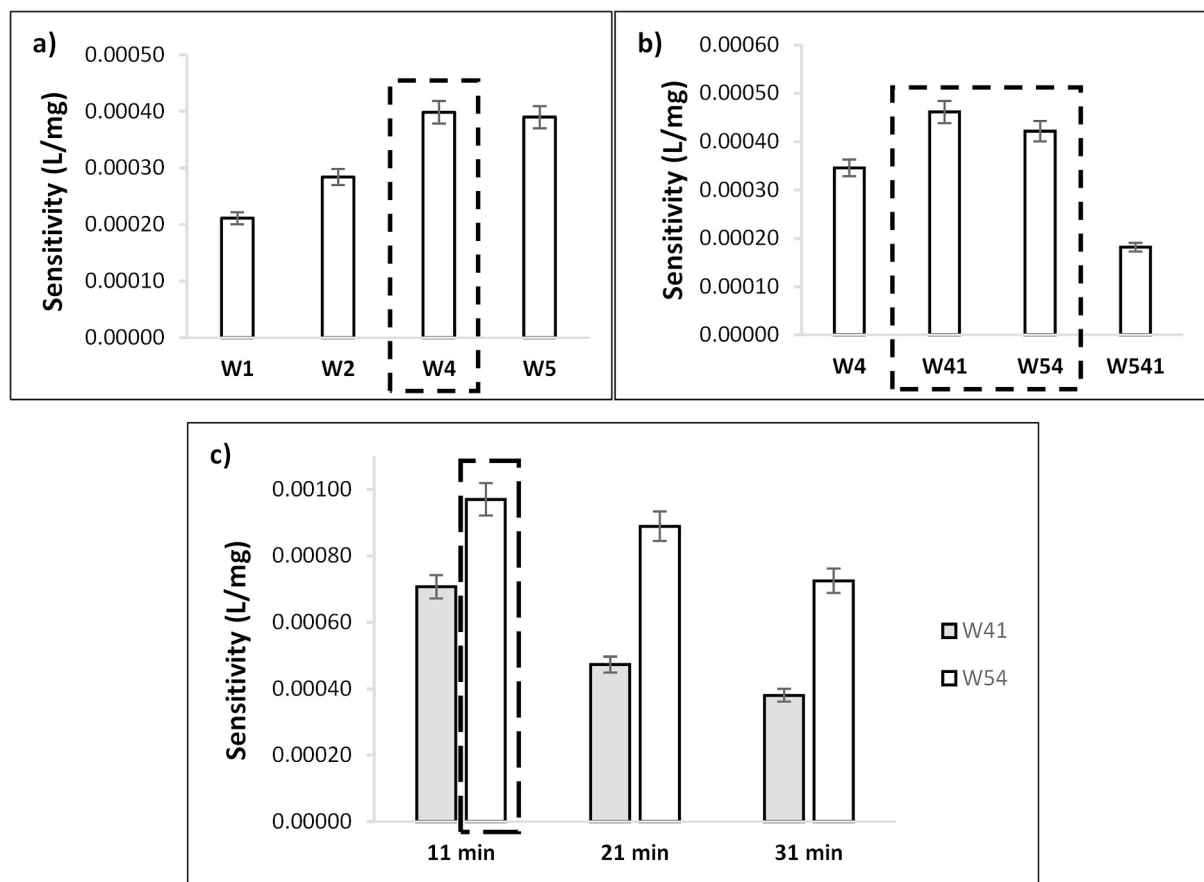


Fig. 2. Study of the top layer filter paper pore size (a) and treatment (b) influence on the sensitivity of the FBBB method calibration curve. WhatmanTM grade 1 was used in the bottom NaOH layer. c) Study of the sensitivity of the calibration curve at different times, using the W3 filter on the bottom layer and different treatment filters on the top layer (41 and 54). The chosen filter paper is marked with a dashed box.

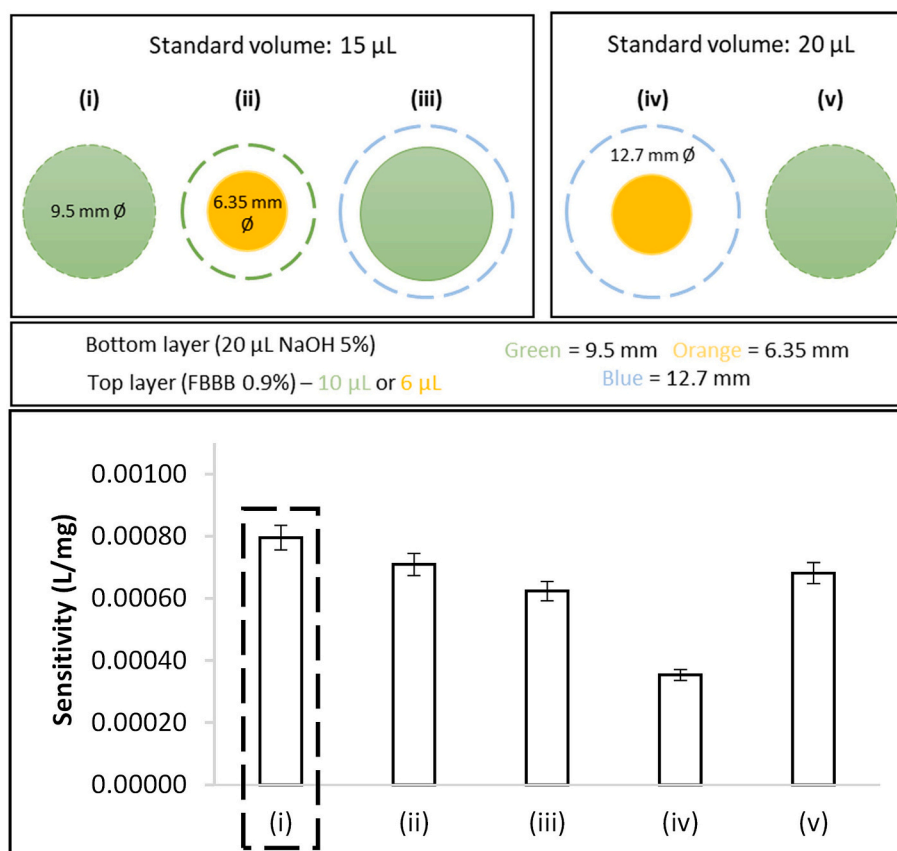


Fig. 3. Schematic representation of the different paper size configurations (top) and their influence on the sensitivity of the calibration curve (bottom). (i) Both layers of 9.5 mm, (ii) a bottom layer of 9.5 mm and a top layer of 6.35 mm, (iii) a bottom layer of 12.7 mm and a top layer of 9.5 mm, (iv) a bottom layer of 12.7 mm and a top layer of 6.35 mm loaded with 20 µL of standard, and (v) both layers of 9.5 mm loaded with 20 µL of standard.

polyphenolic families (flavan-3-ols, hydroxybenzoic, and hydroxycinnamic acids), while non-aromatic reducing species (such as sugars or ascorbic acid) cannot undergo coupling, preventing overestimations. The chosen phenolic compounds were gentisic acid, caffeic acid, syringic acid, trans-ferulic acid, catechin, and epicatechin. A calibration curve could not be established for gentisic acid as all the standards resulted in similar absorbance values. For the other phenolic compounds tested, the calibration standards range, the calibration curves parameters (including the correlation factor) and the determination time were compiled (Table 2).

Table 2
Parameters obtained for different standards.

Standard	Linear range [mg/L]	Calibration curve $A = S \times [\text{mg/L}] + b$	Correlation coefficient (R^2)	Time (min)
Gallic acid	5–200	$0.00080 \times + 0.00503$	0.995	10
Caffeic acid	5–200	$0.00029 \times + 0.03387$	0.992	30
Syringic acid	5–125	$0.00036 \times + 0.00236$	0.996	30
Trans-ferulic acid	5–125	$0.00153 \times + 0.00900$	0.999	30
Catechin	5–125	$0.00156 \times + 0.02580$	0.995	10
Epicatechin	5–125	$0.00150 \times + 0.03166$	0.983	10

Different sensitivities and regression coefficients were achieved for each standard, as well as different reaction times. Syringic and caffeic acid had low sensitivities, while ferulic acid, catechin, and epicatechin showed similar sensitivities and were better than the sensitivity of gallic acid (0.0008). These three compounds also showed much better color reaction stability than GA, as the calibration curves measured at 10 and 30 min did not show statistical differences. For GA, only 15 min can pass since the standard loading.

Considering the different aimed samples, teas, infusions and wines, two phenolic standards were set: Gallic acid for determination in tea and infusion, as the most stable and common phenolic compound in food samples; and catechin for wine samples was chosen because, as mentioned in the introduction, it is more stable in bottled wines than other phenolic compounds.

3.5. Features

The main characteristics of the developed µPAD when using gallic acid and catechin as standards for TPC determination are shown in the table below (Table 3).

After different tests, calibration curves were prepared from 5 to 200 mg/L for gallic acid and 25 to 125 mg/L for catechin. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as three and ten times, respectively, the standard deviation of the average of the intercept ($n = 4$) divided by the slope of the calibration curve ($n = 4$) (Currie, 1995).

The average of different curves is shown in Table 3. Inter- and intra-day precision were evaluated in terms of Relative Standard Deviation

Table 3Analytical parameters of the developed μ PAD.

Analytical parameter	Value	
	Gallic Acid (GA)	Catechin (CA)
Phenolic compound standard		
Linear range (mg/L)	5–200	25–125
Calibration curve	$A = 0.00080 (\pm 0.00008)$	$A = 0.00141 (\pm 0.00008)$
trave $S \times$ [standard]	$[GA] + 0.005 (\pm 0.005)$	$[CA] + 0.033 (\pm 0.018)$
+ b	$R^2 = 0.9995 \pm 0.0003^a$	$R^2 = 0.9988 \pm 0.0014^b$
R^2		
LOD (mg/L)	4.20 ^a	5.29 ^b
LOQ (mg/L)	14.0 ^a	17.6 ^b
RSD _r (%)	4.2 ^a	5.4 ^c
RSD _R (%)	10.3 ^a	5.7 ^b
Reagent	NaOH: 24	
consumption/ μ PAD (mg)	FBBB: 2.16	
Sample consumption (μ L)	60 ^a	
Reaction time (min)	10 (up to 30 min)	10 (up to 60 min)
μ PAD stability (days)	7 (vacuum and 4 °C)	

^a $n = 4$; ^b $n = 5$; ^c $n = 3$.

(RSD %), calibration curves from the same day for RSD_r (repeatability), and calibration curves from different days for RSD_R (reproducibility). In every case, results were below 11%.

The reagent consumption was calculated per μ PAD, and sample consumption corresponds to the four readings (one column of the μ PAD) used for one determination.

3.6. Storage stability of the developed device

The stability of the developed μ PAD was studied by storing devices in different conditions for a period of time. Then, calibration curves were set with the devices stored and compared to freshly prepared devices (fresh). Two devices were stored for two days; both of them were protected from light and at room temperature, one in the presence of air (air) and the other in vacuum (V). Another two devices were stored for seven days in the same conditions mentioned above. Then, another was stored protected from light, in vacuum but in the fridge (4 °C).

The calibration curves' slopes, determination sensitivity, were compared to an average of the freshly prepared devices (Fig. 4).

It was concluded that devices can be stored for two days in any of the tested conditions, as no significant differences were observed (overlapping of the 10% interval of the standard deviation). However, to store

them for a week, vacuum and refrigeration is needed (Fig. 4). Vacuum sealing effectively isolates the immobilized dry reagents from atmospheric oxygen and ambient humidity, preventing oxidative breakdown and paper drying. When combined with refrigeration to suppress thermolysis of the diazonium group, the device maintains its calibration slope within the $\pm 10\%$ tolerance window.

3.7. Sample analysis

Each of the tea and infusion samples analyzed underwent a different processing treatment, and the calibration curve with gallic acid standards (the most stable and common phenolic compound in food samples) was used. For the wine samples analyzed the calibration curve with catechin standards was used.

3.7.1. Sample blank

For sample analysis, a sample blank was performed to avoid the possible interference of the samples' own color. This concept had been previously reported (Mesquita et al., 2023) and consisted of incorporating in the device a column with disc units without the color reagent (FBBB, top layer) and only with 35% ethanol solution (Fig. S2). This would enable the calculation of the sample absorption. In the end, no sample blank subtraction was applied, as the samples did not present intrinsic absorption due to the high dilution factors (Table S1). In pigmented matrices like red wines, applying blank subtraction introduced severe negative bias, increasing relative error from -6% up to -64% .

3.7.2. Wine samples

The TPC of eighteen (#18) red wines and one white wine were analyzed (Table S3). When catechin was used as the standard to establish the calibration curve, none of the wine samples showed statistically significant differences ($p > 0.05$) between the UV-Vis method and μ PAD.

When establishing a linear relationship between the μ PAD and the spectrophotometer, the equation obtained for wine samples (where the values in brackets represent the 95% confidence interval), $[TPC]_{\mu PAD} = 0.981 (\pm 0.165) [TPC]_{UV-Vis} + 77.65 (\pm 275.264)$, proved that the developed method is not significantly different from the comparative method since both the slope and intercept were not statistically different from 1 and 0, respectively. This can also be seen after applying an EJCR test based on ordinary least squares (OLS) analysis of predicted concentrations against nominal concentrations, as the ideal point (*) is inside the red line ellipse shown in supplementary material Fig. S3.

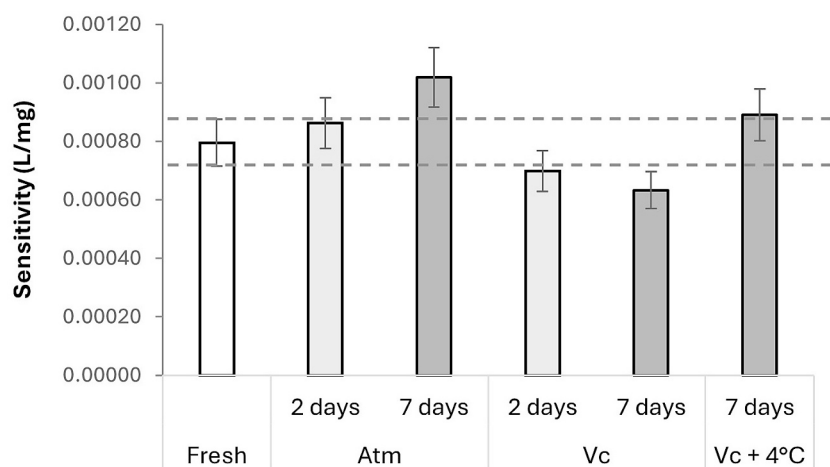


Fig. 4. Stability studies of the developed μ PADs under different atmospheric conditions and periods of time. Atm, in contact with air; Vc, in vacuum; Vc + 4 °C, in vacuum and refrigerated; error bars represent a 10% deviation, and the horizontal dash lines represent the deviation of an average calibration curve slope of the freshly prepared μ PAD.

Table 4

TPC of teas and infusions, expressed in mg GAE/L, analyzed by UV–Vis spectrophotometer and μ PAD. Results show the mean of three replicates and their standard deviation.* Statistically significant differences ($p > 0.05$) were found.

Teas and infusions	UV–Vis	μ PAD	Recovery (μ PAD)			
	mg GAE/ g sample		[mg/L]	Added (mg/L)	Found (mg/L)	%
T1	691 $\pm$	382.68	84.44	50	135.78	103
	122.84 $\pm$	$\pm$ 42.02		100	185.01	101
	483 $\pm$	331.56		50	178.16	80
T2	57.22 $\pm$	$\pm$ 48.23	138.23	100	225.05	87
	212 $\pm$	127.41		50	115.95	108
	57.43 $\pm$	$\pm$ 37.98		100	168.19	106
T3	109 $\pm$	60.24 $\pm$	61.74	50	216.59	70
	11.81	1.57		100	251.66	70
	56.1 $\pm$	43.99 $\pm$		50	172.22	77
I1*	9.43	2.90	133.62	100	210.54	77
	23.5 $\pm$	13.45 $\pm$		50	131.18	98
	5.34	4.21		100	176.72	95

3.7.3. Tea and infusion samples

The TPC of three tea samples and three infusions were also analyzed by the traditional spectrophotometric method and μ PAD, using gallic acid as a standard. Only an infusion sample (I₁) showed statistically significant differences when applying a paired *t*-test. Results are shown in the table below (Table 4).

Few samples were analyzed to perform an EJCR test; therefore, to further evaluate the accuracy, recovery percentages were calculated with the difference in concentrations between the initial samples (initial) and the spiked samples (found) with two different additions (added) of 50 mg/L and + 100 mg/L of gallic acid.

The recovery percentages were calculated following the formula %R = [TPCs]_{added} – [TPCs]_{initial} / [TPCs]_{added} (Table 4). The mean of each spike group measurement was around 90% recovery with a standard deviation of 12%. A paired-samples *t*-test was performed to see if the mean recovery percentage of each spike set was different from 100%, and statistical differences were not detected as the critical *t* value (2.57) was higher than the calculated (*t*_{cal+50mg/L}: 1.82; *t*_{cal+100mg/L}: 1.86).

These results for both sample types, indicate that the developed μ PADs could be successfully used to estimate the TPC using the FBFB method after deciding which phenolic compound is a better fit as a standard for the sample of study.

While comparison with other studies is difficult due to the lack of FBFB- μ PAD research, when comparing the performance of the proposed device with the platforms summarized in Table 1, its practical usefulness for beverage testing becomes evident. While advanced electrochemical platforms (BDDE, ePADs, PtNPs/3DNRGO, MXenes) and techniques (LC-MS/MS) achieve low detection limits, such sensitivity is unnecessary for real beverage matrices (teas, herbal infusions, and wines), where native phenolic levels typically range from 50 to 2000 mg/L. The linear working ranges achieved by our device (5–200 mg/L GAE and 25–125 mg/L CAE) and detection limits (4.2 and 5.3 mg/L, respectively) are perfectly aligned with these matrices following simple operational dilution (1:10 to 1:160), avoiding complex pre-concentration. Thus, the μ PAD offers a unique balance: it matches the portability and low cost of paper devices while introducing the structural selectivity of diazo-coupling, achieving a shelf life of 7 days under vacuum storage.

3.8. Analytical greens (AGREE) index

The transfer of the traditional spectrophotometry to μ PADs demonstrates that this last one is a more sustainable technique as AGREE scores improve. The AGREE scores for each technique are shown in the picture below (Fig. 5). When using UV–Vis, a score of 0.58 is obtained, while when using μ PAD, it increases to a 0.75 value. These scores demonstrate

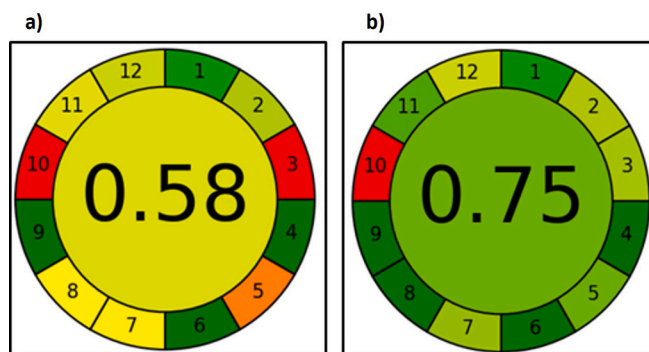


Fig. 5. AGREE scores of the traditional UV–Vis spectrophotometric method (a) and μ PADs (b).

that TPC determination, performed on a paper-based device and analyzed by digital image analysis, is more sustainable than the traditional spectrophotometry method.

The most relevant principles when it comes to achieving a more sustainable method are principle number three and number five. Principle No. 3 considers the feasibility of conducting analysis in situ, and the transportability of the μ PADs makes an on line analysis viable while the spectrophotometer is located in a laboratory outside the line (off line). In principle No. 5, the miniaturization of the method significantly reduces volumes. The less waste generated and the reduced analysis time also help in the improvement of the AGREE score. Otherwise, since employing bio-based reagents is not possible, principle No. 10 presents bad values in both methods.

4. Conclusions

In this research, a novel microfluidic paper-based device was developed for the determination of total phenolic content in different beverages by the Fast Blue BB method. μ PADs, aside from being portable and easy-to-fabricate devices, offer simple, fast, and low-cost analysis while using low volumes, being environmentally friendly, and being easy to dispose of. The limitations of these devices are primarily because they require simple manufacturing, but are somehow tedious when manually aligning the 24 units consisting of two layers.

The previously reported μ PADs for TPC determination use the Folin-Ciocalteu method instead of the FBFB, which only reacts with phenolic compounds, and therefore, the developed μ PAD takes an innovative approach.

Different standards have been chosen depending on the sample to be analyzed, and a range from 5 to 200 mg GAE/L or 25 to 125 mg CAE/L has been obtained with a limit of detection below 7 mg standard equivalent/L in both cases and a precision below 11% RSD. To evaluate the suitability of the device for transportation, stability studies were performed and showed a 7-day stability after assembling and storing it under vacuum conditions. After loading the sample, the device can be scanned within 10 min and up to half an hour if gallic acid is used as the standard, and up to an hour later if catechin is used. The accuracy studies carried out showed no significant differences ($p > 0.05$) between the traditional spectrophotometric method and the μ PAD.

Although the proposed μ PAD platform was successfully validated for complex beverage matrices (teas, herbal infusions, and wines), extending this method to other solid, semi-solid, or turbid food categories (e.g., fruit juices, plant extracts, or vegetable oils) would require an adapted matrix-specific validation protocol. Future implementations should include: (i) optimization of sample extraction or clarification to prevent physical pore obstruction in the paper substrate; (ii) proper dilutions and reference standard selection, evaluating dilution factors for each matrix to ensure analyte concentrations fall strictly within the linear dynamic range of the chosen reference standard while minimizing

background matrix absorption; (iii) individual sample blank evaluation using control devices without FBBB for the new matrix to determine whether intrinsic background color is mathematically negligible at the working dilution or if matrix-specific correction is required; and (iv) reference validation against standard spectrophotometric methods (UV–Vis) to confirm the absence of matrix-induced systematic biases.

CRedit authorship contribution statement

Maider Zugazua: Writing – original draft, Validation, Methodology, Investigation. **Ane Bordagaray:** Writing – review & editing, Supervision. **Miren Ostra:** Writing – review & editing, Supervision. **António O. S.S. Rangel:** Writing – review & editing, Resources, Funding acquisition. **Raquel B.R. Mesquita:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2026.151191>.

Data availability

Data will be made available on request.

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