Design of a microfluidic paper-based device for the quantification of phenolic compounds in wines

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Abstract

In this work, the design and development of a microfluidic paper-based device (μPAD) for the quantification of total phenolic compounds (TPC) in wines is described. The developed μPAD was based upon the vertical flow concept and the colour reaction used was the known Folin-Ciocalteu reaction using gallic acid as reference phenolic compound. After studying operational parameters, namely type of paper, reagents and sample volume, a dynamic range of 5-50 mg L^{-1} was obtained with a limit of detection of 1.2 mg L^{-1}. The described device proved to have good precision (relative standard deviation < 5%) and no significant interferences were observed from known compounds present in wines. Furthermore, the stability of the colour product and of the device itself were assessed; the μPAD was stable for 30 days (in the dark at room temperature) and it could be scanned up to 8 h after sample introduction. The developed μPAD pose as a simple method for TPC quantification and was successfully applied to several wine samples including sparkling and table wines with two different approaches: i) using gallic acid as reference compound with standard addition; and ii) using taniraisin with external calibration. The accuracy of the proposed μPAD method was assessed by comparison with the reference spectrophotometric method according to the International Organisation of Vine and Wine (OIV) recommendations.

Keywords: *cellulose paper, Folin-Ciocalteu, food samples, on-site analysis, phenolic compound.*

1. Introduction

Wine is one of the oldest alcoholic drinks consumed in the world being an economically important global industry for millennia. Wine is mainly composed of water and ethanol. However, other compounds such as glycerol, sugars, organic acids, minerals, volatile compounds and phenolic compounds (PC), among others, are present [1]. PCs are natural substances composed of hydroxyl groups and benzene rings and they have been reported to have anti-oxidant, anti-aging and anti-inflammatory properties [2]. Additionally, PCs are one of the most important groups of chemical compounds for the perceived quality of wines, contributing to sensory characteristics such as colour, flavour, bitterness and astringency, and also to the wine stability through oxidative processes [3]. The total polyphenol content (TPC) can be quite variable depending on grape variety and maturation and winemaking conditions, being higher in red (1-5 $g \cdot L^{-1}$) than in white wines (0.2-0.5 $g \cdot L^{-1}$) [4]. The relationship between the wine quality and its phenolic composition, together with authentication purposes, are two major challenges in the Enology field [5]. As a result, TPC quantification has become one common practice. Nowadays, the globally stablished methods for evaluation of TPC are based on the well-known Folin-Ciocalteu (F.C.) colorimetric assay [6] or by the absorbance measurement at 280 nm [7]. Both methods are widely accepted due to their simplicity and reproducibility. Nonetheless, they require specific laboratory instrumentation with its related costs as well as high volumes and long equilibrium times. In this sense, much effort has been made to develop simple, fast, portable, and cheap methods to determine TPC. In this context, microfluidic devices can be an efficient and interesting approach to meet that aim. They are made from different resources such as polymers, glasses and papers [8,9]. The latter one, also known as microfluidic paper-based analytical device (µPAD), have been gaining popularity from its first appearance in 2007 by Martinez A. *et al.* [10]. There are two main reasons for µPAD popularity: i) the paper nature, providing a cheap hydrophilic support enabling reactions

to occur by allowing the transport of aqueous solutions via capillary forces without the need of external propulsion forces [11,12]; and ii) the advances in the miniaturization concept, detection attained by image capturing [13].

Focusing on μPAD design, it must be composed of two regions: the hydrophilic area, and the hydrophobic area. The former is provided by the cellulosic material, where the reactions usually occur, and the second one has a fencing function to delimit the reaction zone [14]. Different approaches can be carried out to produce these barriers such as photolithography, inkjet methods and wax printing [15]. However, all of them have some drawbacks that must be considered in their production. For instance, wax technology requires an extra heating step and it is relatively expensive due to the printer cost [14] or inkjet printing usually uses toxic organic solvents [11]. To overcome these limitations, a low-cost and eco-friendly μPAD construction is presented in this work using simple stationery materials (punchers, plastic sleeves and a laminator), following a recent trend in the UCP research group [14,16–18].

Concerning μPAD application, these devices present several recognized characteristics that have led to their use on-field analysis in remote locations or in places with few resources [15] as a remarkable alternative. For instance: low cost, portability, ease of miniaturization, low requirements of sample and reagents, etc. being most of them in agreement with the Green Analytical Chemistry [19,20]. They have also been useful as sensors for a variety of applications highlighting food evaluation, clinical analysis, environmental safety, among others [21,22]. However, as far as we known, only few works have been reported in the literature combining μPAD and colorimetric detection in order to stablish TPC in food samples [11,23–27] and only two of them have evaluated stability [23,24], which is undoubtedly one of the most important parameters for on-site applications with no laboratory instrumentation. Furthermore, refrigeration was needed in Puangbanlang's work [23] and also vacuum at 4ºC for the of Calabria's group [24] work in order to maintain the initial μPAD performance.

Among that, additional materials like wax printers [23,25], lab-case, dark boss and smartphone holder [24] or treated cotton [27] were needed to perform the analysis. Consequently, the true potential of these μPADs is still somewhat limited, due to less practicality and the device longevity.

In this work, a novel and simple μPAD method has been designed for quantification of TPC carrying out the renowned F.C. reaction. The strategy was to use the approach recently developed at the UCP research group [14,16–18] based on vertical flow. For this purpose, two individual layers of paper loaded reagents were placed in a 24-fold plastic sleeve. After a heating process with a laminator, the μPAD was ready to use. The resulting analytical platform was properly optimized including not only the chemical part (reagents concentration and volumes), but also the physical aspect such as paper type, thickness, pore size, etc. Once the optimum conditions were stablished, the developed device was successfully evaluated as sensor using different wine samples. The accuracy was validated matching the results with recommended conventional spectrophotometric method.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and Milli-Q water, MQW (resistivity > 18 MΩ cm, Millipore, Bedford, MA, USA).

The Folin-Ciocalteu reagent, F.C. solution, was daily prepared by dilution from a commercial solution of F.C. (2 M respect to acid, Sigma-Aldrich) in MQW to a final concentration of 0.5 M (2 mL final volume).

Sodium carbonate stock solution was prepared weekly dissolving the appropriate amount of anhydrous Na_2CO_3 (Sigma-Aldrich) to obtain a final concentration of 15% in a final volume of 20 mL.

Stock solution of gallic acid (Sigma-Aldrich) 1000 mg L^{-1} was prepared dissolving 100 mg of the solid in 100 mL of MQW and stored at 4°C. Working standards in the range of 1-100 mg L^{-1} were daily prepared from the stock solution and diluted in MQW. For the standard addition approach, the same procedure was followed, the dilutions of the stock solutions were made with diluted samples (see Section 2.5 for further details).

The preparation of taniraisin (Martin Vialatte, France) stock solution (1000 mg L^{-1}) was prepared by weighing 10 mg of solid in 10 mL of MQW. For the preparation of working standards, used in the calibration curve method for the direct determination, proper dilutions of the stock solution in the range 5-50 mg L^{-1} were done with MQW.

In the assessment of potential matrix interferences: ethanol solution (13-20 %) was prepared by dilution from absolute ethanol (Sigma-Aldrich). For the rest, glycerol (1-7 g L^{-1}), sodium sulphite (25-300 mg L^{-1}), D-Glucose (2-20 g L^{-1}) and tartaric acid (3-5 g L^{-1}) (Sigma-Aldrich) were weighed to obtain individual solutions of 10 g L^{-1}, 500 mg L^{-1}, 20 g L^{-1} and 5 g L^{-1}, respectively. Then, proper dilutions from these stock solutions for the further studies were performed, if necessary.

2.2. Scheme of µPAD assembly

The µPAD construction consisted of 48 filter papers discs (two stacked discs per unit) arranged in 4 x 6 units distribution (columns x rows) as 24 hydrophilic units (see Fig. 1A). Information about the different types of paper used in this work can be found in the Table S1. The top layer consisted of 24 papers (Whatman® 541) loaded with 10 µL of 0.5 M F.C. reagent and placed in the oven for 10 min at 50 ºC. The bottom layer consisted of 24 papers discs of (Whatman® 3) loaded with 20 µL Na_2CO_3 15 % m/v and placed in the oven for 20 min at 50 ºC. Both paper discs were cut with a diameter of 9.5 mm (puncher 3/8" EK tools, Lindon, USA). Then, the µPAD was assembled stacking the two layers, unit by unit, in plastic pouches (75 x 110 x 0.125 mm, Q-Connect, Gent, Belgium), previously perforated with 4 mm holes (Knipex puncher, Wuppertal, Germany). Next, a heating process with a laminator (United Office, Cleveland, USA) was carried out and the plastic between the units was melted and combined in a single layer.

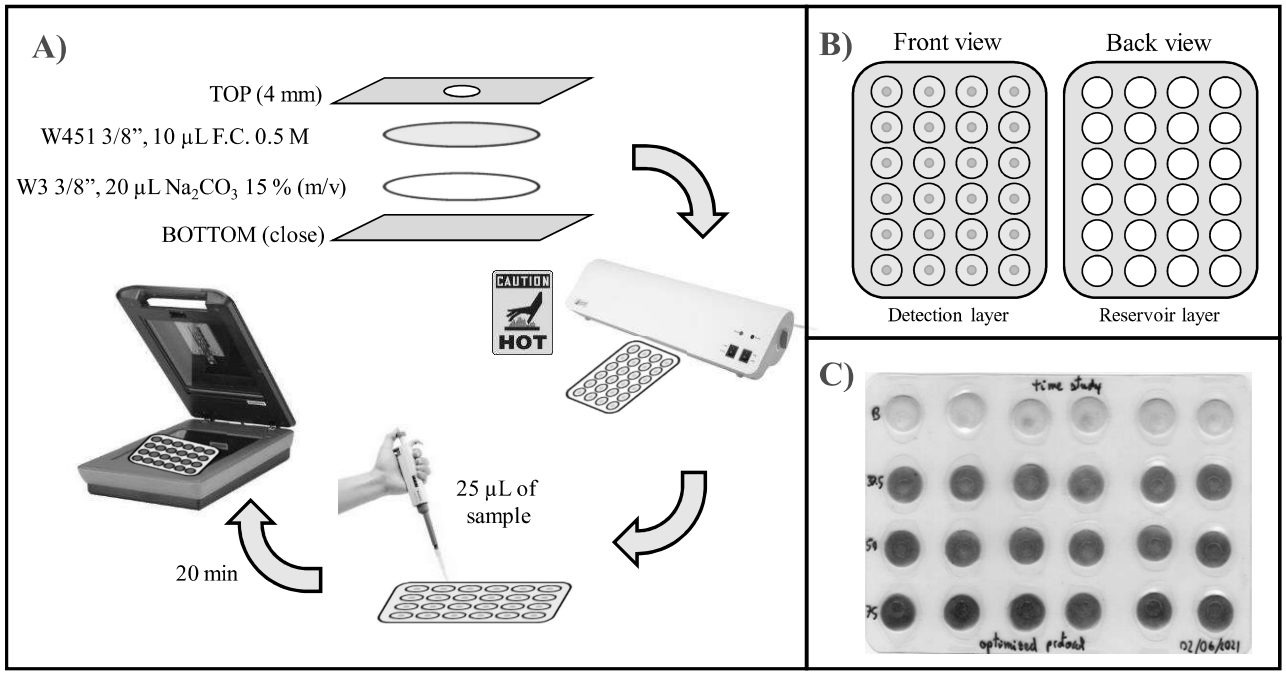

Figure 1 Schematic depiction of the µPAD assembly and the operating procedure (A); views from top and bottom (B); and an example of real µPAD scanning capture after loading standards and wait 20 min (C).

After this process, the μPAD card was finished and ready to use (see Fig. 1B). It should be noted that the space between the different papers acts as hydrophobic area surrounding the reaction/detection zone (physical barrier of approx. 1 cm) as well as avoiding cross-contamination between units,

2.3. TPC quantification

For the quantification of TPC, 25 μL of standard/sample was loaded onto the hole placed over the detection layer (top layer with F.C. reagent). While being absorb through the top paper layer with F.C., the PCs (as reducing substances) reacted forming a blue chromophore of phosphotungsticphosphomolybdenum complex (see Fig. 1C). The intensity of the blue colour is directly proportional to the TPC in the sample. The chromophore production, and consequent blue colour intensity is enhanced at alkali pH, so with sodium carbonate was placed onto the second layer for pH adjustment and to decomposed the F.C. excess.

After standard/sample loading, approximately 10 min were necessary to achieve the complete absorption of the total volume under atmospheric conditions. At that moment, the μPAD was left for another 10 minutes to increase the signal response without exceeding the total analysis time. After 20 minutes, the device top layer was scanned (Epson) and the resulting images processed using free software ImageJ. The images were converted into RBG plots and the intensity counts of circular segments (98 × 98 pixels) obtained using a red filter. The circular segment corresponds to a perfect fit of the reagent paper discs' area. It was stablished 6 replicates for each standard to ensure at least 4 measurements after removing the potential outliers (see Fig. 1C). The intensities values were converted to absorbance following the expression: $A = \log (I_0/I)$, where I is the standard/sample intensity and I_0 is the blank intensity, obtained by loading MQW. Then, calibration curves were plotted with the calculated absorbance for each standard and the F.C. concentration.

In the sample analysis, different approaches can be follow, namely a direct procedure and a standard addition procedure.

2.3.1 Direct determination with taniraisin as model phenolic compound

When taniraisin was used as reference compound, the wine samples were directly loaded into µPAD card, following the previously described procedure. The concentration of TPC in the sample was obtained by absorbance interpolation in the calibration curve established with the taniraisin standards.

2.3.2 Standard addition with gallic acid as model phenolic compound

However, being gallic acid the most used reference compound for TPC analysis, an approach using it was also developed. For the determination using gallic acid a standard addition procedure was established, and gallic standards were prepared in wine sample. Then, the standard addition standards were loaded in the µPAD following the describe determination procedure. The concentration of TPC in the sample was obtained establishing the linear regression of the obtained absorbance and calculating the interception corresponding to no gallic acid addition.

2.4. Reference procedures and in vitro determination

To assess the uPAD accuracy, results obtained with the developed device were compared to results obtained with the reference method from OIV (International Methods of Wine and Must Analysis) Folin-Ciocalteu Index (OIV-MA-AS2-10) [28].

Concisely, 0.2 mL of wine (previously diluted) or standard were mixed with 5 mL of water. Then, 1 mL of F.C. reagent was added, and the mixture was homogenised. Next, the pH was buffered at alkali medium with 4 mL of Na_2CO_3 (20 %, m/v) and the final volume was adjusted

to 20 mL with deionized water. After mixing, the solution was left at room temperature for 30 min and the absorbance was determined at 750 nm through a path length of 1 cm with a spectrophotometer (Helios Gamma UV-Vis Spectrophotometer, Thermo Scientific, Massachusetts, EUA). A blank solution was also prepared with deionized water in place of the wine as reference. The appropriate absorbance for wine samples should be around 0.3 units; otherwise, a proper dilution should be done.

2.5. Sample collection

Different wine samples (Table S2) were purchase, namely sparkling wines (SW1-SW4) and table wines (TW1-TW6), and stored at 4ºC until their use. All wine samples were diluted prior to be analysed (Table S2).

The diluted samples were directly loaded in the μPAD when taniraisin was used as model compound, in a direct determination approach. When using gallic acid as model compound, a standard addition approach was used, so gallic acid standards were prepared in the diluted samples, at least 5 standards with 5 mg L^{-1} intervals (without exceeding the linearity 5-50 mg L^{-1}).

3. Results and discussion

3.1. Preliminary studies

The F.C. assay is the most suitable method to quantify the TPC in wines samples. However, an optimization of reagents, with traditional spectrometric method, was done in order to obtain the best operational conditions. In so doing, the general procedure was followed [28] varying the F.C. and Na_2CO_3 concentrations (Table S3). The highest sensitivity was achieved using 0.75 M of F.C. reagent and 20 % (m/v) of Na_2CO_3 (conditions described in C7 in the Fig. S1). The sensitivity enhancement was set about *ca.* 20 % compared to the initial conditions (C0 in

the Fig. S1 extracted from Hao and collaborators) [25]. Higher values than 1 M of F.C. reagent were discarded since they produced white dispersions after 25 minutes as it has been also indicated elsewhere [29,30].

From these batchwise in vitro studies, 0.75 M of F.C. reagent and 20 % (m/v) of Na_2CO_3 (C7 in the Fig. S1) were selected as starting point for the microfluidic approach. Additionally, two observations were taken into account before initiating the μPAD experiments: i) a single-layer micro-PAD card was discarded since F.C. and Na_2CO_3 are incompatible in direct contact (see Section 2.3) and it would limit the standard/sample volume to 10 μL (it takes about 30 min absorption under atmospheric conditions); and ii) the reading process must be done on the F.C. layer, otherwise no linear relationship between signal and concentration was found (data not shown).

At first glance, several assessments were done in the microfluidic devices for the evaluation of their performance and the results are depicted in the Table S4. From group experience, when 3/8" of diameter disc is used, 10 μL of reagent solution perfectly fits the entire disc. The first assay was directly tested using the conditions previously optimized *in vitro* (C7) and the sensitivity was settled in $1.7 \cdot 10^{-3}$ L mg^{-1}. As it can be appreciated from the results, the lack of sodium carbonate (C2 and C3 conditions) led to a loss of sensitivity around 50% (C1 compared to C2). on the other hand, the addition of sample must be carried out on the F.C. reading layer (C1 compared to C3) to obtain the maximum μPAD performance. In this sense, the C1 conditions were fixed as basis for the optimization process.

3.2. Optimization of developed μPAD for TPC quantification

Several parameters that influence TPC quantification on the μPAD were studied using gallic acid standards. The layer's disposition was set having the F.C. reagent in the top layer and the carbonate solution in the second layer. The sample/standard was loaded through the

F.C. layer which was also the scanned layer. The studies were performed establishing calibration curves for each assessed parameter.

3.2.1. Study of the chemical parameters

First, the F.C. reagent concentrations were studied ranging from 0.05 M to 0.75 M (see Fig. 2A). Although no significant differences in sensitivity were observed between 0.1 - 0.5 M, the best precision and lowest interception were reached when 0.5 M was used, indicating a potentially lower limit of detection (LOD) [14]. Furthermore, it should be considered that, in alkaline environment, the F.C. reagent quickly decomposes, which makes essential to use an excess of the F.C. chemical to obtain a complete reaction without exceeding that can result in white precipitates interfering in the image acquisition. Therefore, 0.5 M was chosen for further studies.

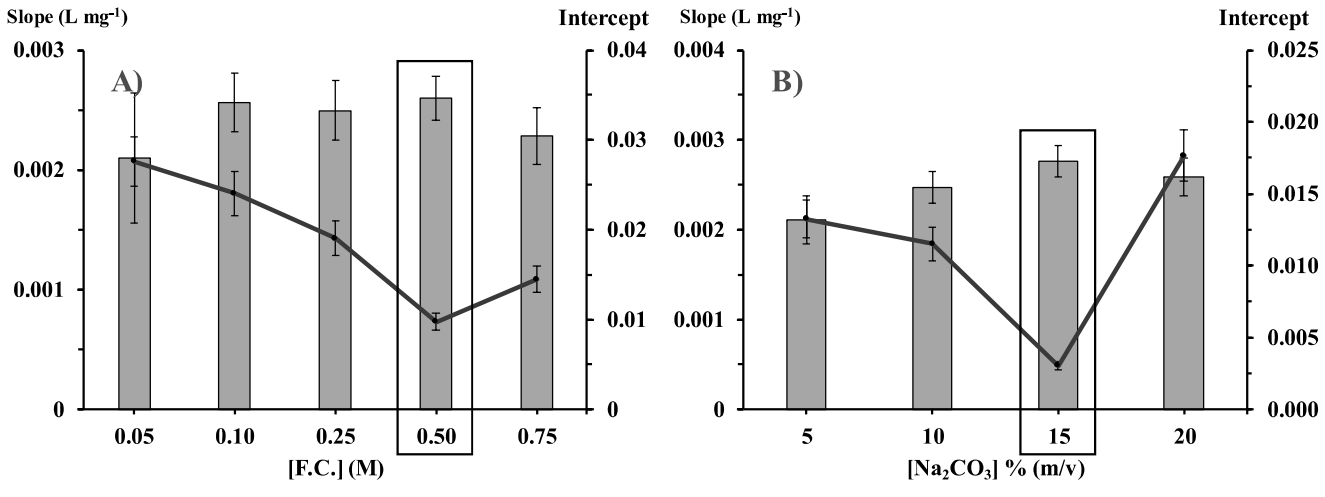

Figure 2 Influence of reagent concentrations on the sensitivity and intercept values. A) optimization of F.C, reagent on the top layer; B) optimization of Na_2CO_3 on the bottom layer. Experimental conditions: W1, 3/8" papers; calibration range 5-100 mg L^{-1} of Gallic acid; sample volume 10 µL; reading time 20 min.

Then, the influence of the sodium carbonate concentration was also studied (see Fig. 2B). In this case, 15 % (m/v) of Na_2CO_3 was chosen from the studied range of 5 to 20 % (m/v)

because it resulted in the best values of the calibration curve parameters (slope and intercept).

3.2.2. Study of the physical parameters – filter paper

The type of filter paper used for the reagent hydrophilic layer was evaluated, testing filter papers with different pore sizes and manufacturing treatments (see Table S1). The linear range for these tests was comprised between 5 and 100 mg L^{-1} and 10 µL of standard was used. Regarding pore size, three qualitative papers were studied namely, W1 (pore size 11 µm), W4 (pore size 20-25 µm) and W5 (pore size 2.5 µm) (see Fig. 3A). The results showed that the higher pore diameter, the better performance, probably due to the favoured fluidity between layers. For this reason, 20-25 µm of pore size was chosen.

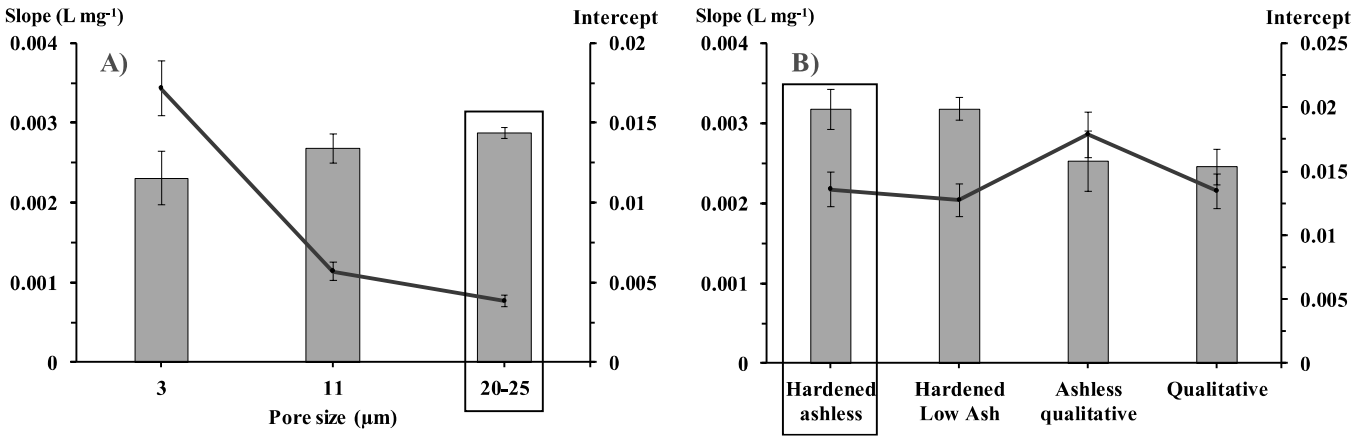

Figure 3 Influence of physical parameter – filter paper nature (top layer) on the sensitivity and interception values. A) diameter pore size; B) manufacturing paper pretreatment. Experimental conditions: W1 bottom layer, 3/8" papers; calibration range 5-100 mg L^{-1} of Gallic acid; sample volume 10 µL; reading time 20 min. Error bars represents the standard deviation (n = 6).

Then, four different treatments were tested using filter paper with similar pore size: W5, qualitative; W42, ashless; W50, hardened; W542 hardened ashless (see Fig. 3B). The highest sensitivity, calibration curve slope, was obtained for W50 and W542 with no statistic difference between the two treatments. Both these papers have hardened treatment and the difference is that W50 is low ash and W542 is ashless. Aiming to make an informed choice between low ash or ashless another experiment with lower linear range, from 0 to 50 mg L^{-1} was performed (data not shown). In this case, the slope value achieved with hardened ashless treatment (W542) was about 8% greater. In the end, based upon both paper testing Whatman® nº 541, a hardened ashless paper with pore diameter of 20-25 μm, was chosen.

3.2.3. Sample volume

Lastly, the sample volume was also tested, and several calibration curves were done with volumes from 10 to 25 μL (see Fig. S2). the increased of the sample volume resulted in the increased of sensitivity without a significant intercept variation. To ensure complete absorption of the sample, for sample volume of 20 and 25 μL a thicker filter paper was used in the second layer. The qualitative Whatman® 1 (W1, 0.18 mm thickness) was replace for qualitative Whatman® 3 (W3, 0.39 mm thickness). Nevertheless, for sample volume above 25 μL, it did not absorb in less than 30 min even with the W3. Therefore, 25 μL was selected as sample volume ensuring the highest sensitivity within a reasonably absorption time (10-15 min).

3.3. Features of the developed μPAD method

3.3.1. Stability studies

The robustness of the present method was assessed in terms of colour and storage stability (see Fig. 4).

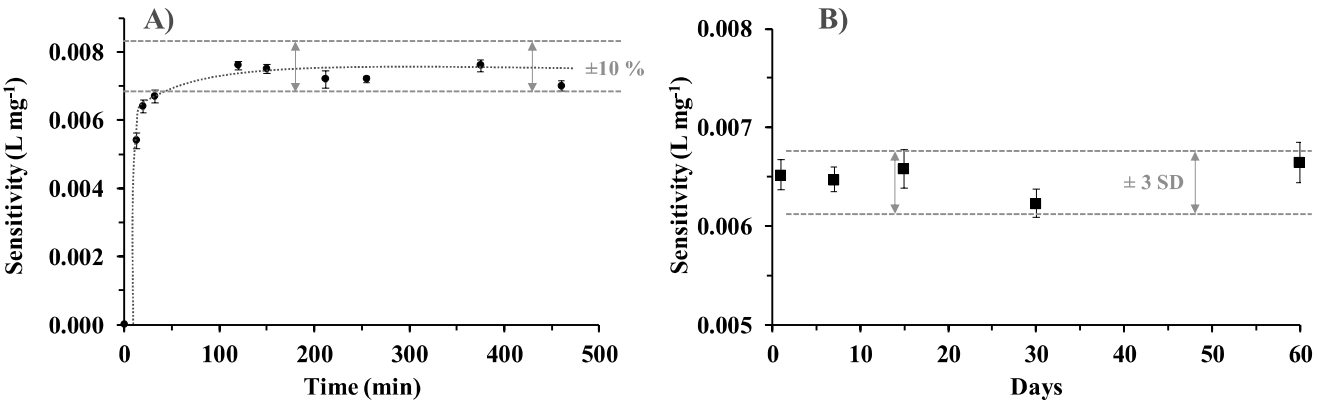

Figure 4 Colour stability in terms of sensitivity of the resulting blue chromophore over time (A) and stability of μ-PAD cards (once they are assembled) during their storage at room temperature (approx. 25ºC) in the darkness (B). Experimental conditions: W541 top, W3 bottom layer, 3/8" papers; calibration range 5-50 mg L^{-1} of Gallic acid; sample volume 25 μL; reading time 20 min. Error bars represents the standard deviation (n = 6).

The former is an important factor due to the possibility of on-site sampling and posterior μPAD reading in the laboratory, meanwhile the latter is a key aspect since it allows the storage and transport of the μPAD before its use. Both synergistically contribute to increase the possibility of sampling in remotes zones and/or low-resources. The stability of the coloured chromophore was investigated scanning the μPAD over the time after loading the standards. From Fig. 4A, it can be seen that the slope value (sensitivity) reached approx. 0.064 L mg^{-1} at 20 min, but the colour intensity was increasing to its maximum at approx. 0.075 L mg^{-1} until 2.5 h. This chromophore intensity enhancement is expected during the time in alkaline medium as it is also described in the Folin-Ciocalteu reaction [6]. At this point, not significant differences (±10%) were found in the following image captures over the time (up to 8 h). Concerning storage stability, μPAD were prepared as previously described in Experimental Section (see Figure 1A and 1B). Then, the devices were stored in the darkness at room temperature (20-25 ºC) without vacuum. After a certain period of time, the μPADs were used and the results compared with freshly prepared ones using in both cases the same set of

standards. As depicted in Fig. 4B, there is not statistical difference between the performance of the different assays. However, at 2 months the upper linear range was decreased from 50 to 25 mg L^{-1}. As consequence, 1 month was fixed as maximum time of μPAD card lifetime (in the mentioned conditions) without loss of its performance.

3.3.2. Analytical characteristics of the developed μPAD

The features of the μPAD method was assessed including the linear range, limit of detection (LOD), limit of quantification (LOQ), precision and stability (Table 1).

Table 1. Analytical performance of developed μ-PAD for the quantification of TPC referred to gallic acid (G.A.): gallic acid; LOD: limit of detection; LOQ: limit of quantification ([a] n=8; [b] n=6; [c] n=4; [d] storage in darkness at r.t. without vacuum).

Analytical parameter, units	Value
Linear range, mg L^{-1}	4.1-50
Calibration equation[a], A = slope ± SD [GA] mg L^{-1} + intercept SD	A = (66 ± 1.6)·10^{-4}·[G.A.] + (8 ± 3)·10^{-3}
LOD, mg L^{-1}	1.2
LOQ, mg L^{-1}	4.1
Precision intra-device, % RSD[b]	3.2 (for 25 mg L^{-1})
Precision inter-device, % RSD[a]	4.8 (for 25 mg L^{-1})
Sensitivity precision intra-day, % RSD[a]	2.2
Sensitivity precision inter-day, % RSD[c]	1.5
Colour stability after sample addition	Up to 8 h
Storage stability[d]	At least 1 month

A good linearity (correlation coefficient higher than 0.99) was achieved in the linear range comprised between 5-50.0 mg L^{-1} using gallic acid standards. The LOD and LOQ were calculated according to IUPAC recommendation [31], corresponding to 3 and 10, respectively, the standard deviation of the intercept. The reproducibility and precision were assessed by

calculation of the relative standard deviation (RSD) of the calibration curves slope and a single gallic acid standard, respectively.

3.3.3. Assessment of potential matrix interferences

The potential interference of relevant components of wine, namely ethanol, glycerol, sulphite (Na_2SO_3), D-glucose and tartaric acid, have been assessed [28]. The studied range of each compound as well as the maximum concentration without interfering in the signal response can be found in Table 2.

Table 2. Effect of concentration of the major compounds in wine samples on the accuracy using the developed µPAD method ([a]EtOH: 20 % (v/v); glycerol: 3 g L^{-1}; Na_2SO_3: 100 mg L^{-1}; D-glucose: 5 g L^{-1}; Tartaric acid: 3 g L^{-1}; [b]standard deviation, n=6).

Interference	Studied range	Max. conc. without interference	Deviation range (SD[b])
EtOH	13-20 % (v/v)	20% (v/v)	from -0.8 to 1.2 %
Glycerol	1-7 g L^{-1}	1 g L^{-1}	-6 %
Na_2SO_3	25-300 mg L^{-1}	200 mg L^{-1}	from -8.5 to -9.7 %
D-glucose	2-20 g L^{-1}	20 g L^{-1}	from -3.3 to 1.8 %
Tartaric acid	3-5 g L^{-1}	5 g L^{-1}	from 3.4 to 10 %
All	mixture[a]	-	-0.6 %

The study was carried out preparing a set of µPADs and loading a standard solution of 25 mg L^{-1} of gallic acid with and without the desired interferer. The average signal from 6 replicates for each test are compared and SD differences lower than 10 % are considered not significant. It should be highlighted that the amount of ethanol, D-glucose and tartaric acid is not problematic when the proposed method is used to quantify TPC. In any case, a mixture of

all of them was done in order to simulate a synthetic wine matrix and the results shown that a signal difference lower than 1 % was achieved, reinforcing the feasibility of the developed method.

3.4. Application to wine samples

The proposed analytical method was applied to the TPC quantification in ten wine samples including six tables wines and four sparkling wines (Table S2). The determination was assessed using the two analytical approaches: direct determination with taniraisin standards and standard addition approach with gallic acid standards prepared in the wine samples. The TPC values obtained with the developed μPAD were compared with the spectrophotometric reference method as recommended by the OIV organization [28] and relative deviation between the sets of results calculated (Table 3). Although gallic acid is the most extended and recognised phenolic compound used as standard for TPC quantification with F.C., when the wine samples were analysed using the calibration curve from these standards there was no correspondence to the OIV method. Then, a calibration curve with taniraisin standards was established and the calculated TPC values for the wine samples were in agreement with OIV method. On the other hand, to overcome the problem of the gallic acid standards, a standard addition approach was performed, and satisfactory results were obtained).

In the end, conventional calibration curve approach could be used with taniraisin standards, and standard addition approach could be used with gallic acid standards. A summary of the results with both approaches has been made (Table 3) and for the accuracy assessment, linear relationships were established between the results obtained with the OIV method and the μPAD method in both approaches (Fig. S3), standard addition with gallic acid ($\mu PAD_{.GA}$) and calibration curve with taniraisin ($\mu PAD_{.T}$). The established relationship proved that there was no difference between the two set of results as the slopes and intercepts were not statistically

different from 1 and 0, respectively: $[TPC]_{OIV} = 0.897(\pm 0.011)\cdot[TPC]_{\mu PAD.T} + 46(\pm 17)$, $R^2 = 0.9990$; and $[TPC]_{OIV} = 0.970(\pm 0.011)\cdot[TPC]_{\mu PAD.GA} + 13(\pm 8)$, $R^2 = 0.9990$; where the values in brackets correspond to the 95% confidence interval [32].

Table 3. Comparison of TPC values in different wine samples using the recommend procedure by the International Organisation of Vine and Wine (OIV) and the two different approaches with the developed µPAD method, the direct determination with taniraisin and the standard addition with gallic acid; the results are presented concentration average ± standard deviation; in vitro assay n= 3; µPAD assay n=6.

Sample type	Sample ID	Gallic acid (mg L^{-1})		RD (%)	Taniraisin (mg L^{-1})		RD (%)
		OIV in vitro assay	µPAD with standard addition		OIV in vitro assay	µPAD direct calibration curve	
Table wines	TW-1	223 ± 10	218 ± 14	-2.6%	412 ± 12	419 ± 5	+1.7%
	TW-2	375 ± 8	376 ± 12	+0.3%	741 ± 9	774 ± 21	+4.5%
	TW-3	367 ± 15	366 ± 22	+0.1%	726 ± 8	647 ± 13	-10.9%
	TW-4	552 ± 11	593 ± 31	+7.5%	1058 ± 7	954 ± 37	-9.8%
	TW-5	240 ± 4	247 ± 10	+3.0%	500 ± 8	463 ± 15	-7.4%
	TW-6	383 ± 9	371 ± 19	-3.4%	756 ± 10	775 ± 39	+2.5%
Sparkling wines	SW-1	232 ± 10	229 ± 16	-1.2%	335 ± 5	360 ± 13	+7.5%
	SW-2	346 ± 15	358 ± 20	+3.7%	758 ± 13	730 ± 48	-3.7%
	SW-3	1980 ± 22	1926 ± 52	-2.7%	4242 ± 15	3852 ± 103	-9.2%
	SW-4	198 ± 6	195 ± 7	-1.8%	424 ± 6	413 ± 20	-2.6%

4. Conclusions

In this work, a simple and robust μPAD method has been developed for TPC quantification in wine samples using the F.C. reaction. For this purpose, the microfluidic device has been optimized in terms of physico-chemical parameters and obtained limit of detection of 1.2 mg L^{-1}, was suitable for the target application to wine samples. The designed μPAD can be used with two analytical approaches, namely calibration curve method with taniraisin standards and standard addition method with gallic acid standards. Both approaches proved to be effective in TPC assessment in several wine samples, namely table and sparkling wines establishing a perfect correlation between values obtained with the spectrophotometric OIV method. The developed device presents a low cost, fast alternative ideally for on-site application since there is no need of expensive equipment nor specialized personnel. Furthermore, both reagent consumption and waste generation were decreased by up to 3 order of magnitude compared to traditional spectrophotometric F.C. assays, which supposes an environmental friendly and accurate alternative for this regard in agreement with Green Analytical Chemistry. The presented μPAD method was compared with other recent reported studies for TPC quantification using μPAD approaches and the F.C. reaction (see Table 4).

Table 4. Comparison between the developed μPAD procedure and similar methods reported in the literature for TPC quantification using the F.C. reaction (*ND: not documented; RSD: relative standard deviation; P and T[a]: pressure and temperature; LFA: later flow assay; PDMS: polydimethylsiloxane; PμZPs: paper microzone plates).

Sample matrix	Sample volume (μL)	Concentration range (mg L^{-1})	RSD (%)	LOD (mg L^{-1})	Storage time (P and T[a])	Notes	Ref.
Tea, wine and beer	2	10-250		2	ND	Hydrophobic barriers were done with pen ink; LFA; 3 detection zones	[11]
Tea, wine and fruit juices	10	85-1021	< 10	85	1 month (atmospheric pressure, 4ºC)	LFA ; 4 detection zones	[23]
Olive oil	20	up to 750	< 4	30 μg g^{-1}	1 month (vacuum, 4ºC)	PDMS microfluidic system with lab-case, dark bos and Smartphone folder; 4 detection zones	[24]
Tea	1	10-100	< 8	ND	ND	LFA; kinetic matching approach; 2 addition steps are needed; 8 or 12 detection zones	[25]
Wine	2	40-1700	< 16	40	ND	PμZPs; 2 addition steps are needed	[26]
Tea	ND	5-100	< 10	ND	ND	Cotton thread combining paper based device	[27]
Tap water	50	4.5-25	< 8	3.0-4.5	2 months (atmospheric pressure, 25ºC)	LFA; 4 detection zones	[33]
Wine	25	5-50	< 5	1.2	1 month (atmospheric pressure, 25ºC)	Vertical flow; 24 detection zones	This work

Concerning sample volume, our method presents the highest value compared to those recently reported [11,23–27,33], allowing to obtain the lowest LOD, which could be really interesting in samples with low TPC as well as when sample dilution is needed due to the high matrix complexity. The proposed μPAD method showed similar precision to other studies (RSD values below 8%) based on polydimethylsiloxane microfluidic system [24] and kinetic matching approach [25] or DNA-copper based method [33] and better to other μPAD approaches [11,23,26,27]. With regards to concentration range, it is true that some works stated higher linear range. For instance, Nuchtavorn and collaborators [11] and Vaher's group [26], although this is to be expected due to the low sample volume used in their approaches. On the other hand, when stability over the time (once the μPAD has been assembled) is compared, few works had studied this parameter [23,24,33] in spite of the robustness and durability importance. As it can be seen from the Table 4, only two μPAD cards (this work and Duc Tran et al. [33]) were able to maintain their performances for up to one/two months of storage from their assembly even at atmospheric pressure and without refrigeration, unlike the other reported stability studies [23,24]. Other strength of our μPAD method is the possibility of processing high volume of samples due to the number of discs for each device and the high number of detection zones. Furthermore, only one sample addition (by a simple dilution) is needed in contrast with other works, where two addition steps are performed [25,26] and sample pre-treatment is needed (e.g., sample extraction or clean-up processes).

Concluding, this work demonstrates that not expensive wax-printers neither toxic organic solvents are needed to build μPAD designs in a rather simple manner and thus this approach can be a smart option to perform field studies not only in wines, but also on other matrices (e.g. grape musts) for the evaluation of TPC, and it opens new areas for advanced screening approaches.

Acknowledgments

This study was supported by the project (RTI2018-095536-B-I00) funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe" by the European Union. This work was also supported by National Funds from FCT - Fundação para a Ciência e a Tecnologia (Portugal) through project UIDB/50016/2020. H. Martínez-Pérez-Cejuela thanks the Ministry of Science, Innovation and Universities for a PhD FPU contract (ref. FPU18/02179) and research stay grant (EST19/00516). This article is based upon work from the Sample Preparation Study Group and Network, supported by the Division of Analytical Chemistry of the European Chemical Society.

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All authors contributed to conceptualization of this manuscript.

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Notes

The authors declare no competing financial interest.

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