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# A novel high-throughput image based rapid Folin-Ciocalteau assay for assessment of reducing capacity

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**ABSTRACT**

The aim of the presented work was to develop a novel high-throughput rapid Folin-Ciocalteau assay for the quantification of reducing capacity based on image scanner (Image-F-C assay). The original rapid F-C assay using a 96-well plate was improved by adding a neutralization step that stabilizes the formed color, enabling image acquisition using a flatbed scanner. The effects of reaction volume, scanning orientation and model of flatbed scanner were assessed based on linearity, sensitivity and reproducibility of gallic acid standard curve. Euclidean distance calculated from R (Red), G (Green) and B (Blue) values was chosen, based on linearity and sensitivity, in order to quantify the reducing capacity. An in-house program using free ImageJ macro language was written to calculate automatically the RGB values of each well. The Image-F-C assay is linear within the range of 0-20 mg $L^{-1}$ of gallic acid ($R^2 \geq 0.9939$). The reducing capacity of herbal infusions using the new method was: tymus (251 ± 11), digestive (166 ± 9), sen (88 ± 7), chamomile (54 ± 3), green tea (615 ± 12), lemon black tea (143 ± 4), coffee (576 ± 20), and fruit juices *Biofrutas mediterraneo* (939 ± 35), pineapple juice (520 ± 27) and apple juice (226 ± 17) and an inter-day relative standard error < 8% was observed. Bland-Altman and correlation analyses showed that there were no significant differences between the reducing capacity values measured by the new Image-F-C and the original rapid F-C assay.

**Keywords**: Rapid Folin-Ciocalteau assay, reducing capacity, image analysis, image scanner

## 1. Introduction

Oxidative stress, defined as the unbalance between reactive oxygen and nitrogen species (ROS/RNS) production and the antioxidant defense, has been proposed to play a key role in different pathophysiological conditions[1, 2]. In this context, the natural antioxidants contained in foods, fruits, beverages, spices, and supplements have received much attention for the prevention or treatment of diseases [3] and therefore antioxidant capacity is widely assessed in food science and nutrition research [4-11].

Chemical-based methods are useful for screening, since they are low cost, high-throughput and yield an index value that allows comparing and ordering different products [7, 8]. Folin-Ciocalteu (F-C) assay is a widespread method in food science and nutrition research and industry owing to its simplicity -to date this assay has 1263 cites on Scopus database-. Although this assay has been used for many years to measure phenolic content, the reaction mechanism is an oxidation/reduction reaction not specific to phenolic compounds [12]. In fact, the F-C assay measures the ability of compounds to reduce the phosphomolybdic/phosphotungstic acid reagent to blue complexes in alkaline medium [12, 13]. However, the original F-C assay is time-consuming (120 min) and its implementation is difficult for routine analysis. Thus, different modifications, such as F-C reagent concentration, alkalinity and temperature of the medium, have been used to reduce the time necessary to attain the maximum color [14, 15]. Recently, a microtiter and rapid F-C assay for routine/screening -reaction time = 3 min- of the reducing capacity has been developed and validated [13]. The applicability of this rapid F-C assay requires an expensive microplate reader, which is not always available in all analytical laboratories. Therefore, the development of a low-cost and rapid version of the F-C assay is very desirable, in order to make it accessible to all researchers in the field.

Digital image-based colorimetry -using a scanner, a digital camera or a mobile phone- has been successfully used as a low-cost alternative to microplate reader in analytical quantification of several compounds [16-21]. Among these, a digital scanner image based Biuret method has been suggested for the quantification of total protein [16]. In addition, glucose, creatinine, triglycerides, total cholesterol and total protein in blood samples measured by image colorimetry showed results similar to those obtained with a photometer [17]. Moreover, a scanner-image based miniaturized immunoassay, widely used in clinical and research laboratories, has been developed as an alternative to conventional procedures [18]. Similarly, a microtiter smartphone-image based colorimetric assay was developed to quantify human C-reactive proteins, horseradish peroxidase and total protein [20]. Recently, a high sensitivity and reproducible method based on digital images was successfully employed to high-throughput quantification of nitrogen dioxide [19].

The aim of the present study was to develop and validate a novel low-cost, high-throughput and image based rapid F-C assay for the assessment of reducing capacity using a flatbed scanner and 96-well plate. In order to make use of the original rapid F-C assay [13], it is necessary to guaranty the stabilization of the formed color longer, since the image acquisition with the scanner is slower than the microplate reader used in the original method [13]. Therefore, the original rapid F-C assay was modified to include a neutralization step to stabilize the formed color. To establish the optimal conditions for the image scanning step, we have evaluated the influence of: 1) reaction volume, 2) scanning orientation and 3) scanner model on linearity and sensitivity. RGB color parameters were evaluated in order to establish which is linearly related to standard gallic acid concentration. The identified color parameter (Euclidean distance) was used

therefore in the new image F-C assay to quantify the reducing capacity of real samples and results were validated against the original rapid F-C assay.

## 2. Experimental

### *2.1. Reagents and solutions*

All chemicals used were of analytical-reagent grade with no further purification. Gallic acid and sodium hydroxide were purchased from Sigma-Aldrich (Madrid, Spain). Folin-Ciocalteau (F-C) reagent, hydrochloric acid and absolute ethanol were acquired from Merck (Barcelona, Spain). Milli Q ultrapure grade water (<18.2mΩ) was used for the preparation of solutions.

The stock solution of gallic acid (500 mg $L^{-1}$) was prepared in ethanol solution and stock solution of sodium hydroxide (1.0 mol $L^{-1}$) was prepared in water. The working solutions of gallic acid, sodium hydroxide (0.35 mol $L^{-1}$) and F-C reagent (1:20 v/v) were prepared from stock solutions by rigorous dilution in water. In the case of gallic acid, working solutions were prepared by serial dilution. Sodium hydroxide solutions were titrated with acid standard solution (Tritisol, Merck).

### *2.2. Samples*

Total reducing capacity of several commercial foods was evaluated by Image-F-C assay. The following samples were assayed: a) Herbals: tymus, digestive, sen and chamomile purchased from local market (Hacendado, Spain); b) Teas: green tea (Tea Ahlan, Spain) and black tea with lemon from the lab hot drinks machine; c) powder coffee (*Marcilla Mezcla*, Spain) and d) Fruit juices: *Biofrutas Mediterraneo* (Pascual, Spain), pineapple juice (Zumosol, Spain) and apple juice (Hacendado, Spain). Herbals and green tea infusions and coffee were prepared with 200 mL of deionized water brewed for 5 min at 85 °C in a thermostatic bath (BA Bunsen, Spain).The samples were centrifuged at 10000xg for 5 min (4 °C) and aliquots of supernatant were collected and stored at -20 °C until analysis.

*2.3. Modification of the rapid F-C assay*

The original rapid microtiter assay for the assessment of F-C reducing capacity [13] was modified by the inclusion of an additional neutralization step in order to improve the color stability and allow the image acquisition using a flatted desktop scanner image, as described below. Fifty microliters of gallic acid standard solution (0, 5, 10, 15, 20 and 25 mg $L^{-1}$) and 50 µL of F-C reagent (1:20 v/v) were placed in each well of a transparent 96-well plate (Nessler, Madrid, Spain). After 2 min at room temperature, 100 µL of NaOH (0.35 mol $L^{-1}$) was added. After 3 additional minutes, 10 µL of hydrochloric acid (1.6 mol $L^{-1}$) was added to neutralize the excess NaOH. The absorbance was read at 760nm every 2 min for 30 min at room temperature in a Synergy HT Multi-Mode microplate reader (Biotek, Rochester, VT, USA). The slopes of standard curves at different reaction times were calculated by linear regression analysis.

*2.4. Image-F-C assay*

*2.4.1. Image acquisition and analysis*

Immediately after the reaction was completed, the 96-well plate was placed on a HP PSC 1510 desktop scanner (Hewlett-Packard Development Company, USA) configured as follow: resolution = 1200 dpi, color depth = 32 bits per point and format file = TIFF compressed format. A specific white cover was designed and printed in a MakerBot 3D printer (NY, USA) to cover the 96-well plate in order to avoid external light interference and to improve the light reflection [16]. The region of the 96-well plate, containing reaction solutions, was selected and image was acquired and stored for analysis.

In the acquired image, quantification was performed as follows: 1) from the center of each well with a circular area (diameter = 0.15, 171 pixels), the mean of Red (R), Green (G) and Blue (B)

values were obtained with the ImageJ (http://imagej.nih.gov/ij/) using the RGB Measure plugin. A specific macro was implemented in ImageJ macro language for the automatic calculation of RGB values in each well and 2) the linear relationship between the concentrations of gallic acid and effective intensity for R, G and B (Eq. 1-3) [16, 22] or Euclidean distance (ED) (Eq. 4) [23] were evaluated by regression analysis.

$$IR = log\left(\frac{R_b}{R_S}\right) \quad (1)$$

$$IG = log\left(\frac{G_b}{G_S}\right) \quad (2)$$

$$IB = log\left(\frac{B_b}{B_S}\right) \quad (3)$$

$$ED = \sqrt{(R_S - R_b)^2 + (G_S - G_b)^2 + (B_S - B_b)^2} \quad (4)$$

Where:

$R_s$, $G_s$, $B_s$ = Mean of R, G and B calculated from wells containing sample or gallic acid, respectively.

IR, IG, IB = Mean of effective intensity for R, G and B, respectively.

*2.4.2. Effect of reaction volume*

In a microtiter assay the reaction volume regulates the optical length and consequently the absorption. Therefore, it might also influence the light reflection and the linearity of scanner-based assays. To test the effect of reaction volume, 315 µL was used to construct the standard gallic acid curves and compared to the volume of 210 µL. For the experiment with 315 µL, 75 µL of gallic acid standard solution (0, 4, 8, 12, 16 and 20 mg $L^{-1}$) and 75 µL F-C reagent (1:20

v/v) were mixed; the reaction was catalyzed by addition of 150 µL of NaOH (0.35 mol $L^{-1}$) and 15 µL of hydrochloric acid (1.6 mol $L^{-1}$) was used to neutralize the reaction. Thereafter, the image was acquired with the scanner as previously described and immediately after, the absorbance was read at 760 nm in a Synergy HT Multi-Mode microplate reader. The slopes and determinant coefficients of standard curves for the digital image and microplate reader were calculated by linear regression analysis from IR, IG, IB and ED and gallic acid concentration.

*2.4.3. Effect of scanning orientation*

Gallic acid solution at different concentrations (0, 4, 8, 12, 16 and 20 mg $L^{-1}$) was put into 96-well plate by triplicate with a layout in rows. The reaction was performed with the modified rapid F-C assay, as described above, using the total volume of 315 µL. Thereafter, the plate was scanned in two different positions, one that allow to acquire the image with scanner light crossing the columns of the plate (column-orientation) and the other with the light crossing the rows of the plate (row-orientation). The effect of scanning orientation on linearity and precision of measurements were evaluated through the determinant coefficients and standard deviation of standard curves, which was constructed using ED and gallic acid concentration.

*2.4.4. Effect of the desktop scanner model*

In addition to the used scanner model mentioned above, the gallic acid standard curve in a 96-well plate was also obtained using a HP 2400 scanner. The slopes and determinant coefficients of standard curves for digital images were calculated by linear regression analysis of ED and gallic acid concentration. In addition, the precision of measurements were evaluated in terms of the standard deviation of each gallic acid concentration.

*2.5. Linearity and sensibility of the Image-F-C assay*

Gallic acid solution at different concentrations (0, 4, 8, 12, 16 and 20 mg $L^{-1}$) in triplicate were prepared by serial dilution from stock solution (500 mg $L^{-1}$) and used to evaluate the linearity of the Image-F-C assay. The slopes and determinant coefficients of standard curves at three different days -to evaluate the inter-day reproducibility- were calculated by linear regression analysis of ED *vs* gallic acid concentration. The sensitivity of the assay was evaluated by limits of detection (LOD) and quantitation (LOQ), which were calculated as follows: LOD = 3.3 σ/S and LOQ = 10 σ/S where σ is the standard deviation for ED of the blank and S is the slope of the standard curve [24, 25].

*2.6. Validation of the Image-F-C assay*

Image-F-C assay was applied to assess the reducing capacity of several commercial samples: herbals infusion, green and black tea infusions, coffee, and fruit juices. All liquid samples were diluted (from 1:10 to 1:100 v/v) with water just before measurement. The image-F-C assay was validated using the reducing capacity of the samples measured in a microplate reader by the correlation and Bland-Altman analyses [6].

## 3. Results and discussion

### *3.1. Modified microtiter and rapid F-C assay*

In the original F-C assay, the carbonate buffer is used to provide an alkaline pH that favors the reduction reaction rate of $Mo^{6+}$ to $Mo^{5+}$(blue complex) by phenolic or reducing compounds completing the reaction after 120 min [12], which makes it difficult to use as a routine analytical procedure. Recently, a rapid F-C reducing capacity assay performed in a 96-well plate by the use of sodium hydroxide instead of sodium carbonate [13]. The reaction was completed after 3 min with a maximal slope of gallic acid standard curve (sensitivity) when the alkalization was performed with sodium hydroxide (0.175 mol $L^{-1}$) and F-C reagent diluted at 1:20 v/v [13]. Despite the fact that the blue complex develops more quickly in a sodium hydroxide solution, it also leads to a rapid destruction of the colored complex [13], which reduces the sensitivity of the assay [26]. We demonstrate that this problem can be solved by a simple neutralization step with hydrochloric acid when the absorbance reaches its maximum value. The concentration of hydrochloric acid was calculated on the basis of the excess of sodium hydroxide, which was estimated to be 0.075 mol $L^{-1}$ [13]. The neutralization step reduces significantly the changes in the slope of gallic acid standard curve with reaction time (Figure 1). The percentage of reduction of the slope was 1.7-2.3 times lower in the neutralized samples compared to non-neutralized samples (water) between 4 to 6 min after the reaction was completed (Figure 1). This improvement of stability of the slope of the standard curve can be relevant for the reproducibility and sensitivity of the image F-C assay, since the scanning acquisition time is slower than the microplate reader. We have observed that under the conditions previously described, at least 4 min are required for the scanning period of the 96-well plate, which includes: preparing scanning, selection of scanning region and scanning process, while a microplate reader in normal

mode requires only 57 s. Therefore, the inclusion of the proposed neutralization step to the rapid F-C assay is well justified.

### *3.2. Novel high-throughput rapid digital image-based Folin-Ciocalteau assay: Image-F-C assay*

#### *3.2.1. Image analysis*

The first step to develop the Image F-C assay was to identify and calculate an empirical and simple image parameter from the scanned image, which should be linearly related to the gallic acid concentration (standard) to guarantee the accuracy of the assessment of reducing capacity in real samples. Four digital image-based parameters were calculated, namely effective intensity for R, G and B channels (IR, IG, IB) and ED by an ImageJ macro, implemented with a specific algorithm (Figure 2). IR, IG and IB showed linear relationship with gallic acid concentration ($R^2$= 0.97, 0.98 and 0.98, respectively). However, better linear relationship were found between IR and metal concentration ($R^2$ = 0.9952) [22], IG and protein concentration ($R^2$ = 0.997) [16], and IG and creatinine concentration ($R^2$ = 0.935) and IB and total cholesterol ($R^2$ = 0.992) [17]. In addition, the slope of the standard curves for Image-F-C assay reflected that IR was approximately twice more sensitive than IG and IB, similar to previous report quantifying chromium and iron using digital image-based colorimetry [22]. On the other hand, Euclidean Distance, a parameter that integrates R, G and B values in a unique parameter, exhibited a higher linear relationship with gallic acid concentration ($R^2 \geq 0.993$). In addition, the sensitivity (slope value) for Euclidean distance was approximately 400 times higher when compared to the slope obtained with IR. In fact, Euclidean distance has been successfully used to find the closest match to the given image for quantification of tetracycline in milk by iPhone-based digital image

colorimeter [27]. Therefore, the Euclidean distance is the parameter of choice to quantify Image-F-C assay.

*3.2.2. Effect of reaction volume*

Considering the optical system, a flatbed scanner is an ideal physical instrument for recording the scattered light reflected by plane solid objects in the visible wavelength range [28]. In addition, the scanner configuration is suitable for colorimetric assays using a 96-well plate, since it measures the transmittance. The incident light passes through the sample, and is reflected by the white surface of the cover to go downwards through the sample again and finally reaches the detector [16]. The absorbance can be estimated from the acquired image as the effective intensity for R, G and B [16, 22], which has a linear relationship with the concentration of analytes, i.e., it obey the Lambert-Beer law [16, 19]. However, data obtained from digital images might be influenced by the type of analytes and the total volume of the sample in the well. For example, using images acquired with a flatbed scanner under the same conditions and the same sample volume (250 µL) it has been demonstrated that standard curves for creatinine showed a $R^2$ = 0.935 [17]. However, the linearity was improved for protein quantification ($R^2$ = 0.997) and nitrogen dioxide ($R^2$ = 0.996) for 300 µL of the sample. To the best knowledge of the authors, there are no previous reports on the effect of volume on linearity using colorimetry and digital scanning. Our data showed that the increase of reaction volume from 210 µL to 315 µL for Image-F-C assay enhanced the linearity of gallic acid standard curve, which was verified by data collected using a microplate reader (Figure 3A and 3B). A plausible explanation could be related to the increase of brightness intensity, which reduces the background color and improve the quality of the acquired image. In fact, the brightness intensity for blank (0 mg $L^{-1}$ of gallic acid) for 315 µL of the sample was 1.18 ± 0.05 times more than that of 210 µL of volume (Figure 3A

and 3B). Similar changes in the brightness intensity were shown with different water volume (Figure 3C), which might be associated with the retention of incident light of the scanner due to a refraction phenomenon produced by the aqueous solution. On the other hand, the optical pathlength in a microplate is not fixed and it is directly related to the sample volume [5], which explains the increases of the slope of the standard curve using a 315 µL volume because the slope is directly related to the optical pathlength and the chemical nature of chromophore [5].

*3.2.3. Effect of scanning orientation*

The influence of scanning orientation on linearity and linear range in colorimetric assays has not been explored previously. Using gallic acid standard curve, we demonstrate that scanning orientation affects both linearity and the linear range (Figure 4). Thus, a linear range between 0 and 20 mg $L^{-1}$ was demonstrated for the scanning orientation A ($R^2$ = 0.9932), whereas the B orientation reduced the linear range to 0-12 mg $L^{-1}$ ($R^2$ = 0.9976). A power model showed a good fit to the entire gallic acid concentration for the B scanning orientation ($R^2$ = 0.9942). Similar effects of scanning orientation were observed for the HP LaserJet scanner model (data not showed). Therefore, the scanning orientation effect is independent of the image acquisition system because HP PSC 1510 scanner has a contact image sensor (CIS) technology and the HP LaserJet model has a charge coupled device (CCD) [28]. The possible explanation for this phenomenon may be related to the structure and the distribution of the light source of the scanner.

*3.2.4. Effect of flatbed desktop scanner model*

Flatbed scanners have different designs of their scanning elements: CIS (contact image sensor), LIDE (LED indirect exposure) or CCD (charge coupled device). CIS scanners contain no optics,

and the light source consists of three groups of RGB light-emitting diodes (LEDs) –mounted on the scanning carriage- and the light reflected by object reach to the photodetector, which is located in close proximity to the scanner glass. LIDE scanners have a powerful tricolor RGB LEDs as light source, which are directed to the object via quartz fibers ensuring uniform exposure over the entire width of the scanning window. Scanners with CCD configuration have lens and mirrors or prisms that project the light flux from the object being scanned on to the photodetector system [28].The influence of scanning elements on digital image colorimetry, has not been explored. Using two scanner models, a HP LaserJet 2400 scanner with a CCD configuration and HP PSC 1510 scanner with a CIS technology, we determined the linearity and sensibility of gallic acid standard curve. The linearity of the standard curve was comparable for two scanner models, with $R^2$ from 0.9947 to 0.9972 for the CCD scanner (Figure 5) and $R^2$ from 0.9943 to 0.9954 for the CIS scanner (Figure 3 and 6). Consequently, those scanner models guarantee a linear relationship between Euclidian distance and gallic acid or reducing compounds concentration for the Image-F-C assay despite the different sensitivity. Interestingly, as opposed to CIS scanners (data not shown), the sensitivity of gallic acid standard curve in the CCD scanner was influenced by the position of the 96-well plate during the scanning (Figure 5). A higher slope value was found for CCD scanner when the plate was put at the center of scanning window (7.5158), compared with a slope of (4.0189) when the plate was placed at the border (Figure 5A and 5B). However, the variability of RGB values for the blank well was significantly higher when placed at the center position (SD = 16.8 ± 1.2) than at border position (SD = 12.2 ± 2.4). This seems to be related to the fact that the optical system in CCD scanners lead to image distortions caused by skew between elements of the optical system leading to non-uniform reading of information along each row [28], which changes with position. A plausible

explanation for the difference in the Image-F-C assay with the plate position in a CCD might be related with enhancement of brightness intensity (1.15 ± 0.04 times more for the center position), which reduces the background color in all the wells of the plate.

*3.2.5. Analytical procedure of Image-F-C assay*

A novel high-throughput and rapid digital image-based Folin-Ciocalteau assay (Image-F-C assay) was performed on a desktop scanner and 96-well plate (Figure 6). Seventy-five microliter of gallic acid standard solutions or samples and 75 µL of FCR (1:20 v/v) were placed in each well and rested for 2 min. Then, 150 µL of NaOH (0.35 mol $L^{-1}$) was added. After 3 min, 15 µL of hydrochloric acid (1.6 mol $L^{-1}$) was added and mixed. Then the image was acquired with a scanner as described in section 2.4.1. To evaluate the intrinsic absorptions of the samples, 75 µL of 0.4 mol $L^{-1}$of acid solution was added instead of the F-C reagent. The reagent blank was evaluated by the addition of 75 µL of water instead of standard compound or samples. All experiments were performed in triplicate at room temperature (25 ±1 °C).

*3.3. Linearity and sensibility of the Image-F-C assay*

The linearity of the novel Image-F-C assay was confirmed for the range of gallic acid concentration between 0 and 20 mg $L^{-1}$ in three independent experiments carried out at different days. The found values of $R^2$ were: day 1 = 0.9939, day 2 = 0.9952 and day 3 = 0.9943. Figure 7 shows a representative gallic acid standard curve. The slope of the standard curve was reproducible with inter-day RSD = 2.3 %, which was comparable to the value obtained by original rapid microplate F-C assay [13]. The LOD and LOQ for Image-F-C assay were 0.5 and 1.6 mg $L^{-1}$ of gallic acid, respectively. When the rapid modified F-C assay was performed on microplate reader, we obtained the following values: LOD = 0.4 mg $L^{-1}$ of gallic acid and LOQ

= 1.1 mg $L^{-1}$ of gallic acid. Similar LOD = 0.25 mg $L^{-1}$of gallic acid has been found by the original rapid microplate F-C assay [13].

*3.4. Reducing capacity of real samples by Image-F-C assay*

The reducing capacity of real samples was estimated by interpolation of Euclidean distance for Image-F-C assay using the following standard linear equation: ED = (5.7994 ± 0.1323) x C ($R^2 \geq$ 0.9939), where ED is the Euclidean distance and C is the concentration of gallic acid (mg $L^{-1}$). The values between brackets are the standard deviation of the parameters corresponding to three calibration curves performed on different days. For comparison purposes, we used a linear relationship between reducing capacity measured by modified rapid F-C assay using digital scanner image and a microplate reader obtaining an intercept = 1.463 ± 8.213, a slope = 0.9990 ± 0.01824, r = 0.9973 and $p < 0.0001$ (Figure 8B). The Bland-Altman analysis showed that the difference measured between reducing capacity values, being all samples within the 95% limit of agreement (Figure 8C). Therefore, there is no evidence for systematic differences between the two sets of results obtained by the Image-F-C assay and modified rapid microplate F-C assay. However, when an unpaired t-test was performed on the data obtained for all samples, two samples showed different reducing capacity: herbal infusion Sen (S) and Chamomile (C) (Table 1). Interestingly, the reducing capacity of above samples was lower when compared to the other samples. They required a lower dilution factor (1:10), which affects the color of the sample and contributes to the RGB values (Figure 8A). Hence, Image-F-C assay can be successfully applied to assess the reducing capacity of samples with medium and high values that required high values of dilution factor or in samples without color. The RSD found for both assays were comparable (Table 1). Image-F-C assay could also be used to measure the total phenolic content after a SPE cleanup procedure [2].

## 4. Conclusions

A novel low-cost image based high-throughput rapid Folin-Ciocalteau assay (Image-F-C assay) was developed to assess reducing capacity using a desktop scanner. The Image-F-C assay uses a modified rapid F-C assay that includes a neutralization step to improve the stability of the color. The Image-F-C assay has been validated to measure the reducing capacity of real samples. This proposed inexpensive method does not require a microplate reader, and is suitable for use in routine screening of reducing capacity in food science and nutritional research. It is worth mentioning that the image processing, analysis and evaluation can be ported for implementation on digital images colorimetry acquired by other means different from scanners.

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## Figure Captions

**Figure 1** Influence of neutralization step with hydrochloric acid (1.6 mol $L^{-1}$) on slope of gallic acid standard curves at different time of the reaction in the rapid F-C assay. Standard curves were prepared with 0, 5, 10, 15, 20 and 25 mg $L^{-1}$ of gallic acid and F-C reagent 1:20 v/v ($R^2$ ≥0.99; n = 3). Water was used as blank to evaluate the effect of neutralization step. Experimental data was fitted to power model by non-linear regression analysis.

**Figure 2** The algorithm for automatic calculation of RGB values from transparent 96-well plate using the ImageJ macro language. A circular region (171 pixels) was selected using MakeOval tool of ImageJ to measure the mean of RGB values.

**Figure 3** The influence of reaction volume on linearity of the gallic acid standard curve in the Image-F-C assay. Linear regression analysis was performed to fit experimental data and calculation of determination coefficient ($R^2$). The original image was acquired in a HP PSC 1510 scanner and different gallic acid solutions, prepared by serial dilution, put into wells of a 96-well plate. Brightness intensity (0.299R + 0.587G + 0.114B) was calculated from the image crop using ImageJ. Data are means of triplicate measurements and RSD was below 7% for a volume = 315 µL and below 14% for a volume = 210 µL.

**Figure 4** The influence of the scanning orientation on linearity of the gallic acid standard curve in the Image-F-C assay. Linear regression analysis and non-linear regression analysis were performed using a power model ($Y = aX^b$) to fit experimental data. The image was acquired in a HP PSC 1510 scanner with 96-well plate with two orientations containing different gallic acid solutions, which were prepared by serial dilution. The image 96-well plate was rotated 90 °. Data are means of triplicate measurements and RSD was below 8% in all cases.

**Figure 5** The influence of scanner model on the linearity and sensitivity of gallic acid standard curves for Image-F-C assay. The images were acquired in two 96-well plate position on scanner at the center (A) and border (B) using a CCD scanner (HP LaserJet 2400). Linear regression analysis was performed to fit experimental data and calculation of determination coefficient ($R^2$). Data are means of triplicate measurements and RSD was below 8% in all cases.

**Figure 6** The novel high-throughput and rapid digital image-based Folin-Ciocalteau assay (Image F-C assay) to assess the reducing capacity. The alkalinization step was performed with sodium hydroxide instead of sodium carbonate. The neutralization was carried out with hydrochloric acid immediately after the reaction was completed. The image can acquired using a CIS or CCD scanner according to scanning orientation and 96-well plate position showed in the procedure. The image analysis was done using a program written in ImageJ macro language, which measured RGB values for each well. RGB values were used to calculated Euclidean distance.

**Figure 7** The standard curve of gallic acid for the Image-F-C assay. The image was acquired on scanner at the border 96-well plate position using a CIS desktop scanner (HP PSC 1510). Linear regression analysis was performed to fit experimental data. Data are means of triplicate measurements and %RSD was below 9% in all cases.

**Figure 8** Validation of the novel Image-F-C assay to assess reducing capacity of herbal infusions: Tymus (T), Digestive (D), Sen (S), Camomile (C), Green tea (GT), Lemon black tea (LT), Coffee (Co) and juices: *Biofrutas mediterraneo* (BF), pineapple juice (PJ) and apple juice (AJ). Correlation analysis (B) and Bland-Altman analysis (C) applied to the measurements of

reducing capacity by the novel Image-F-C assay and a microplate reader. Data are means of triplicate measurements.

## Table Captions

**Table 1** Reducing capacity of real samples assess by Image-F-C assay and modified rapid F-C assay using a microplate reader (n=3).