

A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols

Research Article

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Abstract: Several methods have been described for the determination of phenolic compounds in animal and plant products using the Folin-Ciocalteu (FC) assay. Most of these methods describe the use of this reagent and sodium carbonate in spectrophotometric methods. The macro FC assay was compared with two micro FC assays carried out on a microplate reader. Excellent correlation was obtained among the three assays with a molar extinction coefficient of $5.228 \pm 0.187 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The micro assay may serve as a high throughput method for the rapid determination of polyphenols in various samples.

Keywords: *UV-VIS spectrophotometry • Microplate reader • Methanolic extracts • Tannic acid*

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1. Introduction

Phenolics represent a large number of secondary metabolites present in most fruits and vegetables. These are subsequently divided into the simple phenols or phenolic acids, with one phenol ring and polyphenols, with at least two phenol rings. Typical examples include resorcinol, phloroglucinol, some coumarins, stilbenes, flavonoids, lignans and tannins amongst others.

Several methods have been used for the determination of polyphenols in plants. Typically, high performance liquid chromatography [1,2] and spectrophotometric analysis are frequently quoted. More specifically the Folin-Ciocalteu method has been described by many authors [3]. Originally this method was developed for the colorimetric determination of tyrosine, a phenolic non-essential amino acid [4]. Thereafter, this method has been used for the determination of several compounds including plant phenolics, drugs [5-7], vitamin C [8] and other constituents in a wide range of samples ranging from plant extracts to urine and bee products, in particular. The spectrophotometer-cuvette method is also frequently quoted. Little reference is made to the microanalysis using the FC reagent. Although a

micromethod has been described for wine phenolics, plastic or glass cuvettes were used [9]. However, this method employs micro volumes for the FC reagent and sodium carbonate. A microplate method has been described specifically for the determination of total phenolic compounds in urine [10] and in food products [11]. The latter method gives a short reaction time of 3 minutes. In most experiments, either gallic acid or tannic acid was used as a phenolic standard.

The present study attempts to demonstrate a rapid method for the determination of polyphenols in a high throughput assay when compared to classical FC assays used.

2. Experimental Procedures

2.1 Materials

Tannic acid, methanol, Folin-Ciocalteu reagent and sodium carbonate were purchased from Sigma Chemical Co. (St., Louis, USA). Tannic acid was prepared in five 1 in 2 dilutions, from $960 \mu\text{g ml}^{-1}$ down to $60 \mu\text{g ml}^{-1}$. The FC reagent was diluted 1:10 with de-ionised water, while sodium carbonate was prepared as a 1 M solution [12].

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2.2 Plant materials

Nine methanolic samples were obtained from orange (*Citrus aurantium*) peels, aubergine (*Solanum melongena*) peels and olive (*Olea europaea*) leaves. Briefly, 5 g of plant material were allowed to macerate for 48 h with 30 ml methanol. The resultant mixtures were filtered and the filtrates were mixed with de-ionised water (1:1). All samples were stored in a refrigerator until further analysis.

2.3 Total polyphenols determination

The polyphenolic content of the nine extracts, alongside the tannic acid serial dilutions, were tested with the Folin-Ciocalteu reagent using three different detection methods. The general reaction conditions for the three methods are described below and illustrated in Table 1.

A - Absorbance wavelength and time for the reaction. (a) MacroUV assay. The reaction mixtures were allowed to stand for 15 min and the total phenolic content was determined by colorimetry at 630 and 765 nm (MU630 and MU765, respectively) in quartz cuvettes on a UV-VIS spectrophotometer (WPA light wave S2000). In order to avoid time-related reading errors, the 15-min reactions were spaced by 1 min. (b) MacroMTP assay. The reaction mixture was prepared for all the extracts and tannic acid dilutions, as for the MacroUV test. During the incubation time, the mixtures were mixed thoroughly and microvolumes of 190 μ l were analysed at 630 nm on a Micro titre plate (MTP) reader (BioTek ELx800, Winooski, VT, USA) at the times stipulated below. (c) MicroMTP assay. 10 μ l of diluted extracts or tannic acid dilutions were pipetted in triplicate in wells of a MTP. The repeated volumes of FC reagent and sodium carbonate were transferred by means of a multichannel pipettor (Gilson). The mixtures were allowed to incubate at room temperature for the times stipulated below and then analysed at 630 nm on the MTP reader.

Total phenolic values were expressed in terms of tannic acid equivalents (μ g ml⁻¹). The absorbance readings were taken every five minutes for a period of 60 minutes. The equation $A = \epsilon \cdot c \cdot d$ was applied for the determination of molar extinction coefficient variation between the three assays.

B - Determination of the variation in volume (path length) and absorbance. This was performed for the macroMTP assay. The mixtures were prepared according to the macroUV proportions and mixed thoroughly during the incubation time. Micro-volumes of 300, 250, 190, 150, 100 and 50 μ l were transferred in triplicate to wells of a MTP. After 20 min incubation, the samples were analysed at 630 nm on the MTP reader. The equation $A = \epsilon \cdot c \cdot d$ was used to determine the variation of path length with absorbance for the same reaction mixture (Table 2). The path length was adjusted according to the dimensions of each microtitre plate well and the assay volume used.

C - Detection limit for the reaction. This was conducted for the tannic acid dilutions employing the macroMTP assay. The dilutions considered for this part of the investigation ranged from 960 to 0.002 μ g ml⁻¹ as 1 in 2 serial dilutions. Following a reaction time of 20 min, the absorbance was read at 630 nm on the MTP reader.

D - The tested extracts. The methanolic extracts were tested with the three assays (Table 1). For the macroUV assay, the extracts were tested at 630 and 765 nm (MU630 and MU765). The macroMTP and microMTP assays were tested at 15 and 20 min (MM15, MM20, mM15 and mM20 respectively). The respective tannic acid calibration curve was used to calculate tannic acid equivalents in the extracts.

2.4 Statistical evaluation

Comparative analysis was done using Student's t-test (two-tailed) to compare the FC-tannic acid reactions at different time intervals, different volumes and different experiments, alongside the nine extracts tested at a significance level of $P < 0.05$. The molar extinction coefficients for the three assays for the tannic acid dilutions and the tannic acid equivalence of the nine extracts were investigated with multivariate analysis. The correlation matrix was calculated, giving the correlation coefficients between each pair of variables tested. To identify variability and to reduce the dimensions of the data set, principal component analysis (PCA) was performed, using the XLSTAT Version 2011.5.01 software (Addinsoft, USA).

Method	Reaction vessel	Instrument	Reaction volume (ml)	Folin-Ciocalteu	Sodium carbonate	Extract or standard
MacroUV	Cuvette	UV-VIS spectro-photometer	9.500	5 ml	4 ml	0.5 ml
MacroMTP	Micro Titre Plate	Micro Titre Plate reader	0.190	190 μ l of Macro UV reaction		
MicroMTP	Micro Titre Plate	Micro Titre Plate reader	0.190	100 μ l	80 μ l	10 μ l

Table 1. The general reaction conditions for the three methods.

3. Results and Discussion

3.1 Absorbance wavelength and reaction time

Absorbance wavelength. As described in previous works, the Folin-Ciocalteu reaction with polyphenols produces maximal absorbance between 725 and 765 nm. In this present study, the main peak was determined to be 765 nm. A UV-VIS spectrophotometer is versatile as it can operate at any wavelength between 200 and 950 nm. Usually the standard MTP readers have five fixed wavelength filters and can be operated at these wavelengths only unless the filters are exchanged for different wavelengths. The versatility of the MTP reader is in its ability to read a large number of samples within a very short time interval, as opposed to the UV-VIS spectrophotometer. A suitable wavelength on the MTP reader close to the reaction maximum peak was 630 nm. The macroUV test was carried out with these two wavelengths (630 nm and 765 nm) to determine the correlation between reactions for the tannic acid dilutions for the standard curve and the plant extracts.

The molar extinction coefficient for the reaction at path lengths of 1 cm for the macroUV and 0.5938 cm for the macroMTP and microMTP was $5.228 \pm 0.187 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The extinction coefficients values for each individual test were not significantly different ($P > 0.05$, $v=5$) from each other. From the Principal Component Analysis for the molar extinction coefficients derived from individual determinations for the three assays (*i.e.* three different experiments on three dates), the correlation matrix exhibited positive correlations (Table 3). It is likely that the MicroMTP and MacroMTP correlate well as they are carried out on a small volume scale with a distinct pathlength, while the MicroMTP and Macro UV correlate well, as the former is a downsizing of the latter reaction and therefore the stoichiometry of the reaction is the same in both assays.

Reaction time. Although the first 60 minutes of reactions were considered, beyond the 35-min interval the absorbance values remained relatively stable with no further changes in absorbance with time. For each concentration, the absorbance at 630 nm over the time series indicated that the minimum reaction time in the MTP wells was 20 min compared to the 15 min in the macroUV test.

3.2 Determination of the variation in volume (path length) and absorbance

When using the MTP methods, the path length is not constant as with the UV method, where the reaction mixture is placed in a cuvette of a fixed path length. In the latter case, 10 mm quartz cuvettes were used. In the MTP method, the path length varies with the reaction

Volume of mixture (μl)	Path length (mm)
50	1.56
100	3.13
190	5.94
250	7.81
300	9.38

Table 2. The path length (mm) with mixture volume.

Variables	MacroUV	MacroMTP	MicroMTP
MacroUV	1	0.373	0.859
MacroMTP	0.373	1	0.796
MicroMTP	0.859	0.796	1

Table 3. Correlation matrix for molar extinction coefficients of the three assays.

The values at the level of significance, $\alpha=0.050$ (two-tailed t-test).

volume. MTPs with flat-bottomed wells were used in order to maintain a constant surface area throughout all depths. Mixture depths will vary when using U-shaped or V-shaped well plates. With a fixed surface area, the mixture depth or path length can be determined from the volume, as illustrated in Table 2.

3.3 Detection limit for the reaction

The maximum concentration for the macro UV was for $480 \mu\text{g ml}^{-1}$ TA as the 960 concentration was beyond 2.5 A, which is the upper detection limit for the spectrophotometer. The lower detection limit was determined for the macroMTP method at 630 nm and a 20 min reaction time. The limit was $7.5 \mu\text{g ml}^{-1}$ with tannic acid serving as the reference phenolic compound. There was a good correlation within the 7.5 to $960 \mu\text{g ml}^{-1}$ range ($R^2= 0.9974$, $v=7$).

3.4 The tested extracts

Table 4 represents the percentage (w/w) polyphenols in the plant materials expressed as tannic acid equivalents. The polyphenolic content of the aubergine extracts was low compared to that reported by Jung and co-workers [13], which was 5.519% (w/w). The olive leaf extracts contained a polyphenolic content equivalent to that reported by Ferreira and co-workers [14], which was 1.271% (w/w). The orange peel extracts exhibited mid-range polyphenolic contents when compared to previous studies, *i.e.* 0.284%, 0.559% and 0.670% [15-17].

From the Principal Component Analysis for the percentage polyphenolic content of plant materials, the

	macroUV		macroMTP		microMTP	
	MU630	MU765	MM15	MM20	mM15	mM20
AU1	0.250 ± 0.039	0.247 ± 0.036	0.186 ± 0.002	0.189 ± 0.005	0.175 ± 0.007	0.167 ± 0.008
AU2	0.277 ± 0.024	0.273 ± 0.020	0.219 ± 0.002	0.218 ± 0.006	0.221 ± 0.004	0.215 ± 0.006
AU3	0.277 ± 0.030	0.274 ± 0.026	0.225 ± 0.001	0.224 ± 0.004	0.224 ± 0.004	0.218 ± 0.005
OR1	0.459 ± 0.028	0.452 ± 0.025	0.385 ± 0.004	0.391 ± 0.003	0.344 ± 0.001	0.344 ± 0.007
OR2	0.411 ± 0.044	0.402 ± 0.039	0.275 ± 0.039	0.280 ± 0.044	0.267 ± 0.007	0.265 ± 0.009
OR3	0.494 ± 0.046	0.481 ± 0.041	0.379 ± 0.003	0.385 ± 0.003	0.365 ± 0.016	0.365 ± 0.013
OLC7	1.012 ± 0.002	0.955 ± 0.008	0.924 ± 0.008	0.936 ± 0.013	0.866 ± 0.017	0.864 ± 0.012
OLN7	1.161 ± 0.001	1.084 ± 0.009	1.044 ± 0.004	1.058 ± 0.017	0.998 ± 0.013	0.996 ± 0.017
OLB7	1.348 ± 0.038	1.256 ± 0.043	1.235 ± 0.016	1.250 ± 0.037	1.176 ± 0.017	1.168 ± 0.013

Table 4. The % w/w polyphenols in the plant materials expressed as tannic acid equivalents.

Variables	MU630	MU765	MM15	MM20	mM15	mM20
MU630	1	1.000	0.998	0.998	0.998	0.998
MU765	1.000	1	0.998	0.998	0.997	0.998
MM15	0.998	0.998	1	1.000	1.000	1.000
MM20	0.998	0.998	1.000	1	1.000	1.000
mM15	0.998	0.997	1.000	1.000	1	1.000
mM20	0.998	0.998	1.000	1.000	1.000	1

Table 5. Correlation Matrix for the % w/w polyphenols in the plant materials expressed as tannic acid equivalents.

Values in bold are different from 0 with a significance level $\alpha=0.05$

correlation matrix exhibited strong positive correlations (Table 5). This demonstrates that the extrapolation of plant extract-FC reaction absorbances from the tannic acid calibration curve is consistent for all the conditions mentioned above, mainly at the two wavelengths (630 and 675 nm) for the MacroUV assays, and the reaction times of 15 and 20 minutes for the MacroMTP and MicroMTP assays. The score plot reported in Figure 1 indicates that the olive leaf extracts contained a higher concentration of polyphenols than the orange and aubergine extracts. In fact, the main polyphenols of the olive leaf are flavonoids and secoiridoid glycosides [18,19], while orange and aubergine are likely to contain flavonoids and phenolic acids [20,21].

In conclusion, differences between the UV-VIS assay and the MTP assays depend mainly on path lengths and reaction stoichiometry. As illustrated above, there is a practical problem with the macroUV test, due to the limitation of sample handling within a single batch of experiments. With the spacing of extract readings by 1 minute (due to the repeated discarding of the previous solution and adequate rinsing and filling up of the cuvette

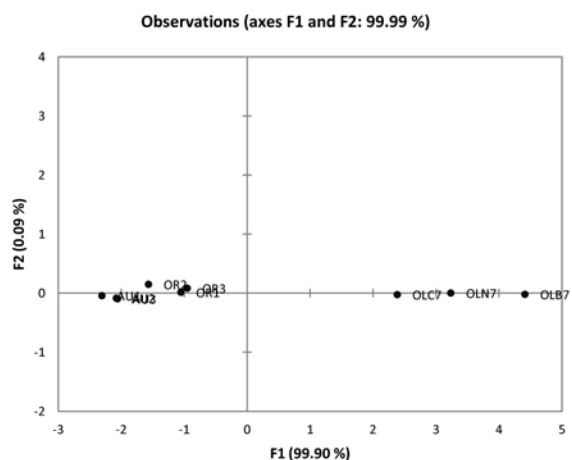


Figure 1. Score plot for the polyphenolic content of olive leaf (OL), orange rind (OR) and aubergine skin (AU) extracts.

with the test solution), only 14 solutions may be tested in a single sequence. Considering that five of these solutions are the standard solutions, only nine extracts can be analysed in such a sequence. To carry out the

testing in triplicate, the sequence has to be run twice more. The MTP assay offers a faster alternative to this cumbersome method. Testing the solutions in triplicate, with 18 wells dedicated to the tannic acid dilutions and blank, 78 wells are dedicated to 26 extracts in a single sequence. Furthermore, for the amount of reagents required to perform the macroUV and the microMTP with the same number of extracts and replicates, the volumes of FC reagent and sodium carbonate solution would be 50 times more for the macroUV than for the microMTP test, *i.e.* 480 ml and 384 ml, and 9.6 ml and 7.68 ml, for the respective tests. The microMTP assay was proven to be better correlated with the macroUV assay as the stoichiometry of the reactants was maintained in both cases. This was observed from the PCA statistical analysis. Furthermore, the optimum reaction time for the microMTP test was 20 minutes, with

a detection limit of 7.5 $\mu\text{g ml}^{-1}$. Finally, prior to testing it is advisable that extracts should be scanned within the 600–800 nm range in order to determine whether other existing compounds could interfere with the chosen Folin-Ciocalteu absorption wavelengths of 630 or 675 nm. In case extracts interfere at the absorption wavelengths, a MTP with extracts (10 μl), de-ionised water (100 μl) and sodium carbonate solution (1 M, 80 μl) should be prepared and read at 630 nm prior to the preparation of the MTP with the reaction mixtures. The absorbance values in the first plate should be subtracted from those of the second plate, to compensate for the background absorbance. Likewise, in case samples contain high amounts of ascorbic acid, especially fruit samples, this oxidation substrate should be determined [22] and subtracted from the total polyphenolic content. This will eliminate possible false positive results.

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