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Microplate Methods for Measuring Phenolic Content and Antioxidant Capacity in Chickpea: Impact of Shaking †

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Abstract: Microplate-based methods are commonly used to conduct spectrophotometric-based assays on large batches of sample extracts as they allow much greater throughput compared to traditional benchtop methods. However, many reported methods have not undergone a thorough method development/optimisation process; thus, the significance of maintaining certain parameters and procedures is often unknown. This study investigated the importance of plate shaking prior to the absorbance measurement step in two common assays: total phenolic content (TPC) measured using the Folin–Ciocalteu method, and total antioxidant activity measured using the Ferric Reducing Antioxidant Power (FRAP) method. A comparison was conducted on 36 methanol extracts of chickpea (*Cicer arietinum*) kernel, which had TPCs ranging from 43 to 111 mg GAEs (gallic acid equivalents)/L and FRAP values ranging from 25 to 67 mg TE (Trolox equivalents)/L. The absorbance of the samples was measured before and after the plate was shaken (300 s); each sample was analysed in duplicate. For the TPC, the unshaken and shaken absorbance values showed a high correlation with one another (R^2 = 0.990); however, a paired samples *t*-test demonstrated a significant increase in absorbance after shaking ($p < 0.001$; mean increase of 10.6%). Similarly, the unshaken and shaken absorbance values for FRAP showed a strong correlation (R^2 = 0.973), but again the shaken absorbance values were significantly higher ($p < 0.001$, mean increase of 12.1%). This demonstrates the importance of plate shaking for ensuring the complete reaction of the well contents prior to measuring their absorbance values. Furthermore, it highlights the need to closely follow the specified procedure when attempting to replicate or set up a microplate-based spectrophotometric method from the literature.

Keywords: 96-well plate; method development; antioxidant activity

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1. Introduction

Chickpea (*Cicer arietinum*) is attracting increased interest due to its potential health-benefitting properties, including antioxidant activity [1], anti-cancer activity [2], hypocholesterolaemic activity [3], hypoglycaemic activity [4], anti-hypertensive activity [5], and anti-inflammatory activity [6]. Many of these beneficial properties are attributed to polyphenols found in this crop, including phenolic acids and flavonoids. Studies from across the globe have demonstrated that the phenolic content of chickpea can vary quite significantly across different chickpea varieties [7–9]; therefore, rapid and high-throughput analytical methods are required to allow the screening of phenolic contents across large numbers of chickpea genotypes.

Microplate-based methods have been extensively reported to measure total phenolic content (TPC) and antioxidant capacity in a range of matrices [10–14]. However, it is worth noting that many of these protocols have not undergone complete validation or standardisation [13]. Part of the challenge stems from insufficient knowledge around the importance

(or lack thereof) of different physical steps in the analytical process (e.g., incubation time, shaking, and wavelength). If a particular step has no significant impacts on the results, it would be logical to eliminate or substantially reduce it to save analysis time.

In this work, our focus was to explore the significance of shaking prior to absorbance reading in microplate methods used for TPC and ferric reducing antioxidant potential (FRAP), which serves as a measure of antioxidant capacity.

2. Materials and Methods

2.1. Sample Details and Reagents

A total of 18 samples of dehulled Desi chickpea kernels (comprising 6 varieties) were used in this work, as described in Johnson et al. [15]. The extraction of polar phenolic compounds was conducted through maceration in 90% methanol, following previously published methods [16]. The resulting 90% methanol extract was used in subsequent work. To ensure reliability, each sample was extracted in duplicate, giving a total of 36 extracts.

2.2. TPC Microplate Method

To conduct the TPC microplate method, 20 µL of sample extract was combined in a 96-well plate with 100 µL of 1:10 diluted Folin–Ciocalteu reagent in each well, followed by 10 min incubation in darkness and the addition of 100 µL of 7.5% aqueous sodium carbonate solution. After a further 10 min incubation in darkness, the absorbance was measured at 750 nm using a microplate reader (Bio-Rad iMark; Hercules, CA, USA). For the experimental treatment, the 96-well plate was shaken for 300 s using the microplate reader (speed setting: mid) prior to the absorbance reading. The results were expressed in gallic acid equivalents (GAEs).

For both TPC and FRAP, each extract was analysed in duplicate, yielding a total of 72 absorbance readings per treatment.

2.3. FRAP Microplate Method

The FRAP microplate method used 10 µL of sample extract in each well, along with 200 µL of FRAP reagent, prepared as previously described [16]. The absorbance was measured at 593 nm using the microplate reader, again either without shaking, or after being shaken for 300 s (speed setting: mid). Results were expressed in Trolox equivalents (TE).

2.4. Data Analysis and Statistics

The absorbance readings were collated and used to compare results for the same samples with and without shaking. One obvious outlier well ($p < 0.01$ using Grubb's test) was removed for the FRAP results. Graphing and statistical testing were conducted in Microsoft Excel and GraphPad Prism 9.5.1.

3. Results

3.1. Effect of Shaking on the TPC Microplate Method

The mean absorbance of the samples without shaking was 0.252 ± 0.051 A, while with shaking treatment this increased to 0.279 ± 0.054 A (n = 72). Overall, this represented a significant increase ($p < 0.001$) in the absorbance by an average of 10.6%. While the specific change in absorbance for individual sample wells ranged from 6.8 to 19.3% (mean = 10.8 ± 2.6%), there was a strong linear correlation between the unshaken and shaken absorbance readings ($r_{70} = 0.995$; see Figure 1).

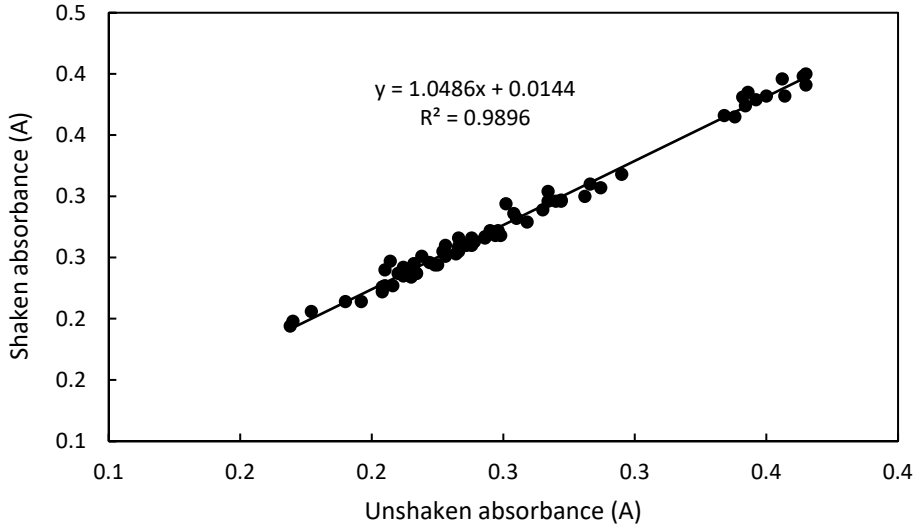

Figure 1. The correlation in absorbance readings between unshaken and shaken TPC measurements on the same sample extracts.

While the increased absorbance in the shaken samples resulted in a ~10% increase in the TPC determined in the original (kernel flour) samples, it did not appear to significantly increase the reproducibility of analysis. The average percentage coefficient of variation (%CV) of duplicate samples was 3.1% using the non-shaken method, while the average %CV for the shaken method was 3.0%.

3.2. Effect of Shaking on the FRAP Microplate Method

As observed with the TPC, the mean absorbance of the samples (0.189 ± 0.023 A) was significantly increased to 0.211 ± 0.025 A (n = 71) with the shaking treatment (paired *t*-test, $p < 0.001$). Again, there was a strong correlation between the unshaken and shaken absorbance readings for each sample ($r_{69} = 0.986$), as can be seen in Figure 2.

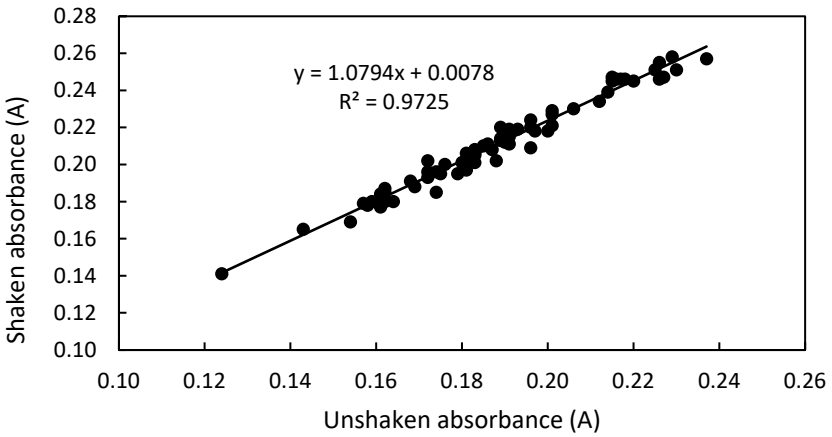

Figure 2. The correlation in absorbance readings between unshaken and shaken FRAP measurements on the same sample extracts.

The individual increases in absorbance after shaking ranged from 6.4 to 17.4% (mean = 12.1 ± 2.2%), very slightly higher than the average increase in absorbance for TPC. Similarly, there was very little reduction in the %CV for the calculated FRAP values for the kernel flour samples, which averaged 7.3% CV for the unshaken analysis and 7.1% CV for the shaken analysis.

Finally, it was noted that the relative increase in absorbance did vary with the sample type (i.e., chickpea variety) for the TPC (Figure 3a; $p < 0.001$ for one-way ANOVA). In

other words, the influence of shaking on the resultant TPC values differed depending on the sample type. This demonstrates the importance of testing a wide range of sample types (ideally covering all the sample types which will be analysed) when developing or validating a microplate-based assay. There were no significant differences in the absorbance increase for the FRAP for different chickpea varieties ($p > 0.05$, Figure 3b).

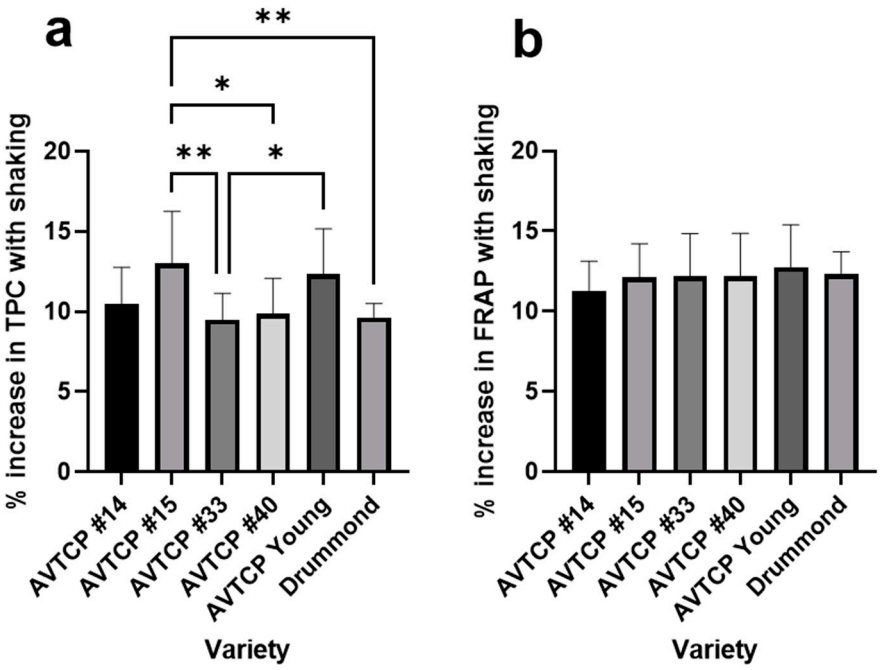

Figure 3. Average increase in absorbance values following shaking for the (**a**) TPC and (**b**) FRAP assays, displayed by chickpea variety. * indicates $p < 0.05$, ** $p < 0.01$.

4. Discussion

In our previous work reporting on a TPC microplate method [13], we presented data on the effects of measurement wavelength, incubation temperature, and incubation time, but did not investigate the impact of shaking prior to measurement.

The results of this work demonstrate the impact of shaking on microplate TPC and FRAP methods, which is to generally increase the absorbance values. This is most likely due to enhanced mixing between the sample and colorimetric reagent, leading to a proportionally increased extent of the reaction. While this would be anticipated to provide more accurate and reliable results by ensuring a complete reaction between the sample extract and the colorimetric reagent, our results did not show any significant improvement in the reproducibility of replicate results when using shaking. However, it is important to note that the response to shaking varied depending on the sample type (for the TPC assay), demonstrating the importance of using numerous different sample types when developing or validating microplate-based assays.

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