Food Chemistry 214 (2017) 156–161

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Continuous method to determine the trypsin inhibitor activity in soybean flour

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ARTICLE INFO

Article history:
Received 23 February 2016
Received in revised form 26 June 2016
Accepted 9 July 2016
Available online 11 July 2016

Keywords:
Soybean flour
Antinutritional factors
Trypsin inhibitor activity
Analytical method

ABSTRACT

The determination of trypsin inhibitor (TI) activity is of importance to evaluate the nutritional value of soybean flours. An analytical method, which involves a continuous spectrophotometric rate determination for trypsin activity against the substrate N-benzoyl-DL-arginine p-nitroanilide, is proposed as an alternative to the standard discontinuous assay. Stopping the reaction with acetic acid and a centrifugation/filtration step to decrease turbidity are not required, thus reducing costs and sample preparation time.

The TI activity of different flour samples, determined by both assays, demonstrated to be statistically comparable, irrespective of the TI concentration level. The coefficients of variation of the novel method did not exceed 8% at any concentration level.

The curves of progress reaction showed a non-linear behavior in samples without TI. A reduction of incubation time from 10 min to 2 min increased the method sensitivity and extended its linear range.

A more economical, faster and simpler assay was developed.

1. Introduction

At present, soybean is the world's most important oilseed, used not only for the extraction of oil but for the recovery of protein as well (Day, 2013). Soybean industry spans continents, feeds millions of livestock, and takes part as ingredient in most of our diet. The high nutritional value of soybean protein is determined by its composition, which includes all the essential amino acids required for growth and by its excellent bioactive properties such as antioxidant, antihypertensive, antithrombotic, hypocholesterolemic (Alibhai, Mondor, Moresoli, Ippersiel, & Lamarche, 2006; Coscueta et al., 2016; Isanga & Zhang, 2008; Wang, Mejia, & Gonzalez, 2005). However, like other beans and grains, soybean contains several components (lectins, protease inhibitors, etc.) related to protection and immune mechanisms in the plant, which prevent the utilization and digestibility of soybean protein (Bajpai, Sharma, & Gupta, 2005; Becker-Ritt, Mulinari, Vasconcelos, & Carlini, 2004; Norton, 1991). Among these anti-nutritional factors, the trypsin inhibitors (TI) are the major components reported to cause retardation of growth and digestive and metabolic diseases (Gatel, 1994; Norton, 1991; Westfall & Hauge, 1948). These factors are heat labile, therefore, they are able to be removed totally or partially by different treatments during soybean industrialization (Akande & Fabiyi, 2010; Babar, Chavan, & Kadam, 1988; Elsheikh, Fadul, & El Tinay, 2000; van den Hout, Pouw, Gruppen, & van't Riet, 1998). The determination of TI activity is of importance to evaluate the nutritional value of soybean flour after processing stages in order to provide high-quality products. At present, the standard method is based on the ability of extracts of soybean flours to inhibit the activity of trypsin towards the chromogenic substrate N-benzoyl-DL-arginine p-nitroanilide (Kakade, Rackis, Mc Ghee, & Puski, 1974). The amount of the product p-nitroaniline, formed during a 10 min incubation step, is determined through absorbance measurements in the presence and absence of soybean extract, thus giving differences related to the TI activity. Results are expressed in trypsin inhibitor units (TIU) per gram of initial soybean sample, each TIU being defined as the amount of inhibitor that causes a change of 0.01 in absorbance units at 410 nm per 10 mL of reaction mixture under the experimental conditions (AOCS, 2009). It is a discontinuous or stopped assay in which the enzyme (trypsin) is inactivated after

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http://dx.doi.org/10.1016/j.foodchem.2016.07.056
0308-8146/

the incubation time (10 min) by lowering the pH with the addition of acetic acid. The overall assay is complicated and does not fulfill the requirements for quality control in industrial processing of soybean derivatives. It presents several shortcomings (Liu & Markakis, 1989; Stauffer, 1990), as addressed bellow. The stopping reactant (acetic acid) causes the denaturation not only of the trypsin but also of the extract proteins, thus producing the formation of turbidity which must be reduced by a later filtration/centrifugation step. A blank, in which the substrate is added after the acid, is required to be prepared in order to subtract the remained turbidity. Each determination involves the preparation of several trial dilutions, attempting to arrive at a concentration in which the sample inhibition is between 40% and 60% of total trypsin activity in order to minimize the relative standard deviation. Time delay steps between additions are required to reach identical reaction conditions (incubation/stopping times), thus limiting the number of samples able to be analyzed simultaneously. Finally, this assay presents a disadvantage proper of any discontinuous one, the shape of the progress curve is not revealed and any irregularity in curvature is not able to be detected (Meyers, 1995).

In this context, the goal of this work was to overcome the above mentioned disadvantages by developing an improved continuous assay as a potential replacement of the current one. An adequate treatment of the data was proposed to inform the results in TIU units comparable with those obtained from the standard stopped method. Furthermore, the analysis of the progress of reaction with time was assessed to determine the optimal conditions that assure a linear relationship between the measurements and the TI activity in samples.

2. Materials and methods

2.1. Materials

Crystallized, salt free trypsin (bovine) (TRP) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were purchase from Sigma Chem. Co. and used without further purification. All other reagents were of analytical quality.

Different soybean flours were analyzed. Defatted soybean flours, both deactivated and non-deactivated samples, were obtained from the food processing company Molinos Río de la Plata SA (San Lorenzo, Argentina). An additional commercial sample of soybean flour was purchased from a local market.

2.2. Procedures

2.2.1. Deactivation

Several soybean flours were subjected to mild and moderate deactivation treatments in our laboratory in order to obtain samples with different TI activity and extend the analyzed range. Heating protocols were carried out by incubating the samples in an oven at two temperatures (80, 100 °C) for different periods of time (1, 1.5 and 2.5 h). Each sample and its treatment are described below:

- Sample 1: Defatted soybean flour without any thermal deactivation treatment, supplied by Molinos Río de la Plata SA.
- Sample 2: Defatted soybean flour supplied by Molinos Río de la Plata SA, subjected to oven dry heat at 80 °C for 1 h.
- Sample 3: Defatted soybean flour supplied by Molinos Río de la Plata SA, subjected to oven dry heat at 100 °C for 1.5 h.
- Sample 4: Defatted soybean flour thermally deactivated by Molinos Río de la Plata SA.
- Sample 5: Commercial defatted soybean flour.
- Sample 6: Commercial defatted soybean flour subjected to oven dry heat at 100 °C for 2.5 h.

2.2.2. Preparation of TI extracts

The extraction of TI was performed by mixing 1.00 g of soybean flour (treated/non-treated) with 50.0 mL of 0.01 M NaOH and agitating the resultant suspension for 3 h at room temperature according to the standard method proposed by Kakade et al. (1974) and later modified (AOCS, 2009). A final centrifugation step for 10 min at 3500 rpm allowed separating the supernatant (soybean flour extract) for the TI assay.

2.2.3. Discontinuous TI assay

The reaction between TRP and BAPNA in absence and presence of TI (soybean flour extracts) was performed for 10 min at 37 °C and stopped by acetic acid. The reaction mixture was centrifuged for 15 min at 10,000 rpm and filtered through a Watman No. 2 paper in order to obtain a clear supernatant. The extension of the reaction was followed by the absorbance of the released reaction product, the p-nitroaniline, at 410 nm. The preparation of TRP and BAPNA solutions, all the protocol steps and the final calculations were carried out by following exactly the AOCS method (AOCS, 2009). Results were expressed in trypsin inhibitor units (TIU) per gram (g) of soybean flour, TIU/g.

Each TIU is arbitrarily defined as the change of 0.01 absorbance units at 410 nm per 10 mL of reaction mixture after 10 min of reaction in Tris buffer pH 8.20, 0.050 M at 37 °C.

2.2.4. Continuous TI assay

The modified proposed method involves a continuous spectrophotometric rate determination for trypsin activity against the substrate BAPNA, the reaction taking place directly in the spectrophotometer cuvette with a reduction of volume from 10 to 2.5 mL. Stopping the reaction with acetic acid and clarification step to reduce turbidity were not required. Nevertheless the final concentrations (in cuvette) of all the reactants -enzyme, substrate and buffer- were the same of the standard method, the concentrations and volumes of working solutions were modified to improve the procedure and make it simpler. All the details of the modified protocol are given below:

2.2.4.1. Reactants. Stock and working solutions of TRP and BAPNA were prepared according to the following directions.

The *TRP stock solution* was prepared by dissolving 10 mg of TRP in 1 mL of 0.001 M HCl acid. It was stored at −18 °C until use and mixed gently when defrosting. The *TRP working solution* was prepared fresh daily by a 1:100 dilution of *TRP stock solution*, defrosted and gently mixed with Tris buffer 0.050 M pH 8.20. This solution was maintained in an ice-water bath during the assay.

The *BAPNA stock solution* was prepared by dissolving 100 mg of solid BAPNA in 2.3 mL dimethyl sulfoxide. It was maintained at −18 °C until use. The *BAPNA working solution* was prepared fresh daily by a dilution 1:100 of the *BAPNA stock solution*, previously defrosted and gently mixed, with Tris buffer 0.050 M pH 8.20.

2.2.4.2. Rate measurements. Each TI determination required of two conditions to be measured: Control (trypsin activity without inhibitor) and Sample (trypsin activity in the presence of inhibitor). The volumes (mL) of reagents/samples and their sequence of addition are indicated in Table 1.

Immediately after mixing, the Absorbance at 410 nm was monitored for 10 min recording measurement readings at time intervals of 10 s or less. The reaction rate (Abs units/min) was obtained from the slope (m) of Absorbance vs. time plot at both conditions ($m_{Control}$, m_{Sample}). The assays were performed at constant temperature of 37 °C.

Notice that the standard method states that the rate at Sample condition must be 0.4–0.6 times the value of the Control one to

Table 1
Procedure for measuring TI activity by the continuous method.

Reagents	Control[a]	Sample[a]
Tris buffer 0.050 M, pH 8.20	1.00	0.72
TRP *working solution*	0.20	0.20
Supernatant conveniently diluted	–	0.28
Mix by inversion and equilibrate to 37 °C for 2 min., then add		
BAPNA *working solution* (pre-incubated at 37 °C)	1.30	1.30

[a] Values correspond to volume in mL.

minimize errors. Consequently, when the continuous method was carried out by recording data for 10 min, the supernatant was conveniently diluted in order to reach this condition, the dilution factor (D), being included in the calculation expression.

Absorbance measurements were carried out in a JASCO V-550 spectrophotometer by using a thermostated cell of 1 cm pathlength.

2.2.4.3. Calculation. The results were expressed in trypsin inhibitor units per gram of soybean flour (TIU/g) in order to be comparable with those provided by the standard method (discontinuous). The calculation was made by the following expression:

$$\mathrm{TIU/g} = \frac{100 \quad 2.5(\mathrm{m_{Control}} - \mathrm{m_{Sample}})}{0.280} \mathrm{D}\ 50 \qquad (1)$$

where *100* is the factor to convert 0.01 u. Abs in TIU units; $m_{Control}$-m_{Sample}, the difference between the slopes of progress curves in absence and presence of TI respectively; *D*, the dilution factor of supernatant, calculated as the final volume divided by the amount of aliquot taken to dilute the extract; *50* is the extraction volume (mL) of 0.01 M NaOH used per gram of soybean flour; *0.280*, the aliquot (mL) used in the current assay and *2.5*, the final reaction volume (mL) in the cuvette.

2.2.4.4. Additional modifications. The continuous method was also evaluated by shortening the recording time from 10 to 4 and 2 min but maintaining the rest of the conditions.

2.2.5. Statistical analysis

Each determination was performed in triplicate and the results were expressed as the mean values (M) with standard deviations (SD) and coefficients of variation (CV) in percentage. The mean values were analyzed statistically by analysis of variance followed by the Tukey's post-hoc test (Neter, Kutner, Nachtsheim, & Wasserman, 1996; Tukey, 1949). Separation of means was conducted by using the least significant difference at the 5% level of probability.

The comparison of the standard and proposed techniques was performed by the regression of the TIU/g values, obtained by the proposed method, against those provided by the standard method. Considering that both y (value obtained by proposed assay) and x (value obtained by standard assay) are affected by random errors of comparable magnitude, the bivariate least-squares regression (BLS) procedure was carried out to find the slope and intercept respectively. Once these parameters were obtained, their comparison with the ideal ones (0 and 1 respectively) was carried out by calculating their elliptic joint confidence region (EJCR) with the equation reported by Franco, Mantovani, Goicoechea, and Olivieri (2002).

Major statistical analysis was carried out with the aid of Statgraphics Centurion XVI.I software. BLS and EJCR analysis was carried out with the aid of Matlab R2015a (MathWorks).

3. Results and discussion

3.1. Proposed method vs. standard one

Flour samples containing TI were analyzed simultaneously by both the standard and the proposed continuous method to assess if their results were comparable. Commercial and industrial soybean flour products were subjected to thermal deactivation processes in order to reduce their TI activity and consequently, make the analytical TI activity range wider. Results obtained are presented in Table 2.

The values obtained by the standard assay showed clearly that those samples either subjected to mild deactivation conditions or non-treated presented the higher TI activity. The obtained values, around 40,000 TIU/g, were found to be in concordance to the results reported by other authors (Gupta, 1987; Machado et al., 2008; Olguin et al., 2003; Qin, Verstegen, & Van der Poel, 1998). Worthy of considering is that the available literature data presents a wide range of results reported for the antitrypsin activity depending on the bean species, the stage of ripening and the weather conditions during the vegetative season. Although the samples subjected in our laboratory to dry-heat processing for larger periods of time and higher temperatures presented significantly reduced TI activity, the flour deactivated by the food company (Mol*), showed to be the most efficiently deactivated with the lowest TI activity.

Samples 1, 2, 3, 5 and 6 had to be diluted conveniently (1:20) while sample 4 was not diluted in order to achieve the condition of Sample rate being 0.4–0.6 times the value of Control. This condition, stated by the standard method to minimize errors (AOCS, 2009), required conducting several experiments thus consuming time and reactants.

When the continuous method was performed, the curves of reaction progress followed a pattern similar to that of Fig. 1. As expected, the slope of the Sample plot (m_{Sample}) showed to be lower than the Control one ($m_{Control}$) in all the assayed flours due to the inhibition of trypsin caused by the TI presence in the sample. These values introduced in Eq. (1) allowed us to recover the TI activity in TIU/g similarly to the discontinuous method. When comparing these results with those provided by the standard method by ANOVA analysis with Tukey's post-hoc test, no significant differences for most of the assayed samples were observed. For the sample 4, it was noteworthy that no significant differences

Table 2
Comparison of TIU/g values obtained by the proposed (continuous) and standard (discontinuous) method.

Sample	Treatment[a]	Assay[b]					
		Standard			Proposed		
		M	SD	CV	M	SD	CV
1. – Mol	Without	43,420	1091	2.5	41,193	2363	5.7
2. – Mol	80 °C, 1 h	39,874	642	1.6	41,122	1822	4.4
3. – Mol	100 °C, 1.5 h	30,310[c]	508	1.7	23,960[c]	966	4.0
4. – Mol*	Without	1352	92	6.8	2673	212	7.9
5. – Com	Without	31,463	773	2.5	33,067	991	3.0
6. – Com	100 °C, 2.5 h	25,340	341	1.3	26,862	1002	3.7

Mol, flour defatted, non-deactivated supplied by Molinos Rio de la Plata SA; Mol*, flour defatted and deactivated by Molinos Rio de la Plata SA; Com, flour purchased in a local market.
M, SD and CV: mean, standard deviation (TIU/g) and coefficient of variation (%) of triplicates.
[a] Specifications (temperature and incubation time) of deactivation processes carried out in our laboratory.
[b] Standard assay: reaction conditions described in Section 2.2.3. Proposed assay: reaction conditions described in Section 2.2.4, reaction time 10 min.
[c] Values within the row differ significantly with Tukey's test ($p < 0.05$).

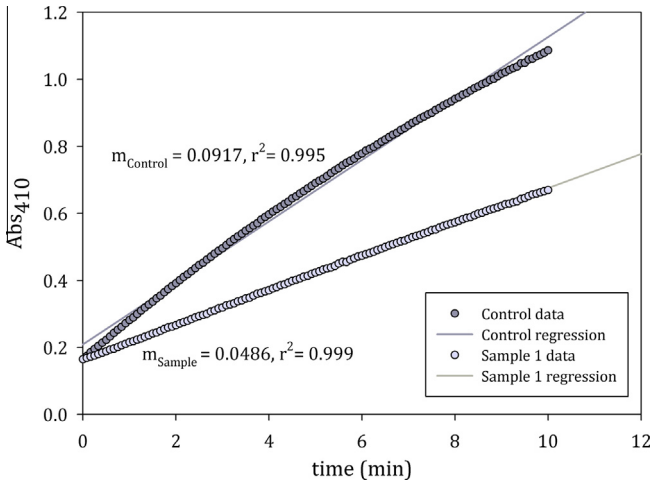

Fig. 1. Typical curve of the reaction progress with the proposed continuous method for the sample (flour sample number 1) and control conditions. Procedure details are indicated in Table 1.

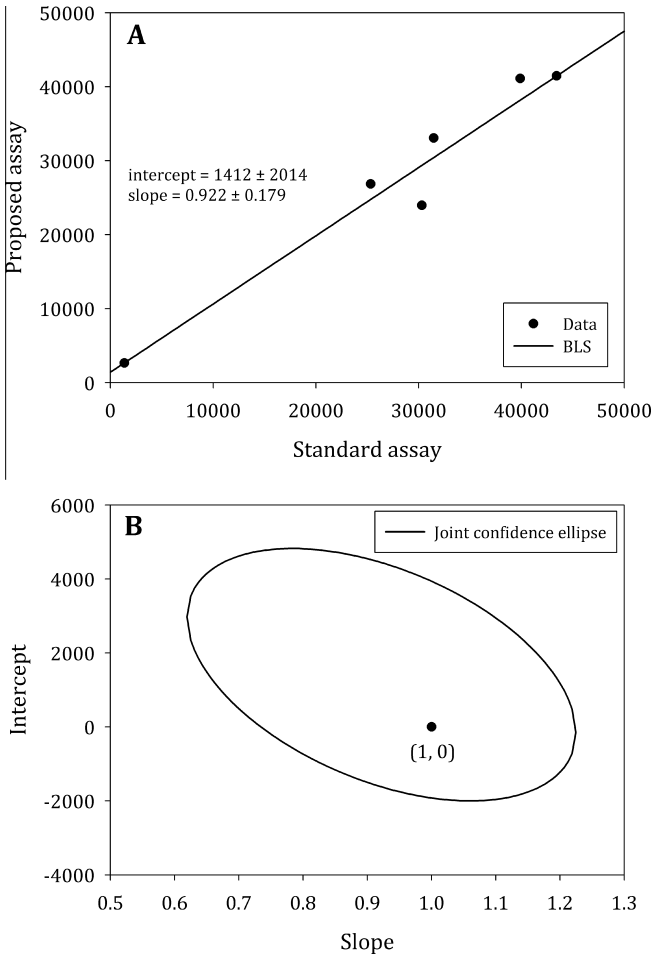

Fig. 2. (A) TI activity of flour samples observed by both standard and proposed methods (dots) and BLS regression line (solid line) along with its parameters (slope and intercept) and confidence intervals (0.95 confidence level). (B) Joint confidence region based on BLS method (0.95 confidence level) and representation of the dot determined by the theoretical slope and intercept (1, 0).

were found by Tukey's test despite the high differences between the two means (from continuous and discontinuous methods) and their small SD values. To clarify this point, it is important to note that this test adjusts the confidence intervals to allow multiple comparisons among all pairs of means. It uses the Tukey's *t* instead of the Student's *t* for the calculations, the Tukey's *t* being equal to $(1/\sqrt{2})$ times the Studentized range distribution. Tukey's procedure is more conservative than, for example, the Fisher LSD procedure, since it makes it harder to declare any particular pair of means to be significantly different (Kuehl, 2000).

In addition to said comparison between individual estimates, a further analysis was carried out to assess the comparability of both assays (see Section 2.2.5).

Fig. 2A shows the regression plot, performed by the BLS method, in which the abscissa represents the TI activity values obtained by the discontinuous assay and the ordinate, those obtained by the continuous one. The individual confidence intervals for the slope and the intercept of this plot (see inset Fig. 2A) showed to contain the theoretical slope and intercept values (1 and 0 respectively), thus suggesting the comparability of standard and proposed methods. However, this could be an erroneous conclusion since it ignored an eventual correlation of each slope and intercept (Franco et al., 2002). Therefore, Franco et al. (2002) recommended to calculate and plot the elliptic joint confidence region (EJCR) for these parameters (Fig. 2B). From this plot it could be inferred that constant and proportional bias were absent since the point (1; 0) was contained within the mentioned region. This indicated that the slope and intercept did not differ statistically from 1 and 0 thus allowing us to confirm that both methods were comparable. Furthermore, Van de Velde, Pirovani, Cámara, Güemes, and Bernardi (2012) stated that when the optimal point (1; 0) is included in EJCR, a good accuracy has been reached with the proposed methodology.

Table 2 also shows the coefficients of variation corresponding to the different TI measurements. It can be clearly appreciated that the CV (%) corresponding to the proposed method, representative of the measurement precision, were larger than the ones obtained using the standard method. This behavior could be attributed to the different operating conditions of continuous and discontinuous methods. Commonly, continuous methods are carried out sequentially (i.e., each measurement implies a run), while discontinuous ones are performed by running a given number of samples simultaneously. As a consequence, the latter methods allow achieving identical operating conditions for the samples of a run (15 determinations/runs in our case), thus minimizing the variability derived from unstable reagents and environmental factors that may change over time. Taking into consideration these comments, the CV (%) of continuous method could be reduced by utilizing spectrophotometers with multiple cells or microplate readers, capable of reading as many as 96 samples almost simultaneously. The increased CV (%) value of the continuous method could be also attributed to the slight turbidity of samples under working conditions, this turbidity being higher at lesser diluted samples. In the standard method there is clarification step that removes these interferences. Regardless of the mentioned causes, it is worth to notice that the CV% of the proposed method did not exceed 8% at any concentration level (Tables 2 and 3). This value is far less than the 15%, this being the maximum value acceptable for the validation of a given bioanalytical method (EMA, 2011).

3.2. Analysis of the curve of reaction progress and assay linearity

When analyzing the shape of the progress curve (Fig. 1) it was noticeable that Abs vs. time plot resulted linear in presence of TI (Sample) but showed to be slightly curved in absence of TI (Control). When we performed the statistical analysis on six replicates it was observed a lack of fit for the linear model with a confidence level of 95%. This suggested considering a polynomial of higher

Table 3
Comparison of the linearity range of proposed (continuous) method, at different time assay, and standard (discontinuous) method.

*Sample dilution	Assay											
	Proposed									Standard		
	2 min			4 min			10 min					
	M	SD	CV	M	SD	CV	M	SD	CV	M	SD	CV
0.6	53,068	2073	3.9	49,799	2936	5.9	36,463[a]	2294	6.3	45,067[a]	1554	3.4
1.0	52,390	1527	2.9	48,764	916	1.9	39,664[b]	429	1.1	41,687[a]	858	2.0
1.4	54,705	3292	6.0	52,089	2845	5.5	45,139[a,b]	455	1.0	48,264[a,b]	477	1.0
1.8	52,988	1034	2.0	50,299	1088	2.2	41,706[a,b]	1349	3.2	43,980[b]	446	1.0

M, SD and CV: mean, standard deviation (TIU/g) and coefficient of variation (%) of triplicates.
[*] Dilutions of sample 1, calculated as the ratio between the supernatant volume assayed and that corresponding to the protocol of each method.
[a] Values within the column differ significantly with Tukey's test ($p < 0.05$).
[b] Values within the column differ significantly with Tukey's test ($p<0.05$).

degree, finding a proper fit on a quadratic polynomial ($r^2 = 0.998$) as follows:

$$Abs = 0.0278521 + 0.0020113t - 7.41739\,10^{-7}t^2 \quad (2)$$

where Abs is the absorbance and t, the time in s.

The non-linearity of Control plot suggested that the degradation of BAPNA by trypsin did not take place under saturating conditions over the 10 min of incubation. In presence of TI, however, the Sample plot did not show lack of fit to the linear model. Under this later operating condition, TI binds to trypsin and the free enzyme, capable of reacting against BAPNA, is low enough to make the substrate working-concentration being saturating. The non-linearity of the progress curve and its possible causes have been reported by other authors (Stauffer, 1990). Irrespective of such causes, the mentioned curvature is expected to conduce to underestimated results and therefore, to a narrower linear range. A further analysis and reformulation of volumes, purity characteristics and concentrations of reactants should be developed.

In an attempt to achieve the linear condition without changing most of experimental conditions, we evaluated the effect of shortening the incubation times on the TI results. For this purpose, all the data corresponding to the progress curves obtained previously for 10 min were reprocessed by considering only 4 and 2 min of reaction respectively (Fig. 3). In the analysis of the Control curve, it was observed that the data corresponding to 4 min, despite being able to fit a linear model, continued to adjust better to a quadratic one. However, by considering 2 min of reaction, the ANOVA for variables demonstrated that the linear relationship was the most appropriate.

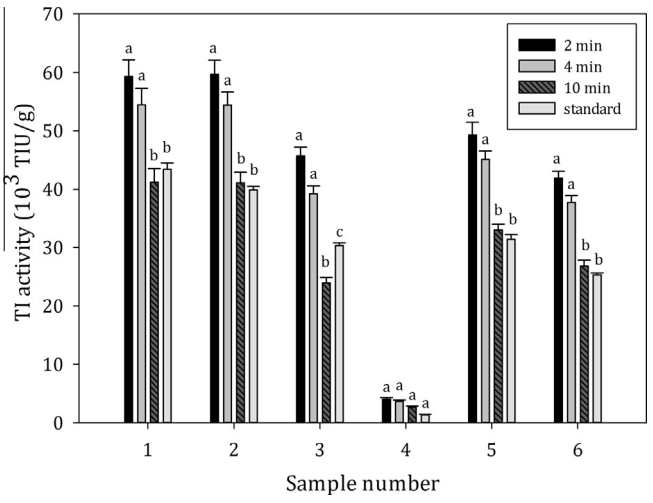

Fig. 3. Effect of reducing the incubation time on the TI results obtained by the continuous and discontinuous methods. Flour samples numbers according to Table 2. Bars sharing the same letter (a, b, c) within a sample number do not differ significantly with Tukey's test ($p > 0.05$).

As expected, the TI activity values increased significantly when the incubation time decreased. Except for sample 4 (Fig. 3), this trend was observed for all other assayed flour samples. This could be due to sample 4 having a TI activity value close to the quantification limit of the method. Our calculations demonstrated that observed enhancement of TI activity values mainly resulted from the increased slopes of Control plots ($m_{Control}$) since the Sample plots were practically constant. Increases in TI results at shorter incubation times (4 min) were also observed by applying the discontinuous assay (data not shown).

At this point, we assessed if shortening incubation times could imply changes in the linear range of the method. The standard assay (incubation time 10 min) and the proposed one at different incubation times (2, 4 and 10 min) were applied on a wide range of dilutions (0.6, 1, 1.4 and 1.8) of a given sample (number 1). Results, considering these dilutions (Table 3) indicated that only the continuous assay performed for 2 or 4 min conduced to TI activity values statistically equivalent for all the dilutions. On the other hand, incubation of 10 min led to values significantly different for both the continuous and discontinuous method. According to these findings, shortening incubations could be considered a good alternative, not only to reduce the overall experimental time but also to make the linear range of the method wider by increasing method sensitivity to change response per unit of activity, i.e. a larger slope for the regression line.

4. Conclusions

On the basis of our results, we consider that the proposed continuous method *fulfills the requirements for quality control in industrial processing of soybean flour and* constitutes a good alternative to replace the current standard method (discontinuous). An appropriate treatment of the data allows informing the results in TI units, similarly to the standard assay, this being especially important to compare results from different laboratories. The proposed method does not involve the addition of acetic acid to stop the reaction. Consequently, it does require neither of filtration/centrifugation steps to reduce turbidity or performing blanks. This is of practical importance since it leads to reduce cost and experimental time.

In addition, a reduction of incubation times (2 min) is proposed since it demonstrated to extend the linear range of the method.

To conclude, the proposed method is more economical, faster and simpler than the standard one.

Acknowledgement

This work was supported by a grant from CONICET PIP0551/12. Author E.R. Coscueta acknowledges BiValBi – Biotechnologies to Valorise the regional Biodiversity in Latin America (Ref[a] PIRSESGA-2013-611493 BI_1) – for the research fellowship administered for funding part of this work and CONICET for PhD fellowship.

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