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DETERMINATION OF TRYPSIN INHIBITOR ACTIVITY OF MICROWAVE-HEATED BEAN SEEDS USING BROMOCRESOLE PURPLE INDEX (BCPI)

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The usefulness of a new analytical method called bromocresole purple index (BCPI) for determination of trypsin inhibitor activity (TIA) of microwave-heated bean seeds was tested. The study was conducted on bean seeds of "Jaś" cultivar which were microwave heated using one of ten variants of that process intensity. Each of the three radiation power levels (350, 500, or 650W) corresponded to three different processing times (60, 120, or 180 s), and one of the samples remained untreated (*i.e.* unheated). Each sample was analysed using the BCPI method and the TIA method (with a synthetic substrate BAPA – N-α-benzoyl-DL-arginine-p-nitroanilide). The comparison of those two analytical methods (BCPI and TIA) used to analyse the microwave-heated bean seeds samples indicates the superiority of bromocresole purple index method in terms of time consumption (τ_{TIA}=2.5 h, τ_{BCPI}=1.5 h), distinguishability (ρ_{TIA}=51.11%; ρ_{BCPI}=71.11%), accuracy of determination (lower coefficient of variation; π_{TIA}=3.02–9.27; π_{BCPI}=1.22–5.88) and detectability (detectable minimum μ_{TIA}=10.32 and μ_{BCPI}=5.01), whereas the TIA assayed proved superior in terms of method sensitivity (χ_{TIA}=0.40; χ_{BCPI}=0.16). The statistical analysis of experimental data indicates also that the results obtained for microwave-heated bean seeds using BCPI and TIA-BAPA methods are highly correlated (correlation coefficient r=95.28%), moreover both those traits may be related by mathematical functions TIA= f(BCPI). The usefulness of some of those traits in the analysis was confirmed statistically; based on high coefficients of determination of these equations to experimental data (*e.g.* R^2=90.72% for linear equation and R^2=91.11% for the IV^o polynomial equation). Due to the specificity of quick, routine tests performed at an industrial laboratory, the application of the simplest linear regression equation: TIA=f(BAPA) seems to be the most justified, whereas its coefficient of determination R^2 (in description of experimental data) should assure the reliability of calculations.

SYMBOLS AND ABBREVIATIONS

BAPA – N-α-benzoyl-DL-arginine-p-nitroanilide, BCPI – bromocresole purple index, $BCPI_{P.D.M}$ – bromocresole purple index (expressed per gram of protein in dry matter of seeds), CV – coefficient of variation, χ – method sensitivity, Ln – natural logarithm, μ – detectable minimum, NIR – the lowest significant difference, π – precision of determination, r – coefficient of correlation, R^2 – coefficient of determination, ρ – discrimination, SD – standard deviation, τ – time consumption of analysis, TIA – trypsin inhibitor activity (TIA-BAPA – trypsin inhibitor activity determined with synthetic substrate BAPA), and TUI – trypsin inhibitor activity units.

INTRODUCTION

Bean seeds, due to favourable chemical composition (first of all abundance of carbohydrates, proteins with a high nutritive value, vitamins and mineral salts), constitute an important component of food [Korus *et al*., 2006; Gyori *et al*., 1998; Balandran-Quintana *et al*., 1998]. Consumption of bean seeds is, however, limited for they contain antinutritional factors, above all hemagglutinin activity and antitrypsin factors [Jasińska & Kotecki, 1999; Valdebouze *et al*., 1980]. The trypsin inhibitor activity (TIA) of bean seeds is linked with the content of four proteins with a similar molecular weight comparable to the molecular weight of the Bowman-Birck soybean trypsin inhibitor. They are characterised by similar properties both in terms of isoelectric point (pH=4.6–5.09), and amino acids content, among which threonine, cysteine, serine, asparagine and proline are predominant, methionine, glycine and alanine and aromatic amino acids occur in low quantities, whereas tryptophan and valine have not been detected at all [Wu & Whitaker, 1990; Whitaker & Sgarbieri, 1981]. Three of those inhibitors demonstrate inhibiting activity towards trypsin only, while one is additionally able to inhibit chymotrypsin activity [Wu & Whitaker, 1990].

On account of numerous, thermolabile antinutritional factors contained in bean seeds, their dietary application depends on different forms and means of their heating, resulting in a decreasing activity of antinutrients and increasing bioavailability of nutrients [Balandran-Quintana *et al*., 1998].

The microwave heating, combining high yield and significant efficiency, is one of newer concepts in modern food processing [Regier & Schubert, 2001]. A drawback of microwave processing of raw food material (including processed bean seeds) is the risk of reducing the nutritional value of a product, connected with its excessive heating that results from signifi-

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cant uncontrolled fluctuations in parameters of the production process *e.g*. upon voltage fluctuations of power grid of the microwave heating installation. Under those conditions, an appropriate control system of the quality of product obtained from bean seeds as a result of microwave heating becomes essential. The system should enable continuous monitoring of its properties with special consideration of the nutritional value. It should also be based on appropriately sensitive, quick, accurate and reliable and at the same time adequately easy analytical methods, to facilitate its utilization in usually modest conditions of an industrial laboratory.

To date, the methods used in that scope are most often based on the assessment of the activity of selected antinutritional factors (trypsin inhibitor and hemagglutinin activities) [Kakade *et al*., 1974] and due to time consuming and highly complicated analytical procedure, they do not meet the requirements for quality control in industrial processing of bean seeds.

The undertaken study is a continuation of broader scope studies, which were discussed also in previous papers [Szmigielski, 1999, 2002, 2004; Szmigielski & Matyka, 2002, 2004] considering various aspects of applying bromocresole purple index ($BCPI_{P.D.M.}$) for the determination of selected quality attributes of legume seeds and their products obtained as a result of different forms of heating.

The aim of the study was to assess the usefulness of a new analytical method called the bromocresole purple index (BCPI) in determining the influence of microwave processing on trypsin inhibitor activity (TIA-BAPA) in beans. A hypothesis has been adopted in the study that there is a relationship between the results obtained with:

– classical method of trypsin inhibitor activity TIA (using N-α-benzoyl-DL-arginine-p-nitroanilide – BAPA) and

– method of bromocresole purple index BCPI (using 5', 5"-dibromo-3', 3"-dimethylphenylsulphophtalein – commonly called bromocresole purple)

in studies performed on microwave-heated bean seeds.

MATERIALS AND METHODS

The study was carried out on bean seeds of Polish cultivar "Jaś" with moisture content of 5.61%. The seeds contained 22.93% of crude protein, 1.59% of crude fat and 3.02% of ash in dry matter. Determinations of moisture, crude protein, crude fat and ash content of seeds were carried out according to procedures recommended in Polish standards (three independent measurements were performed and mean values calculated were presented in Tables 1 and 2).

Ten samples (25 g each) were picked up from bean seeds, then 9 of them were placed (separately) in the geometrical centre of a microwave oven Vip 20 in order to subject them to microwave heating (radiation with working frequency of 2450 MHz), under conditions of one out of nine variants of processing intensity in which each of the three levels of radiation power (350, 500 or 650 W) corresponded to three durations of thermal processing (60, 120 or 180 s). The last, tenth sample remained unprocessed and was further referred in the text as a raw sample or a reference sample.

Analyses using bromocresole purple index method (BCPI) were performed according to the procedure postulated by Szmigielski [1999; 2004] (in the variant enabling the maximal test sensitivity, *i.e.* the concentration of bromocresole purple at the level of 0.13 mg/mL and acidity of the solution of 0.10 mol HCl/L). This analytical procedure involved: grinding the bean seeds and sieving them through 0.20 mm mesh, then transferring (separately) 100-mg portions of each sample (obtained through different processing) to 100 mL conical flasks to which 50 mL of bromocresole purple working solution (at the concentration of 0.13 mg/mL and acidity of 0.03 mol HCl/mL) were added, and finally stirring the mixtures using a magnetic stirrer for 30 min.

Afterwards, the content of the flask was centrifuged for 10 min (at 3000 rad/min), and next 1 mL of clear extract was transferred to the tube containing 20 mL of 0.02 mol NaOH, mixed and after 10 min absorbance of the solution was measured at 590 nm against distilled water as a reference.

The described procedure (BCPI) was applied for each of ten variants of bean seeds heating, in five independent measurements.

The mass of adsorbed active substance – bromocresole purple (equal to the value of the so-called bromocresole purple index – BCPI for each sample) was calculated as a difference between its quantity in the solution before and after contact with the ground sample.

Determination was carried out by the measurement of absorbance of bromocresole purple colour solution using the proportional relationship between its concentration and absorbance at the wavelength of 590 nm.

The quantity of dye adsorbed – S (mg/g), which equals the value of BCPI for each sample was calculated using equation (1), and expressed per protein mass unit in dry matter of seeds ($BCPI_{P.D.M.}$).

$$S = (E_o - E_b) \times C \times V / (E_o \times m) \quad (1)$$

where: E_o – absorbance of working solution; E_b – absorbance of extract; C – concentration of dye solution (mg/mL), (0.13 mg/ mL); V – volume of dye solution (mL) (50 mL); and m – weight of sample (g).

The determination of trypsin inhibitor activity (TIA) was performed using the classic method by Kakade *et al*. [1974] with some modifications postulated by Korol & Przegalińska [1994]. The method is based on the reaction of trypsin with a synthetic substrate N-α-benzoyl-DL-arginine-p-nitroanilide – BAPA. As a result of that reaction yellow p-nitroanilin is formed, and its maximum absorbance at 410 nm is proportional to its concentration. The calibration curve is plotted which describes the relationship between the absorbance of the solution (at 410 nm) and trypsin concentration, under optimal conditions for this enzyme.

The trypsin inhibitor activity is calculated on the basis of that calibration curve and is expressed as the quantity of trypsin, whose activity was inhibited (commonly called trypsin inhibitor activity unit – TUI) per milligram of dry matter of seed (for example beans).

According to the described procedure (TIA), the bean samples obtained as a result of 10 different processing variants were studied, in five independent replications.

The data obtained for each sample from the analysis of values of the traits examined (TIA and $BCPI_{P.D.M.}$) were subjected to a statistical analysis, involving calculation of mean value, standard deviation and coefficient of variation for those measurements.

The samples' distinguishability (ρ) was evaluated as the significance of differences between the mean values of each tested trait (TIA and $BCPI_{P.D.M.}$) for each sample, and expressed as the percent of the significant correlations in reference to all tested ones [Szmigielski, 1999, 2004; Szmigielski & Matyka, 2004]. The significance of differences of the results was established by analysis of variance method (at the significance level of 5%) and determining the lowest Tukey's differences – NIR [Oktaba, 2000].

Furthermore, on the basis of the results obtained, the coefficient of correlation (r) between the analysed traits was calculated (TIA, $BCPI_{P.D.M.}$), which enabled verifying the possibility of their correlation. When the coefficient turned out to be high, regression functions were established between experimental data obtained by BCPI and TIA /TIA = f(BCPI)/ methods and respective algorithms were used. The goodness of fit of the equations was assessed using a coefficient of determination (R^2) for each mathematical formula [Oktaba, 2000].

The time consumption (τ) of the performed analysis (BCPI, TIA) was estimated as the time necessary for making one replication of a test for one seed sample according to the adopted analytical procedure [Szmigielski, 1999, 2004; Szmigielski & Matyka, 2004].

RESULTS AND DISCUSION

The trypsin inhibitor activity (TIA-BAPA) of raw bean seeds (Table 1) was found to be at the level similar to the results reported by Gyori *et al*. [1998], *i.e.* at variability range of this antinutrient typical of the species and the stage of ripening. Worthy of notice is also that the extent of changes in trypsin inhibitor activity of raw, ripe bean seeds is very wide and, probably, dependent on genetic traits and weather conditions during the vegetative season.

The available literature data presents a wide range of results reported for the antitrypsin activity (from very high, comparable to that of soybean seeds [Szmigielski, 1999], through medium [Gyori *et al*., 1998], to hardly detectable [Pastuszewska, 1988; 1997 after Jasińska & Kotecki, 1999]).

TABLE 1. Trypsin inhibitor activity (TIA) of microwave-heated bean seeds ($TUI/mg_{D.M.}$).

Type of thermal processing	Parameters of thermal processing			
	Radiation power (W)	Thermal processing time (s)		
		60	120	180
Microwave heating	350	23.25±0.70 (3.02)*	23.02±0.56 (2.43)	22.93±0.91 (3.97)
	500	21.94±0.77 (3.51)	21.63±1.16 (5.36)	16.97±0.53 (3.12)
	650	20.85±1.80 (8.60)	17.48±1.62 (9.27)	7.81±0.23 (2.94)
Control (raw bean seeds)	23.89± 0.94 (3.93)			
NIR= 3.16				

*coefficients of variation of the results obtained were provided in parentheses (%).

The microwave heating significantly decreased the trypsin inhibitor activity of bean seeds. The values of TIA-BAPA were observed to decrease along with the increasing intensity of microwave heating, yet this descending tendency appeared to be regular for the heating variants of low intensity, for which most of the arithmetic means of results of determinations obtained for the extreme variants of microwave heating (at the adopted time or power of radiation) approximated the value obtained for the intermediate variant (Table 1). The increasing intensity of the heating process was understood as increasing the power of microwave radiation at the adopted time of sample exposure or as elongation of exposure time at the adopted radiation power, or as simultaneous elongation of the exposure time and increase of the power of microwave radiation applied.

For the variants of microwave heating with the highest intensity intended in the experimental procedure (*e.g.* a significant number of microwave heated samples with radiation power of 500 W and 650 W), that regularity of decreasing trypsin inhibitor activity was disturbed, which was undoubtedly reflected by their decreased distinguishability. The distinguishability of the samples was relatively low (ρ=51.11%, Tables 1 and 3). The TIA values obtained for intensively heated seeds turned out to be non-distinguishable. For those samples the trypsin inhibitor activity was reduced to a very low level. For that reason further microwave heating does not cause changes in TIA-BAPA corresponding to energy input, which consequently leads to diminished distinguishability of the samples and shows the TIA-BAPA method to be useless for their assessment. Similar conclusions were drawn on the basis of TIA-BAPA results for autoclaved and heated by unforced convection soybean seeds of Polan cultivar [Szmigielski, 2002], and based on analogous studies performed on bean seeds [Szmigielski & Matyka, 2002] and autoclaved chickling vetch seeds [Szmigielski & Matyka, 2004]. Only the analyses of microwave-heated soybean seeds [Szmigielski, 2004] demonstrated high 100% distinguishability at a significantly greater range of the results obtained.

It should be emphasized, however, that in the latter study the very low trypsin inhibitor activity was obtained only for samples subjected to microwave heating with the highest intensity (650W for 180 s).

The TIA-BAPA method, proved useless in the evaluation of the samples exposed to intensive heating, may yield material and economic losses that result from overheating the raw material and the resultant reduced availability of its nutrients during the production process. Experimental data obtained in this study indicate that results of the TIA-BAPA test for the intensively-heated samples should be subjected to in-depth analysis because the results obtained may in part diverge from the real properties of the product, while the discrepancy results from the specificity of the assay.

The analysis carried out by the TIA-BAPA method is additionally characterised by relatively low accuracy. It applies particularly to the results obtained for seeds subjected to intensive heating, for which the results noted were often comparable to standard deviation of the mean (Tables 1 and 3).

TABLE 2. Bromocresole purple index (BCPI) of microwave-heated bean seeds (mg/g protein $_{D.M.}$).

Type of thermal processing	Parameters of thermal processing			
	Radiation power (W)	Thermal processing time (s)		
		60	120	180
Microwave heating	350	68.44±2.93 (4.28)*	73.49±1.11 (1.51)	75.00±1.93 (2.57)
	500	71.46±1.94 (2.71)	78.68±1.92 (2.44)	124.26±1.92 (1,.55)
	650	76.03±1.11 (1.46)	97.54±1.91 (1.96)	156.97±1.90 (1.21)
Control	65.45±3.85 (5.88)			
NIR= 6.33				

*coefficients of variation of the results obtained were provided in parentheses (%).

TABLE 3. Time consumption, distinguishability, accuracy of determination, sensitivity, detectability of analytical methods used for the assessment of effectiveness of microwave heating of bean seeds.

Criteria of compared traits		Analytical method	
		$BCPI_{P.D.M.}$ (mg/g protein$_{D.M.}$)	TIA (TUI/mg)
Time consumption	τ (h)	1.5	2.5
Distinguishability	ρ (%)	71.11	51.11
Accuracy of determination	π (CV) (%)	1.21–5.88	3.02-9.27
Sensitivity	χ	0.16(*)	0.40(*)
Detectability – minimum detectable level	μ (min)	5.01(*)	10.32(*)

(*) – Data according to Szmigielski & Matyka [2002].

Similar conclusions were drawn by Szmigielski [2002] in a research on thermally-processed soybean seeds of Polan cultivar and in analogous studies performed on bean seeds [Szmigielski & Matyka, 2002] and on autoclaved chickling vetch seeds [Szmigielski & Matyka, 2004].

The value of bromocresole purple index ($BCPI_{P.D.M.}$) for raw bean seeds was similar to literature data [Szmigielski & Matyka 2002], being at the level characteristic for seeds of that species and for the ripening stage (however direct comparison between the previously obtained results requires expressing the results per mass of protein in dry matter of seeds).

The microwave heating (under conditions of the highest intensity of the process, *i.e.* radiation power of 650 W for 180 s) caused a considerable, almost twofold increase in the $BCPI_{P.D.M.}$ value compared to the raw seeds (Table 2). Changes in the $BCPI_{P.D.M.}$ values were, to a great extent, characterised by regularity of increasing tendency, correspondingly to the increasing intensity of seeds heating, which was confirmed by high distinguishability of the samples (ρ=71.11%, Tables 1 and 3), thus enabling their reliable identification. The increase noted in results of assays conducted with the $BCPI_{P.D.M.}$ method was found regular owing to the fact that most of the arithmetic results of the determinations obtained for the extreme variants of microwave heating (at the adopted time or power of radiation) approximated the value obtained for the intermediate variant (Tables 1 and 3).

Similar results were obtained in the studies carried out by Szmigielski [2002] on autoclaved and heated with unforced convection soybean seeds and autoclaved chickling vetch seeds [Szmigielski & Matyka, 2004] and heated bean seeds [Szmigielski & Matyka, 2002].

The characteristic trait of the determinations carried out with the $BCPI_{P.D.M.}$ method is their high accuracy and low time consumption (nearly twice lower than that of TIA-BAPA) which make them useful for quick, routine analyses conducted in industrial laboratories (Table 3).

Similar conclusions were drawn Szmigielski [2002] in a research on soybean seeds of Polan cultivar subjected to heating as well as in analogous studies with bean seeds [Szmigielski & Matyka, 2002], and chickling vetch [Szmigielski & Matyka, 2004].

A high correlation coefficient (r=0.9528, Table 4), calculated on the basis of experimental data obtained using TIA-BAPA and $BCPI_{P.D.M.}$ methods, indicates the linear correlation between results of those determinations conducted for microwave-heated bean seeds, and enables constructing mathematical formulas useful for mutual recalculations of results of those analysis. High correlation coefficients between the results of TIA-BAPA and $BCPI_{P.D.M.}$ determinations were also obtained for soybean, bean and chickling vetch seeds [Szmigielski, 2002; Szmigielski & Matyka, 2002, 2004].

TABLE 4. Application of bromocresole purple index (BCPI) for the assessment of trypsin inhibitor activity (TIA) of microwave-heated bean seeds (regression equation: TIA = f(BCPI).

Dependent variable	Independent variable	Correlation coefficient (r)	Type of equation		Equation	Determination coefficient (R^2) (%)
TIA	$BCPI_{P.D.M.}$	- 0.9528	Polynomial	IV°	$TIA = -0.027(BCPI)^4 + 0.5316(BCPI)^3 - 3.5591(BCPI)^2 + 8.4554(BCPI) + 17.908$	91.11
				III°	$TIA = -0.0617(BCPI)^3 + 0.7831(BCPI)^2 - 3.4113(BCPI) + 27.164$	85.47
				II°	$TIA = -0.2355(BCPI)^2 + 1.2866(BCPI) + 21.868$	79.92
			Linear		$TIA = -1.3042(BCPI) + 27.049$	90.72
			Exponential		$TIA = 29.951\ e^{-0.0819\ (BCPI)}$	54.78
			Logarithmic		$TIA = -4.549\ Ln(BCPI) + 26.747$	47.15
			Power		$TIA = 28.948(BCPI)^{-0.2759}$	36.39

Some of the suggested mathematical functions, prepared as regression equations TIA-BAPA = f($BCPI_{B.D.M.}$) (Table 4), are characterised by high determination coefficients, which indicates their high reliability and enables replacing the time-consuming and complicated TIA-BAPA method with a quick, simple and sensitive $BCPI_{P.D.M.}$ method useful in analyses of microwave-heated bean seeds.

CONCLUSIONS

1. Bromocresole purple index ($BCPI_{P.D.M.}$) turned out to be useful in describing changes in trypsin inhibitor activity – TIA of microwave heated bean seeds (determined with the BAPA method – the so-called TIA-BAPA).

2. Due to the specificity of quick, routine tests carried out in an industrial laboratory, the application of the simplest linear regression equation (TIA = -1.3042(BCPI) + 27.049; R^2 = 90.72) seems to be more justified, whereas its determination coefficient R^2 should assure the reliability of calculations.

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