EVALUATION OF BLUE VALUE IN DIFFERENT PLANT MATERIALS AS A TOOL FOR RAPID STARCH DETERMINATION

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(Received: February 4, 1991. Accepted: November 15, 1993)

ABSTRACT

In order to determine the concentration of starch in plant materials from the intensity of the blue iodine complex, it is necessary to know the Blue Value (B.V.), which is defined in this paper as the absorbancy of 100 mg of a starch-iodine complex in 100 ml of aqueous solution. An adequate amount of plant material is treated with a hot CaCl_2 solution for 1/2 hour and the solute is diluted to 25 ml with CaCl_2 . This basic solution serves to measure absorbancy, as well as for starch determination. The first measurement is done by the dilution of a proper amount of basic solution with water and after adding a diluted iodine-iodide solution the reading of B.V. is taken off. The second measurement is done by the precipitation of a starch iodine complex from a proper amount of the basic solution, which is then purified, destroyed by Na_2SO_3 solution, and starch is determined by the anthrone method. These two readings serve for the establishing of B.V. for the starch. Once established, B.V. can be used for starch determination in the proper plant material. A high degree of variation of the B.V. was found. The highest B.V. was obtained for wrinkled pea seeds (17.4); walnut, potato, smooth pea and pear gave values from 12.6 to 11.0, common bean and broad bean – 10.3 and 9.7, *Triticale*, carrot, rye, wheat and garden parsley from 8.7 to 8.0 and maize, oat, normal rice from 7.6 to 6.2. The B.V. for amylose was 25.3, for potato starch 12.4, soluble starch 11.9, wheat starch 8.8 and for *Triticale* and rye starches, 8.7.

KEY WORDS: Blue Value, starch determination, starch-iodine complex, starch in plant materials, starch dissolution in CaCl_2

INTRODUCTION

There are several methods of quantitative determination of starch in plant material. Some of them are adapted to certain species of plants, others can be used in materials with high starch content, and still others have been specially developed for microdetermination of this polysaccharide. Substances which interfere during determination can be a major obstacle in obtaining absolute results.

A simple, fast and inexpensive method is needed for routine determination. Such a method should be able to cope with a wide variety of plant materials and numerous samples that are to be assayed in a short time.

In this context it is interesting to discuss, first of all, the methods based on starch hydrolysis.

Acid hydrolysis has been used for many years both in scientific research and in industrial work. As main disadvantage is the need to remove the interfering substances (e.g., soluble sugars, phenols, etc) and to hydrolyze the starch under conditions ensuring complete hydrolysis of this polysaccharide. According to Banks et al. (1973) α -1, 4-bonds are broken down by 2 hours at 100°C with 0.75 M H_2SO_4 . In the case of α -1, 6-bonds, which are, as a rule, present in starch, hydrolysis should be extended to 3 hours. The procedure is thus a long one. According to Mitchell (1990), starches of different origin differ in terms of the completeness of acid hydrolysis.

On the other hand, enzymic hydrolysis is claimed to be selective. The main obstacle is to have the proper set of enzymes, for example a heat stable α -amylase, amyloglucosidase etc (see Rasmussen and Henry 1990). The enzymic procedure should be tested for each type of plant material to obtain quantitative results. The differences among various

enzymic procedures can be sometimes so great that the method cannot be accepted (Perez et al. 1990). In addition, enzymic procedures are time-consuming and expensive.

Perchloric acid is very often used for solubilization of starch. In this case it is necessary to remove low molecular sugars, but other polysaccharides can interfere in quantitative examination. Therefore, it is very often necessary to precipitate solubilized starch with an iodine solution, which complicates the procedure (see Rose et al. 1991). According to Matsui et al. (1990) cold HClO_4 does not solubilize starch quantitatively, so it was necessary to raise the temperature even to 80°C. The recovery of starch was 57% when using cold HClO_4 and 95% after hot extraction at 80°C.

Concentrated CaCl_2 solution has been used for many years for starch solubilization and is recommended by many authors (Whistler 1964, Sochocka 1969). It does not degrade starch molecules, so it can be used in colorimetric determinations with iodine. Preparation of such a solution and colorimetric readings of the blue complex are very fast and easy to perform. Therefore, we decided to try this procedure for quantitative starch determination in plant material.

In the classical work of McCready (1943) it was shown that starch, as well as its linear component – amylose, can be estimated quantitatively using a colorimetric procedure. Unfortunately, the iodine affinities for amyloses as well as amylopectins of different origin vary considerably. Thus, iodine affinity (expressed as mg I_2 absorbed by 100 mg of amylose) is highest for potato starch amylose and constitutes 21.2% (Singh and Nath 1956), much lower for tapioca amylose (19.7%) and quite low for *Sorghum* – 9.7% (Ohashi 1957). It is well known, too, that the ratio of amylose to amylopectin in granules of various starches is different. Thus, a

special standard curve, prepared on the starch extracted from the examined tissue is needed for every quantitative starch determination. To obtain pure starch for this purpose it is sometimes necessary to use a complicated procedure and the product can be degraded to some extent. Such degradation influences the colour intensity of the iodine complex.

The presence of complexing agents in some materials (e.g., fatty acids, proteins, vanillin etc, Rutgers 1955) can diminish absorbancy to a certain extent, interfering in the quantitative determination (Morrison and Langnelot 1983).

The colorimetric determination depends also on the method of preparing the starch solution. The concentration and the I₂ to KI ratio also influence colour intensity. The relationship between absorbancy and starch concentration is not linear in some methods and hence, does not obey the Lambert-Beer law. It is necessary to add that the complex is temperature-sensitive: the absorbance of the blue solution decreases with increasing temperature.

In this paper we have addressed the following question. How to avoid the above mentioned difficulties and develop a simple and rapid procedure for starch determination in plant materials? Our proposal is to use, first of all, the standard procedure for preparation of a starch solution from different plant materials and secondly, to determine the so-called Blue Value (B.V.), which will be used in the calculations. This can be done by determining the absorbancy of the iodine complex as well as the starch concentration in the same solution. Starch can be precipitated and purified as an iodine-iodide complex, and after it, the concentration of the polysaccharide can be determined by the anthrone method. Having these determinations (absorbancy of blue polysaccharide complex and starch concentration) one can complete the Blue Value. We have chosen a concentrated CaCl₂ solution used at elevated temperatures from the different reagents for starch solubilization. It does not degrade starch molecules and can also be used in the iodine precipitation of starch. We defined B.V. as the absorbancy of the starch-iodine complex at a starch concentration of 100 mg per 100 ml of a solution that has been obtained according to the standard procedure (see – Materials and Methods). Once established, the B.V. for a certain species or strain can be used to calculate the starch concentration from the absorbancy of starch-iodine complex.

MATERIALS AND METHODS

Plant material – Several samples of plant material with high and low starch content were used. In the case of materials with high water contents the analyses were performed both on the raw material as well as after removing the free water (drying in a draft oven at 60°C to obtain crispy material). Dried samples were ground and sieved through a 60-mesh sieve (= 0.089 mm). Fresh material was homogenized with an excess of distilled water in a UNIPAN homogenizer to obtain a slurry, which was then centrifuged off and washed three times with distilled water. The wet „cake” was used for preparation of the starch solution.

The experiments were also performed with chemically pure amylose (Jansen, Belgium, 150 000 D) and with starches obtained from potato tuber (Superior quality), rye, wheat and Triticale (obtained by courtesy of Dr. H. Nowotny, University of Agriculture, Kraków). Soluble starch according to Lintner (Polskie Odczynniki Chemiczne, Gliwice) was also used.

Basic reagents – The CaCl₂ solution was prepared according to Lau (1988): 300 g CaCl₂ · 2 H₂O was diluted with 350 ml H₂O and after filtration the pH was brought to 2.5 ml with

0.2 M HCl. Overtitration can be corrected with an 0.2 M NaOH solution.

The diluted iodine contained 0.200 g I₂ and 2.000 g KI in 100 ml of water.

The concentrated iodine solution for starch precipitation contained 3 g I₂ and 3 g KI in 100 ml water.

Statistics – All of the statistical calculations were based on pseudo-test t (u-Lord's test) (Lord 1947).

Basic starch solution – The sample (0.1 to 0.5 g of material with a high and low starch content) was transferred to a 50 ml centrifuge tube and low molecular substances were extracted with cold distilled water (10 minutes extraction with 10 ml H₂O, washing twice with 5 ml of distilled water). Twenty mls of CaCl₂ solution were added to the precipitate and the mixture was heated in a boiling water bath for 30 minutes, occasionally mixed with a glass rod. The heating was prolonged to 60 minutes in same experiments. After cooling to 20°C the mixture was centrifuged and the supernatant containing solubilized starch was transferred to a 25 ml volumetric flask. The precipitate was washed with 5 ml CaCl₂ solution and the supernatant transferred to the same flask. The thoroughly mixed solution was filtered to a conical flask and used for starch determination by the anthrone method and for developing of blue colour with the iodine-iodide solution.

Development of the blue colour – One half ml of the basic solution was transferred to a 50 ml volumetric flask, which was filled with about 40 ml distilled water. After thorough mixing, 0.5 ml of diluted iodine solution was added and the volume was made up to 50 ml with distilled water. If colour intensity is below 0.1 absorbancy, more starch solution can be added, even up to 2.5 ml to obtain optimal absorbancy (about 0.4-0.6). If the readings are too high, the basic solution can be diluted with the CaCl₂ solution. After 5 minutes the readings are taken up a 1 cm cuvette at 620 nm (SPEKOL photometer) against a blank. It is important to keep the temperature of the solution at about 20°C.

Starch precipitation – Five ml of basic starch solution were transferred to a 25 ml centrifuge tube, 1 ml of concentrated iodine solution was added and mixed with a glass rod. After 15 minutes on an ice bath the mixture was centrifuged, the precipitate washed twice with 5 ml of 80% ethanol. The excess of iodine was decomposed with a few drops of 0.5% Na₂SO₃ in 10% NaCl, washed again twice with 5 ml 80% EtOH, decomposed with a few drops of 0.5% Na₂SO₄ in 10% NaCl, washed twice with 5 ml 80% EtOH and warmed in a boiling water bath after adding 10 ml of 1 M H₂SO₄. When the starch was completely dissolved, the solution was cooled and diluted with water to 50 ml. After appropriate dilution of this solution with distilled water the starch concentration was determined by the anthrone method.

Starch determination with anthrone – One ml of diluted starch solution (0.6-3.0 mg/100 ml) was mixed in a test tube with 2 ml of anthrone reagent (0.1% solution in concentrated H₂SO₄) in a cold water bath using a glass rod, flattened at the end. The test tube was heated in a boiling water bath for 10 minutes, cooled under running water and the reading was taken at 625 nm against a blank in 1 cm cuvette. The reading was compared with 2 mg/100 ml glucose (analytical grade) and the result converted to starch concentration using the factor 0.9.

Calculation of B.V. – The following example explains the calculation of B.V. The standard procedure was used to obtain a starch solution from 0.2 g dried and powdered smooth pea seeds (basic starch solution). Blue colour was developed

after 100-fold dilution with water of 0.8% basic solution. The absorbancy of this 0.008% solution was 0.405.

Five ml of basic solution were used for starch precipitation and purification. The basic solution was also diluted 100-fold in this case (0.008%). The starch concentration in this solution was 3.44 mg/100 ml. Hence, in 100 mg/100 ml starch solution the absorbancy is 11.8 = B.V.

The absorbancy of 8 mg/100 ml (= 0.008%) solution of smooth peas was 0.405, hence 100 mg/100 ml solution gives an absorbancy of 5.062. 100 mg/100 ml starch solution equals 11.8 absorbancy, so 5.062 absorbancy gives 42.9 mg per 100 mg of pea seeds, which is 42.9% starch.

RESULTS

The effect of temperature – Potato starch prepared according to the basic procedure was used in the experiments on the influence of temperature on the intensity of the blue colour of the complex (Table 1). There is a reciprocal linear relationship between temperature and colour intensity between 10 and 40°C. The average difference is 0.019 per 5°C, which means that the temperature of the solution has a substantial influence on the readings. Our experiments confirmed those of Morrison and Langnelot (1983).

TABLE 1. The effect of temperature on colour intensity of the blue complex of potato starch solution

Temperature °C	10	15	20	25	30	35	40
Absorbancy	0.470	0.445	0.430	0.410	0.385	0.375	0.355
5°C difference	0.025	0.015	0.020	0.025	0.010	0.020	
Average difference per 5°C			0.019				

Proportionality of blue color intensity – Three preparations of starch: soluble, potato and *Triticale* were used to check the proportionality of blue colour intensity. Basic starch solutions were taken in three different amounts: 0.5, 1.0 and 1.5 ml and were diluted to 50 ml with distilled water. Full proportionality was observed in every case, and the solutions obeyed the Lambert-Beer's law (Table 2).

Additional CaCl₂ was added to the second series of the above solutions: 2 ml to the lowest concentration, 1.5 ml to the intermediate one, and 1 ml to the highest concentration. There was no change in the absorbancy of the solutions with and without added CaCl₂. This means that CaCl₂ does not influence colour intensity in this procedure.

TABLE 2. Colorimetric readings of the blue colour intensity of starch solutions

Mls of basic solution in 50 ml	Soluble starch		Potato starch		Triticale starch	
		calculated per 1 ml		calculated per 1 ml		calculated per 1 ml
0.5	0.215	0.430	0.178	0.357	0.165	0.330
1.0	0.433	0.433	0.353	0.353	0.325	0.325
1.5	0.650	0.433	0.530	0.358	0.492	0.328

Influence of the pH of the CaCl₂ solution – The pH of the CaCl₂ solution was checked for its effect on colour intensity, using potato and soluble starch. Four different pH values were tested: 1.0, 1.5, 2.0, 2.5. The heating time was 0.5 hour. The lowest results for absorbancy and B.V. were seen at pH 1.0 (Table 3). At pH 1.5, 2.0 and 2.5 the readings were higher than for pH 1.0 but they did not change substantially. From the statistical point of view even the lowest values in the series (pH = 1.0) cannot be accepted as a gross error. The conclusion is that a pH ranging from 1.0 to 2.5 does not influence the results of B.V. determination, but it is better to keep the pH not lower than 1.5. All of the solutions of potato starch had a deep blue colour, while the solutions of soluble starch were violet-blue. It is supposed that lintnerization partly degrades starch molecules, but this process does not change iodine affinity in the proposed procedure.

TABLE 3. Influence of pH of CaCl₂ solution during solubilization of starch sample. Starch concentration = 0.4%, dilution = 100 times.

Starch		pH of CaCl ₂ solution			
		1.0	1.5	2.0	2.5
Potato Soluble potato	absorbancy	0.397	0.464	0.487	0.470
	absorbancy	0.423	0.446	0.466	0.458
Potato Soluble potato	absorbancy	9.92	11.51	12.17	11.75
	absorbancy	10.57	11.15	11.11	11.45

Blue Value of amylose and various starches – The blue values of amylose and potato, soluble potato, wheat, *Triticale* and rye starches were compared (Table 4). The highest B.V. was found for pure amylose (25.3), intermediate – for potato (12.4) and soluble potato (11.9) and low values for cereal (normal) starches (8.7-8.8). The precision was typical for colorimetric measurements.

TABLE 4. Blue Value of amylose, soluble potato, potato, wheat, rye and *Triticale* starches as determined by standard procedure

Starch	Blue Value	Standard deviation for 4 parallel determinations	Coefficient of variation (%)
Amylose	25.3	0.07	0.29
Potato	12.4	0.12	0.98
Soluble potato	11.9	0.11	0.92
Wheat	8.8	0.07	0.83
<i>Triticale</i>	8.7	0.097	1.12
Rye	8.7	0.05	0.56

Ageing and colour intensity – According to our earlier investigations (Samotus 1961) some degree of starch disaggregation occurs after prolonged storage of starch preparations. Therefore, we compared two samples of potato starch: freshly prepared from young potatoes and a preparation extracted from young potatoes 40 years ago. In both cases we obtained almost the same B.V.: 12.4 for freshly extracted starch and 12.0 for old starch.

Removal of soluble constituents – The removal of low molecular constituents is time-consuming and, in the case of starch determination, some losses of starch can take place. So, it is interesting to compare the absorbancy of the samples before and after such a procedure. Four plant materials were used in this experiment: dried apple, dried pear, dried carrot and wrinkled pea. The pea and carrot tissues were extracted with distilled water only, apple and pear tissues were extracted with 20 ml of 80% EtOH and twice with 20 ml of distilled water. No difference was found in absorbancy when comparing the samples before and after removal of low molecular substances (Table 5). However, in this paper we have chosen the more laborious procedure to avoid the risk of overestimating the starch concentration using the anthrone method. Even some negligible impurities of the sample by sugars other than starch could falsify the results.

TABLE 5. Absorbancy of starch-iodine solution before and after removal of low molecular substances

Plant material	Absorbancy	
	Before treatment	After treatment
Wrinkled pea	0.490	0.485
Carrot	0.310	0.310
Apple	0.200	0.200
Pear	0.300	0.310

Heating duration – The plant material used in this paper was analyzed after 0.5 hour as well as after 1 hour of heating in a boiling water bath. The B.V.'s obtained in these two series were identical. However, in the case of wrinkled pea, starch extraction was better after 1 hour heating than after 0.5 hour. The difference was significant (24.97 and 28.77%). It is supposed that the solubilization process is difficult in the presence of starch-protein complexes in pea tissue, thus, prolonged time of heating facilitates starch extraction.

TABLE 6. Blue Values of different plant materials

No	Plant tissue	Blue Value	Standard deviation for 4 parallel determinations	Coefficient of variation, %
1.	Wrinkled pea (seed)	17.4	0.24	1.38
2.	Walnut (seed) defatted	12.6	0.25	1.98
3.	Potato (tuber)	11.9	0.21	1.76
4.	Smooth pea (seed)	11.8	0.24	2.03
5.	Pear (fruit)	11.1	0.25	2.25
6.	Apple (fruit)	11.0	0.25	2.27
7.	Common bean (seed)	10.3	0.24	2.33
8.	Broad bean (seed)	9.7	0.25	2.58
9.	Triticale (meal)	8.7	0.24	2.76
10.	Carrot (root)	8.6	0.25	2.91
11.	Rye (meal)	8.5	0.25	2.82
12.	Wheat (meal)	8.5	0.25	2.94
13.	Garden parsley (root)	8.0	0.25	3.11
14.	Maize (meal)	7.6	0.25	3.29
15.	Oat (flakes)	7.3	0.25	3.49
16.	Rice (normal) (seed)	6.2	0.24	3.87
Average		9.95	0.24	2.61

Blue Values of different plant materials – The results of B.V. are presented in Table 6 according to decreasing values. One can observe various B.V.'s for the examined plant material: from 17.4 (wrinkled pea) to 6.2 (rice). A certain correlation can be seen between B.V. and amylose content in the starch. High-amylose wrinkled pea seed, which is constituted by 64.7 to 66% amylose (Larson and Gillio 1953, Potter et al. 1953) has the highest B.V. Smooth pea seed, examined by the same authors has a much lower amylose content (35%) and B.V. is also lower in our experiments. Normal rice is 18.5% amylose (Anderson and Greenwood 1955) and its B.V. is three times lower (6.2) in comparison to wrinkled pea. It should be stressed, however, that this coincidence can be accidental as it is seen, e.g., on potato tuber starch (B.V. = 11.9), which, in this case, should give twice as low a B.V. in comparison to wrinkled pea starch, because its amylose content, according to literature data (Anderson and Greenwood 1955) is 21-22.9%.

In the case of walnut seed removal of fatty constituents was necessary (8 hour extraction in a Soxhlet apparatus) and the B.V. reached values as high as 12.6. This value is comparable to potato tuber starch in our experiments.

The standard deviation and coefficients of variation were higher in the case of B.V. determined in plant material than in the case of pure starches (Table 4). This is understandable, because the analytical procedure is more complicated than in the case of B.V. determination of pure starches. However, the precision is sufficient for colorimetric analysis.

CONCLUSIONS

It was shown that the intensity of the blue-iodine complex can be used to overcome the disadvantages that appear during starch determination in plant material. A standard procedure was developed by which different plant materials can be examined in a simple and fast way. The main points can be summarized as follows:

- 1) Plant material can be prepared for starch determination by solubilizing it in a hot CaCl_2 solution. This reagent does not degrade starch molecules, can be used directly for colorimetric readings after iodine complexing, and can serve for starch determination with the anthrone method. That last step is required for determining the B.V. Having the B.V. for the pertinent plant material, one can quickly determine the starch concentration colorimetrically.
- 2) The determination of blue colour intensity should be done at a fixed temperature (e.g., 20°C), because the complex is temperature-sensitive.
- 3) The conditions of blue colour development permit the use of a constant B.V. for a given plant species, which is not possible in other methods previously presented (compare for example: Whistler 1964).
- 4) It was also found that the pH of the CaCl_2 solution is not as important a factor as was stressed in some papers (e.g., Lau 1988). However, it is advisable to keep the pH between 1.5-2.5.
- 5) The process of starch ageing, which changes the colloidal properties of this polysaccharide (especially in the case of potato starch, does not change the colour intensity of the complex.
- 6) Removing solubles from the tissue is not a necessary step before solubilization of the material in hot CaCl_2 . However, some constituents (e.g., fatty substances) can interfere in the determination. This problem will be addressed in further investigations.

- 7) It was found that 0.5 hour heating with CaCl_2 is sufficient, but in some cases it is advisable to extend the time to 1 hour.
- 8) The method allows the starch to be determined in materials with both high and low starch concentrations, thus it can be used for a very wide range of plant material.

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OSZACOWANIE NIEBIESKIEGO INDEKSU W RÓŻNYCH MATERIAŁACH ROŚLINNYCH, NIEZBĘDNEGO DO SZYBKIEGO OZNACZANIA SKROBI

STRESZCZENIE

Celem oznaczenia stężenia skrobi w materiałach roślinnych na podstawie intensywności niebieskiego kompleksu z jodem należy znać tzw. niebieski indeks (B.V.), który został zdefiniowany w tej pracy jako absorbancja 100 mg kompleksu jodo-skrobiowego w 100 ml wodnego roztworu. Określoną ilość materiału roślinnego traktuje się gorącym roztworem CaCl_2 przez 1/2 godziny, a solut rozcieńcza się do 25 ml tym samym chlorkiem wapnia. Ten podstawowy roztwór służy do pomiaru absorbancji, a także do ilościowego oznaczenia skrobi. W pierwszym przypadku odpowiednią ilość roztworu podstawowego zadaje się wodą destylowaną, dodaje rozcieńczonego roztworu jodu w jodku potasu i oznacza B.V. fotometrycznie. W drugim zaś, wytrąca się kompleks jednoskrobiowy z odpowiedniej ilości roztworu podstawowego, uwalnia od płynu macierzystego, rozkłada kompleks za pomocą Na_2SO_3 , a uwolnioną skrobię oznacza metodą antronową. Te dwa odczyty pomiarowe służą do wyznaczenia B.V. dla danego rodzaju skrobi. Otrzymany w ten sposób B.V. służy do oznaczania skrobi w danym materiale roślinnym. Otrzymano znaczne zróżnicowanie wartości B.V.; najwyższą wartością cechowała się skrobia otrzymana z nasion grochu zmarszczonego (17.4), skrobie z orzecha włoskiego, ziemniaka, gładkiego grochu i gruszki wykazały wartości od 12.6 do 11.0, zwykła fasola i bób dały wartości od 10.3 do 9.7, *Triticale*, marchew, żyto, pszenica i pietruszka ogrodowa pomiędzy 8.7 do 9.0, natomiast kukurydza, owies, zwykły ryż od 7.6 do 6.2. Wartość B.V. dla amylozy wyniosła 25.3, dla skrobi ziemniaczanej 12.4, skrobi rozpuszczalnej 11.9, pszennej 8.8, a dla *Triticale* i ryżu 8.7.

SŁOWA KLUCZOWE: Niebieski Indeks (B.V.), oznaczanie skrobi, kompleks jednoskrobiowy, skrobia w materiałach roślinnych, rozpuszczanie skrobi w CaCl_2