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EFFECT OF LIPIDS ON STARCH DETERMINATION THROUGH VARIOUS METHODS

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This study investigated the influence of lipids on amylose, amylopectin and total starch content in barley. Comparisons of various methods of single-wavelength colorimetric procedure (SWC) for amylose, dual-wavelength colorimetric method (DWC) for amylose, amylopectin and total starch, and polarimetric method by calcium chloride dissolution (PCC) for total starch content were conducted as well in barley. The results showed that defatted samples measured by SWC had higher amylose than non-defatted ones ($P<0.05$). Several samples exhibited significant difference for amylose content when defatted and non-defatted samples were analyzed by DWC ($P<0.05$). However, for amylopectin, the defatted samples determined by DWC were significantly lower than that of non-defatted ones ($P<0.05$). The total starch of defatted samples measured by DWC was much lower than those with fat ($P<0.05$). According to various methods, the amylose contents of defatted samples measured by SWC were higher than those measured by DWC ($P<0.05$). The non-defatted samples measured by PCC had lower starch than those measured by DWC, but had higher starch than the defatted samples measured by DWC ($P<0.05$). Collectively, defatting should not be omitted in determining starch content, and DWC is a suitable method for the measuring of barley starch.

Keywords: Barley, starch, lipid, dual-wavelength colorimetric method, amylose, amylopectin

INTRODUCTION

Barley (*Hordeum vulgare* L., 2n=14) is the fourth major cereal crop produced in the world, and has been utilized mainly for malting and brewing and as animal feed (Kling, 2004). Starch, the main constituent of barley grains (60-64% of kernel dry weight), is the main object of saccharification and thereby affects the quantity and quality of beer (Borém *et al.*, 1997). Starch granules consist of two types of glucose polymers, the essentially linear amylose and the highly branched amylopectin, in a ratio ranging 20–30%/70–80% (Fukunaga *et al.*, 2002; James *et al.*, 2003; Zeeman *et al.*, 2007). High-amylose starches can be processed into 'resistant starch', which is the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals and has nutritional benefits (Bird *et al.*, 2000). In addition, they are also used in adhesive products and in the production of corrugated board and paper (Jobling, 2004). Amylopectin can be used as a stabilizer and thickener in food products and as an emulsifier for salad dressings (Jobling, 2004). Moreover, the ratio of amylose to amylopectin in barley endosperm is one of the key factors in determining malting and food and feed quality (Bhatty, 1993; Swanston *et al.*, 1995). Recently, with the release of the waxy barley in the world, the determination of amylose contentin barley became an important issue for breeders and industries (Jansen *et al.*, 2012).

Up to now, many procedures have been reported in starch analysis, such as polarimetric by calcium chloride dissolution (PCC) (Clendenning, 1942), by acid hydrolysis (Ewers, 1908), various enzymatic methods (Thivend *et al.*, 1965), and several others. PCC was evaluated as one of the most reliable and reproducible method for total starch content (Mitchell, 1990). For the determination of amylose, various methods have also been developed in the last decades including single-wavelength colorimetric (SWC) procedure (Chrastil, 1987), multi-wavelength colorimetric (MWC) method (Jarvis and Walker, 1993; Séne *et al.*, 1997), high-performance size-exclusion chromatography; HPSEC (Grant *et al.*, 2002), differential scanning calorimetry; DSC (Mestres *et al.*, 1996), enzymatic (Riley *et al.*, 2009), near-infrared reflectance spectrophotometry: NIR (Bao *et al.*, 2007), thermogravimetric: TG (Stawski, 2008), and orthogonal-function spectrophotometry: OFS (Wang *et al.*, 2011). However, all of these methods suffer from some drawbacks. For example, the accuracy of SWC is limited by the interference of amylose with lipids (McCready and Hassid, 1943; South *et al.*, 1991), moreover, the fatty acids are hydrophobic molecules and can be fixed within the coil of the amylose double-helix and thus reduce its iodine-

binding capacity (Martinez and Prodolliet, 1996; Hoover and Ratnayake, 2001). HPSEC is a costly method while MWC is a complicated procedure. Dual-wavelength colorimetric (DWC) method (Hovenkamp-Hermelink *et al.*, 1988) was found to be the most precise and most generally applicable a method in detecting amylose content when compared to DSC, HPSEC, and the enzymatic method (Zhu *et al.*, 2008). In addition, the DWC method can also be used to simultaneously determine amylopectin content (Hovenkamp-Hermelink *et al.*, 1988). Due to these advantages, DWC has been widely used and developed for the determination of starch. Although above-mentioned methods have been exploited to determine starch content of various cereals, few of them are applied specially to determining barley starch. It is reported that lipids content of barley with various amylose contents range from 4.7 to 6.8% (Li *et al.*, 2001). Our knowledge that whether lipids affect the measurement of barley starch is limited.

In this study, employing thirteen barley lines, our objectives are to(1) investigate the effect of fat on the estimation of amylose, amylopectin and total starch content; (2) compare several methods of determination of starch.

MATERIALS AND METHODS

***Materials, apparatus and reagents*:** Thirteen barley lines (Table 1) were grown in the field in October, 2010 and harvested in June, 2011. The accessions with GSHO, CIho and PI numbers were provided by USDA-ARS (http://www.ars-grin.gov) (Ma *et al.*, 2014). NA40 (CDC Candle) was from Canada and deposited at Triticeae Research Institute, Sichuan Agricultural University, China. Among them, GSHO 908, GSHO 1828 and NA40 are waxy barley (low amylose content) (Ma *et al.*, 2010; Ma *et al.*, 2013). Total starches of samples were isolated in our lab (Triticeae Research Institute). Both amylose (Type III) and amylopectin extracted frompotato were purchased from Sigmacompany. All other solvents and reagents were of analytical grade. Instrument used were UV-visible spectrophotometer (model UV-1800, Shanghai MAPADA Co., China) with six 1-cm matched quartz cells and automatic polarimeter (model WZZ-1, Shanghai Yice Apparatus & Equipments Co., China). All weighing was done on Sartorius analytical balance (model BS 124 S, Sartorius Mechatronics Co., LTD, Beijing).

***Moisture content determination*:** Moisture contents were determined according to Approved Method 44-15A(AACC, 2000).

***PCC in determining total starch*:** The contents of barley total starch were determined by PCC modified from Clendenning and Wright (1945). Weighed 2 .0 gof the dry 140 mesh gluten into a 250-mL conical flask. Added10mL calcium chloride solution (specific gravity was adjusted to 1.3, pH 2.3 byacetic acid) to wet and resolve the sample, then another 50 mL of calcium chloride solution was added to accelerate the decomposition of the sample. Immediately whipped the contents of the flask gently until lumps were completely disintegrated and a uniform suspension was obtained. Afterwards, the solution was steadily boiled for 25 min and then cooled to room temperature in running tap water. The contents of the flask were then transferred into a 100 mL volumetric flask containing 1 mL 30%zinc sulfate solution and 1 mL 15% potassium ferrocyanide solution. Destroyed any foam by adding two drops of 95% ethyl

Table 1. Amylose, amylopectin and starch content* (% dry base) of 13 defatted and non-defatted samples by SWC, DWC and PCC

Accession	SWC (amylose content)		DWC (amylose content)		DWC (amylopectin content)		DWC (starch content)		PCC (starch content)
	Non-defatted	Defatted	Non-defatted	Defatted	Non-defatted	Defatted	Non-defatted	Defatted[f]	Non-defatted
GSHO 754	8.76 ± 0.00	47.27 ± 0.84#	23.95 ± 0.24#	22.84 ± 0.04§	43.09 ± 1.02#	12.65 ± 0.49	67.04 ± 0.82#	34.77 ± 0.52†	53.95 ± 0.01‡
GSHO 1853	nd	0.60 ± 0.07	9.83 ± 0.24	8.91 ± 0.18§	25.16 ± 0.80	25.86 ± 0.35	34.99 ± 1.00	34.06 ± 0.48†	25.21 ± 0.02‡
GSHO 1957	nd	13.28 ± 0.6	12.64 ± 0.14#	16.37 ± 0.11§	35.77 ± 0.68#	19.81 ± 1.44	48.41 ± 0.82#	35.69 ± 1.34†	38.83 ± 0.02
GSHO 2468	nd	24.79 ± 0.13	18.7 ± 0.13	18.82 ± 0.14§	37.06 ± 0.84#	17.65 ± 0.86	55.75 ± 0.85#	35.78 ± 0.71†	44.14 ± 0.02‡
GSHO 753	nd	----	11.74 ± 0.00#	13.50 ± 0.15§	23.77 ± 0.59#	17.27 ± 1.06	35.51 ± 0.59#	31.30 ± 1.20†	25.89 ± 0.03‡
GSHO 1852	5.68 ± 0.13	33.08 ± 0.31#	24.4 ± 0.14#	25.83 ± 0.15§	47.33 ± 0.67#	19.78 ± 0.57	71.73 ± 0.81#	45.43 ± 0.68†	51.27 ± 0.01‡
GSHO 1975	2.77 ± 0.20	13.00 ± 0.29#	20.16 ± 0.34	21.47 ± 0.38§	41.80 ± 0.82#	22.11 ± 1.12	61.96 ± 0.99#	45.24 ± 0.94†	48.27 ± 0.01
GSHO 2474	1.32 ± 0.34	3.35 ± 0.94#	15.95 ± 0.00	16.55 ± 0.16§	32.26 ± 0.46#	10.77 ± 1.28	48.21 ± 0.46#	28.20 ± 1.33†	36.79 ± 0.02‡
GSHO 908	nd	nd	5.98 ± 0.08#	4.81 ± 0.09§	22.96 ± 0.23#	20.67 ± 0.57	28.94 ± 0.24#	25.27 ± 0.64†	49.62 ± 0.02‡
GSHO 1828	nd	nd	6.65 ± 0.05#	6.15 ± 0.08§	25.96 ± 0.62#	19.07 ± 0.69	32.60 ± 0.66#	24.27 ± 0.67†	56.13 ± 0.01‡
CIho 15773	13.12 ± 0.13	39.09 ± 0.97#	24.69 ± 0.12#	27.67 ± 0.15§	49.07 ± 0.59#	14.03 ± 0.00	73.76 ± 0.54#	41.55 ± 0.15†	55.81 ± 0.01‡
PI 483237	10.51 ± 0.39	39.67 ± 1.36#	24.11 ± 0.31#	26.56 ± 0.11§	43.71 ± 0.83#	16.73 ± 0.41	67.81 ± 1.04#	42.81 ± 0.33†	58.86 ± 0.02‡
NA40	nd	nd	4.93 ± 0.14#	3.07 ± 0.25§	19.16 ± 1.07#	13.13 ± 0.20	24.09 ± 0.98#	15.85 ± 0.31†	51.14 ± 0.02‡

*: The contents were expressed as mean ± standard deviation; nd: not detected; ----: sample was not enough to be tested; #: significant difference (P<0.05) was detected between defatted and non-defatted values for a given sample; §: significant difference (P<0.05) was detected between amylose contents measured by DWC and SWC; †: significant difference (P<0.05) was detected between starch content by DWC (defatted) and PCC (non-defatted); ‡: significant difference (P<0.05) was detected between starch content by DWC (non-defatted) and PCC (non-defatted).

alcohol. The volume was made accurately with the calcium chloride solution, shaken by hand vigorously for three minutes and allowed 10 mL to run through a fluted 15 cm Whatman No. 12 filter paper, taking care to wet the whole filtering surface thoroughly. Discarded this first filtrate, and then filtered the remainder of the solution without suction. Polarize the crystal clear filtrate in a 2 dm tube. The starch content of the crude gluten was calculated from the formula, where α=observed optical rotation in degrees, L= length of tube (dm), W= weight of samples (g), H = moisture content of samples (%), and 203 is the specific rotation.

$$\frac{\alpha \times 10^6}{LW(100-H)\times 203} = \%\,\text{starch}$$

***SWC in determining amylose*:** Total amylose contents from barley starch were determined using a traditional single-wavelength method by described the Zhu *et al.* (2008) with minor changes. Firstly, samples were prepared according to the following procedure: Isolated starches were defatted for 4 h using methanol and a Soxhlet extraction apparatus. Then the defatted starches were kept in an oven at 30-50ºC to a constant weight. Defatted starches containing 100.0±0.1 mg of dry substance were weighed and transferred into a 100 mL volumetric flask. Ethyl alcohol (1 mL) was added to wet the sample. Then 9 mL of 1mol/L sodium hydroxide solution was added, dissolved by heating for 15 min in a boiling water bath. This solution was cooled as soon as it dissolved. Then it was diluted to volume (100 mL) with distilled water. Then we measured the amylose content of the prepared samples as follows. The starch extraction dilution (5 mL) was pipetted to another 100-mL volumetric flask. Water (≈50 mL), 1mL of 1mol/L acetic acid, and 1mL of 0.2% iodine solution (2.0 g of potassium iodide and 0.2 g of iodine diluted to 100 mL with distilled water) were added in sequence and mixed well (final pH≈3.5). The flask was filled to volume (100 mL). This final solution was allowed to sit for 25 min for development of the color. The absorbance of a sample of this solution in a 1-cm path length quartz cell was measured at 620 nm against the blank (distilled water).

Finally, the standard curve was plotted as follows. 1 mg/mL of amylose and amylopectin standard solution were made according to the method mentioned above. Then the standard curve was plotted for mixtures of amylose and amylopectin solution containing 0, 5, 10, 20, 30, and 40% amylose. Standard curve was created using % amylose as the *x*-axis and absorbance as the *y*-axis. The regression equation was: $y = 0.0086x + 0.2225$ ($R^2 = 0.9996$, n=6).

***DWC in determining amylose and amylopectin*:** Amylose and amylopectin contents of barley samples were measured by the dual-wavelength colorimetric method revised from Jain *et al.* (2010) and Zhu *et al.* (2008). The detailed procedure was as follows. At first, we plotted the absorption spectrum of amylose and amylopectin using the method as below. 1 mg/mL of amylose and amylopectin standard solution were obtained as mentioned in the previous subsection. Subsequently 2.6 mL and 4.0 mL of 1 mg/mL of amylose and amylopectin, respectively, were pipetted to a 100-mL beaker respectively. Then, 25 mL distilled water was added, and the pH was adjusted to 3.5 by the addition of 0.1 mol/L of hydrochloric acid, and for the higher pH levels by addition of 1 mol/L sodium hydroxide. The reason for adjusting pH to 3.5 is that the measurement value of starch is much steadier under this acid and alkali circumstance (Dai *et al.*, 2008). Lastly, the solution was transferred to a 50 mL volumetric flask; 0.5 mL of 0.2% iodine solution was added and then the flasks were filled to volume with distilled water. This final solution was allowed to sit 25 min for development of the color. The solutions were subsequently scanned through the visible and short-wave near infrared regions (400~1000 nm at 20-nm intervals) against the blank (distilled water). The absorption spectrum was drawn in a spreadsheet using Microsoft Excel (Fig. 1).

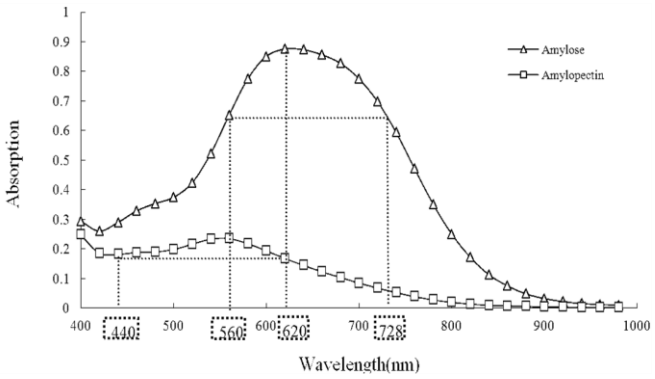

Figure 1. Absorption spectra of iodine complexes
Dotted lines illustrate the process of selection of wavelengths by drawing. Virtual box denotes the selected wavelengths used in DWC.

Wavelengths of amylose and amylopectin were obtained by drawing according to the testing principle of dual-wavelength colorimetric method described by Jain *et al*. (2010). The wavelengths of 620nm and 440nm were selected for the determination of amylose, and 560nm and 728nm were selected for measuring amylopectin.

We then plotted the standard curve of amylose and amylopectin as follows. According to the method mentioned above, the amylose standard series were obtained containing 0.0054, 0.012, 0.02, 0.028, 0.036, 0.044, and 0.052 mg/mL amylose, and the amylopectin standard series containing 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, and 0.2 mg/mL amylopectin were prepared. The standard curves were created using the content of amylose (amylopectin) as the *x*-axis and the difference of absorbance at 620nm and 440nm (560nm and 728nm) as the *y*-axis. The regression equation was: $y = 12.853x - 0.0880$ ($R^2 = 0.9996$, n=7) (for the calculation of

amylose content), and $y = 2.6179x + 0.0335$ ($R^2 = 0.9926$, n=7) (for the calculation of amylopectin content).

The contents of amylose and amylopectin were determined according to the following method. Samples were prepared as the method in previous section ("SWC in determining amylase"). Then 5 mL of the starch extraction dilution was used to measure the contents of amylose and amylopectin in accordance with the method in this section. The starch content was expressed as amylose plus amylopectin. When the starch content of non-defatted samples was determined, the procedure was the same as that for defatted samples except that the defatting step with ethanol was omitted.

***Statistical analysis*:** We evaluated the precision of a method by calculating the standard deviation (SD) (Jain *et al.*, 2010). We conducted triplicate measurements to determine amylose, amylopectin and starch, and the results were given as the mean± SD. Statistical analysis was performed using Microsoft Excel and t-test using SPSS 11.0 (SPSS Inc., Chicago, IL) for windows. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

***Effect of fat on amylose, amylopectin and total starch*:** The results of the contents for the amylose, amylopectin and total starch in the 13 samples analyzed by SWC and DWC (Table 1) indicated that the amylose content of defatted starch was significantly higher than that of non-defatted starch when it was measured by SWC ($P<0.05$). Similar results were reported by Martinez and Prodolliet (1996), Morrison and Laignelet (1983) and McGrance *et al.* (1998). This may be explained by the fact that fatty acids are hydrophobic molecules and can be fixed within the coil of the amylose double-helix and thus reduce its iodine-binding capacity (Martinez and Prodolliet, 1996; Hoover and Ratnayake, 2001). The significant difference only appeared in several samples when DWC was used to determine amylose content. It seems that DWC can, more or less, remove interference of the lipid. In addition, we found that amylose of some samples cannot be detected by SWC whether or not they are defatted. We hold that the amylose contents of these samples were very low and the interference by lipids cannot be completely removed (Knutson, 2000). Thus, the fat severely interferes with the formation of the amylose-iodine complex but we were unable to test this. Also we can see from this phenomenon that DWC can detect the amylose content of waxy barley which amylose content is rather low (<10 %) while SWC cannot, which indicated that DWC has a higher sensibility than SWC.

Moreover, amylopectin content of defatted starch is lower than that of non-defatted starch significantly (Table 1). We think that not only lipids combine with amylose, but also this complex absorbs light at a wavelength similar to that of the amylopectin-iodine complex, which thus causes an overestimation of the amylopectin content. In addition, it can be clearly seen from Table 1 that non-defatted total starch content was much higher than defatted total starch ($P<0.05$), which was mainly caused by the overestimation for amylopectin. Our results and the discussions above indicate that defatting should not be omitted when amylose, amylopectin and total starch are measured by means of DWC.

***Comparison of SWC and DWC in determining amylose*:** The differences of SWC and DWC in determining amylose content were shown in Table 1. Amylose content of some defatted samples measured by SWC was higher than that by DWC ($P<0.05$), which was in accordance with the results of Zhu *et al.* (2008), Himmelsbach *et al.* (2001) and Morrison and Laignelet (1983). This can be explained that a complex can be formed between iodine and long chain amylopectin polymers, absorbing light at a wavelength similar to that of the amylose-iodine complex. Furthermore, the existence of intermediate-sized polymers can also affect the iodine-binding results (Morrison and Laignelet, 1983; Himmelsbach *et al.*, 2001) which consequently gave an inflated value in the iodometric determination of amylose when SWC was used. In contrast, some of the defatted samples had lower amylose measured by SWC than DWC. This might be explained by the same reason mentioned in a previous subsection. That is they had lower amylose and the lipid cannot be removed completely, thereby reducing the iodine-binding capacity of amylose (Martinez and Prodolliet, 1996; Knutson, 2000; Hoover and Ratnayake, 2001) and giving rise to the lower determined value of amylase content. Additionally, Table 1 shows that SD of DWC was lower than that of SWC, indicating that DWC is a more precise method than SWC. This is consistent with the results of Zhu *et al.* (2008). From the results shown above it can be concluded that DWC can remove interference of other components with higher precision and accuracy.

***Comparison of DWC and PCC in determining total starch*:** Measurement of the total starch of non-defatted barley samples by PCC, according to the method of starch determination (see "materials and methods") has shown that defatting can be omitted in cereals with low lipid, such as barley, wheat, and rice. That is, the low fat might have little or no effect on the determination of total starch by PCC. The differences of PCC and DWC in determining total starch content were summarized in Table 1. The non-defatted samples measured by PCC had obviously lower starch than those by DWC except in four samples with lower amylose ($P<0.05$). Most likely the fat has a less effect on the starch tested by PCC than that by DWC. Nevertheless, the content of non-defatted starch determined by PCC was increased appreciably compared with that of defatted starch determined by DWC ($P<0.05$) which is regarded as the currently most acceptable method for amylose and amylopectin content determination. Thus, we inferred that

lipid, to some extent, affects the determination of starch although it is not significant, and that many other components interfere mostly with the results when PCC is used. It is reported that PCC can be influenced by many factors such as precipitating of protein, salt acidity and concentration, extraction temperature, and sorptive effects of filter paper (Clendenning, 1945; Clendenning and Wright, 1945). The procedure of PCC is cockamamie and time-consuming. The results implied that, comparing with PCC, DWC is a simpler and more accurate method.

***Precision and accuracy of DWC*:** When DWC was adapted to determination of starch content, amylose and amylopectin should be determined within 20~30 min and 15~25 min, respectively. The value would become unstable below or above the range of this measured time (Table 2). The precision of DWC was also tested (Table 3) and the results indicated that DWC has a high precision with a relative standard deviation (RSD) less than 2.6%. The accuracy of DWC recovery studies showed that the percentage recoveries of defatted amylose (Table 4) was significantly higher than that of non-defatted amylose. For amylopectin, contrary results were obtained although the difference was not significant (P>0.05). These results were in accordance with that of amylose and amylopectin content determination (see previous section). The results are satisfactory with recoveries ranging from 77 to 128 % only when the starch is defatted. The results further demonstrated that DWC is precise and accurate, and can be used to determine barley amylose and amylopectin content.

***Conclusions*:** This study showed that defatted samples measured by SWC had higher amylose than non-defatted ones. While several samples exhibited significant difference for amylose content when defatted and non-defatted samples were analyzed by DWC. For amylopectin, the defatted samples determined by DWC were significantly lower than that of non-defatted ones. Thus care should be taken to determine starch content with regard to effects of lipids.

Table 2. Absorbance of amylose and amylopectin at different time

Contents of determination	Time of determination (min)*						
	10	15	20	25	30	35	40
Amylose**	1.098	1.094	1.083	1.084	1.083	1.079	1.073
Amylopectin***	0.059	0.053	0.053	0.051	0.050	0.048	0.045

*: Timing started at the addition of 0.2% iodine solution, and 3 mL of standard amylose or amylopectin was used to measure. **: measured at 620 nm. ***: measured at 728nm.

Table 3. Results of repeated experiments of DWC

Contents of determination	Results of determination (% dry base)						Mean	SD	RSD (%)
Amylose	24.01	24.20	24.88	25.63	24.35	24.63	24.62	0.58	2.38
Amylopectin	43.47	41.84	43.68	43.62	42.09	41.15	42.64	1.09	2.55
Starch	67.48	66.05	68.55	69.25	66.44	65.78	67.26	1.42	2.11

SD:standard deviation. RSD: relative standard deviation

Table 4. Recovery studies of DWC

Sample	Amount of starch added (mg/mL)	Recovery of non-defatted starch (%)	Recovery of defatted starch (%)
Amylose*	0.5	30.99	77.28
	0.5	30.73	114.37
	0.5	42.79	121.63
	0.3	36.70	127.94
	0.3	39.29	119.30
	0.3	33.46	121.03
Amylopectin**	1.5	108.48	94.65
	1.5	102.63	86.16
	1.5	97.02	78.52
	1.0	115.87	98.68
	1.0	110.56	95.50
	1.0	101.44	105.68

*: means a significant difference between defatted and non-defatted starch (P<0.05).

**: means no significant difference between two groups (P>0.05)

Compared with SWC and PCC, DWC cannot only simultaneously determine amylose and amylopectin content thus obtain starch content, but also can remove interference of lipid and other components. Thus, DWC is more simple, sensitive and accurate than other methods. It is suitable for the determination of barley amylose, amylopectin and total starch.

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