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Rapid Characterization of Wheat Low Molecular Weight Glutenin Subunits by Ultraperformance Liquid Chromatography (UPLC)

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ABSTRACT: Low molecular weight glutenin subunits (LMW-GS), as important seed storage proteins, together with HMW-GS significantly define unique dough viscoelastic properties. In this study, a rapid ultraperformance liquid chromatography (UPLC) method for the separation and characterization of LMW-GS in wheat was optimized and established. The fast, reproducible, and high-resolution UPLC separation of LMW-GS could be obtained by gradually increasing eluting gradient from 21 to 47% in 30 min at flow rate of 0.55 mL/min and 60 °C for separation temperature. By this method, analysis of one sample could be completed in <20 min, significantly less time than the traditional reversed-phase high-performance liquid chromatography (RP-HPLC) method. Under the optimized conditions, the genetic features of LMW-GS and genotype × environmental interaction were successfully analyzed, leading to a fast identification of 17 main LMW-GS alleles that were related to different quality properties in wheat. The results demonstrated that UPLC could be a powerful and alternative tool for genetic and proteomic studies of wheat grain proteins and fast identification or screening of desirable LMW-GS alleles in wheat quality improvement.

KEYWORDS: *bread wheat, LMW-GS, alleles, RP-UPLC*

INTRODUCTION

Glutenins and gliadins are the two major storage proteins deposited in the seed endosperm of wheat and are the determinant factors of the viscoelasticity and extensibility of dough, respectively.[1] According to their molecular weights, glutenins are divided into high (70000−90000 Da) and low (20000−45000 Da) molecular weight glutenin subunits (HMW-GS and LMW-GS), which are the major determinants for unique elasticity and viscosity of wheat dough, respectively. The glutenin subunits can be polymerized by inter- and intramolecular disulfide bonds and form the largest protein molecules with molecular weights up to tens of millions. The polymerization of glutenin subunits significantly affects the rheological properties of wheat dough.[2]

LMW-GS are encoded by *Glu-A3*, *Glu-B3*, and *Glu-D3* loci on the short arm of group 1 chromosomes.[3] The estimated number of LMW-GS gene copies varies from 10−15[4] to 35−40[5,6] in individual hexaploid wheat genotype. The allelic variations of LMW-GS were found to be related to different dough properties in wheat cultivars.[7,8] In particular, LMW-GS fractions were significantly correlated with dough resistance.[9−12]

Therefore, it is important to develop more powerful methods to separate and characterize LMW-GS for better understanding of their complexity and heterogeneity in compositions.[13,14] For the present, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC) are the most commonly used methods in separating and identifying wheat glutenin subunits.[15−17] In the 1990s, capillary electrophoresis (CE), a method with various electrophoresis modes, was developed, which could satisfy the requirements of different protein separations and characterizations for fast operation, high resolution, and small amount of samples.[18−20] Since then, it has been successfully used to separate and characterize wheat seed proteins, including water-soluble proteins,[21,22] gliadins,[19,23−25] HMW-GS[15,17,26,27] and LMW-GS.[28] In recent years, proteomics, two-dimensional electrophoresis (2-DE), and various mass spectrometry techniques, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), have been developed to isolate wheat grain proteins with higher resolution and accuracy.[29−32] However, it is clear that there are still some disadvantages in these separation techniques, although they have different advantages. For instance, traditional SDS-PAGE is generally inefficient and labor-intensive, and its resolution and repeatability are not so satisfactory, even leading to incorrect results when applied to subunit identification.[17] RP-HPLC usually needs longer separation time and consumes more analytical reagents, and its resolution is still not so high.[16] CE is also sensitive to separation conditions, and its resolution and reproducibility often are not as high as expected.[17,19] The 2-DE and various MS techniques have significantly improved the resolution and accuracy. However, their utilization is limited because they are sophisticated and expensive.

Recently, reversed-phase ultraperformance liquid chromatography (RP-UPLC), a new technology based on RP-HPLC, has been developed.[33] Using fine particle size fillers (1.7 μm) and small diameter columns, this system can produce a column performance as high as up to 100,000−300,000 theoretical plates/m. Compared with RP-HPLC, more rapid separation, higher resolution, and greater sensitivity could be obtained by RP-UPLC analysis.[34] UPLC has received more and more attention, and it has been successfully applied to food, chemical,

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and medicine analyses.[35,36] More recently, we have established a rapid and valid UPLC method for water-soluble protein analysis in wheat grains.[37] In this work, we intended to further optimize the separation conditions and procedures and then develop a practical UPLC method for the fast separation and characterization of wheat LMW-GS and alleles at different loci. We expect that it could serve as a more powerful tool for grain proteome studies and quality improvement, especially for rapid screening of desirable LMW-GS alleles in wheat.

MATERIALS AND METHODS

Plant Materials. The experimental materials used in this study included three common wheat varieties, six nullisomic-tetrasomic (NT) lines, two substitution lines, two pairs of LMW-GS near-isogenic lines (NIL), and a set of Aroona NILs (Table 1), along with two durum wheat LMW-GS variants. The common wheat varieties Chinese Spring (CS), Zhongyou 9507, and Ningchun 4 were collected from the Chinese National Germplasm Collections in the Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS). The CS NT lines N1AT1B, N1BT1D, N1DT1B, N6AT6D, N6BT6D, and N6DT6A as well as CS were kindly provided by Prof. Z. Liu, China Agricultural University. The substitution lines CS-1C(1A) and CS-1S^{l}(1B) were developed and provided by Dr. Hsam, Department of Plant Breeding, Technical University of Munich, Germany, in which the chromosomes 1A and 1B were replaced by 1C from *Aegilops caudata* ($2n = 2x = 14$, CC) and 1S^{l} from *Aegilops longissima* ($2n = 2x = 14$, S^{l}S^{l}), respectively. A set of Aroona NILs (Table 1)[38] was kindly provided by Prof. X. Xia, CAAS. The two pairs of LMW-GS near-isogenic lines of spring wheat CB037 and CB037-n and CS and CS-n, were developed in our laboratory. CB037 and CB037-n have the same HMW-GS (1, 17 + 18, 5 + 10) and LMW-GS (*Glu-A3c*, *Glu-B3h*, and *Glu-D3d*) compositions except for *Glu-B3h* silenced in CB037-n, whereas CS and CS-n contain the same HMW-GS (null, 7 + 8, 2 + 12) and LMW-GS (*Glu-A3a*, *Glu-B3a*, and *Glu-D3a*) except for *Glu-A3a* silenced in CS-n. The two durum wheat LMW-GS variants, Lira 45 with LMW-2 type subunit and Lira 42 with LMW-1 type subunit, were kindly provided by Prof. D. Lafiandra, University of Tuscia, Italy.

Table 1. *Glu-1*and *Glu-3* Glutenin Allele Compositions and Their Donor Parents in Aroona Near-Isogenic Lines (NILs)

no.	NIL	*Glu-A1*	*Glu-B1*	*Glu-D1*	*Glu-A3*	*Glu-B3*	*Glu-D3*	donor parent
1	Aril15-4 (*Glu-A3a*)	a	c	a	**a**	b	c	Chinese Spring
2	Aril16-1 (*Glu-A3b*)	a	c	a	**b**	b	c	Gabo
3	Aril18-5 (*Glu-A3d*)	a	c	a	**d**	b	c	Orca
4	Aril19-2 (*Glu-A3e*)	a	c	a	**e**	b	c	LermaRojo
5	Aril20-1 (*Glu-A3f*)	a	c	a	**f**	b	c	Bungulla
6	Aril21-2 (*Glu-B3a*)	a	c	a	c	**a**	c	Chinese Spring
7	Aril23-4 (*Glu-B3c*)	a	c	a	c	**c**	c	Halberd
8	Aril24-3 (*Glu-B3d*)	a	c	a	c	**d**	c	Orca
9	Aril26-2 (*Glu-B3f*)	a	c	a	c	**f**	c	Gawain
10	Aril27-6 (*Glu-B3g*)	a	c	a	c	**g**	c	Millewa
11	Aril28-4 (*Glu-B3h*)	a	c	a	c	**h**	c	Sonalica
12	Aril29-4 (*Glu-B3i*)	a	c	a	c	**i**	c	Jufy 1
13	Aril30-1 (*Glu-D3a*)	a	c	a	c	b	**a**	Chinese Spring
14	Aril36-2 (*Glu-D3b*)	a	c	a	c	b	**b**	Bungulla
15	Aril33-1 (*Glu-D3d*)	a	c	a	c	b	**d**	Jufy 1
16	Aril35-1 (*Glu-D3f*)	a	c	a	c	b	**f**	India 115

Field Planting, Identification, and Sampling. To investigate the variations of LMW-GS in different growing environments, two spring wheat varieties, CB037 and Ningchun 4, were planted in three locations, each representing a different ecological district of spring wheat production in China, that is, Yingchuan, Ningxia province (north); Xining, Qinghai province (northwest); and Beijing (Jingjintang areas). In each location and for each cultivar, a plot of 20 m^2 and three replications were planted under the same conditions of cultivation and management as local wheat field production. The mature seeds were harvested and dried and then used for protein analysis.

LMW-GS Extraction. Grain flours (30 mg) were first incubated in 1000 μL of 70% ethanol (v/v) for 1 h. Then 500 μL of 55% isopropanol (v/v) was added at 65 °C in a water bath, following by centrifuging at 13000 rpm for 10 min. This step was repeated three times. The precipitate was extracted with 100 μL of buffer containing 50% isopropanol (v/v), 1 M Tris-HCl (pH 8.0), and 1% dithiothreitol (DTT). The pellets were mixed completely and placed in a 65 °C water bath. Subsequently, another 100 μL of extraction buffer with 1.4% 4-vinylpyridine (v/v) instead of 1% DTT was added and mixed. After centrifugation at 13000 rpm for 10 min, the supernatants were collected and added to cold (−20 °C) acetone and then stored at −20 °C overnight. Different acetone densities were tested, and the precipitated proteins were harvested by centrifugation at 10000 rpm for 10 min, then resuspended with 500 μL of ethanol, and centrifuged once again. The extracted proteins were precipitated with cold acetone containing 0.07% β-mercaptoethanol (v/v) and centrifuged at 10000 rpm for 5 min. This step was undertaken twice. Finally, the protein pellet was freeze-dried and stored at −80 °C for RP-UPLC analysis.

SDS-PAGE. LMW-GS separation and identification were performed by SDS-PAGE, following the method of Yan et al.[15]

RP-UPLC. RP-UPLC was performed on an Acquity UPLC (Waters Corp.) with a Waters 300SB C18 column (1.7 μm). All chemicals used for RP-HPLC were of high purity, without needing further purification. Methanol and acetonitrile (ACN) were purchased from Fisher Scientific (USA), and trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO, USA). Ultrapure water was produced in a Milli-Q purification system (Millipore, Bedford, MA, USA). Four elution buffers were used: solution A, ultrapure water with 0.06% TFA; solution B, ACN with 0.06% TFA; solution C, ultrapure water; and solution D, methanol. Column balance was performed by increasing the concentration of solution B from 21 to 47% in 15 min. The ratios of solution A to B for weak washing and strong washing needles were 79:21 and 53:47%, respectively. Sample washing was done with solution A from 95 to 5% and solution B from 5 to 95% in 5 min. Final washing was completed by turning off the lamp with solution C from 90 to 10% and solution D from 10 to 90% three times within 30 min. At the end, 10 μL of solution D was injected into the column.

To obtain high resolution and reproducibility for LMW-GS separation, the separation conditions were optimized by testing various temperatures, flow rates, and elution gradients. The resolution, reflecting the separation degree between two adjacent protein peaks, was calculated with the formula $2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 represent the migration times of two adjacent peaks and w_1 and w_2 indicate the widths of two adjacent peaks.[39]

RESULTS AND DISCUSSION

Optimization of UPLC Separation Conditions. To obtain better separation results, different UPLC parameters were tested and optimized. According to our experiments, many factors could influence UPLC separation time and resolution of LMW-GS, of which elution time, temperature, and flow rate were the three most important parameters.

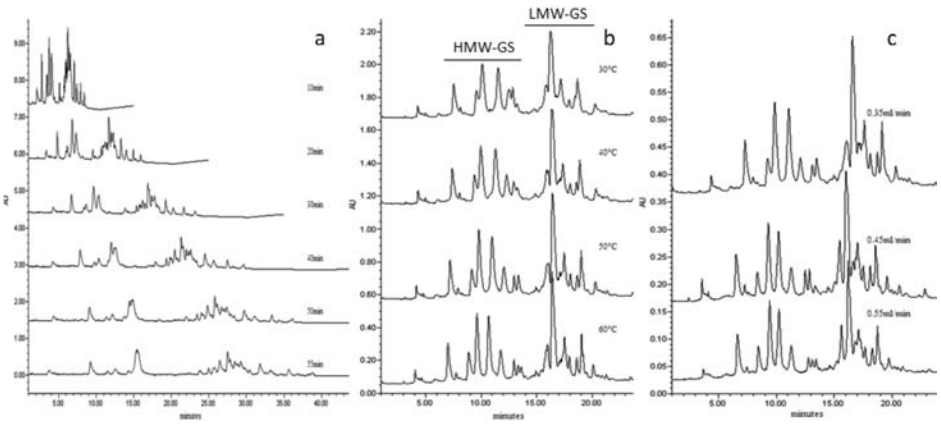

Figure 1. Optimization of UPLC separation conditions for LMW-GS from Chinese bread wheat cultivar Zhongyou 9507: (a) separation flow rate; (b) separation temperature; (c) separation elution time.

The elution times had significant effects on both separation time and resolution of glutenin proteins. Six different gradient elution times (10, 20, 30, 40, 50, and 60 min) were tested to separate LMW-GS. As shown in Figure 1a, HMW-GS eluted earlier than LMW-GS, indicating that HMW-GS had higher surface hydrophobicities than LMW-GS, consistent with a previous result with RP-HPLC.[16] When the concentration of solution B was increased gradually from 21 to 47%, the migrations of both HMW-GS and LMW-GS were significantly delayed along with the increasing elution times. The data of separation time and resolution indicated that the optimal gradient elution time for LMW-GS was 30 min with solution B being increased from 21 to 47%.

In general, a high separating temperature is needed due to the complex structures of LMW-GS subunits. However, although higher temperatures could enhance the separation selectivity and reduce separation time, the working life of the column would be shortened if the temperature exceeded 70 °C. Thus, four different separation temperatures (30, 40, 50, and 60 °C) were selected to test the effects of temperature on separation patterns. In the result (Figure 1b), only slight changes of UPLC patterns were found, but the resolution of LMW-GS exhibited significant differences under different separation temperatures (Table 2). The highest resolution (4.98) with a shorter separation time (13.99 min) was obtained at 60 °C. Therefore, we propose that 60 °C should be the optimal temperature for the separation.

Although a flow rate of 0.35 mL/min was suggested by the instructions of the column manufacturer (Waters Corp.), our testing did not provide satisfactory results for LMW-GS separation. Thus, three different flow rates (0.35, 0.45, and 0.55 mL/min) were tested and compared. The results demonstrated a UPLC pattern similar to those of different separation temperatures. As shown in Figure 1c, as the flow rate was increased from 0.35 to 0.55 mL/min, the migration times of LMW-GS were slightly delayed, but their resolution was significantly increased (Table 2). At 0.55 mL/min, the highest resolution (4.98) with a separation time of 13.99 min could be obtained.

Table 2. Migration Time and Resolution of LMW-GS Separated by UPLC at Different Separation Temperatures and Flow Rates

	mean migration time[a] (min)	resolution[a]
temperature (°C)		
30	12.55a	2.47a
40	13.96b	1.78b
50	14.15c	1.50b
60	13.99b	4.98c
flow rate (mL/min)		
0.35	13.42a	2.42a
0.45	13.91b	3.15b
0.55	13.99b	4.98c

[a]Different letters indicate a significant difference at $P = 0.05$.

To analyze the reproducibility of UPLC, 15 consecutive runs were performed for the separation of LMW-GS from Chinese Spring under the optimized conditions of elution time, temperature, and flow rate as described above (Figure 2). The averages, standard deviations, and relative standard deviations (RSD%) of migration time, peak height, and peak area of 13 representative LMW-GS are listed in Table 3. The results showed that a high reproducibility of separation was produced under the optimized conditions, with RSD% values of <0.1 for migration time, <4.0 for peak height, and <5.0 for peak area, respectively. Therefore, a high reproducibility as well as a rapid separation and high resolution for LMW-GS separation could be obtained by using the optimized method. Analysis for one sample could be completed in about 22 min, and LMW-GS generally eluted in 13−22 min (Figure 2b). It is clear that UPLC is much faster than traditional HPLC, in which the glutenin separation for a sample needs at least 50 min.[16]

Genetic Control of LMW-GS Determined by UPLC. To explore the potential application of the optimized UPLC method in wheat genetic study and quality improvement, we used it to investigate LMW-GS genetic controls, environmental effects, and allele identification.

The LMW-GS from six nullisomic-tetrasomic (NT) lines of Chinese Spring (N1AT1B, N1BT1D, N1DT1B, N6AT6D, N6BT6D, and N6DT6A) were analyzed by the optimal UPLC separation method (Figure 3a). By comparing the separation patterns of different CS NT lines, the chromosomal locations of the genes encoding different LMW-GS could be determined. The results showed that 14 main LMW-GS in CS were clearly isolated, and the genes encoding different subunits could be assigned to specific chromosomes. For instance, the genes for subunits 1, 2, 3, 4, and 8 were assigned to 1A; those for subunits 5, 7, 9, 11, and 12 to 1B; those for subunits 6, 10, and 14 to 1D; and that for subunit 13 to 6B.

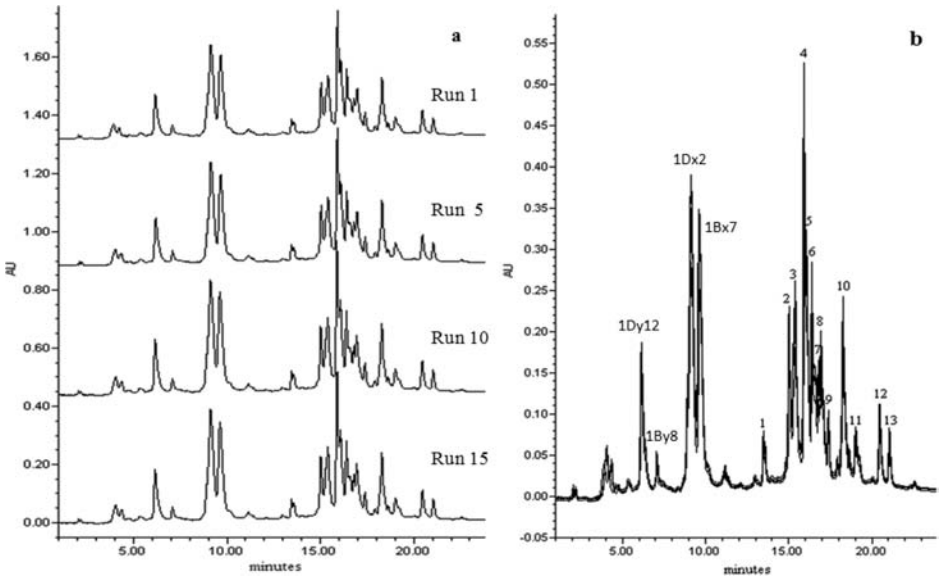

Figure 2. Reproducibility of consecutive UPLC separations of LMW-GS in Chinese Spring with optimized conditions: (a) 15 consecutive runs by optimized UPLC; (b) overlap of 15 consecutive runs.

Table 3. Repeatability of LMW-GS in Chinese Spring Separated by UPLC

peak[a]	migration time (min)	RSD%	peak height (1000 uV)	RSD%	peak area (1000 uV/S)	RSD%
1	13.474 ± 0.003	0.022	62.354 ± 2.011	3.225	647.312 ± 30.211	4.667
2	15.024 ± 0.006	0.040	205.723 ± 7.125	3.463	2317.186 ± 105.169	4.539
3	15.403 ± 0.010	0.065	235.685 ± 8.046	3.414	4015.277 ± 181.449	4.519
4	15.907 ± 0.011	0.069	487.907 ± 18.105	3.711	3853.277 ± 173.152	4.494
5	16.063 ± 0.009	0.056	293.736 ± 10.109	3.442	3132.030 ± 144.577	4.616
6	16.393 ± 0.007	0.043	256.456 ± 8.322	3.245	3485.708 ± 43.208	1.234
7	16.803 ± 0.006	0.036	142.313 ± 5.188	3.646	1017.448 ± 45.101	4.433
8	16.958 ± 0.008	0. 047	177.675 ± 6.173	3.474	2457.460 ± 121.180	4.931
9	17.383 ± 0.006	0.035	84.708 ± 2.101	2.480	873.688 ± 33.798	3.868
10	18.282 ± 0.006	0.033	217.486 ± 5.679	2.611	2554.631 ± 127.232	4.980
11	19.022 ± 0.007	0.037	63.994 ± 2.065	3.227	1060.896 ± 51.844	4.887
12	20.468 ± 0.007	0.034	92.699 ± 3.080	3.323	1023.901 ± 44.629	4.359
13	21.038 ± 0.007	0.033	65.949 ± 2.223	3.371	614.463 ± 28.819	4.690

[a]Peak numbers are the same as those indicated in Figure 2b.

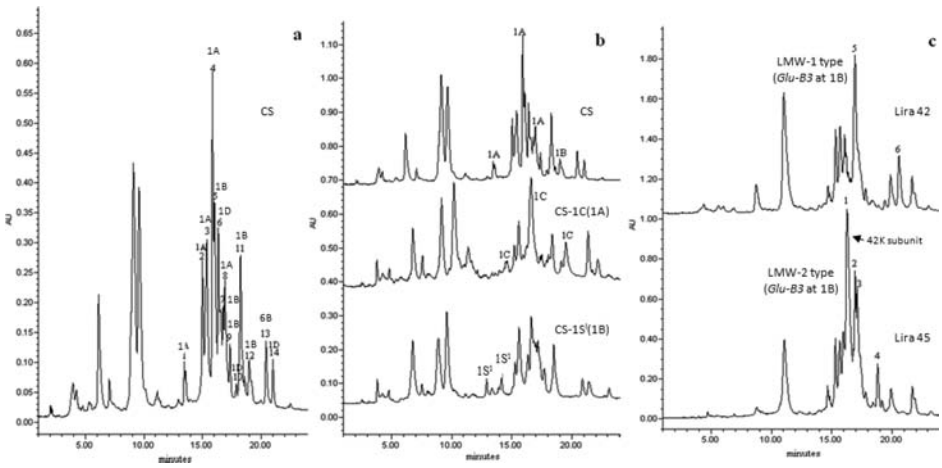

Figure 3. Genetic controls of the genes encoding LMW-GS revealed by UPLC: (a) chromosomal locations of LMW-GS from six NT lines of Chinese Spring; (b) chromosomal locations of LMW-GS from Chinese Spring and its substitution lines CS-1C(1A) and CS-1S^{l}(1B); (c) genetic controls of LMW-1 and LMW-2 type subunits from Lira 42 and Lira 45.

Previous study showed that the substitution line CS-1S^{l}(1B) had better gluten quality than CS,[40] suggesting that the S^{l} genome could contain genes for potential storage protein subunits related to superior quality. Our recent analyses

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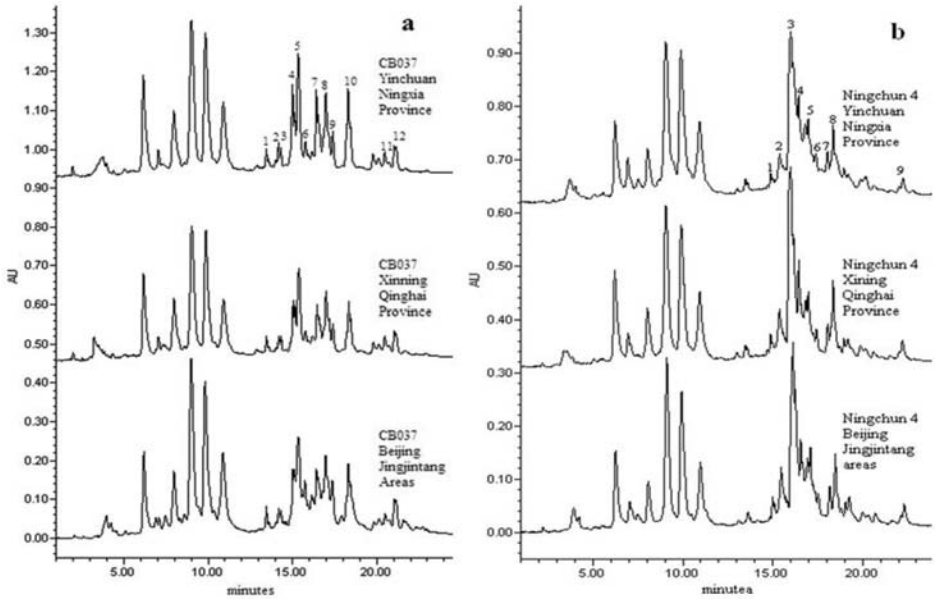

Figure 4. LMW-GS variations of Chinese Spring wheat variety CB037 and Ningchun 4 from three growing locations of China (Yinchuan, Xining, and Beijing) detected by UPLC.

Table 4. Effects of Three Growing Locations (Yinchuan, Xining, and Beijing) on LMW-GS in CB037 and Ningchun 4 Revealed by UPLC

peak[a]	migration time (min)	RSD%	peak height[b] (1000 uV)	RSD%	peak areas[b] (1000 uV/S)	RSD%
CB037						
1	13.469 ± 0.006	0.045	51.648 ± 5.947**	11.514	346.006 ± 35.450**	10.245
2	14.186 ± 0.010	0.070	55.305 ± 12.168**	22.002	375.372 ± 87.451**	23.297
3	14.324 ± 0.007	0.049	49.241 ± 5.088**	10.333	330.306 ± 34.598**	10.475
4	15.014 ± 0.019	0.127	167.939 ± 42.901**	25.546	1462.474 ± 419.724**	28.700
5	15.362 ± 0.021	0.137	248.473 ± 42.076**	16.934	3617.819 ± 453.935**	12.547
6	15.774 ± 0.017	0.108	83.067 ± 35.036**	42.178	885.189 ± 392.762**	44.370
7	16.431 ± 0.021	0.128	159.623 ± 41.144**	25.776	1154.008 ± 291.502**	25.260
8	16.985 ± 0.017	0.100	179.822 ± 20.605**	11.459	2586.329 ± 533.083**	20.612
9	17.382 ± 0.015	0.086	97.237 ± 26.171**	26.915	1027.194 ± 461.996**	44.977
10	18.307 ± 0.016	0.087	165.725 ± 37.679**	22.736	1507.500 ± 358.913**	23.808
11	20.499 ± 0.014	0.068	43.882 ± 3.567*	8.129	528.660 ± 22.979*	4.347
12	21.047 ± 0.002	0.010	65.846 ± 9.378**	14.242	500.396 ± 71.974**	14.383
Ningchun 4						
1	14.913 ± 0.063	0.422	40.160 ± 7.151**	18.703	262.769 ± 38.422**	14.622
2	15.408 ± 0.053	0.344	85.925 ± 17.385**	20.233	1268.016 ± 285.964**	22.552
3	16.035 ± 0.066	0.412	328.927 ± 26.499*	8.056	3999.152 ± 438.965**	10.976
4	16.482 ± 0.071	0.431	168.969 ± 12.266*	7.259	1727.074 ± 609.950**	35.300
5	17.026 ± 0.070	0.411	128.912 ± 15.632**	12.126	1454.145 ± 150.372**	10.341
6	17.397 ± 0.035	0.201	55.818 ± 13.951**	24.994	523.976 ± 272.406**	51.988
7	18.099 ± 0.068	0.376	64.192 ± 9.371**	14.598	678.894 ± 266.042**	39.188
8	18.387 ± 0.068	0.370	128.446 ± 5.033*	3.918	1480.538 ± 181.930**	12.288
9	22.261 ± 0.055	0.247	33.129 ± 4.714**	14.229	384.197 ± 42.771**	11.133

[a]Peak numbers are the same as those indicated in Figure 4. [b]* and ** indicate the significance level between different growing locations at $P = 0.05$ and 0.01, respectively.

indicated that CS-1C(1A) substitution line also had superior quality properties (data unpublished). This means that the C genome might possess some candidate genes of glutenin subunits which could be used to improve wheat flour quality. The UPLC patterns of LMW-GS from two substitution lines as well as CS (Figure 3b) demonstrated that the genes encoding three protein subunits indicated in CS-1C(1A) were located on the 1C chromosome, whereas the encoding genes for other two subunits showed in CS-1S^{l}(1B) were located on the 1S^{l} chromosome. In addition, compared with the two substitution lines, CS carried different genes on 1A and 1B chromosome, which encoded three subunits and one subunit indicated in CS, respectively. This result was well consistent with those from the analysis of CS NT lines (Figure 3a).

In durum wheat, LMW-2 type subunits are associated with superior pasta-making properties, whereas LMW-1 type subunits had negative effects on pasta quality.[41] The LMW-2 type subunits also exhibited a positive effect on gluten strength in

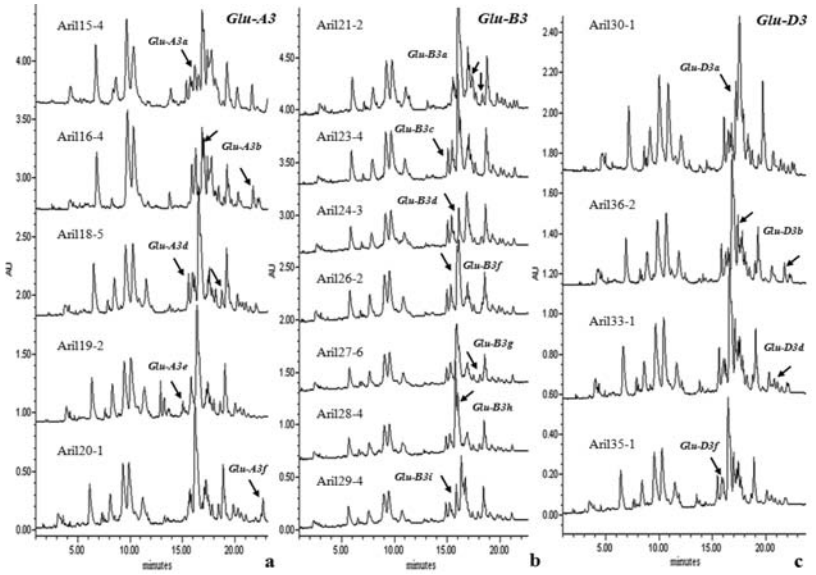

Figure 5. Identification of LMW-GS alleles from a set of Aroona near-isogenic lines by UPLC: (a) alleles at *Glu-A3*; (b) alleles at *Glu-B3*; (c) alleles at *Glu-D3*.

hexaploid tritordeum, an amphiploid derived from the hybridization between *Triticum durum* and *Hordeum chilense*.[42] The major difference of LMW-GS between the two types was reflected in the presence of the largest 42K subunit in LMW-2 group, which was encoded by the *Glu-B3* locus on chromosome 1B.[43] Therefore, LMW-2 type subunits could be used as useful biochemical markers for wheat quality improvement. The UPLC separation patterns of LMW-1 type subunits in Lira 42 and LMW-2 type subunits in Lira 45 are shown in Figure 3c. The specific LMW-GS peaks could be readily differentiated by UPLC. Lira 45 with LMW-2 type subunits possessed four specific peaks (1−4), whereas Lira 42 with LMW-1 type subunits had two specific peaks (5 and 6) (Figure 3c). The largest 42K subunit, encoded by *Glu-B3* on chromosome 1B and with the highest amount of expression in LMW-2 type, was eluted at 16 min and clearly differed from other LMW-GS.

Effects of Growing Environments on LMW-GS Compositions Revealed by UPLC. The LMW-GS compositions of wheat varieties CB037 and Ningchun 4 from three growing locations (Yinchuan, Xining, and Beijing) of China were analyzed by the optimized UPLC method. The three growing places represent three main spring wheat production districts (north, northeast and northwest) in northern China, with different ecological conditions. In general, northern China has less rainfall and drier weather compared to southern areas. Figure 4 shows that 12 and 9 clear LMW-GS peaks were isolated in CB037 and Ningchun 4, respectively. Statistical analysis indicated that the peak migration times exhibited few variations between different growing locations (Table 4), suggesting that the component compositions of LMW-GS were stable and less affected by growing environments. However, both peak heights and areas of all protein subunits showed highly significant variations between growing places, indicating that the environmental factors had significant effects on the synthesis and accumulation of LMW-GS.

Rapid Identification of LMW-GS Alleles by UPLC. The allelic variations at *Glu-3* loci are highly related to gluten quality. A total of 20 LMW-GS alleles in common wheat were previously identified and nominated, namely, 6 at *Glu-A3*, 9 at *Glu-B3*, and 5 at *Glu-D3*.[3] Some of the alleles, such as *Glu-A3b*, *Glu-B3b*, *Glu-D3a*, *Glu-D3b*, *Glu-D3d*, and *Glu-D3e*, showed positive effects on dough properties.[3,44] Recent research using a set of Aroona NILs demonstrated that the alleles *Glu-A3b*, *Glu-A3d*, *Glu-B3g*, and *Glu-D3f* were significantly contributive to mixograph properties.[38] Therefore, a method applicable to rapid and effective identification of LMW-GS alleles is very helpful for wheat quality improvement. Although 2-DE and MALDI-TOF-MS could well separate and identify some LMW-GS alleles,[31] they are sophisticated and expensive and thus are not widely used in wheat improvement programs.

SDS-PAGE has been successfully used in the identification of some major LMW-GS alleles.[45] However, it is not easy to accurately discriminate specific alleles at different loci by SDS-PAGE gels because some LMW-GS bands have similar molecular weights. To validate and detect the potential of tge UPLC method, a set of Aroona NILs (Table 1) was used to identify LMW-GS alleles at different loci by the optimized UPLC method, and the results are presented in Figure 5. All 16 LMW-GS alleles at three *Glu-3* loci could be identified by comparing their characteristic peaks. At *Glu-A3* locus, 5 alleles (*Glu-A3a*, *Glu-A3b*, *Glu-A3d*, *Glu-A3e*, and *Glu-A3f*) were readily differentiated (Figure 5a); 3 alleles had two characteristic peaks (15.5 and 16 min for *Glu-A3a*, 16.5 and 18 min for *Glu-A3b*, and 15.2 and 18.5 min for *Glu-A3d*) and 2 alleles had a characteristic peak (15 min for *Glu-A3e* and 22.7 min for *Glu-A3f*). Of 7 LMW-GS alleles at the *Glu-B3* locus, *Glu-B3a* had two minor characteristic peaks at 18 and 19 min and the other 6 alleles (*Glu-B3c*, *Glu-B3d*, *Glu-B3f*, *Glu-B3g*, *Glu-B3h*, and *Glu-B3i*) exhibited only one characteristic peak (Figure 5b). At the *Glu-D3* locus, 4 alleles were identified, of which *Glu-D3b* had two characteristic peaks at about 18 and 22 min, and the other 3 alleles showed one characteristic peak (*Glu-D3a* at about 17 min, *Glu-D3d* at 21 min, and *Glu-D3f* at 16 min as shown in Figure 5c).

Two pairs of LMW-GS near-isogenic lines of Chinese spring wheat, CB037 and CB037-n and CS and CS-n, have been recently developed in our laboratory. Analysis of flour quality properties by mixograph testing (data not shown) indicated that the silencing of *Glu-B3h* in CB037-n and *Glu-A3a* in CS-n could significantly reduce the flour-mixing quality, suggesting that both alleles have an important influence on wheat quality. The results

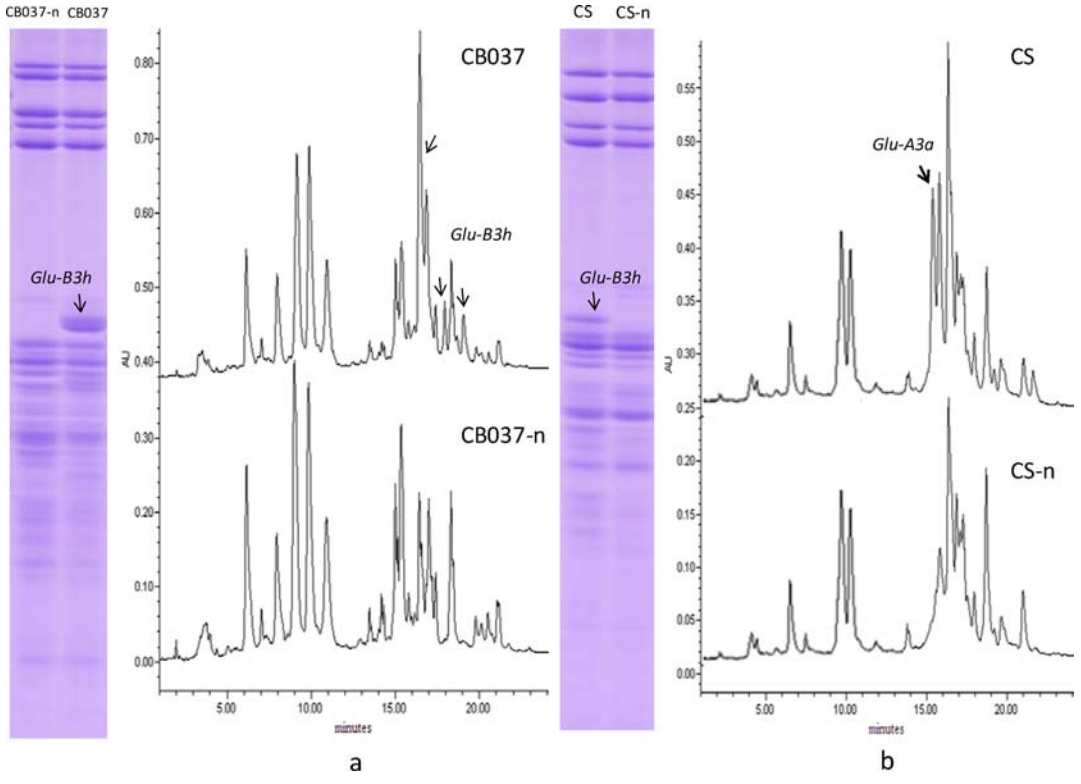

Figure 6. Identification of *Glu-B3h* and *Glu-A3a* in two pairs of LMW-GS near-isogenic lines of CB037 and Chinese Spring by UPLC.

also demonstrated that both alleles could be readily identified by UPLC analysis (Figure 6). The allele *Glu-B3h* exhibited three characteristic peaks eluted at 16.5, 18, and 19 min, respectively (Figure 6a), whereas *Glu-A3a* presented two characteristic peaks eluted at 15.5 and 16 min (Figure 6b).

Conclusions. Different UPLC separation conditions were tested and optimized for separating and characterizing wheat LMW-GS in this study. Under the conditions of increasing the eluting gradient gradually from 21 to 47% in 30 min at a flow rate of 0.55 mL/min and separation temperature of 60 °C, fast, high-resolution, and reproducible separations for LMW-GS could be achieved with a small amount of sample and reagent consumptions in <20 min. Both separation time and resolution of UPLC were much more desirable than those of traditional HPLC. The optimized UPLC could be effectively used for studies on genetic controls of LMW-GS genes and environmental effects on LMW-GS synthesis and rapid identification of LMW-GS alleles that are associated with different quality properties. In particular, our results demonstrated the separation efficiency of the optimized UPLC in the separation of LMW-GS and its application potential for quickly identifying and screening desirable LMW-GS alleles in the early generations of wheat quality improvement. Therefore, UPLC could be used as a powerful and alternative tool for wheat genetic studies and practical breeding programs as well as grain proteomics.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACN, acetonitrile; CE, capillary electrophoresis; HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; RP-HPLC, reversed-phase high-performance liquid chromatography; RP-UPLC, reversed-phase ultraperformance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

REFERENCES

(1) Payne, P. I. Genetics of wheat storage protein and the effect of allelic variation on pan bread quality. *Annu. Rev. Plant Physiol.* **1987**, *38*, 141−153.

(2) Wrigley, C. W. Giant proteins with flour power. *Nature* **1996**, *381*, 738−739.

(3) Gupta, R. B.; Shepherd, K. W. Two-step one-dimensional SDS-PAGE analysis of LMW subunits of glutelin. 1. Variation and genetic control of the subunits in hexaploid wheats. *Theor. Appl. Genet.* **1990**, *80*, 65−74.

(4) Harberd, N. P.; Bartels, D.; Thompson, R. D. Analysis of the gliadin multigene loci in bread wheat using nullisomic-tetrasomic lines. *Mol. Gen. Genet.* **1985**, *198*, 234−242.

(5) Sabelli, P. A.; Shewry, P. R. Characterization and organization of gene families at the *Gli-1* loci of bread and durum wheat by restriction fragment analysis. *Theor. Appl. Genet.* **1991**, *83*, 209−216.

(6) Cassidy, B. G.; Dvorak, J.; Anderson, O. D. The wheat low-molecular-weight glutenin genes: characterization of six new genes and progress in understanding gene family structure. *Theor. Appl. Genet.* **1998**, *96*, 743−750.
(7) Skeritt, J. H. Gluten proteins: genetics, structure and dough quality − a review. *AgBiotech News Inf.* **1998**, *10*, 247−270.
(8) Nagamine, T.; Kai, Y.; Takayama, T.; Yanagisawa, T.; Taya, S. Allelic variations of glutenin subunit loci *Glu-1* and *Glu-3* in southern Japanese wheat and their effects on dough and gluten properties. *J. Cereal Sci.* **2000**, *32*, 129−135.
(9) Gupta, R. B.; Shepherd, K. W. Low-molecular-weight glutenin subunits in wheat: their variation, inheritance, and association with physical dough properties. In *Proceedings of the 7th International Wheat Genetics Symposium*, Cambridge, UK; Miller, T. E., Koebner, R. M. D., Eds.; Institute of Plant Research: Cambridge, UK, 1988; pp 943−949.
(10) Gupta, R. B.; Singh, N. K.; Shepherd, K. W. The cumulative effect of allelic variation in LMW and HMW glutenin subunits on dough properties in the progeny of two bread wheats. *Theor. Appl. Genet.* **1989**, *77*, 57−64.
(11) Metakovsky, E. V.; Wrigley, C. W.; Bekes, F.; Gupta, R. B. Gluten polypeptidesas useful genetic markers of dough quality in Australian wheats. *Aust. J. Agric. Res.* **1990**, *41*, 289−306.
(12) Gupta, R. B.; MacRitchie, F.; Shepherd, K. W.; Ellison, F. W. Relative contribution of LMW and HMW subunits of glutenin to dough strength and dough stickiness of bread wheat. In *Gluten Proteins*; Bushuk, W., Tkachuk, R., Eds.; American Association of Cereal Chemistry: St. Paul, MN, 1991; pp 71−80.
(13) D'Ovidio, R.; Masci, S. The low-molecular-weight glutenin subunits of wheat gluten. *J. Cereal Sci.* **2004**, *39*, 321−339.
(14) Appelbee, M. J.; Mekuria, G. T.; Nagasandra, V.; Bonneau, J. P.; Eagles, H. A.; Eastwood, R. F.; Mather, D. E. Novel allelic variants encoded at the *Glu-D3* locus in bread wheat. *J. Cereal Sci.* **2009**, *49*, 254−261.
(15) Yan, Y.; Hsam, S. L. K.; Yu, J.; Jiang, Y.; Ohtsuka, I.; Zeller, F. J. HMW and LMW glutenin alleles among putative tetraploid and hexaploid *T. spelta* progenitors. *Theor. Appl. Genet.* **2003**, *107*, 1321−1330.
(16) Dong, K. C.; Hao, Y.; Wang, A. L.; Cai, M. H.; Yan, Y. M. Characterization of HMW glutenin subunits in bread and tetraploid wheats by reversed-phase high-performance liquid chromatography. *Cereal Res. Commun.* **2009**, *37*, 65−72.
(17) Gao, L.; Ma, W. J.; Chen, J.; Wang, K.; Li, J.; Wang, S. L.; Bekes, F.; Appels, R.; Yan, Y. Characterization and comparative analysis of wheat high molecular weight glutenin subunits by SDS-PAGE, RP-HPLC, HPCE, and MALDI-TOF-MS. *J. Agric. Food Chem.* **2010**, *58*, 2777−2786.
(18) Bietz, J. A.; Schmalzried, E. Capillary electrophoresis of wheat gliadin: Initial studies and application to varietal identification. *LWT−Food Sci. Technol.* **1995**, *28*, 174−184.
(19) Yan, Y.; Surlan-Momirovic, G.; Prodanovic, S.; Zoric, D.; Liu, G. Capillary zone electrophoresis analysis of gliadin proteins from Chinese and Yugoslav winter wheat cultivars. *Euphytica* **1999**, *105*, 197−204.
(20) Bean, S. R.; Lookhart, G. L. Ultrafast capillary electrophoresis analysis of cereal storage proteins and its application to protein characterization and cultivar differentiation. *J. Agric. Food Chem.* **2000**, *48*, 344−353.
(21) Bean, S. R.; Tilley, M. Separation of water-soluble proteins from cereals by high-performance capillary electrophoresis (HPCE). *Cereal Chem.* **2003**, *80*, 505−510.
(22) Wang, A.; Gao, L.; Pei, Y.; Zhang, Y.; He, Z.; Xia, X.; Appels, R.; Ma, W.; Huang, X.; Yan, Y. Rapid separation and characterization of grain water-soluble proteins in bread wheat cultivars (*Triticum aestivum* L.) by capillary electrophoresis. *Can. J. Plant Sci.* **2008**, *88*, 843−848.
(23) Yan, Y.; Hsam, S. L. K.; Yu, J. Z.; Jiang, Y.; Zeller, F. J. Genetic polymorphisms at *Gli-D*^{t} gliadin loci in *Aegilops tauschii* as revealed by acid polyacrylamide gel and capillary electrophoresis. *Plant Breed.* **2003**, *122*, 120−124.
(24) Lookhart, G. L.; Bean, S. R. Separation and characterization of wheat protein fractions by high-performance capillary electrophoresis. *Cereal Chem.* **1995**, *72*, 527−532.
(25) Lookhart, G. L.; Bean, S. R. Improvements in cereal protein separations by capillary electrophoresis: resolution and reproducibility. *Cereal Chem.* **1996**, *73*, 81−87.
(26) Yan, Y.; Yu, J.; Jiang, Y.; Hu, K.; Cai, M.; Hsam, S. L. K.; Zeller, F. J. Capillary electrophoresis separation of high molecular weight glutenin subunits in bread wheat (*Triticum aestivum* L.) and related species with phosphate-based buffers. *Electrophoresis* **2003**, *24*, 1429−1436.
(27) Yan, Y.; Jiang, Y.; Sun, M.; Yu, J.; Xiao, Y.; Zheng, J.; Hu, Y.; Cai, M.; Li, Y.; Hsam, S. L. K.; Zeller, F. J. Rapid identification of HMW glutenin subunits from different hexaploid wheat species by acidic capillary electrophoresis. *Cereal Chem.* **2004**, *81*, 561−566.
(28) Li, J.; Wang, S.; Yu, Z.; Li, X.; Guo, G.; Feng, S.; Ma, W.; Yan, Y. Optimization and development of capillary electrophoresis for separating and identifying wheat low molecular weight glutenin subunits. *J. Cereal Sci.* **2012**, *55*, 254−256.
(29) Zhang, Q.; Dong, Y.; An, X.; Wang, A.; Zhang, Y.; Li, X.; Xia, X.; He, Z.; Yan, Y. Characterization of HMW glutenin subunits in common wheat and related species by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). *J. Cereal Sci.* **2008**, *47*, 252−261.
(30) Liu, L.; Wang, A.; Appels, R.; Ma, J.; Xia, X.; Lan, P.; He, Z.; Bekes, F.; Yan, Y.; Ma, W. A MALDI-TOF based analysis of high molecular weight glutenin subunits for wheat breeding. *J. Cereal Sci.* **2009**, *50*, 295−301.
(31) Liu, L.; Ikeda, T. M.; Branlard, G.; Peña, R. J.; Rogers, W. J.; Lerner, S. E.; Kolman, M. A.; Xia, X.; Wang, L.; Ma, W.; Appels, R.; Yoshida, H.; Wang, A.; Yan, Y.; He, Z. Comparison of low molecular weight glutenin subunits identified by SDS-PAGE, 2-DE, MALDI-TOF-MS and PCR in common wheat. *BMC Plant Biol.* **2010**, *10*, 124.
(32) Cozzolino, R.; Di Giorgi, S.; Fisichella, S.; Garozzo, D.; Lafiandra, D.; Palermo, A. Proteomics of gluten: mapping of subunit 1Ax2* in Cheyenne cultivar by matrix-assisted laser desorption/ionization. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1129−1135.
(33) Wu, Y.; Engen, J. R.; Hobbins, W. B. Ultra performance liquid chromatography (UPLC) further improves hydrogen/deuterium exchange mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 163−167.
(34) Swartz, M. E. UPLC: an introduction and review. *J. Liq. Chromatogr. Relat. Technol.* **2005**, *28*, 1253−1263.
(35) Zhou, J. H.; Zhao, J.; Yuan, H. C.; Meng, Y.; Li, Y.; Wu, L. M.; Xue, X. F. Comparison of UPLC and HPLC for determination of *trans*-10-hydroxy- 2-decenoicacid content in royal jelly by ultrasound-assisted extraction with internal standard. *Chromatographia* **2007**, *66*, 185−190.
(36) Fekete, S.; Fekete, J.; Ganzler, K. Validated UPLC method for the fast and sensitive determination of steroid residues in support of cleaning validation in formulation area. *J. Pharm. Biomed. Anal.* **2008**, *49*, 833−838.
(37) Yu, Z.; Han, C.; Wang, S.; Lv, D.; Chen, G.; Li, X.; Jiang, G.; Yan, Y. Fast separation and characterization of water-soluble proteins in wheat grains by reversed-phase ultra performance liquid chromatography (RP-UPLC). *J. Cereal Sci.* **2012**, DOI: 10.1016/j.jcs.2012.11.008.
(38) Jin, H.; Zhang, Y.; Li, G.; Mu, P.; Fan, Z.; Xia, X.; He, Z. Effects of allelic variation of HMW-GS and LMW-GS on mixograph properties and Chinese noodle and steamed bread qualities in a set of Aroona near-isogenic wheat lines. *J. Cereal Sci.* **2013**, *57*, 146−152.
(39) Poul, C. S. *The HPLC Solvent Guide*, 2nd ed.; Wiley: Canada, 2002.
(40) Wang, S.; Shen, X.; Ge Pei, Li J.; Subburaj, S.; Li, X.; Zeller, F. J.; Hsam, S. L. K.; Yan, Y. Molecular characterization and dynamic expression patterns of two types of γ-gliadin genes from *Aegilops* and *Triticum* species. *Theor. Appl. Genet.* **2012**, *125*, 1371−1384.
(41) Payne, P. I.; Jackson, E. A.; Holt, L. M. The association between γ-gliadin 45 and gluten strength in durum wheat variaties. A direct causal effect on the result of genetic linkage. *J. Cereal Sci.* **1984**, *2*, 73−81.

(42) Alvarez, J. B.; Campos, L. A. C.; Martin, A.; Martin, L. M. Influence of HMW and LMW glutenin subunits on gluten strength in hexaploid tritordeum. *Plant Breed.* **1999**, *118*, 456−458.
(43) Ruiz, M.; Carrillo, J. M. Relationships between different prolamin proteins and some quality properties in durum wheat. *Plant Breed.* **1995**, *114*, 40−44.
(44) Zhao, X.; Xia, X.; Liu, L.; He, Z.; Sun, Q. Review on low-molecular-weight glutenin subunits and their coding genes. *Sci. Agric. Sin.* **2007**, *40*, 440−446.
(45) Wrigley C. W.; Bekes F.; Bushuk W. *The Gluten Composition of Wheat Varieties and Genotypes*; AACC International: St. Paul, MN, 2006; ISBN 1-891127-51-9.