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# Rapid separation and identification of wheat HMW glutenin subunits by UPLC and comparative analysis with HPLC

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**Abstract**

High molecular weight glutenin subunits (HMW-GS) are among the major determinant on wheat bread-making quality. Rapid ultra-performance liquid chromatography (UPLC) methods with high resolution and reproducibility for HMW-GS separation were established in this work. A sample could be completely analyzed in less than 12 min by using the optimized UPLC conditions: gradually increasing elution gradient from 21% to 47% in 30 min at flow rate 0.55 ml/min and separation temperature 55$^{o}$C. The results from 15 consecutive runs showed that UPLC patterns of HMW-GS including peak migration time, height and peak area displayed high reproducibility. In addition, the analysis consumes small amounts of reagent. A total of 34 subunits from 111 wheat cultivars and gene bank accessions were well separated and showed clearly different UPLC patterns. Thus all subunits could be readily identified, especially for 1Dx and 1By subunits that were not separable by traditional HPLC. The results from different growing environments demonstrated that the subunit elution times were highly stable, displaying significant changes in the subunit peak heights and areas, reflecting their expression amounts. In comparison with the state-of-the-art HPLC method, UPLC has obvious advantages in separation time, resolution and reagent consuming, which provides an alternative powerful technique for wheat storage protein studies and quality improvement.

**Keywords**: Bread wheat; Related species; HMW-GS; UPLC.
**Abbreviations:** ACN_Acetonitrile; HPCE_High performance capillary electrophoresis; HMW-GS_high molecular weight glutenin subunits; LMW-GS_low molecular weight glutenin subunits; MALDI-TOF-MS_matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RP-HPLC_reversed-phase high-performance liquid chromatography; RP-UPLC_reversed-phase ultra-performance liquid chromatography; SDS-PAGE_sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA_trifluoracetic acid.

## Introduction

The storage proteins in wheat endosperm consisting of polymeric glutenins and monomeric gliadins are the major determinants of wheat quality properties (Payne, 1987). The polymeric glutenins are subdivided into high- and low-molecular weight glutenin subunits (HMW-GS and LMW-GS), which mainly determine dough elasticity and viscosity, respectively (Payne et al., 1987; Shewry et al., 1992). It is known that HMW-GS are encoded by two tightly linked genes (x- and y-type) at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci on the long arms of chromosomes 1A, 1B and 1D, whereas LMW-GS are encoded by genes at the *Glu-3* loci on the short arms of chromosomes 1A, 1B, and 1D (Payne et al., 1987). Genetic analysis showed that there are extensive allelic variations in HMW-GS compositions, which confer different wheat bread-making qualities. For example, the subunit pair 1Dx5+1Dy10 correlated with superior dough quality while 1Dx2+1Dy12 were related to poor dough property (Payne et al., 1987). Some subunits and subunit pairs also display positive effects on bread-making quality, including 1Ax1, 1Ax2*, 1By8, 1Bx13+1By16 and 1Bx17+1By18 (Branlard and Dardevet, 1985; Yan et al., 2009; Liu et al., 2012). In recent years, through combining with traditional gel electrophoresis and chromatography methods as well as capillary electrophoresis and mass spectrometry, some new HMW-GS in common wheat and related wild species have been identified, including 1Ax2.1*, 1Bx13*+1By19* and 1Bx6.1+1By22.1 in *Triticum spelta* (Yan et al., 2003a); 1Dx1.6$^{t}$, 1Dx3$^{t}$, 1Dx4$^{t}$, 1Dy12.1$^{t}$ in *Aegilops tauschii* (Yan et al., 2004; Zhang et al., 2008; An et al., 2009; Wang et al., 2012); 1By16* in *Triticum dicoccoides* (Jin et al., 2012); ASy15*, AKx1*, AKx3*, AKx2.3, AKy20* and Ky8* in *Aegilops speltoides* and *Aegilops kotschyi* (Ma et al., 2013); Sx2.3* and Sy18* in *Aegilops longessima* (Wang et al., 2013). These new subunits are expected to extend the genetic diversity of HMW glutenin subunits in breeding programs for wheat quality improvement. HMW-GS generally have highly repetitive sequences and similar molecular weight as well as similar surface hydrophobicity, which may result in wrong identification of some subunits by traditional gel electrophoresis such as 1Bx20+1By20 and 1Bx14+1By15 (Gao et al., 2010). Furthermore, the extensive allelic variations at *Glu-1* loci make accurate identification of some subunits quite difficult. Thus, it is highly important to develop new methods for separation and identification of HMW-GS during wheat quality improvement. So far, sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) has been the most widely used method to identify wheat glutenin subunits (Yan et al., 2003b; Gao et al., 2010). The routine SDS-PAGE, however, is relatively slow, labor-intensive and poor reproducibility. In the 90s of last century, capillary electrophoresis (CE) was developed as a powerful tool for wheat storage protein analysis due to its fast, efficient and automated separation (Bietz and Schmalzried, 1995; Li et al., 2012). However, it is generally difficult to maintain a very high resolution and reproducibility when CE is used for analysis of a large number of samples in the practical application based on our experiments. Although two dimensional electrophoresis (2-DE) and various mass spectrometry techniques developed recently, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can obtain a high resolution and accurate determination of subunit molecular mass (Yan et al., 2003a, 2004; Zhang et al., 2008; Liu et al., 2010; Gao et al., 2010), sophisticated experimental operation and high equipment cost limit their wide applications. Since 1980s, reversed-phase high-performance liquid chromatography (RP-HPLC), which has the advantages of automatically separation and quantitative analysis of glutenin subunits, with its initial application in wheat cultivar identification has been routinely applied in cereal chemistry (Bietz, 1983; Huebner and Bietz, 1985; Marchylo et al., 1988). Afterward, it was improved and used for identifying particular glutenin subunits related to bread-making quality (Marchylo et al., 1989; Dong et al., 2008; Gao et al., 2010). However, the disadvantages of RP-HPLC are still obvious, such as longer separation times, more chemical consumes and limited separation resolution. In order to overcome these problems, an revolutionary improvement in chromatographic performance has been achieved by the introduction of ultra-performance liquid chromatography (UPLC), which allows faster and more efficient separation by using small particle-packed column with small diameter that has a positive effect on both system efficiency and analysis time (Nováková et al., 2006). Until now, UPLC has been extensively applied to pharmaceutical, toxicological, clinical analysis and in separation chemistry (Wren, 2005; Swartz, 2005; Fekete et al., 2009). Recently, we have conducted a serious of investigations on UPLC separation and characterization of wheat seed proteins and the results on UPLC separation of wheat water-soluble proteins and LMW-GS have been reported (Yu et al., 2013a,b). In this work, we further optimized and established an efficient UPLC separation method for rapidly separating and characterizing wheat HMW-GS. In addition, a comparative analysis with traditional RP-HPLC was carried out.

## Results and discussion

### *Optimization of UPLC experimental conditions*

In order to get a rapid, high-resolution separation for HMW-GS, different UPLC parameters including elution time, temperature and flow rate were tested according to Yu et al. (2012). The results showed that the similar UPLC conditions for the separations of both HMW-GS and water-soluble proteins were optimized, by which a fast, higher resolution and reproducible separation for HMW-GS could be obtained by using gradually increasing elution gradient from 21% to 47% in 30 min at flow rate 0.55 ml/min and separation temperature 55$^{o}$C. As shown in Fig. 1, four HMW-GS (7+8, 2+12) from Chinese Spring were well separated and reproducible. Analysis of a sample could be completed in about 12 min and HMW-GS generally eluted in 5-11 min. The averages, standard deviations and relative standard deviations (*RSD%*) of peak migration time, peak height and peak area of four HMW-GS in 15 consecutive runs were listed in Table 1. The data showed that high reproducibility of HMW-GS separation was produced under the optimized UPLC conditions and the *RSD%* for migration time, peak height and peak area were less than 0.2%, 2.5% and 3.0%, respectively.

### *Fast characterization of HMW-GS by optimized UPLC*

The optimized UPLC method was used to separate and characterize HMW-GS from bread wheat and related species. In total, 34 HMW-GS from 111 bread wheat cultivar and related germplasms were successfully identified, including some new subunits present in wheat related species (Table 1 and Suppl. Table 1). The elution order of HMW-GS based on their surface hydrophobicities was 1Dy → 1By → 1Dx → 1Bx → 1Ax, same as that separated by RP-HPLC (Fig. 2). Most HMW-GS from hexaploid bread wheat identified by Payne and Lawrence (1983) and several new subunits in spelt wheat reported by Yan et al. (2003) could be well separated and readily differentiated (Fig. 2). Some HMW-GS from *Aegilops caudata*, *Aegilops longissima*, *Triticum dicoccum* and durum wheat were separated and identified by both SDS-PAGE (Fig. 3a) and UPLC (Fig. 3b, c). Two pairs of novel subunits, Cx2**+Cy3* from *Aegilops caudata* encoded by 1C and Sx2.3*+Sy18* from *Aegilops longissima* encoded by 1S$^{l}$ demonstrated clearly different UPLC patterns from 1B and 1D-encoded 7+8 and 2+12 subunits from Chinese Spring (Suppl. Fig. 1b). Three novel subunits from *Triticum dicoccum* (1Ax2.1*, 1Bx14.1+1By22*) also showed clear UPLC patterns different from 1Ax2*, 1Bx6, 1Bx7 and 1By8 subunits (Fig. 3c). In general, 1Ax, 1By and 1Dy subunits could be well separated and readily differentiated. The peaks of 1Dx and 1Bx subunits in some wheat cultivars were slightly closer but they were still differentiable based on their migration times such as 1Dx5 and 1Bx7 (Fig. 4a, c) and 1Dx5 and 1Bx14 (Fig. 4b). Elution times of 34 HMW-GS and their changes in different cultivars and accessions are shown in Table 2. The results demonstrated that all subunits separated displayed different elution times, rendering them to be readily identified. Particularly, superior quality subunits 1Dx5+1Dy10 were readily differentiated from poor quality subunits 1Dx2+1Dy12. The other subunits and subunit pairs that positively affect gluten quality were still separable from other subunits such as 1Ax1, 1Bx13+1By16 and 1Bx17+1By18 based on their elution times (Table 2).

### *Comparative analysis of UPLC and traditional HPLC*

The results of comparative analysis between UPLC and HPLC for the separation and identification of HMW-GS are given in Fig. 4. It is obvious that UPLC had higher resolution and shorter separation time than traditional HPLC. As shown in Fig. 4a, the separation time of HMW-GS from a sample by HPLC was about 28 min, much longer than UPLC (< 12 min). Another disadvantage of HPLC was poor resolution, particularly for 1Dx and 1By subunits. For example, almost all 1Dx and 1By subunits could not be clearly separated by HPLC, such as 1Dx2 and 1By8, 1Dx5 and 1By9 (Fig. 4a), 1Dx5 and 1By15, 1Dx2 and 1By20 (Fig. 4b) and 1Dx5 and 1By18, 1Dx2 and 1By15 (Fig. 4c). This agrees well with previous report by Dong et al. (2009). However, all 1Dx and 1By subunits studied in this work could be clearly separated, and therefore they were readily identified by UPLC (Fig.

**Table 1.** Repeatability of HMW-GS in Chinese Spring separated by UPLC.

| HMW-GS | Migration time (min) | *RSD%* | Peak height (1000 uV) | *RSD%* | Peak areas (1000 uV/S) | *RSD%* |
|---|---|---|---|---|---|---|
| 1Dx2 | 9.12±0.01 | 0.04% | 365.99 ±9.11 | 2.49% | 7317.20±214.58 | 2.93% |
| 1Bx7 | 9.64±0.02 | 0.19% | 321.90±7.36 | 2.29% | 6021.14±161.72 | 2.69% |
| 1By8 | 7.07±0.01 | 0.14% | 41.21±1.01 | 2.45% | 492.41±11.80 | 2.40% |
| 1Dy12 | 6.16 ±0.01 | 0.16% | 167.89±3.45 | 2.06% | 2445.64±61.56 | 2.52% |

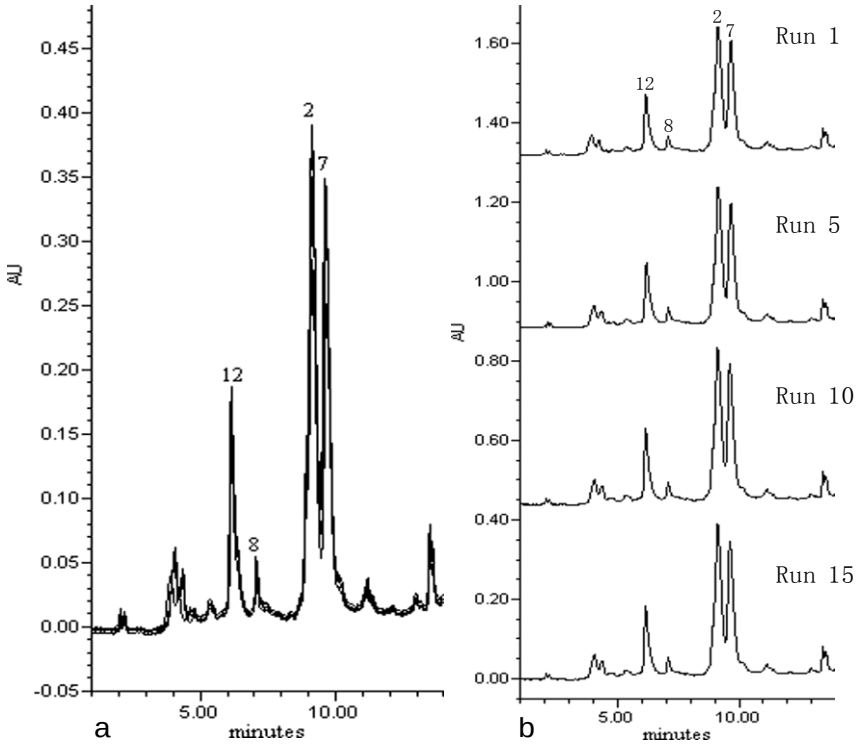


**Fig 1.** Reproducibility of HMW-GS from Chinese Spring separated by UPLC with optimized conditions (gradually increasing elution gradient from 21% to 47% in 30 min at flow rate 0.55 ml/min and separation temperature 55$^{o}$C). HMW-GS compositions were indicated. a. The overlap of 15 consecutive runs. b. 15 consecutive runs.

4a-c). In addition, UPLC only used fewer amount of samples and consumed less reagent compared to traditional HPLC. Thus, UPLC is a more rapid and economically feasible for HMW-GS separation and identification and suitable for fast identifying and screening of desirable subunits at early generations of wheat quality improvement.

***UPLC pattern changes of HMW-GS from different growing locations***

In order to understand the stability of UPLC patterns of HMW-GS, three spring wheat cultivars (CB037, CB037-B and Ningchun 4) were planted at three locations in China (Yingchuan, Xining and Beijing) and their HMW-GS were separated by the optimized UPLC method (Fig. 5). The results showed that the elution times of 9 HMW-GS (1, 17+18, 7+8, 2+12, 5+10), which serves as qualitative parameters, displayed few changes, demonstrating a high stability of glutenin component compositions. However, as quantitative parameters, the peak heights and peak areas of all subunits displayed greater changes (Suppl. Table 2). These results suggest that the growing environments have few effects on HMW-GS compositions, but significantly affect their expression amounts.

**Materials and Methods**

***Plant materials***

A total of 111 common wheat cultivars and germplasms from related species were used in this work, including 70 bread wheat cultivars, 30 spelt wheat cultivars, 2 durum wheat cultivars (Simeto and Bidi 17), 3 cultivated emmer accessions (*T. dicoccum*, AABB, 2n=4x=28), 1 wild emmer accession KU1952 (*T. dicoccoides*, AABB, 2n=4x=28), 3 *Aegilops tauschii* ($D^tD^t$, 2n=2x=14) accessions and 2 Chinese Spring (CS) substitution lines CS-1C(1A) and CS-1$S^l$(1B) developed at the Division of Plant Breeding and Applied Genetics, Technical University of Munich, Germany. In these lines the chromosome 1A and 1B genomes of CS wheat were replaced by 1C chromosome from *Aegilops caudata* (2n=2x=14, CC) and 1$S^l$ chromosome from *Aegilops longissima* (2n=2x=14, $S^lS^l$), respectively. HMW-GS compositions containing 34 different subunits from 111 genotypes were initially identified by SDS-PAGE and their detailed information were listed in Suppl. Table 1.

**Table 2.** Elution time of 34 HMW glutenin subunits from bread wheat and related species detected by UPLC.

| Type | HMW -GS | Elution Time (min) | RSD % | Genotype number | Type | HMW -GS | Elution Time (min) | RSD% | Genotype number |
|---|---|---|---|---|---|---|---|---|---|
| Ax | 1 | 10.95±0.16 | 1.46 | 73 | | 16 | 8.41±0.14 | 1.67 | 9 |
| | 2* | 10.85 | - | 1 | | 16* | 8.21 | - | 1 |
| | 2** | 10.90 | - | 1 | | 18 | 8.00±0.11 | 1.38 | 6 |
| | 2.1* | 10.54±0.15 | 1.42 | 3 | | 18* | 7.45 | - | 1 |
| Bx | 2.3* | 9.56 | - | 1 | | 19* | 8.59±0.12 | 1.40 | 5 |
| | 6 | 9.52±0.11 | 1.16 | 3 | | 20y | 8.64±0.08 | 0.93 | 13 |
| | 6.1 | 10.19±0.19 | 1.87 | 10 | | 22.1 | 7.92±0.13 | 1.64 | 11 |
| | 7 | 9.71±0.13 | 1.34 | 47 | | 22* | 7.24±0.12 | 1.66 | 3 |
| | 13 | 9.27±0.17 | 1.83 | 11 | Dx | 2 | 9.13±0.12 | 1.31 | 57 |
| | 13* | 10.99±0.18 | 1.64 | 5 | | 3 | 8.60±0.10 | 1.16 | 4 |
| | 14 | 10.01±0.13 | 1.30 | 3 | | 3* | 7.20 | - | 1 |
| | 14.1 | 9.62±0.12 | 1.25 | 2 | | 4 | 9.82±0.12 | 1.22 | 6 |
| | 17 | 9.92±0.23 | 2.32 | 6 | | 5 | 9.11±0.12 | 1.32 | 36 |
| | 20x | 10.86±0.12 | 1.11 | 13 | Dy | 10 | 6.28±0.11 | 1.75 | 35 |
| By | 8 | 7.06±0.17 | 2.41 | 33 | | 12 | 6.18±0.10 | 1.62 | 67 |
| | 9 | 7.93±0.12 | 1.51 | 15 | | 12.1 | 6.15 | - | 1 |
| | 15 | 8.22±0.15 | 1.83 | 3 | | 12.2 | 6.12 | - | 1 |

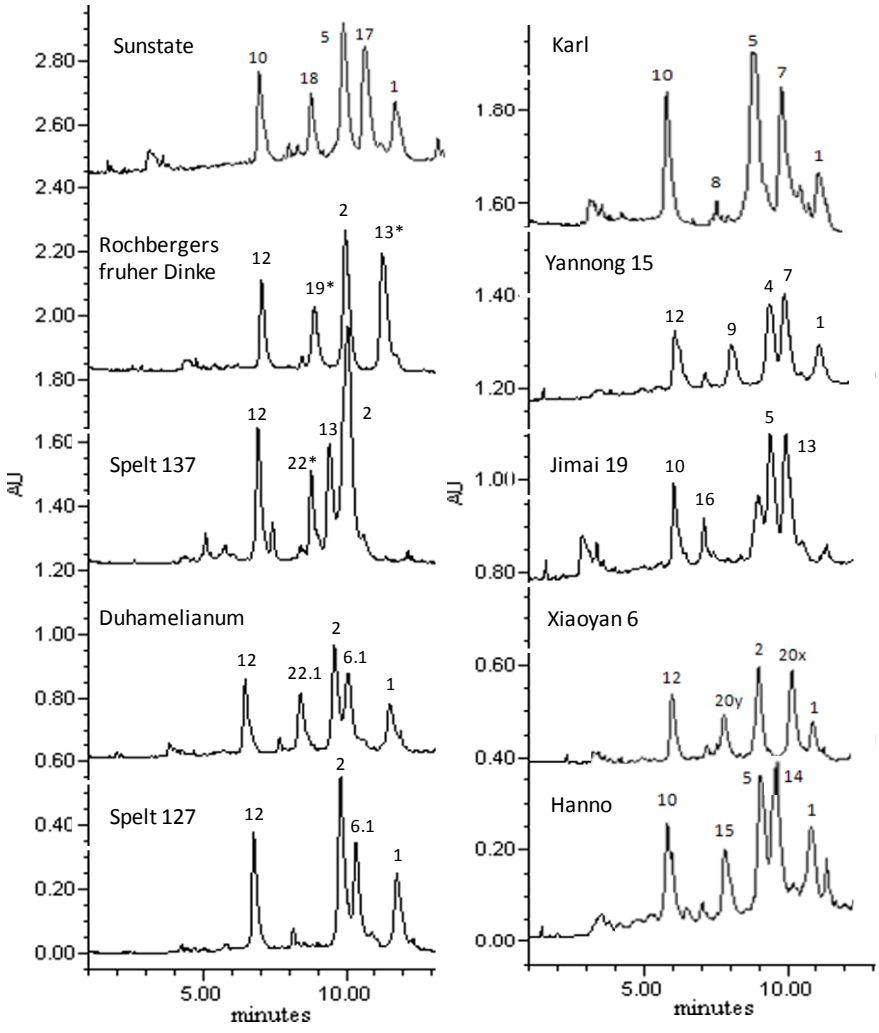


**Fig 2.** Separation and identification of HMW-GS from 10 bread wheat and spelt wheat cultivars by the optimized UPLC. Different HMW-GS were indicated.

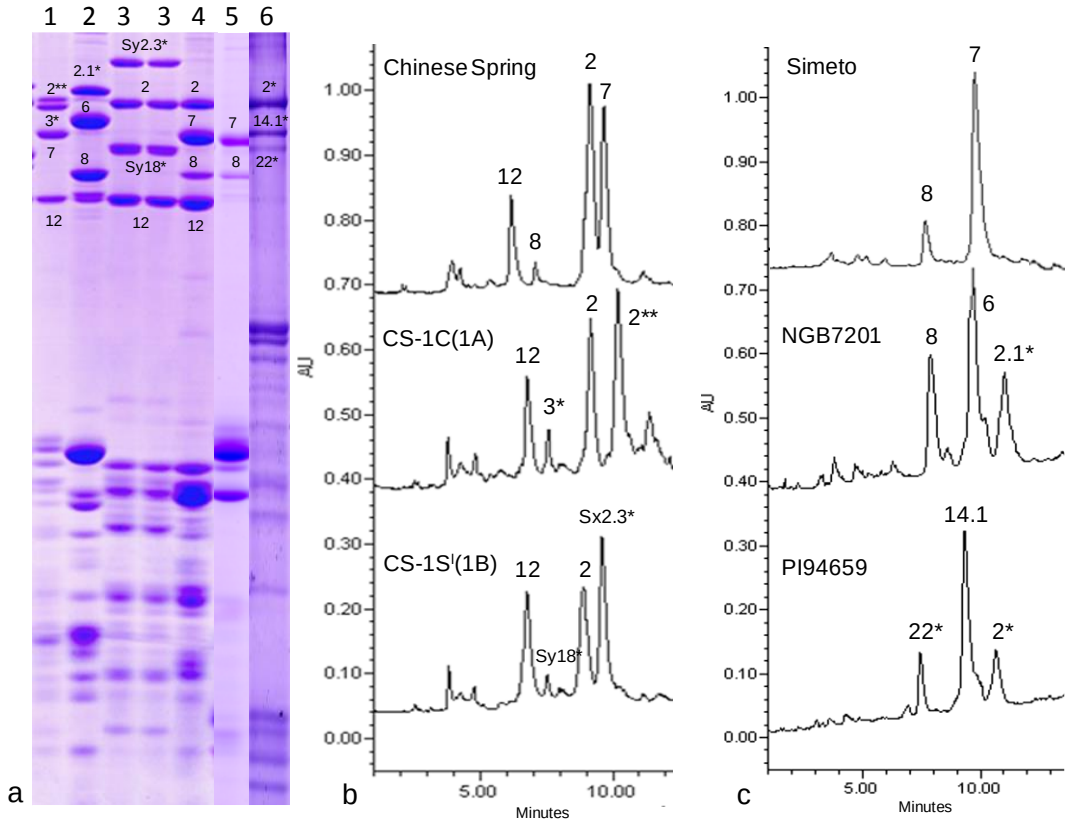


**Fig 3.** Characterization of HMW-GS from Chinese Spring - Aegilops 1C(1B), 1S$^l$(1B) substitution lines and tetraploid germplasms by both SDS-PAGE and the optimized UPLC. a. SDS-PAGE, b. UPLC identification of HMW-GS from substitution lines CS-1C(1B) and CS-1S$^l$(1B), c. UPLC identification of HMW-GS from Italy durum wheat cultivar Simeto and cultivated einkorn accessions PI94659 and NGB7201. Different HMW-GS were indicated.

***Field planting and sampling***

To investigate HMW-GS variations in different growing environments, 3 spring wheat varieties, CB037 (1, 17+18, 2+12), CB037-B (1, 17+18, 5+10) and Ningchun 4 (1, 7+8, 2+12) were planted in three locations, each representing a different ecological district of spring wheat production in China, i.e., Yingchuan, Ningxia province (North), Xining, Qinghai province (Northwest), and Beijing (Jingjintang area). 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 (grain moisture about 10%), and then used for protein analysis.

***HMW-GS extraction***

HMW-GS were extracted from wheat grains according to Zhang et al. (2008) with minor modifications. Wheat grains without embryo were crushed into powder and 30 mg flour was used for HMW-GS extraction. The water-soluble proteins and monomeric gliadins were first removed by continuous four time extractions with 55% isopropanol for 30 min at 65$^o$C. Subsequently, glutenins from the residues were extracted with 100 µl extraction buffer, including 50% isopropanol, 80 mM Tris-HCl pH 8.0 with freshly added 1% dithiothreitol (DTT) by stirring at 65°C for 30 min, and then alkylated by stirring with an equal volume of extraction buffer replacing 1% DTT with freshly added 1.4% 4-vinylpyridine (v/v) under the same water incubation conditions. After centrifugating at 15700g for 15 min, the supernatants were moved to new tubes for SDS-PAGE analysis. HMW-GS were precipitated from glutenin extracts with the addition of acetone to a final concentration of 40% v/v. Precipitated HMW-GS were redissolved in 0.2 mL of 21% v/v acetonitrile (ACN), 0.1% m/v trifluoracetic acid (TFA) and centrifuged for 10 min at 13 000*g*, and then used for RP-HPLC and RP-UPLC analysis within 24h of extraction.

***SDS-PAGE***

SDS-PAGE was carried out by using the method described by Yan et al. (2003b) with minor modifications, and performed on a Bio-Rad Mini-PROTEAN III cell. Sample buffer 8 µl containing 2% SDS, 0.02 % bromophenol blue, 0.08 M Tris-HCl pH 8.0 and 40 % glycerin was mixed with the same volume of protein sample. The mixture was incubated at 65°C for 30 min. After centrifugation at 13000*g* for 10 min, protein samples were electrophoresed at 20 mA for 8 h, and the gels were stained with 1 % Coomassie brilliant blue for 1 h and then destained with solution containing 10 % ethanol and 10 % acetic acid. Finally, the gels were scanned by using a GS-800 Calibrated Densitometer (Bio-Rad, USA).

***RP-HPLC***

RP-HPLC analysis based on Dong et al. (2008) was performed on Agilent 1100 instrument with reversed-phased

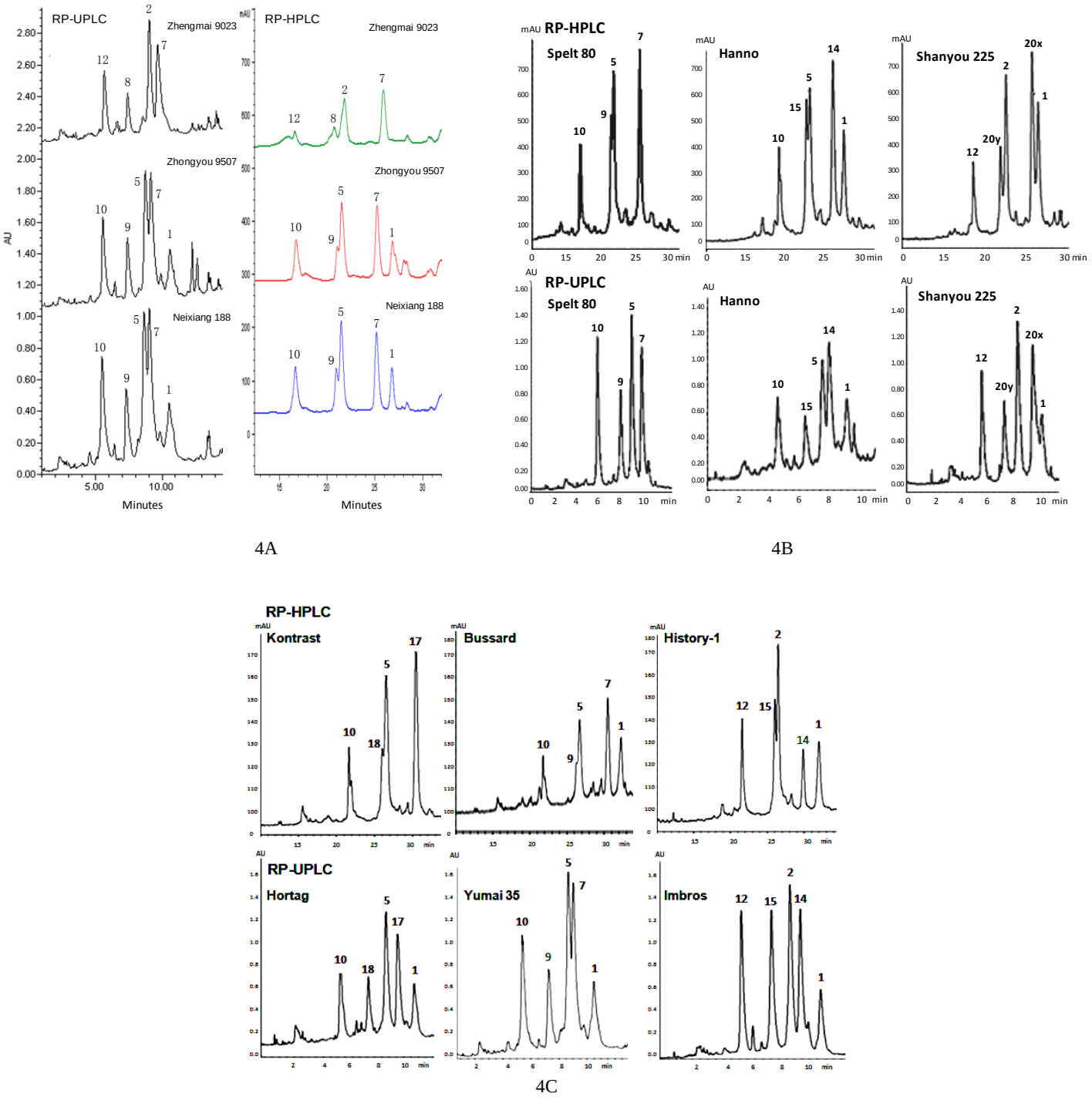


**Fig 4.** Comparison between UPLC and HPLC for the separation and characterization of wheat HMW-GS. a. Three Chinese bread wheat cultivars (Zhengmai 9023, Zhongyou 9507 and Neixiang 188). b. Two bread wheat (Hanno and Shanyou 225) and one spelt wheat (Spelt 80) cultivars with different HMW-GS compositions. c. Six bread wheat cultivars (Kontrast and Hortag, Bussard and Yumai 35, History-1 and Imbros had same HMW-GS compositions).

column (ZORBAX 300SB-C18 Stable Band Analytical 4.6 x 250 mm, 5-Micron). The major analytical parameters performed were set as 60°C for column temperature, 1.00 ml/min for flow rate, 20 µl for sample volume, eluting gradient and the variable concentrations of ACN with 0.06 % TFA gradually growing from 21 % to 47 % (v/v) in 55min. Column washing time between two adjacent samples is 15 min.

The proteins were detected by measuring UV absorbance at 210 nm.

***RP-UPLC***

RP-UPLC was performed on Acquity UPLC$^{TM}$ (Waters Corporation) according to Yu et al. (2012).

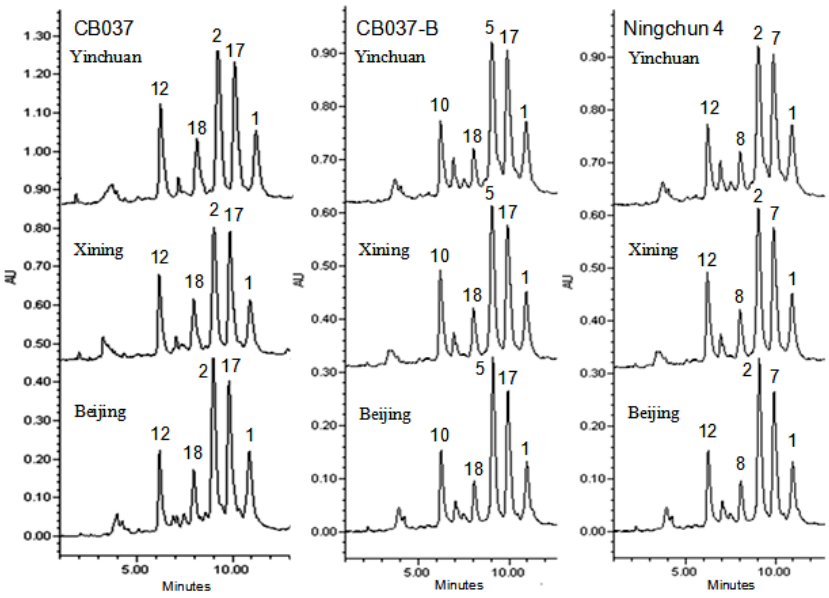


**Fig 5.** UPLC pattern changes of HMW-GS from three Chinese spring wheat cultivars CB037, CB037B and Ningchun 4 growing in three different locations of China (Yingchun, Xining and Beijing) separated by UPLC.

The mobile phases were dd$H_2O$ containing 0.1 % TFA and ACN with 1% TFA, respectively, and UPLC grade solvents were used in all cases. The dissolved HMW-GS samples (10 µl) were injected and eluted with a linear gradient of 21 % to 47 % ACN containing 0.1 % TFA at 0.2-0.6 mL/min flow rates and elute times from 10 to 55 min. A ACQUITY UPLC™ BEH 300 C18 column (1.7 µm particle size, 2.1 mm x 50 mm i.d., Waters, USA) was used with a column temperature from 30 to 60°C, and the protein peaks were detected by UV with absorbance areas at 210 nm.

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