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DETECTION OF QUALITY DIFFERENCES AMONG WHEATS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Proteins extracted with various solvents from small wheat flour samples were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) and size-exclusion HPLC (SE-HPLC). RP-HPLC analysis of gliadin proteins can identify wheat varieties and, for durum wheats (used for pasta products), can rapidly determine quality. After lower-molecular-weight proteins are extracted, high-molecular-weight glutenin proteins can be reduced, alkylated, isolated and analyzed by RP- or SE-HPLC. The ratio of high- to low-molecular-weight glutenin subunits can be used to determine the quality of wheats used to make bread.

INTRODUCTION

Wheat producers have a continuing need to grow new, improved varieties, due to susceptibility of old varieties to diseases and insects. There is also a continuing desire to improve flour quality for bread making, and protein quality for better nutrition. To develop improved varieties, a breeder must make thousands of crosses between wheats, and increase each to test their quality[1]. At present, production of new varieties requires up to 10–12 years, and fairly large samples are needed for testing[2]. Recently, more sensitive methods of testing wheat flour for baking quality have been proposed. One such method involves a sedimentation test with sodium dodecyl sulfate (SDS) in the solution[3]. Another relates polyacrylamide gel electrophoresis (PAGE) patterns of extracted gliadin proteins to durum wheat quality[4,5]. Such rapid and sensitive methods, capable of analyzing very small samples, save both much time and expense.

The rheology of dough and pasta and the quality of the flour or semolina from which it is derived are highly dependent upon the composition and properties of wheat flour proteins. Classically, the proteins have been defined as water-soluble albumins, salt-soluble globulins, 70% ethanol-soluble gliadins, and acid- or alkali-soluble glutenins[6]. Albumins, globulins, gliadins, and glutenins are all heterogeneous. In addition, glutenins are high-molecular-weight proteins consisting of subunits, linked by disulfide bonds, which differ in molecular weight (MW) and charge.

With the advent of high-performance liquid chromatography (HPLC)[7,8], fur-

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ther improvements in protein analytical methodology are now becoming possible. Bietz and co-workers[7,9] first analyzed gliadins extracted from numerous wheat varieties by reversed-phase high-performance liquid chromatography (RP-HPLC). Nearly every variety had a unique elution pattern, conistent with separations achieved by PAGE[10,11], which can be related to quality characteristics. When pedigrees are similar, however, only slight quantitative differences may occur. RP-HPLC may be carried out on single kernels, but, since some genotypic variability may exist within varieties, analysis of composite flour samples is generally preferable.

Recent electrophoresis studies have indicated posible relationships between hexaploid wheat gliadins and baking quality[5,12]. Since resolution of RP-HPLC is generally at least equal to that of one-dimensional electrophoresis, it is likely that similar relationships can be detected by RP-HPLC. For durum wheats, specific differences among gliadins which differentiate varieties having good and poor qualities for pasta products have already been identified by RP-HPLC[8]. Results of these analyses confirm those first found by electrophoresis[4]. With RP-HPLC, however, since the gliadins involved are eluted near the end of the chromatogram, a rapid method could be developed which permitted analysis of up to 130 early-generation durum wheats per day to determine their qualities. With this technique, undesirable types can be discarded, while germ ends of desirable genotypes can be propagated to develop new varieties, since RP-HPLC is nondestructive of germplasm.

Wheat quality may also be better understood because of the preparative capabilities of RP-HPLC. Significant enrichment of gliadins was achieved when RP-HPLC was the sole preparative method[7,8]. but, because of gliadin's heterogeneity, combining RP-HPLC with methods which separate proteins by size or ionic charge gave fractions having significantly better purity[13]. RP-HPLC is particularly valuable since it complements these other methods, and is extremely rapid. Similar procedures can be used to fractionate wheat albumins and globulins, as well as reduced and alkylated glutenin subunits[14].

RP-HPLC of reduced and alkylated glutenin subunits has also been found capable of differentiating wheat varieties on the basis of high molecular weight subunits, thus predicting breadmaking quality[14]. We here describe procedures developed for rapid, efficient, small-scale extraction of glutenin subunits and unreduced gluten proteins, and for their analysis by RP-HPLC and size-exclusion HPLC to relate proteins to wheat quality.

EXPERIMENTAL*

Materials

Wheat flour samples were obtained from V. Youngs, USDA Spring and Durum Wheat Quality Lab, Fargo, ND, U.S.A. These wheats were grown in four locations in test plots in N. Dakota, Arizona, and California in 1983. They were previously analyzed for protein content, mixing time, loaf volume and general bread score. Organic solvents used were HPLC grade; other chemicals were reagent grade or better, as described previously[7].

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* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Sample preparation

Flour samples (50 mg) were extracted in 10-ml capped polypropylene centrifuge tubes with appropriate solvents, as described below, for 15–30 min with continuous agitation on a Buchler Vortex evaporator (Buchler, Fort Lee, NJ, U.S.A.). Extractions were performed at room temperature, unless stated otherwise. Samples were centrifuged for 15–60 min in a Beckman model L centrifuge (Beckman, Fullerton, CA) in a type 40 rotor at speeds of 12000 rpm for some initial fractions and 25000 rpm (50000 *g*) for the final extraction. This high-speed centrifugation ensures that all gel-like partially soluble protein, which could clog HPLC column frits and filters, is removed.

To obtain a total protein extract, flour was extracted for 30 min with 1.5 ml of 0.1 *M* sodium phosphate buffer, 2% SDS (pH 6.9).

To obtain glutenin subunits, the following procedure was carried out. Flour was first extracted with 5 ml of 0.04 *M* sodium chloride for 20–30 min and centrifuged. The residue was then extracted with 5 ml 70% ethanol for 30 min and centrifuged. The remaining residue was agitated with 2 ml 0.1 *M* acetic acid for 15 min, whereupon ethanol was added to 70% by volume and extraction was continued for an additional 30 min. The mixture was then adjusted to pH 6.5 with sodium hydroxide to precipitate glutenin, cooled in an ice bath for 1 h, and centrifuged to remove any remaining soluble gliadin[15]. Glutenins were then reduced and extracted from the residue for 1.5 h with 0.1 *M* sodium phosphate (pH 7.5), containing 2% SDS and 0.1% dithiothreitol (DTT), and treated for 45 min with sufficient 4-vinylpyridine to alkylate all sulfhydryls of both the protein and DTT[14]. The reaction mixture was then acidified to *ca.* pH 3.0 with 3 *M* hydrochloric acid and centrifuged. The clear solution was used for HPLC.

Instrumentation

The apparatus used included a Spectra-Physics (San Jose, CA, U.S.A.) SP8700 solvent delivery system with a Rheodyne (Cotati, CA, U.S.A.) sample injector or a WISP 710B automatic sample injector and a Schoeffel SF770 spectroflow monitor (Kratos, Ramsey, NJ). Columns used were a 600 × 7.5 mm TSK G4000SW size-exclusion HPLC (SE-HPLC) column (Varian Assoc., Palo Alto, CA, U.S.A.) and a 250 × 4.1 mm SynChropak RP-P(C_{18}) (SynChrom, Linden, IN, U.S.A.) reversed-phase HPLC (RP-HPLC) column. Eluted components were generally detected at 210 nm at 0.1 AUFS/10 mV, recorded on an OmniScribe (Houston Inst., Austin, TX, U.S.A.) recorder and simultaneously stored in a ModComp (Ft. Lauderdale, FL, U.S.A.) computer system for subsequent integration and replotting. A TSK G3000SW (75 × 7.5 mm) guard column was used for SE-HPLC, and a 0.5-μm in-line prefilter (No. A-103, Upchurch Sci., Oak Harbor, WA, U.S.A.) was also used for both SE-HPLC and RP-HPLC[7]. Solvents used for the RP-column were (A) 15% acetonitrile and (B) 80% acetonitrile, each containing 0.1% trifluoroacetic acid (TFA). For SE-HPLC, the solvent was 0.1 *M* sodium phosphate (pH 6.9), containing 0.1% SDS; SDS is necessary to dissociate fully high-molecular-weight gluten proteins.

RESULTS

RP-HPLC of reduced glutenin subunits

Earlier SDS-PAGE studies of glutenin subunits suggested molecular weights of 10000 to 130000[16], but more recent studies suggest that the reported molecular weights of some subunits were too high[17]. To analyze glutenin by RP-HPLC, it is necessary to reduce disulfide bonds and alkylate the resulting sulfhydryls, thus dissociating glutenin to its subunits[14]. For the variety Chinese Spring, and presumably for all other wheats, the high-molecular-weight glutenin subunits are associated with longer dough mixing times and, to some extent, bread quality. Since high-molecular-weight subunits are eluted before low-molecular-weight saubunits[14], their quantitative determination may serve as an important new method of determining wheat baking quality. Studies of glutenin subunits by SDS-PAGE have demonstrated that high-molecular-weight subunits are related to wheat baking quality[18]. Fig. 1 shows the elution patterns obtained by RP-HPLC of glutenin subunits of two wheat varieties having long (Yecora Rojo, 4.5 min) and short (Iuanillo 86, 2 min) mixing times. Both qualitative and quantitative differences occur between glutenins of these wheats. When we express protein subunits eluted between 18 and 27.5 min (found to be the high-molecular-weight subunits by SDS-PAGE[14] as a percentage of total subunits

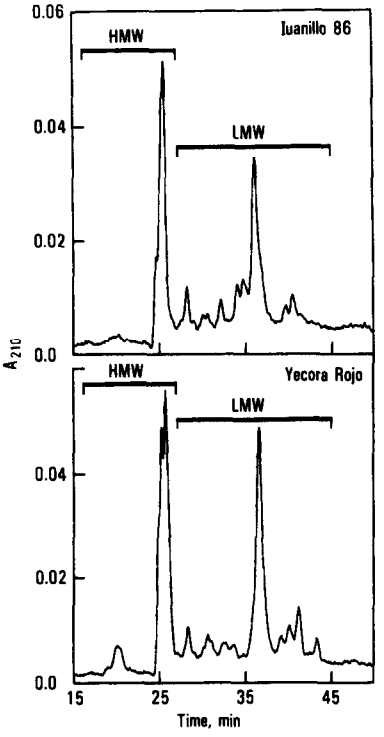

Fig. 1. RP-HPLC of reduced and alkylated glutenin subunits from hard red winter wheats. Iuanillo 86 and Yecora Rojo had mixing times of 2.0 and 4.5 min, respectively. Proteins were eluted with a 2-step linear gradient from 0 to 20% B in 3 min and 20 to 48% B in 42 min. Areas used to calculate the percent of eluted protein are shown by bars at top. HMW, LMW = high- and low-molecular weight subunits.

(between 18 and 45 min), we find a direct relationship of percent high-molecular-weight subunits to good wheat quality. These quantitative determinations are based on the absorbance at 210 nm, which gives a reasonably accurate determination of protein concentration based on peptide bonds, and is much less dependent on specific amino acids than is the absorbance at 280 or 254 nm.

Fig. 2 relates, for six wheats (two of which were shown in Fig. 1), the percent high-molecular-weight glutenin subunits to mixing times, loaf volumes, and general bread scores. Correlation coefficients were determined for percentage of high-molecular-weight glutenin subunits *vs.* mixing time (0.867), loaf volume (0.658), and general score (0.940). These data strongly suggest that the percentage of high-molecular-weight glutenin subunits in wheat is a good predictor of both general score and mixing time, but is less closely related to loaf volume. General score is perhaps most closely related to over-all bread quality, with a score of 4.0 being best. There are known relationships between molecular weight distributions of native glutenins and mixing time[19], but these results demonstrate that the actual amounts of reduced high-molecular-weight glutenin subunits are also related to mixing time. A high loaf volume is generally desirable in bread, but this is perhaps less important than texture, since overly large holes will reduce quality. These results, then, for the first time, strongly indicate statistically significant relationships between various bread quality parameters and the percentage of high-molecular-weight glutenin subunits; thus, for the samples in Fig. 2, Numbers 3, 4 and 6, each having glutenins containing more than 40% high-molecular-weight subunits, are clearly indicated as being best. The general validity of this method as a predictive tool for bread wheat quality obviously needs further confirmation, and is being tested with a greater number of diverse samples.

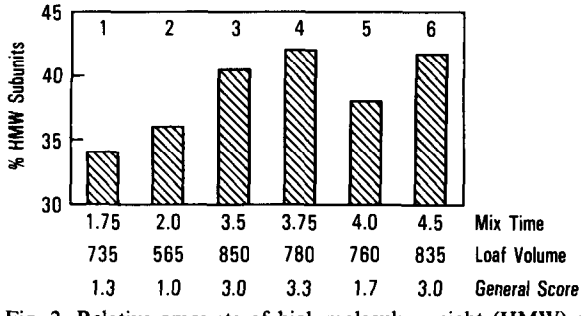

Fig. 2. Relative amounts of high-molecular-weight (HMW) glutenin subunits from 6 hard red winter wheats of various qualities: (1) Anza' (2) Iuanillo 86; (3) Probrand 771; (4) Probred; (5) Westbred 911; and (6) Yecora Rojo. Grown at Imperial Valley, CA, U.S.A. A general score of 1 = no promise; 2 = little promise; 3 = some promise; and 4 = good promise. Loaf volumes are expressed in ml.

SE-HPLC of unreduced wheat proteins

The various classes of native wheat proteins differ in solubilities and molecular weight[19]. For hexaploid wheats, baking quality and mixing times are determined by absolute amounts or ratios of these classes[19]. Most methods used to quantitate amounts of wheat protein classes employ either gel chromatography or sequential extraction, which are tedious, insensitive, lengthy, poorly reproducible, and difficult

to quantitate. SE-HPLC has the potential of greatly reducing sample size and analysis time (less than 35 min per analysis), while increasing reproducibility and giving better quantitative data. Fig. 3 shows results for unreduced protein extracts from 3 wheats, chromatographed on a TSK 4000SW column. The patterns appear somewhat similar, but major differences in percentage of the first peak (2.5–6%), which is related to dough-mixing time, are observed. The amount of the first peak is much smaller than previously found by other procedures[19]. This may result from incom-

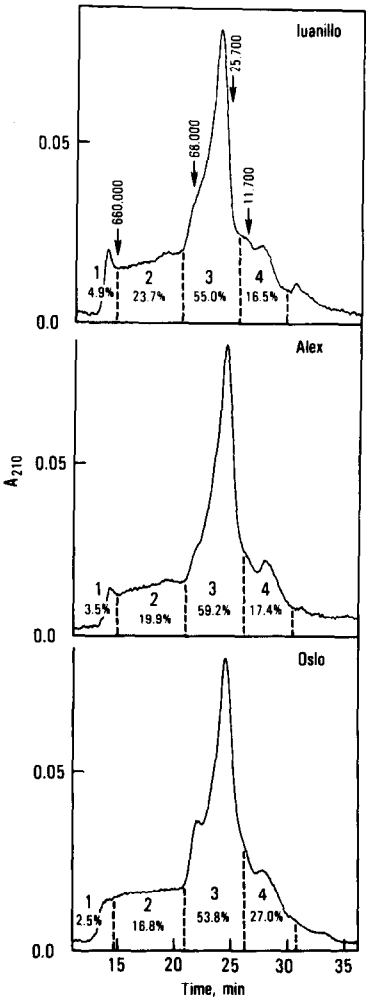

Fig. 3. SE-HPLC of unreduced proteins, extracted with 0.1 *M* sodium phosphate (pH 6.9), containing 2% SDS, from the wheat varieties Iuanillo, Alex, and Oslo. Column solvent was 0.1 *M* sodium phosphate (pH 6.9), containing 0.1% SDS. Flow-rate was 0.85 ml/min. Elution positions of unreduced protein standards [thyroglobulin (660 000), bovine serum albumin (68 000), chymotrypsinogen A (25 700), and cytochrome *c* (11 700)] are indicated. Divisions of graph indicate areas used to calculate percentages of fractions corresponding to HMW glutenin (Fraction 1, MW > 750 000), LMW glutenin (Fraction 2, MW 90 000–750 000), gliadins (Fraction 3, MW 25 000–90 000), and water-soluble proteins (Fraction 4, MW 8000–25 000).

plete extraction of all less-soluble glutenin of higher molecular weight. Alternatively, SDS may dissociate glutenin to relatively low-molecular-weight complexes (Fig. 3). The first possibility seems more likely, since samples were centrifuged at high speed for an extended time to remove poorly-soluble material, which may otherwise clog the column. This material may be glycoprotein in native glutenin[20].

The size of Peak 1 (Fig. 3) is directly related to dough-mixing time, but considerable variability exists. These results appear to disagree partially with earlier SE-HPLC results[21], which indicated that the percentage of the first peak was inversely related to mixing time. However, they agree with previous findings by Sepharose gel chromatography[19]. In earlier SE-HPLC studies, however, it is likely that primarily low-molecular-weight, easily-solubilized glutenins were examined; such proteins are known to be inversely related to quality[22]. Also, in earlier SE-HPLC studies, no special precautions were taken to prevent changes from occuring between time of extraction and analysis by HPLC.

SE-HPLC should be the quickest and simplest method for determining wheat protein molecular weight distributions, since only one extraction is required. Nearly all wheat proteins are extracted, with the exception that 10–20% of highly insoluble glutenin (residue protein) may remain insoluble. Thus, data do not totally represent the least-soluble high-molecular-weight glutenin proteins of wheat. Another problem is that the porosity of most available SE-HPLC columns is too small to fractionate the largest native glutenin species. Also, associated polysaccharides[20] may make some native glutenins poorly soluble in SDS, and they may be removed by high-speed centrifugation.

Another problem with our present SE-HPLC method may be that proteases or other enzymes, extracted with 0.1 *M* sodium phosphate + 2% SDS at pH 6.5–7.0[23,24] along with glutenin, may remain active. High-molecular-weight glutenins could thus be degraded to smaller species, thereby rapidly changing the molecular-weight distribution. Thus, samples stored for a few days at room temperature or even at −17°C exhibit decreased size (up to 30% in 5 days at room temperature) of Peak 1. In addition, samples do not seem to change at the same rate, possibly indicating variation in quantity of enzymes present or differences in associative tendencies. Thus, at present, samples are stored frozen until immediately prior to analysis in order to minimize such changes (less than 10% in 7 days). Obviously, further studies are necessary to eliminate this problem and improve the reliability of the method.

SE-HPLC of glutenin subunits

Another potential way of relating the molecular weight distribution of wheat protein to quality is to extract first all low-molecular-weight proteins, then to reduce and alkylate glutenins, and finally to analyze glutenin subunits by SE-HPLC. SE-HPLC, in the presence of SDS, reveals two major distinct fractions, corresponding to high-molecular-weight and low-molecular-weight glutenin subunits[14]. As we showed with RP-HPLC, the ratio of high-molecular-weight and low-molecular-weight glutenin subunits is related to wheat quality characteristics; this ratio may also be rapidly estimated by SE-HPLC.

Fig. 4 shows results for four wheats, varying in mixing time. For these samples, all saline-soluble and 70% ethanol-soluble proteins were first extracted. Glutenin soluble in 2% SDS at room temperature (30-min extraction) was then dissolved, and

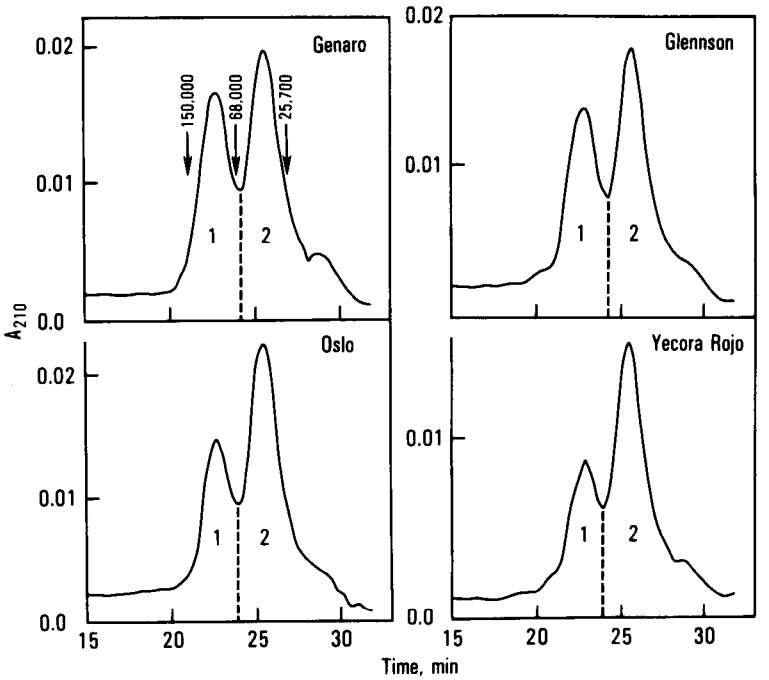

Fig. 4. SE-HPLC of reduced and alkylated insoluble residue protein. Chromatographic conditions are as in Fig. 3. Wheat varieties are Genaro, Glennson, Oslo, and Yecora Rojo. Unreduced protein standards used are the same as in Fig. 3, plus immunoglobulin F (MW 150000).

removed by centrifugation. Finally, the remaining insoluble residue protein, consisting of high-molecular-weight native glutenin, was reduced and alkylated, as described previously. Subunits of this residue protein fraction were analyzed on a TSK 4000SW column (Fig. 4 and Table I). There is a positive relationship ($r = 0.79$ between mixing time and the quantity of Fraction 2. However, other SE-HPLC analyses (not shown) of subunits of the entire glutenin fraction showed a positive relationship between mixing time and the quantity of high-molecular-weight subunits; a similar relationship was apparent from RP-HPLC of glutenin subunits (Figs. 1 and 2). This suggests significant differences in subunit composition among native glutenins, the more soluble fraction having a higher content of high-molecular-weight subunits.

TABLE I

COMPARISON OF PERCENTAGE GLUTENIN SUBUNITS TO MIXING TIME

Wheat variety	*Mixing time (min)*	*Fractions (%)*	
		1	*2*
Genaro	2.75	43.0	57.0
Glennson	2.75	42.5	57.5
Oslo	3.0	34.3	65.6
Yecora Rojo	4.75	32.0	68.0

DISCUSSION

Our results demonstrate that several HPLC methods are now available for analyzing wheat proteins and for estimating wheat quality. The unique nature and the complexity of wheat proteins permit formation of doughs having the visco-elastic properties necessary to produce a good loaf of bread; among other cereals, only rye may form a somewhat similar gluten. However, the complexity and variability of gluten may also be associated with problems in milling and baking. Thus, there is a continuing need for fast, simple, and accurate methods for analysis of wheat proteins.

Results to date clearly demonstrate that wheat varieties can be identified by RP-HPLC of gliadins, and show that durum quality may be predicted by RP-HPLC. However, it is not yet possible to estimate bread wheat quality by RP-HPLC of gliadins. RP-HPLC of reduced glutenin subunits may be useful for determining wheat quality. Such reduced and alkylated extracts are stable for at least one month. This facilitates reproducibility of the method[14]. Preparative RP-HPLC is also useful for purifying wheat proteins quickly and in significant quantity.

Examination of whole wheat protein extracts by SE-HPLC is easy and rapid, but instability of such samples extracted by SDS, and problems involving incomplete extraction, may limit the usefulness of this method. However, by examining only reduced and alkylated glutenin subunits by SE-HPLC (or RP-HPLC) relationships between subunit molecular weight distribution and mixing time or bread quality have been demonstrated.

Even though methods relating protein size to dough mixing time have been found, mixing time is not the only factor related to bread quality, which may be better indicated by a "general score". This score is dependent on many factors but difficult and time-consuming to determine. Our results, based on RP-HPLC for estimating the high-molecular-weight subunits in glutenin from various wheats reveal the highest correlation between percentage of high-molecular-weight glutenin subunits and general score, suggesting the potential usefulness of this rapid method for quality prediction. Previous chromatographic methods lacked the speed, sensitivity, and accuracy to permit reliable predictions. It is obvious that additional studies, based on a greater number of diverse samples, are necessary to ascertain the general validity of HPLC methods for prediction of bread-making quality, as well as for determining how fractions interact to produce a good loaf of bread. It is also apparent that HPLC has become and will remain a highly significant analytical tool for wheat protein analysis and for quality prediction.

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