Rice Cultivar Identification by High-Performance Liquid Chromatography of Endosperm Proteins

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ABSTRACT

Rice varietal identification is important for breeding new varieties and for marketing varieties for specific uses. Previous identification methods were not satisfactory. We therefore analyzed rice prolamins (soluble in high concentrations of alcohol), salt-soluble proteins, and glutelins by reversed-phase high-performance liquid chromatography (RP-HPLC) to find a better method. Using improved extraction and RP-HPLC conditions, glutelins, rice's major storage proteins, best differentiate varieties. RP-HPLC also reveals that amounts of protein classes differ among varieties.

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Identification of rice varieties is important during breeding and in commerce because varieties vary in cooking quality, taste, and nutritional value. Varieties cannot usually be identified by physical appearance, so other methods, such as those based on protein analysis, have been developed.

Milled rice contains 1.7–7.6% alcohol-soluble prolamins, 0.3–9.9% albumins, 1.6–14.0% globulins, and 73–94% glutelins (depending on analytical method, genotype, and environment) (Cagampang et al 1966, Houston et al 1968, Lee et al 1968). In contrast, prolamins predominate in some other cereals.

Investigators have characterized rice proteins by gel electrophoresis (Mandac and Juliano 1978, Villareal and Juliano 1978, Chauhan and Nanda 1984 Kusama et al 1984, Sarkar and Bose 1984), two-dimensional electrophoresis and electrofocusing (du Cros et al 1979, Guo et al 1986, Chen et al 1987), gel filtration chromatography (Iwasaki et al 1982), and reversed-phase (RP-) high-performance liquid chromatography (HPLC) (Lookhart et al 1987, Hussain et al 1989). Isozyme analysis may also identify varieties (Glaszmann 1987). Of these methods, two-dimensional electrophoresis best resolves proteins and differentiates genotypes but is difficult and slow.

RP-HPLC effectively separates alcohol-soluble prolamins of many cereals, permitting varietal identification (Bietz et al 1984, Lookhart 1985, Marchylo and Kruger 1984). In rice, however, such analyses appear less useful, because prolamins are a minor fraction. Lookhart et al (1987) found close similarity among chromatograms of varieties analyzed by HPLC. An alternative extraction procedure was devised by Hussain et al (1989), permitting RP-HPLC differentiation of many varieties; in this work, proteins from all classes may be present. Because nonprolamin proteins are most plentiful in rice, we explored other methods for their analysis by RP-HPLC. We also extracted proteins by other solvents in our search of an improved method of varietal identification.

MATERIALS AND METHODS

Materials

Twenty-seven samples of short, medium, long, and extra long rice cultivars (mainly U.S. commercial varieties, plus a few foreign and specialty rices) were from Beaumont, TX (1985). Kernels were harvested and prepared as before (Webb et al 1986). Milled kernels (8–12) of each sample were pulverized for 20 sec in a Wig-L-Bug (Crescent Dental Mfg. Co., Lyons, IL). The powder was sieved to under 250 μm for analysis.

Six samples from B. Juliano (Table I) were milled flours prepared by first dehulling rough rice with a Satake THU-35A test husker, then milling the resulting brown rice in a Satake TM-05 grain testing mill, and grinding it in a Udy cyclone mill with 60-mesh sieve.

Organic solvents, including acetonitrile (ACN) and trifluoroacetic acid (TFA), were HPLC grade. Distilled water was further purified with a Barnstead Nanopure system. Other chemicals were reagent grade or better (Bietz 1983, Huebner and Bietz 1984).

Sample Preparation for HPLC

All extractions were done in 10-ml capped polypropylene centrifuge tubes (Huebner and Bietz 1984) at room temperature. Flour (150 mg) was first extracted for 20 min with 4 ml of *n*-butanol/ethanol (50:50, v/v) to remove lipids, which interfere with RP-HPLC when sodium dodecyl sulfate (SDS) is in the extracting solvent. Samples plus extractant were mixed in a Buchler Vortex-Evaporator (Buchler Inst., Fort Lee, NJ) at sufficient speed to keep the flour suspended. The mixture was then centrifuged for 12 min at 17,000 × *g*. The extractant was discarded.

Prolamins were first extracted from duplicate samples of defatted flours with 60% *n*-propanol for 30 min by vortexing as above. This extraction procedure was suggested by G. Lookhart (*personal communication*) and Sugimoto et al (1986), because *n*-propanol is a better prolamin extractant than 70% ethanol. Centrifugation was at 17,000 × *g* for 15 min. Albumins plus globulins were then extracted with buffered (pH 7.5) 1.0*M* NaCl for 30 min using the vortex mixer as above, with centrifugation as for prolamins. Finally, glutelins were extracted at pH 8.0 with 3% SDS + 0.5% dithiothreitol (DTT) for 30 min on the vortex mixer. After centrifugation for 20 min at 47,000 × *g*, clear supernatants were transferred to vials for HPLC analysis. Insoluble material can also be removed by filtration (0.2 or 0.45 μm)

TABLE I
Milled Rice (*Oryza sativa* L.) Samples of International Varieties from IRRI[a]

Variety	Pedigree	Stature	Source
H4	indica	tall	Sri Lanka, IRRI crop
Milyang 49	indica × japonica	semidwarf	Korean, IRRI crop
IR42	indica	semidwarf	Philippines, IRRI crop
Rojolele	bulu or javanica	...	Indonesia
Koshihikari	japonica	short	Japan
Mahsuri	indica × japonica	tall	Malaysia

[a] Philippine varieties from B. Juliano, International Rice Research Institute (IRRI).

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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.

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before HPLC. Before HPLC of the NaCl extract, SDS (2%, w/v) and 0.2% (w/v) DTT were added.

RP-HPLC

The HPLC apparatus included a Spectra-Physics (San Jose, CA) SP8700 solvent delivery system and an SP8780XR autosampler. Proteins were detected at 210 nm (0.1–0.2 absorbance units full scale) with an SF770 Spectroflow monitor with a variable attenuation range of 0.01–2.0 absorbance units full scale (Kratos, Ramsey, NJ). A Vydac C_4 column (250 × 4.1 mm; Separations Group, Hesperia, CA) was used. It was preceded by a 22 × 3.5 mm SynChrom RSC guard column and a 0.5-μm in-line prefilter (A-103, Upchurch, Oak Harbor, WA).

Chromatographic solvents (solvent A: H_2O containing 0.11% TFA; solvent B: ACN containing 0.085% TFA) were deaerated by vacuum filtration (0.45 μm) and sparged with helium during use (Huebner and Bietz 1986).

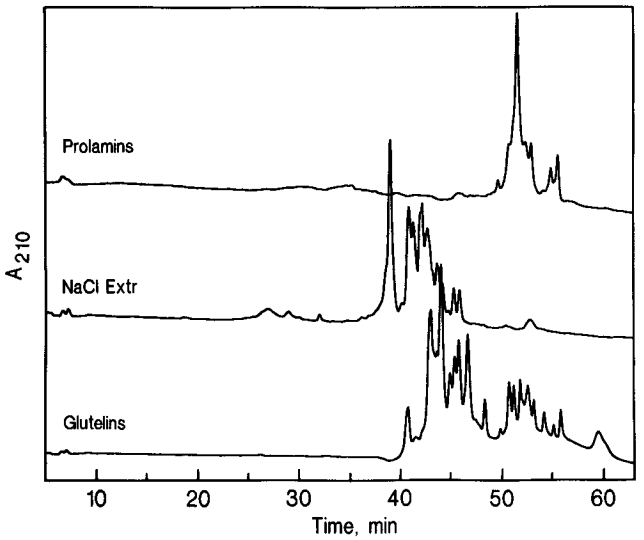

Fig. 1. Reversed-phase high-performance liquid chromatography of Starbonnet prolamins, albumins + globulins (NaCl extract), and glutelins on a Vydac C_4 column at 60°C. Solvent A was water containing 0.11% trifluoroacetic acid, and solvent B was acetonitrile containing 0.085% trifluoroacetic acid. The gradient began at 15% B and increased linearly to 49% B at 50 min; the acetonitrile concentration was then maintained at 49% B until 55 min.

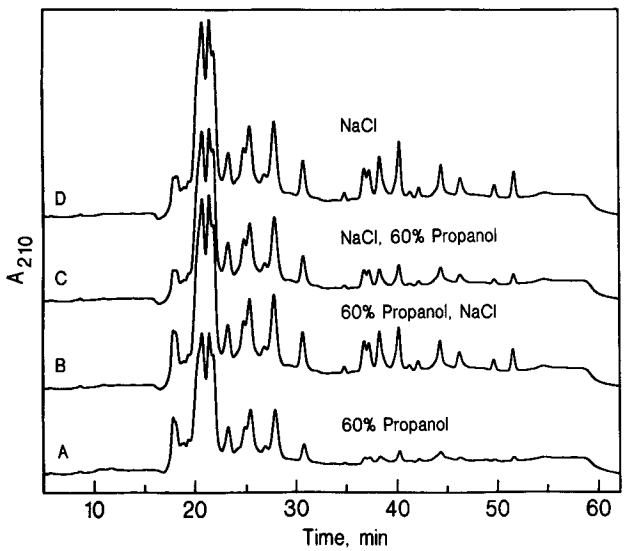

Fig. 2. Reversed-phase high-performance liquid chromatography of Starbonnet glutelins solubilized after extraction with 60% *n*-propanol (**A**), 60% *n*-propanol followed by NaCl (**B**), NaCl followed by 60% *n*-propanol (**C**), and only NaCl (**D**). Reversed-phase high-performance liquid chromatography conditions as in Fig. 5.

Samples (8–40 μl) were analyzed at a solvent flow rate of 1.0 ml/min on columns kept at 60°C with a CH-20-C column heater (Scientific Systems, State College, PA). Gradients were linear but often had more than one step to elute proteins more quickly and improve resolution. Gradients were optimized for each protein class as described in figure legends.

Data Analysis

Data were recorded on an Omniscribe recorder (Houston Inst., Austin, TX) and stored in a ModComp computer system (Ft. Lauderdale, FL) for later integration and replotting. Chromatograms could be redisplayed on a video screen for operator-assisted integration or could be integrated automatically by specifying time. Each method can correct for increasing or decreasing baselines.

RESULTS AND DISCUSSION

Extraction of cereal proteins is usually sequential, using methods similar to those of Osborne (1907) or Landry and Moureaux (1981). However, cereals vary in amounts of protein classes, so extraction conditions must be optimized for each grain. The method we developed for rice evolved from previous studies

TABLE II
Percentages of Protein Classes in Milled Rice[a]

Variety[b]	Prolamins	NaCl Soluble	Glutelins
U.S. extra long grain			
Leah	10	7	83
U.S. long grain			
Bluebelle	9	7	84
CP-231	11	8	81
Dawn	5	6	89
Della	9	9	82
Labelle	8	7	85
LA-110	5	6	89
Lemont	10	10	80
L-202	9	10	81
Newbonnet	9	10	81
Newrex	6	8	86
RU8303169	7	7	86
Starbonnet	6	4	90
Toro-2	9	9	82
International long grain			
Basmati 370	10	5	85
Cica-6	7	7	86
IR-28	7	5	88
IR-36	9	9	82
Jojutla	16	6	78
U.S. medium grain			
M-201	9	7	84
Mars	8	8	84
Nato	8	10	82
International medium grain			
Kwang-chang-di	7	6	87
Ribe	6	9	85
Taichung Native 1	6	9	85
U.S. short grain			
Calmochi 202	10	6	84
S-201	10	8	82
International varieties from IRRI			
H4	10	4	86
IR-42	7	5	82
Koshihikari	11	6	83
Mahsuri	10	5	85
Milyang 49	18	5	77
Rojolele	9	5	86

[a] Determined by reversed-phase high-performance liquid chromatography for equal volumes of samples, extracted at a constant weight/volume ratio. Percentage of each protein class is based on the sum of areas representing the three protein classes. Results are averages of two analyses from different flour samples of the same varieity. Results varied by up to 2% between runs, especially for prolamins.

[b] Except for those from IRRI, rice samples were from varieties grown at Beaumont, TX, in 1985.

of wheat (Bietz 1983, Huebner and Bietz 1985) and rice proteins (Iwasaki et al 1982, Sugimoto et al 1986, Chen et al 1987).

Figure 1 shows RP-HPLC elution profiles of rice albumins plus globulins, prolamins, and glutelins under the same gradient conditions.

Hydrophobicities of most proteins are fairly high and fall in a narrow range for each protein class. Most proteins elute between 37 and 49% ACN, about 5% higher than for most wheat proteins. The glutelin pattern is especially complex, containing components with hydrophobicities similar to both prolamins and salt-soluble proteins. To compare varieties, gradients were modified for each protein class to further improve separations.

Our extraction procedures differ from those previously used. Only single extractions were used, since there was minimal overlap between fractions.

The second major difference was using SDS to extract proteins for RP-HPLC. Urea, often used as a glutelin extractant, may gelatinize starch. This is usually not a problem below 38°C, but short-grained varieties have lower gelatinization temperatures (Normand and Marshall 1989). Thus, SDS extraction of rice proteins was tested. Besides being a good protein extractant, it enhanced some separations. SDS may modify protein retention in RP-HPLC and is compatible with columns (Bietz 1983).

The residue after glutelin extraction was not analyzed, since Kusama et al (1984) found that SDS-containing solvents extract 95–98% of rice protein.

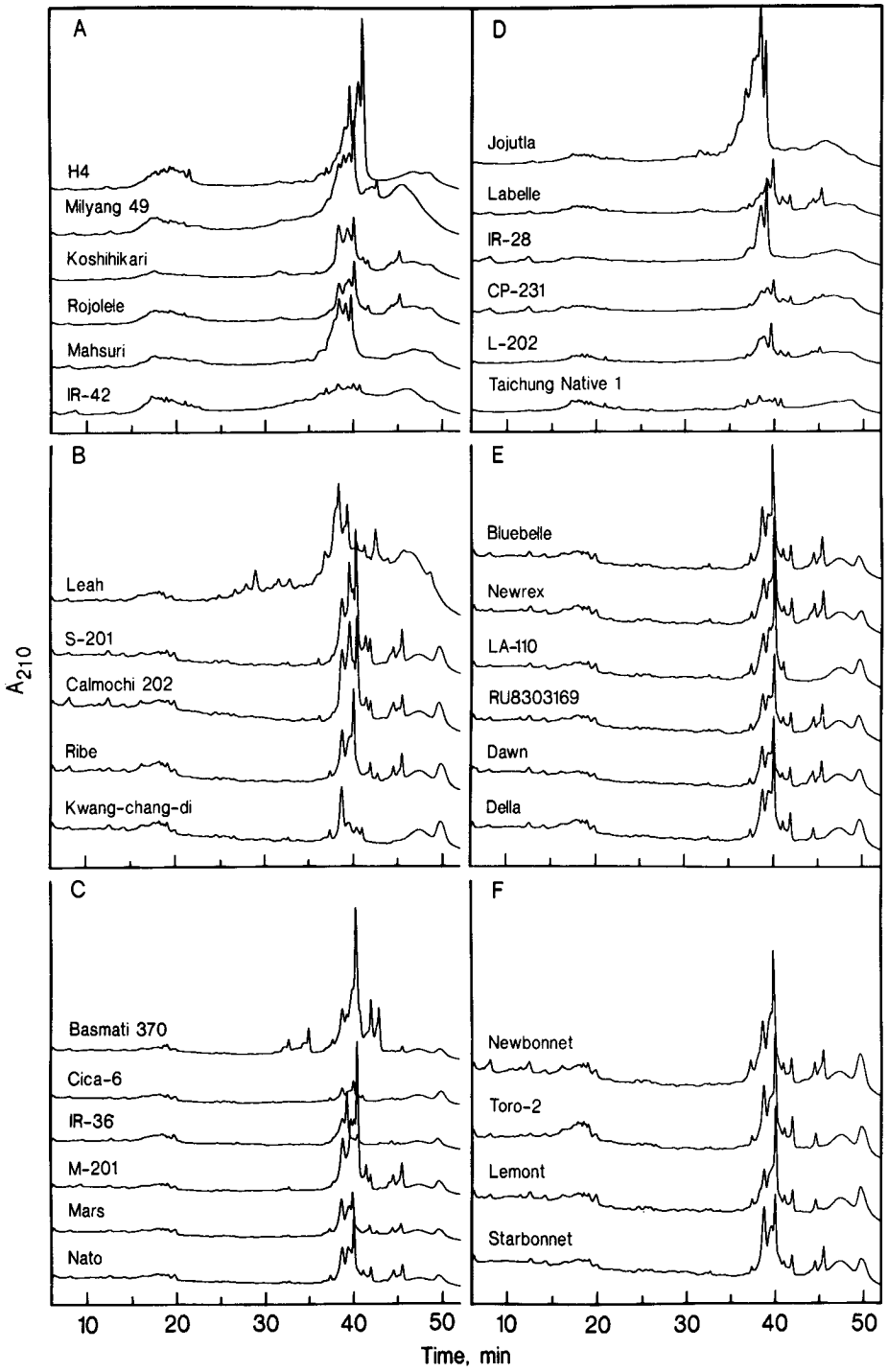

Fig. 3. Reversed-phase high-performance liquid chromatography of prolamins from 33 rice varieties: **A**, varieties from the International Rice Research Institute; **B**, extra long-, short-, and medium-grain varieties; **C** and **D**, long- and medium-grain rice; and **E** and **F**, long-grain varieties. Gradient: initially 20% acetonitrile (ACN), increasing to 34% ACN at 12 min and to 48% ACN at 40 min and held for 4 min. Other conditions as in Fig. 1.

We used SDS plus DTT to dissociate and solubilize glutelins. To extract albumins and globulins without glutelins, however, SDS and DTT must be left out of the extraction solvent. The resulting extracted proteins often resolved poorly upon RP-HPLC, however, so SDS and DTT were added before HPLC. DTT appears especially important for providing good separations of albumins and globulins.

Most sequential extraction procedures first solubilize albumins plus globulins with saline, and then prolamins with alcohol. We found, however, that reversing these steps improved chromatograms of glutelins (Fig. 2). Proteins eluting after 35 min were poorly extracted (about 77%) if NaCl extraction preceded *n*-propanol extraction (Fig. 2C), when compared with results in which the order of these solvents was reversed (Fig. 2b). If salt extraction is omitted (Fig. 2A), extracted glutelin also decreases markedly (61%). Surprisingly, however, if alcohol extraction is not done (Fig. 2D), results for glutelin are qualitatively and quantitatively identical to those in which alcohol extraction precedes NaCl extraction (Fig. 2b). Thus, for varietal differentiation by glutelin analysis, possibly only NaCl extraction need precede solubilization of glutelin.

Figure 3 shows RP-HPLC separations of rice prolamins. Chromatograms are grouped by grain length as in Table II. Some differences occur between chromatograms, but most varieties were

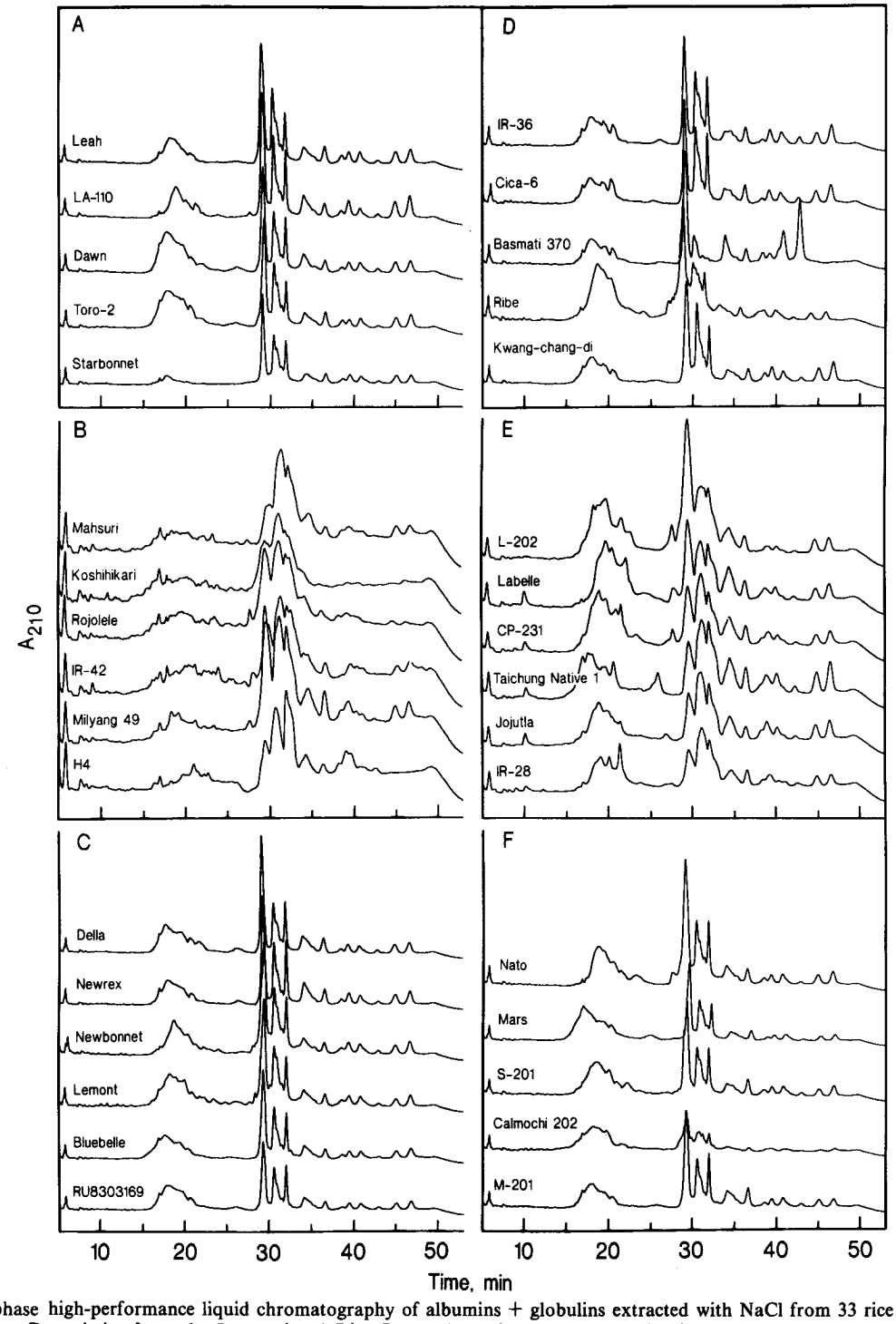

Fig. 4. Reversed-phase high-performance liquid chromatography of albumins + globulins extracted with NaCl from 33 rice varieties: **A**, extra long- and long-grain rice; **B**, varieties from the International Rice Research Institute; **C**, long-grain rice; **D** and **E**, long- and medium-grain rice; and **F**, medium- and short-grain rice. Gradient: initially 18% acetonitrile (ACN), increasing to 26% ACN at 4 min, to 33% ACN at 11 min, to 39% ACN at 40 min, and to 40% at 44 min. Other conditions as in Fig. 1.

similar, as found by Lookhart et al (1987). Duplicate chromatograms of prolamins and other protein classes exhibited good reproducibility.

To resolve prolamins well in a reasonable time, gradients began at 20% ACN. The column was equilibrated to a low ACN concentration to prevent least hydrophobic proteins from eluting in the void volume. This can occur when large samples contain high concentrations of organic solvents (Marchylo and Kruger 1988). After samples were applied, the ACN concentration was increased to 34% at 12 min, and then to 48% ACN at 40 min. This gradient enhanced separation and visibility of peaks between 15 and 21 min (Fig. 3) compared with their resolution in Figure 1. Because few components elute between 21 and 37 min, the gradient can be further shortened.

Figure 4 presents RP-HPLC chromatograms of rice albumins and globulins. Peaks elute over the entire range of hydrophobicity and are well resolved, except for material between 16 and 26 min, which may be similar to early eluting prolamins (Figs. 1 and 3). Many cereal proteins are slightly soluble in different solvents. As with prolamins, differences between chromatograms are slight for many varieties.

The major rice protein fraction (glutelin) remains insoluble after extraction of albumins, globulins, and prolamins. Its disulfide bonds must be reduced before it can be solubilized and separated by HPLC (Fig. 5). Because no glutelins eluted before 38 min (Fig. 1), the gradient began at a higher initial ACN concentration

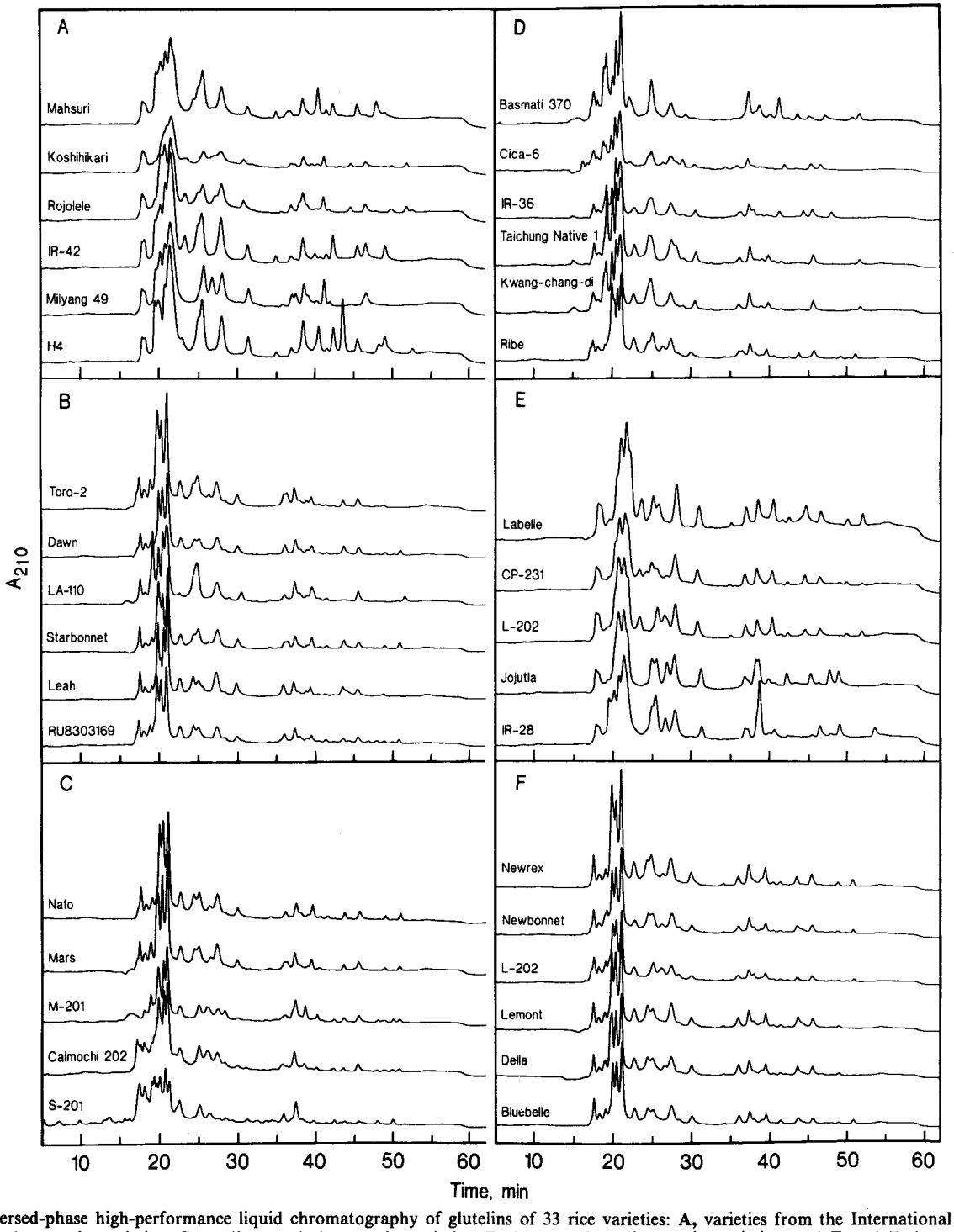

Fig. 5. Reversed-phase high-performance liquid chromatography of glutelins of 33 rice varieties: **A,** varieties from the International Rice Research Institute; **B,** long-grain varieties; **C,** medium and short grain varieties; **D,** long- and medium-grain varieties; and **E** and **F,** long-grain varieties. Gradient: initially 28% acetonitrile, increasing to 36% at 5 min and to 47% at 50 min and held for 4 min. Other conditions as in Fig. 1.

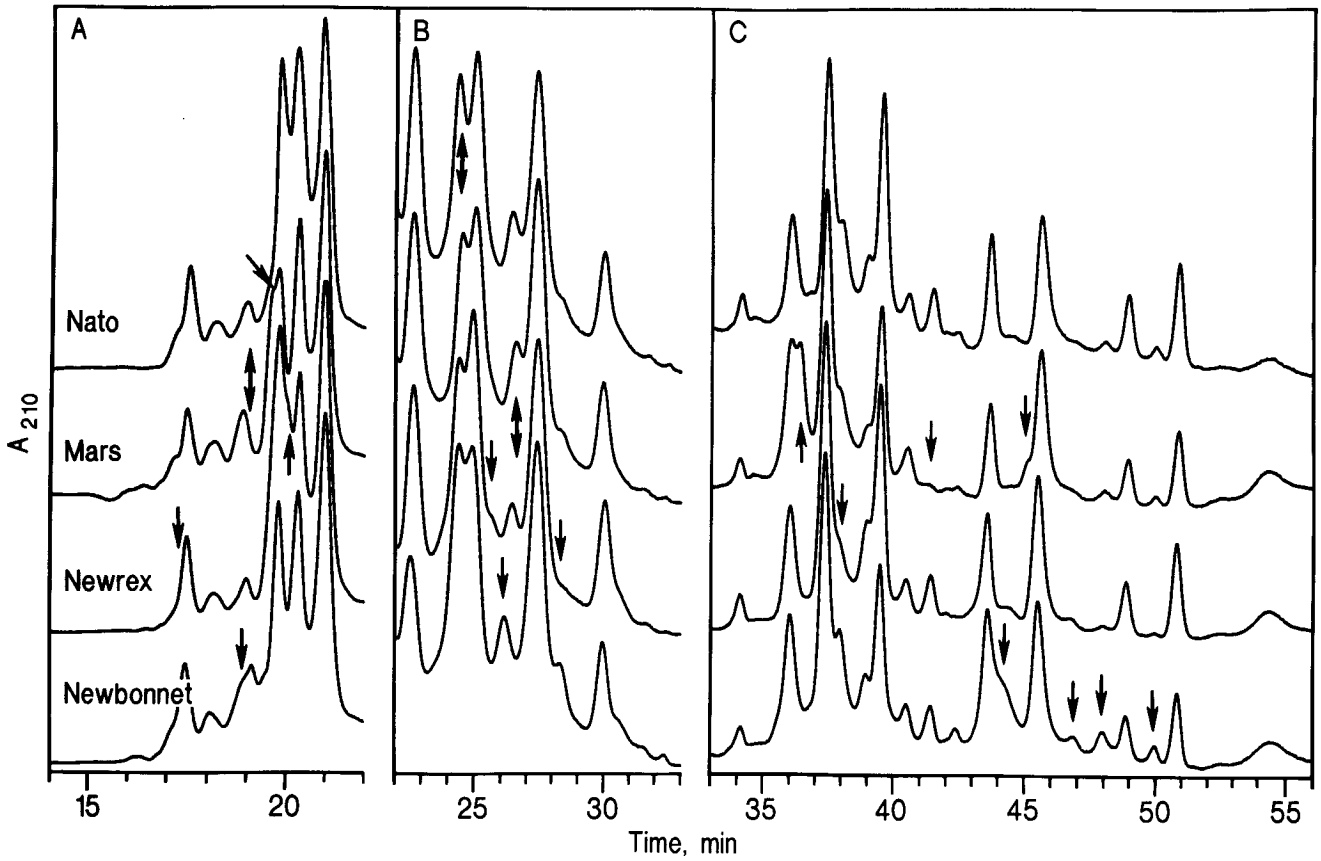

Fig. 6. Differentiation of closely related rice varieties by reversed-phase high-performance liquid chromatography. Portions of chromatograms (Fig. 5) are expanded, emphasizing minor differences. Arrows point to significant qualitative and minor quantitative differences.

and was more shallow. Glutelin subunits of most varieties then resolved into more than 20 peaks (plus additional shoulders and minor peaks).

Most varieties had qualitatively unique and reproducible glutelin chromatograms (Fig. 5). For closely related varieties, however (e.g., Mars, Nato, Newbonnet, and Newrex or Lemont, Della, and Bluebelle), differences were small. For such varieties, computer scale expansion (as in Fig. 6 for Mars, Nato, Newbonnet, and Newrex) better shows minor qualitative differences and some apparent quantitative differences. Identification of rice cultivars based on differences in amounts of individual components is not advisable, however. Environment during kernel development may selectively influence quantities of individual rice proteins, as it does in wheat (Huebner and Bietz 1988).

Other quantitative relationships revealed by RP-HPLC may, however, be valuable in varietal identification. Total amounts of prolamins, albumins plus globulins, and glutelins estimated from absorbance at 210 nm differed significantly (Table II). Prolamins varied by over 300% (ranging from 5% for Dawn and Lemont to 16 and 18%, respectively, for Jojutla and Milyang 49). Salt-soluble proteins varied by more than 100% (4% for Starbonnet and H4 to 10% for L-202, Nato, Lemont, and Newbonnet). These results, though preliminary, do suggest major differences in amounts of protein classes between varieties. Such determinations are also faster, easier, and more sensitive than previous methods used to determine amounts of rice protein classes (Cagampang et al 1966, Houston et al 1968, Lee et al 1968).

CONCLUSIONS

Our results confirm the demonstration by Lookhart et al (1987) and the recent studies of Hussain et al (1989) that RP-HPLC can identify most varieties of rice. There appear to be four major advantages to our procedure for varietal identification, however.

1) Glutelin analysis appears preferable to that of prolamins (Lookhart et al 1987) or total proteins (Hussain et al 1989). There are differences in all protein classes, but glutelins, rice's major storage proteins, differ most among varieties.

2) We report improved extraction and RP-HPLC procedures for rice polypeptides. Glutelin extraction with solvents containing SDS is quantitative, avoids starch gelatinization, and enhances resolution. Glutelin resolution is also enhanced if alcohol extraction is omitted, or if extraction with alcohol precedes NaCl extraction. Resolution of albumins and gobulins is enhanced by reduction of disulfide bonds.

3) Reprocessing of stored chromatographic data, as in scale expansion, facilitates varietal identification by emphasizing minor qualitative differences between genotypes.

4) Quantitation of RP-HPLC data permits rapid estimation of total amounts of each protein class, which differ among many varieties.

These advantages, combined with the speed, sensitivity, resolution, and reproducibility of RP-HPLC, suggest usefulness of the procedure in breeding, marketing, and processing. RP-HPLC may prove as useful for predicting the quality of rice as it is for wheat quality (Burnouf and Bietz 1984; Huebner and Bietz 1985, 1986, 1988). Further correlations of rice protein compositions with quality are necessary to achieve this goal.

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