Effect of Solvent Extraction, Environment, and Genetic Background on Differentiating Rice by Reversed-Phase High-Performance Liquid Chromatography[1]

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ABSTRACT

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The effects of solvent (protein fraction) and location of growth (environmental) were studied on milled U.S. and non-U.S. rices extracted by four separate solvent systems: 60% 1-propanol, 70% ethanol, 60% 1-propanol plus 1% dithiothreitol, and 5% acetic acid. The high-performance liquid chromatography (HPLC) patterns of the alcohol extracts of five U.S. varieties (including short-, medium-, and long-grain rices) grown in five states showed minor but consistent differences depending on location of growth. HPLC patterns of 5% acetic acid extracts of the

same varieties differed more than did the patterns of the alcohol extracts among locations for the U.S. varieties. Therefore, identifying varieties from different environments requires the use of alcohol extracts. HPLC patterns for the Philippine rice IR36 also showed environmental effects of extraction solvent similar to those for U.S. rices. Prolamin patterns of international rices of different genetic backgrounds differed. In 10 lines from the International Rice Research Institute, the prolamin HPLC patterns of progeny usually were similar to those of one parent.

Varietal identification of cereal grains is commercially important. In processed nonviable products such as milled rice, from which some proteins are removed in pearling, varietal identification is more difficult than it is in ground grain. Separation of prolamin proteins by polyacrylamide gel electrophoresis (Wrigley et al 1982) and high-performance liquid chromatography (HPLC) (Bietz et al 1984, Lookhart et al 1986) produces characteristic genotypic "fingerprints" for most wheat cultivars.

Rice prolamins are located in the large, spherical protein bodies of the grain endosperm (Tanaka et al 1980) and are easily extracted by aqueous alcohols. Reversed-phase HPLC patterns of milled rice prolamins differentiated the 29 U.S. rice cultivars analyzed in a previous study (Lookhart et al 1987). For 19 of the 29 rices, peaks eluting at 13–15 min were most useful for defining five subgroups with a common ancestor in their genetic backgrounds. Huebner et al (1990) differentiated U.S. rices by HPLC of the glutelin proteins.

According to the U.S. Rice Council (*personal communication*), international rices of inferior quality have been mixed with and/or sold as high quality U.S. rices. Therefore, considerable commercial interest has been generated in differentiating U.S. rices from those grown overseas. Lookhart et al (1987) were among the first to show that rices from different countries varied; the HPLC pattern of the Philippine variety IR36 was quite different from that of any major U.S. variety. Hussain et al (1989) also found differences among rices from various countries by HPLC analysis of acetic acid extracts.

In this article, we demonstrate through HPLC protein analysis the effect of environment on the proteins extracted from U.S. and Philippine rices by 60% 1-propanol, 70% ethanol, 60% 1-propanol plus 1% dithiothreitol (DTT), and 5% acetic acid. We also present a method that differentiates U.S. and Philippine rices on the basis of their different genetic backgrounds.

MATERIALS AND METHODS

Rice Samples

Samples of the U.S. rice *Oryza sativa* L. were obtained from

B. D. Webb; the Philippine samples were from B. O. Juliano. Rices from the International Rice Research Institute were dehulled in a Satake THU-35A dehuller and milled in either a McGill mill or a Kett Pearlest sample mill. At the U.S. Grain Marketing Research Laboratory, prolamins were extracted from these rices and analyzed by reversed-phase HPLC. All rices were obtained as milled rices.

Sample Preparations

The milled rices were ground into flours using a Udy cyclone mill equipped with a 0.5-mm sieve. Rice flour (250 mg) was extracted with 750 μl of 60% 1-propanol, 70% aqueous ethanol, 60% 1-propanol plus 1.0% DTT, or 5% acetic acid for 30 min at room temperature and centrifuged at 4,000 $\times$ g (Beckman J-6B) for 10 min at 25°C (Lookhart et al 187). The supernatant was decanted and stored at room temperature until analysis by reversed-phase HPLC.

HPLC

The HPLC procedure was a modification of those of Lookhart et al (1986, 1987). Extracts (10 μl) were analyzed on a Hewlett-Packard 1090 chromatography data system by elution from a Vydac (218TP54) RP C18 column (4.6 $\times$ 250 mm) at 1 ml/min. Elution was performed with a multistep linear gradient beginning at 25% aqueous acetonitrile (containing 0.1% trifluoroacetic acid). The acetonitrile concentration increased linearly to 35% at 5 min, to 50% at 10 min, to 75% at 17 min, and to 85% at 18 min; at 19 min it returned to the initial conditions (25% acetonitrile). The total run time from injection to injection was 30 min, including an 11-min reequilibration step between runs. A Hewlett-Packard 1040A diode-array detector was used to detect the eluted components at 210 nm (0.500 absorbance units full scale). A data point was stored every 640 msec on a Hewlett-Packard (9000-310) computer for subsequent integration, replotting, and comparison. All samples were analyzed within four days of extraction.

To reduce some of the variation in protein concentration of the sample and the extract, some of the chromatograms were normalized over the areas of interest. This makes the largest peak in the range of interest the same size for all patterns in a figure.

RESULTS AND DISCUSSION

U.S. Rices

Extraction conditions. The effects of various (optimum) extraction conditions were determined by HPLC analysis of five U.S. rices extracted with 60% 1-propanol, 70% ethanol, 60% 1-propanol plus 1% DTT, and 5% acetic acid (Fig. 1). The patterns of the materials extracted by each solvent have four peaks in common that are most likely solvent-related chromatography peaks: the void volume peak eluting at 3 min and the three peaks eluting at 18, 19, and 21 min. The peaks eluting after 15 min are in

the fast elution area, where the gradient is steep and therefore is quickly cleaning off the column.

Addition of the reducing agent to 1-propanol caused changes in the patterns, especially in the ranges of 3–4 and 14–16 min. The size of the peak eluting at 3–4 min is much larger than that of the broad peaks eluting at 14–16 min. However, it is feasible that the 14–16-min material is reduced by the DTT and therefore elutes earlier as expected of more hydrophylic peptides along with other reduced proteins. The major peak in each chromatogram of Fig. 1A is the early eluting void volume peak eluting near 3 min. Since the normalization procedure makes the largest peak in each chromatogram the same size, the smaller peaks that are necessary for varietal identification are not easily seen in Fig. 1A.

Therefore, the chromatograms were replotted over the 5–30-min range (Fig. 1B).

It is apparent that the consistent portion of the chromatograms from the alcohol solvents 60% 1-propanol, 70% ethanol, and 60% 1-propanol plus 1% dithiothreitol are in the 10–13-min range, whereas the interesting region of the 5% acetic acid extract is in the 5–10-min range. The amount of material extracted by acetic acid appears less than that extracted from the three alcohol solvents, and it elutes in a different region. This is to be expected, as the alcohol solvents extract prolamins, whereas dilute acetic acid extracts more polar (hydrophylic) albumins, globulins, and glutelins. It is apparent that the most polar of those proteins must have eluted in or near the void volume around 3 min, where a doublet appears in the 5% acetic acid solvent (Fig. 1A).

The patterns of the alcohol extracts in Figure 1B are similar to those reported by Lookhart et al (1987) in that the peaks in the 10–13-min range are useful for identification in this study, whereas the peaks in the 13–15-min range were used for subgrouping the varieties in the 1987 study, and each chromatogram shows about 14 peaks in each study. Other than that, the patterns are not similar. Possible reasons include the use of different chromatographic systems for gradient elution and the use of different columns. The most likely reason is the major difference in the design of the chromatographic pumps for producing and mixing the gradients.

Figure 1C shows the same chromatograms normalized over the 5–13-min range. The qualitative and quantitative differences of minor components in each extraction solvent are more easily seen. Adding the reducing agent DTT increased the intensity of the minor peak at 11.7 min. The similarity of the patterns with and without DTT indicates that rice prolamins contain few disulfide bonds; DTT would reduce them, making the prolamins more soluble. Since 60% 1-propanol extracts as much or more prolamin as do any of the other solvents tested (Fig. 1A), and
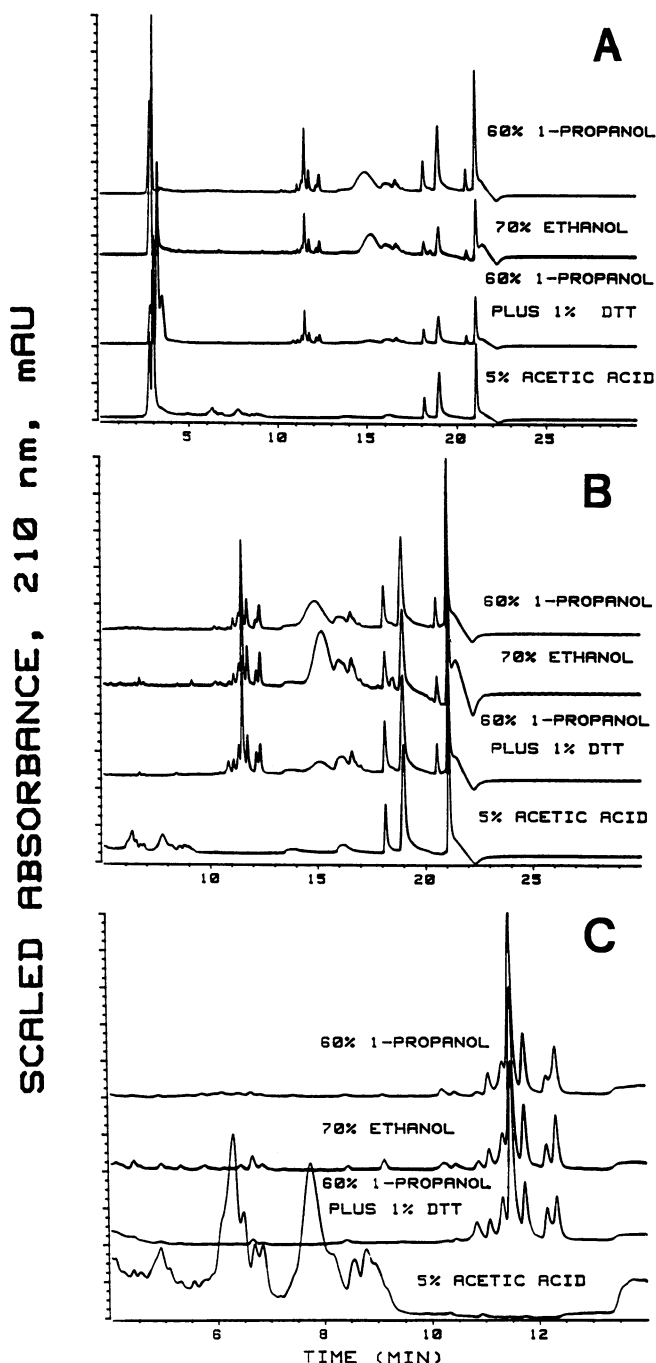

Fig. 1. High-performance liquid chromatographic patterns of U.S. rice variety L202 extracted by one of four solvents. The chromatograms were plotted at 5–30 min (A), 5–30 min (B), and 1–13 min (C). The patterns are normalized, making the largest peak for each pattern in the given time range the same height. mAU = milliAbsorbance Units.
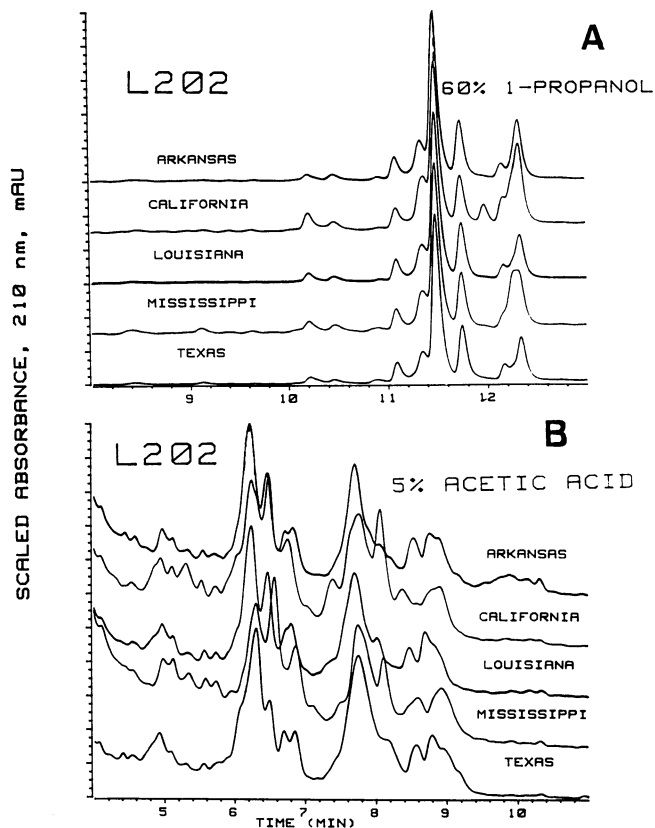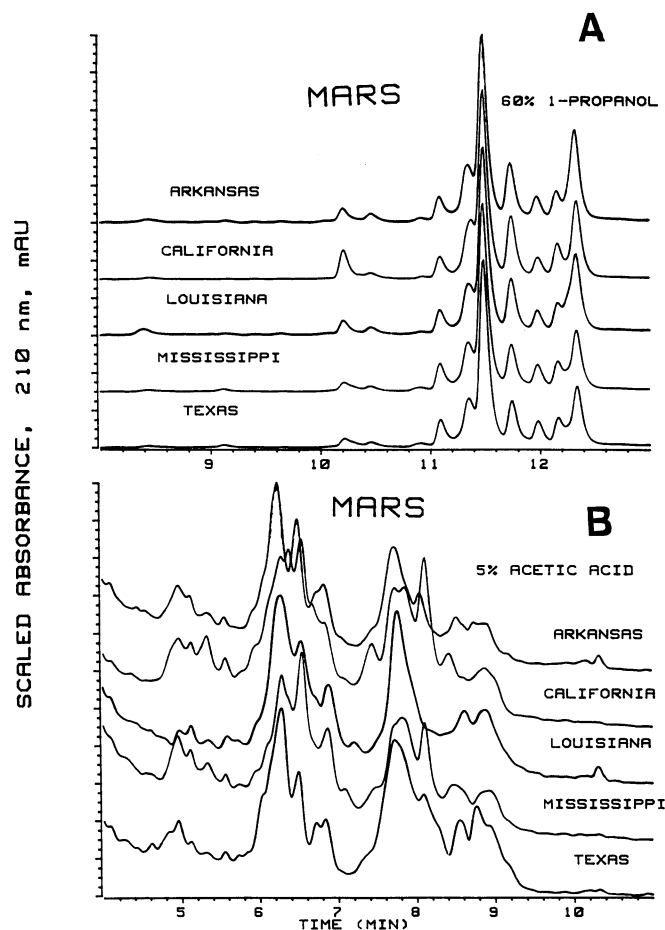

Fig. 2. Prolamin high-performance liquid chromatographic patterns of U.S. rice variety L202, from five growing locations, extracted with 60% 1-propanol (A) and 5% acetic acid (B). The patterns are normalized, making the largest peak for each pattern in the given time range the same height. mAU = milliAbsorbance Units.

since acetic acid extracts a completely different group of proteins (Fig. 1C), those solvents were used to extract the five U.S. varieties for each location.
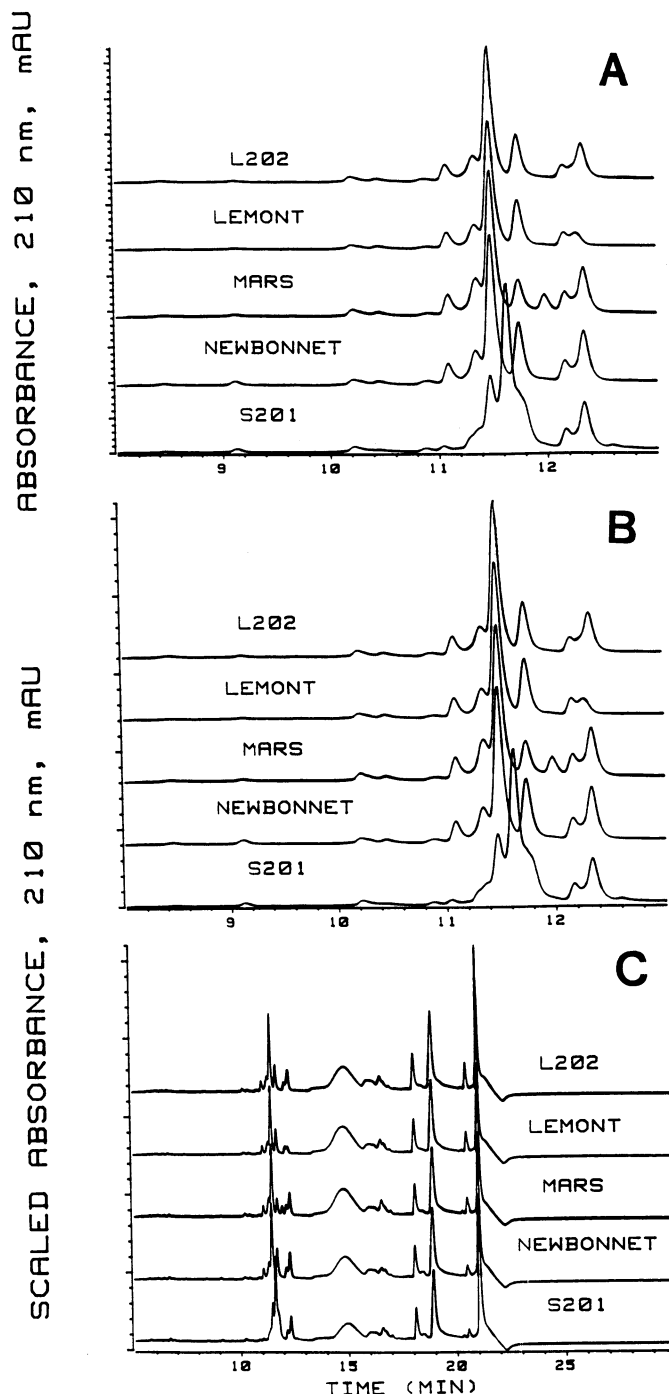
Effect of environment. The effects of environment on each of the five varieties (L202, Lemont, Mars, Newbonnet, and S201) grown in five locations (Arkansas, California, Louisiana, Mississippi, and Texas) were determined by analyzing the HPLC patterns of each variety at each location. Figure 2A shows the effect of location on the 60% 1-propanol extracts of variety L202. The peaks are in the same positions for all locations; only the heights of the peaks differ, even on the normalized patterns shown. The difference in height is most likely accounted for by agronomic practices. In this study, the Texas-grown rice consistently produced larger (more intense) peaks (nonnormalized data is not shown), which would be expected from optimum fertilization. The normalized patterns (Fig. 2A) partially eliminate that effect. The peak positions are still consistent, and most peak heights are similar. The major differences in the patterns are in the intensity of the 12.4-min peak and the resolution of the 12.2-min peak. A minor peak at 10.2 min is present in L202 for all locations but is largest in the California sample. Since the patterns were normalized to make the largest peak the same size for all locations, any differences in intensity are probably an environmental effect. It appears that California-grown samples can be determined by examining the intensity of the peak at 10.2 min. This was true for all other California-grown samples analyzed in this study.

The patterns of the acetic acid extracts of the same samples (L202) are shown in Figure 2B (also normalized). Major differences in peak position and intensity are easily seen. Similar

information can be readily seen from the analysis of the HPLC patterns of the 1-propanol (Fig. 3A) and acetic acid (Fig. 3B) extracts of the medium-grain rice Mars. The 1-propanol extracts of the California-grown Mars had the largest 10.2-min peak (as discussed above) for California-grown samples. Among locations, the patterns of the alcohol extracts were very similar, whereas the acetic acid extracts showed great variation. Aqueous acetic acid extracts nonprolamin proteins, particularly albumins, globulins, and glutelins. The patterns of the acetic acid extracts corroborate the variability of albumins and globulins with environment, as reported by Doekes (1968) and Konarev et al (1979). Therefore, extraction with 5% acetic acid does not seem useful for rice varietal identification, and only the HPLC patterns of

Fig. 3. Prolamin high-performance liquid chromatographic patterns of U.S. rice variety Mars, from five growing locations, extracted with 60% 1-propanol (A) and 5% acetic acid (B). The patterns are normalized, making the largest peak for each pattern in the given time range the same height. mAU = milliAbsorbance Units.

Fig. 4. Prolamin high-performance liquid chromatographic patterns of five U.S. rice varieties grown at Beaumont, Texas, extracted with 60% 1-propanol. The patterns in A are not normalized; those in B and C are normalized, making the largest peak for each pattern in the given time range the same height. mAU = milliAbsorbance Units.

1-propanol extracts are shown in the rest of this article. Results for the other three rice varieties (data not shown) were similar.

Effect of variety. The 60% 1-propanol extracts of the varieties L202, Lemont, Mars, Newbonnet, (all grown in Texas) show considerable similarity at 10–13 min (Fig. 4A, not normalized; Fig. 4B and 4C, normalized). Overall patterns of these U.S. long- and medium-grain varieties are similar, but the peak of the short-grain S201 shifted to 11.5 min. L202 and Newbonnet have nearly identical patterns and are not easily differentiated by this technique. Use of the chromatographic pattern at 5–25 min allows differentiation of Newbonnet and L202 on the basis of quantitative differences in peak heights eluting at 18.5 and 20.6 min (Fig. 4C). It appears that protein peaks eluting during the column washout period may be useful for varietal identification. Some of those peaks were used by Lookhart et al (1987) for differentiating U.S. rices. L202 is a recent release and was not included in that study.
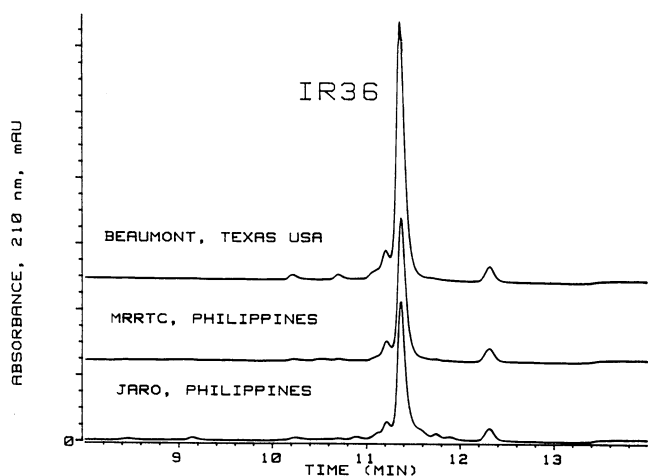
Philippine Rices

The effect of environment on IR36 samples extracted with 60% 1-propanol is shown in Figure 5. These samples were grown at two locations in the Philippines (VAES Jaro, Iloilo, and MRRTC Muñoz, Nueva Ecija) and in Texas (Beaumont). The patterns are essentially identical; however, patterns of the 5% acetic acid soluble fractions of those IR36 lines were quite different (data not shown).

HPLC patterns of 10 IRRI rice lines extracted with 60% 1-propanol appear to adequately differentiate most of these related IRRI lines (Fig. 6, Table I). Each of two sets of closely related lines (IR36, IR50, IR1561-228-3-3, and IR42, IR62) exhibited nearly identical prolamin HPLC patterns. Closely related lines usually exhibit similar patterns (Lookhart et al 1987). However, IR36 is a sister line of IR42, but their patterns are quite different, and IR36 is a parent of or is related to many IRRI varieties. Of 50,308 crosses made at the International Rice Research Institute from 1962 to 1984, 38% of the females were derived maternally from Cina, including IR36 and IR42 (Hargrove et al 1985). The male parent of IR50 and IR62 is IR36. The patterns of IR36 and IR50 appear nearly identical (Fig. 6A and 6B), whereas the pattern of IR62 is very similar to that of IR42, differing only in the 8–10-min region, where IR62 has an additional peak at 8.4 min.
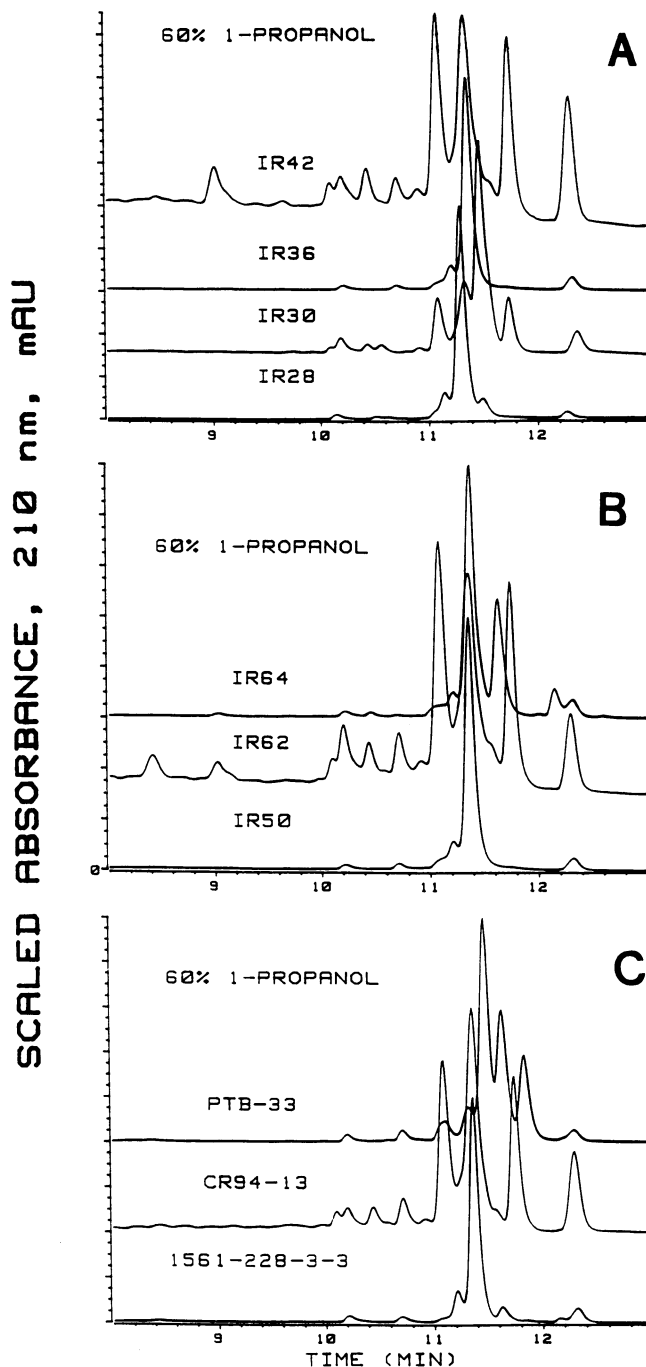
The HPLC pattern of IR1561-228-3-3 (Fig. 6C) is also very similar to that of IR36, but it has a very small additional peak at 12.2 min. IR36 and IR42, which are sister lines through IR2071, exhibit contrasting HPLC prolamin patterns in the 9–13-min elution period: IR36 had a prominent peak at 11.4 min, whereas IR42 had that peak plus three additional peaks at 11.1, 11.8, and 12.3 min (Fig. 6A). Only IR1561-228-3-3 and CR94-13 were

available as parental material of IR36 and IR42 (IR1561-228-1-2 and IR1737 were not available). IR1561-228-3-3 had an IR36-type pattern, and CR94-13 had an IR42-type pattern (Fig. 6C). Of the two IRRI varieties with IR36 as male parent, IR50 (Fig. 6B) showed a pattern similar to that of IR36 (Fig. 6A), but IR62 (Fig. 6B) had the pattern of IR42 (Fig. 6A). One other parent of IR50, IR28 (Fig. 6A), had a pattern similar to that of IR36 (Fig. 6A) but with one peak at 11.3 rather than 11.4 min. Since IR50 is the cross of IR2153-14-1-6-2 (which was unavailable) with IR28 and has IR36 as the male parent, one would expect its pattern to be similar to that of one of its parents. A sister line of IR2153-14-1-6-2, IR30 (Fig. 6A) (IR2153-159-1-4), showed a combination of the IR36 and IR42 patterns.

IR62 exhibited major prolamin HPLC peaks eluting at 11.1,

Fig. 6. Prolamin high-performance liquid chromatographic patterns of rice varieties from the International Rice Research Institute, grown in the Philippines, extracted with 60% 1-propanol. The patterns are normalized, making the largest peak for each pattern in the given time range the same height. mAU = milliAbsorbance Units.

Fig. 5. Prolamin high-performance liquid chromatographic patterns of Philippine rice variety IR36, from three growing locations, extracted with 60% 1-propanol. mAU = milliAbsorbance Units.

TABLE I
Parentage and Prolamin High-Performance Liquid Chromatography Peak Patterns and Elution Times
of 10 Milled Rice Genotypes from the International Rice Research Institute

Sample	Parentage (female/male)	Peak Patterns (Elution Time, min)
IR28 (IR2061-214-3-8-2)	IR833-6-1-1-1/IR1561-149-1//IR1737	IR28 (11.3)
IR30 (IR2153-159-1-4)	IR154-102-6-3//IR20/O. nivara	IR30 (11.5)
IR36 (IR2071-625-1-252)	IR1561-228-1-2/IR1737//CR94-13	IR36 (11.4)
IR42 (IR2071-586-5-6)	IR1561-228-1-2/IR1737//CR94-13	IR42 (11.1, 11.4, 11.8, 12.3)
IR50 (IR9224-117-2-3)	IR2153-14-1-6-2/IR28//IR36	IR36 (11.4)
IR62 (IR13525-43-2-3)	PTB 33/IR30//IR36	IR42 (11.1, 11.4, 11.8, 12.3)
IR64 (IR18348-36-3-3)	IR5657-33-2-1/IR2061-465-1	IR64 (11.4, 11.6)
IR1561-228-3-3	IR579-48-1-2/IR747B2-6-3	IR36 (1.4)
CR94-13	Indian variety	IR42 (11.1, 11.4, 11.8, 12.3)
PTB 33	Selection from Arikari	PTB 33 (11.4, 11.6, 11.8)

11.4, 11.8, and 12.3 min. However, none of the parents of IR62 (IR36, PTB-33, and IR30) showed HPLC patterns similar to the IR42-type pattern of IR62 (Fig. 6B). The prolamin HPLC patterns of IR30 (Fig. 6A) and PTB-33 (Fig. 6C) exhibited peaks in the same positions but with very different intensities of nonnormalized peaks (data not shown).

The prolamin HPLC pattern of IR64 (Fig. 6B), which is not derived from IR36, showed a major peak at 11.4 min plus a smaller peak at 11.6 min, and, as such, was different from both IR36 and IR42.

CONCLUSIONS

The solvent of choice for extracting rice prolamins is 60% 1-propanol. The proteins it extracts are nearly invariant to environmental conditions. Rice prolamins (proteins extracted by 1-propanol) are easily extracted, and HPLC analysis of them can be used to differentiate U.S. and non-U.S. varieties because of differences in their patterns that are related to their genetic history. The patterns of related lines are usually similar to each other.

LITERATURE CITED

BIETZ, J. A., BURNOUF, T., COBB, L. A., and WALL, J. S. 1984. Wheat varietal identification and genetic analysis by reversed-phase high-performance liquid chromatography. Cereal Chem. 61:129.

DOEKES, G. J. 1968. Comparison of wheat varieties by starch-gel elec-trophoresis of their grain proteins. J. Sci. Food Agric. 19:169-176.

HARGROVE, T. R., CABANILLA, V. L., and COFFMAN, W. R. 1985. Changes in rice breeding in 10 Asian countries: 1965-84. Diffusion of genetic materials, breeding objectives, and cytoplasm. IRRI Res. Paper Ser. 11. Int. Rice Res. Inst.: Los Baños, Laguna, Philippines.

HUEBNER, F. R., BIETZ, J. A., WEBB, B. D., and JULIANO, B. O. 1990. Rice cultivar identification by high-performance liquid chroma-tography of endosperm proteins. Cereal Chem. 67:129.

HUSSAIN, A., SCANLON, M. G., JULIANO, B. O., and BUSHUK, W. 1989. Discrimination of rice cultivars by polyacrylamide gel electro-phoresis and high-performance liquid chromatography. Cereal Chem. 66:353.

KONAREV, V. G., GAVRILYUK, I. P., GUBAREVA, N. K., and PENEVA, T. I. 1979. Seed proteins in genome analysis, cultivar iden-tification, and documentation of cereal genetic resource: A review. Cereal Chem. 56:272-278.

LOOKHART, G. L., ALBERS, L. D., and BIETZ, J. A. 1986. Com-parison of polyacrylamide gel electrophoresis and high-performance liquid chromatography analyses of gliadin polymorphism in the wheat cultivar Newton. Cereal Chem. 63:497-500.

LOOKHART, G. L., ALBERS, L. D., POMERANZ, Y., and WEBB, B. D. 1987. Identification of U.S. rice cultivars by high-performance liquid chromatography. Cereal Chem. 64:199.

TANAKA, K., SUGIMOTO, T., OGAWA, M., and KASAI, Z. 1980. Isolation and characterization of two types of protein bodies in the rice endosperm. Agric. Biol. Chem. 44:1633.

WRIGLEY, C. W., AUTRAN, J. C., and BUSHUK, W. 1982. Iden-tification of cereal varieties by gel electrophoresis of the grain proteins. Pages 211-259 in: Advances in Cereal Science and Technology. Vol. 5. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.