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Variation in kafirin and alcohol-soluble glutelin chromatograms of sorghum inbred lines revealed by reversed-phase high-performance liquid chromatography

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Summary. Separations of kafirin and alcohol soluble glutelin proteins by reversed-phase high-performance liquid chromatography (RP-HPLC) from 7 inbreds and one hybrid of sorghum [*Sorghum bicolor* (L.) Moench] and one source of Johnsongrass [*Sorghum halapense* (L.) Pers.] were compared. Objectives were to assess the stability of protein profiles for seed sources produced at different locations and in different environments to examine the potential of RP-HPLC to provide genotypic profiles for sorghum. Analyses of variance data showed that levels of variation due to environments and locations were small; the majority of variation (93%) was among genotypes. Associations among inbreds revealed by multivariate and cluster analysis showed similarity with those that would be expected on the basis of pedigree. A chi-square analysis showed no deviation in the hybrid profile from the expected 2:1 ratio of peaks from the female and male inbred parents, respectively. Improvements in the ability to correctly assign common peaks are necessary before associations among numerous sorghum genotypes can be reliably demonstrated by analysis of data from reversed-phase high-performance liquid chromatography (RP-HPLC).

Key words: Taxonomy – Germplasm identification – Varietal identity – Environmental interaction – Genetics – Multivariate analysis

Introduction

Cultivar identification is necessary for the efficient breeding and production of varieties, inbred lines, and hybrids. Separations of proteins by electrophoresis (Tanksley and Orton 1983; Cooke 1984; Draper and Cooke 1984) or by reversed-phase high-performance liquid chromatography (RP-HPLC), (Bietz 1983, 1986; Smith 1986; Smith and Smith 1987) has allowed the characterization of cultivars for many crop species.

Contrary to the situation found with most major crops, there are few examples of successful distinction between elite inbreds or hybrids of sorghum [*Sorghum bicolor* (L.) Moench] having been provided through biochemical means. Isoelectric focusing of soluble protein and esterase isozymes allowed male sterile lines to be distinguished, but additional discrimination between lines was not achieved (Tripathi et al. 1982, 1983). Using electrophoresis of soluble proteins and isozymes of esterase and malate dehydrogenase, Shechter and DeWet (1975) were able to characterize and show racial groupings between 7 local African cultivars. Paulis and Wall (1979) distinguished electrophoretically an inbred line, an unrelated hybrid, and an Ethiopian variety. Taylor and Schussler (1984) concluded that electrophoresis could be useful in distinguishing between cultivars, however, little variation was apparent. Sastry et al. (1986) revealed significant differences between 3 elite U.S. inbred lines using either isoelectric focusing or RP-HPLC. A fourth line was similar to an inbred to which it was related by pedigree. Isoelectric focusing and RP-HPLC also provided unique identification of 7 varieties of diverse germplasm from Africa, India, and China (Sastry et al. 1986).

Biochemical characters can only be useful genotypic descriptors if they are free from environmental effects, reproducible, and are readily comparable. An examination of chromatographic profiles from seed sources grown in different environments is, therefore, necessary to assess their reliability and usefulness as descriptors. These studies are necessary because of 1) the heterogenic nature of sorghum proteins (Virupaksha and Sastry 1968); 2) the exceptionally high resolving power of RP-

Table 1. Provenance data for the seed sources used in the present study showing the location in which seed source was last increased

Source no.	Pedigree	Cytoplasm[a]	Location	Environment
1	PH104	Feterita?	Hutchinson, KS	Dry
2		Feterita?	Hutchinson, KS	Irrigated
3		Feterita?	Lone Star, TX	Irrigated
4		Feterita?	Plainview, TX	Irrigated
5	PH210	Kafir?	Hutchinson, KS	Dry
6		Kafir?	Hutchinson, KS	Irrigated
7		Kafir?	Planview, TX	Irrigated
8		Milo	Midland, TX	Limited Irrigation
9		Milo	Plainview, TX	Irrigated
10	PH113	Kafir	Hutchinson, KS	Dry
11		Kafir	Hutchinson, KS	Irrigated
12		Kafir	Plainview, TX	Irrigated
13		Milo	Midland, TX	Limited Irrigation
14		Milo	Plainview, TX	Irrigated
15	PH132	Kafir?	Hutchinson, KS	Dry
16		Kafir?	Hutchinson, KS	Irrigated
17		Kafir?	Plainview, TX	Irrigated
18		Milo	Midland, TX	Limited Irrigation
19		Milo	Plainview, TX	Irrigated
20	TX2737	Kaura?	Hutchinson, KS	Dry
21		Kaura?	Hutchinson, KS	Irrigated
22		Kaura?	Plainview, TY	Irrigated
23		Kaura?	South Plains, TX	Irrigated
24	Redlan	Kafir	Plainview, TX	Irrigated
25		Milo	Plainview, TX	Irrigated
26	TXS214	Milo	Plainview, TX	Irrigated
27	Amber	Amber?	Plainview, TX	Irrigated
28	Johnsongrass		Plainview, TX	Irrigated

[a] All inbreds "in" Milo cytoplasm are male sterile and are "A" lines; lines "in" Kafir cytoplasm are "B" lines; lines "in" Feterita and Kaura cytoplasm are "R" lines

HPLC (Bietz 1983); 3) the qualitative and quantitative nature of data obtained from RP-HPLC (Marchylo and Kruger 1984); 4) the qualitative and quantitative effects of environment on seed protein content (Higgins 1984); 5) possible unpredictable interactions mediated through the structural and regulatory genes of a complex multigene system (Bietz 1982; Soave and Salamini 1983); and 6) differences in gene expression or protein extractability (Sastry et al. 1986). This study, therefore, reports upon the stability of RP-HPLC chromatographic profiles of kafirin and alcohol soluble glutelin proteins for inbred lines of sorghum grown in different environments.

Materials and methods

Approximately 5 g of seed from each source, produced in the summer of 1984, (Table 1) were ground to flour in an Udy cyclone hammermill. Initial experiments to test the effects or pre-extraction (defatting) and extraction solvents were investigated in a factorial experiment using flour of inbred PH104[1], source no. 1. Pre-extraction conditions were, 1) no defatting; 2) 1-butanol; 3) acetone; 4) petroleum ether; and 5) hexanes. Three 10 min washes of each solvent in a 1 : 25 (w/v) ratio were followed by drying at 25 °C under a gentle stream of helium. Three extraction solutions were used; 1) 55% 2-propanol, 1% 2-ME; 2) 60% t-butanol, 1% 2-ME; and 3) 70% ethanol, 0.5% NaOAc, 5% 2-ME. All extracts were made in a 1 : 10 ratio (w/v) at 25 °C with vigorous shaking. Following centrifugation at 13,000 g for 3 min, supernatant was filtered through a 0.45 μM membrane and used directly in chromatography. Twenty microliters were injected thereby initiating a linear gradient from 45% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) to 50% ACN, 0.1% TFA during 45 min followed by isocratic conditions at 53% ACN, 0.1% TFA for 10 min at a flow rate of 1 ml/min. A Brownlee RP-300 C_8 column was used at 70 °C and equilibrated at 45% ACN, 0.1% TFA for 15 min prior to each injection. Protein was detected spectrophotometrically at 210 nm. Data were

[1] PH104 is an inbred line which is a proprietary product of Pioneer Hi-Bred International, Inc. All lines designated with the prefix 'PH' that are subsequently mentioned are also proprietary products of Pioneer Hi-Bred International, Inc.

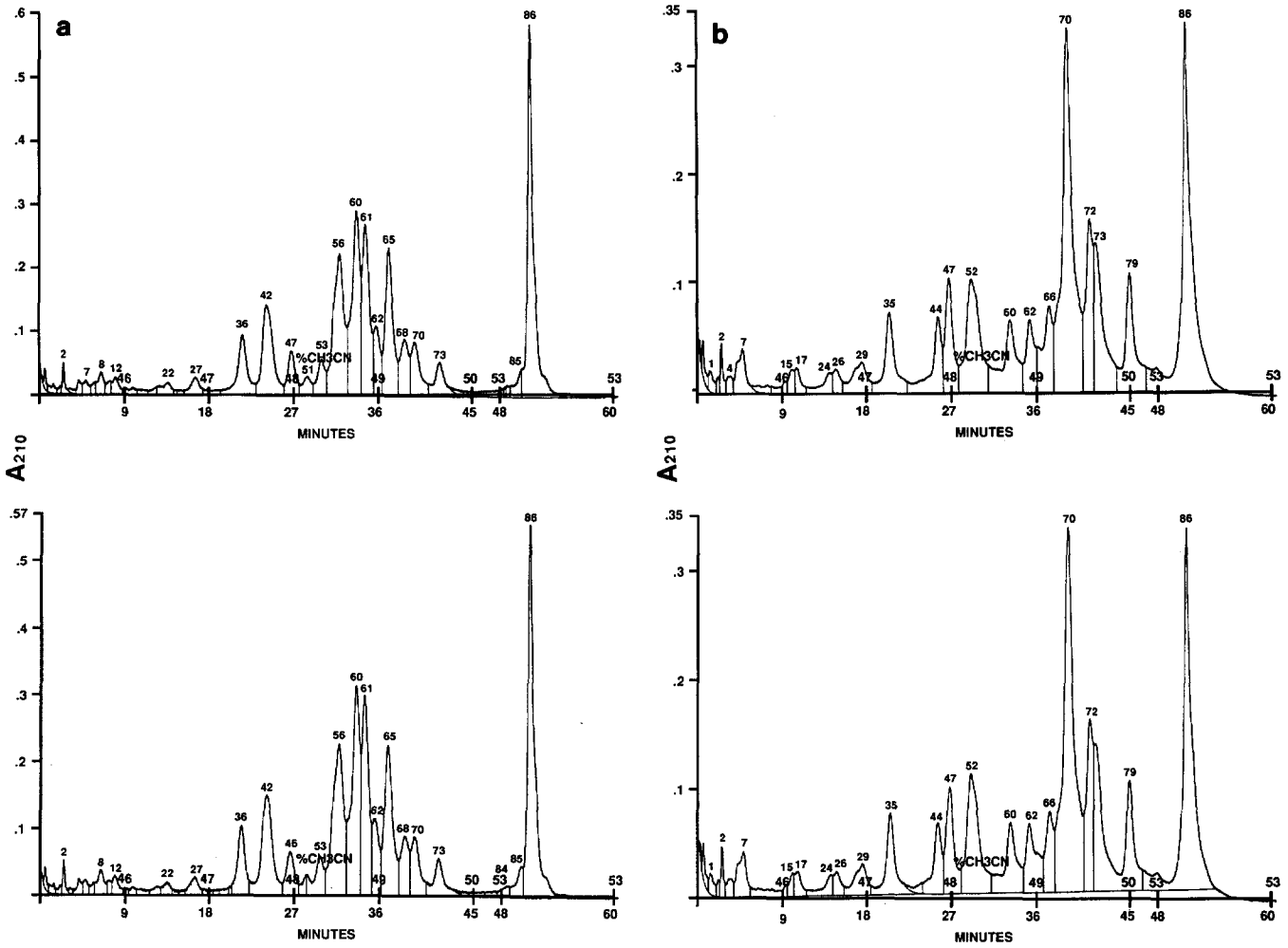


Fig. 1. Chromatograms resulting from replicate injections **a** of PH104 source 3, and **b** of PH210 source 5

collected at 0.5 s intervals and integrated using a version 3.5 software package provided by Nelson Analytical.

The impact of environment, location, extraction, and injection was tested using different seed sources (1–23) of 5 inbred lines (Table 1). Flour was defatted by three 10 min washes of petroleum ether in 1:25 (w/v) ratio of flour to solvent followed by drying at 25 °C. Defatted flour was shaken vigorously for 2.25 h at 25 °C in 60% t-butanol, 2% 2-ME in a 1:10 (w/v) ratio. Following centrifugation at 13,000 g for 10 min, supernatant was filtered through a 0.45 μM membrane. Fifteen microliters of sample were injected and chromatographic protocols were as described previously. Replicate pairs of injections were made for all sources of lines PH104 and PH210 (Table 1). Additional replicate injections for PH104 (source 2) and PH210 (source 9) were made 41 and 33 h, respectively, after the initial replicate pairs were injected. Injections from replicate extractions were made using 3 sources; PH113 (source 12), PH132 (source 17), and TX2737 (source 20). Single injections of all other sources (Table 1) were made. One injection combining equal extract volumes from one source each of inbreds TX2737, PH113, PH104, PH210, and PH132 was made and analyzed visually.

Peak areas were converted to percent of total area after disregarding all peaks eluting prior to 5 min (solvent peaks). Peak numbers were assigned to peaks after adjusting each run such that the time interval between the solvent front and a major peak present in all samples (no. 86) was constant for each run, and the time of this major peak was constant for each run. Peak numbers were then assigned based on elution time and peak size; peaks that contributed $\leq 0.5\%$ of the eluted area were dropped from further analysis.

The variation among samples was partitioned into first, the variation due to differences in peak areas from replicate injections from the same sample, with $\sum_{i=1}^{g} e_i^* p(r-1)$ degrees of freedom (df), where g is the number of genotypes, p is the number of peaks, e is the number of seed sources, and r is the number of replicates. Second, the variation in the differences in peak areas from samples of the same genotype produced in different environments, i.e., the source of variation labeled Env (Genotype Peak), which is a measure of the variation due to the production environment and has $\sum_{i=1}^{g} (e_i-1)p$ df. Third, the variation due to differences in peaks of different genotypes [Genotype (Peak)] with $g(g-1)$ degrees of freedom. Differences are pooled over peaks since the variation in the peak areas appeared uniform across peaks. However, some of the minor peaks were scored as having zero area if the actual area was less than 0.5%.

Results

All peaks contributing $\geq 0.5\%$ of eluted proteins were considered to be of sufficient magnitude to contribute to the chromatographic profile; individual samples revealed from 16 to 23 such peaks. A total of 92 peaks were recognized over all chromatograms following analysis of all elution times. No qualitative differences in chromatograms could be attributed to the various defatting treatments. All defatted flour resulted in the improved resolution of major ($\geq 5\%$ eluted proteins) peaks, although 3 minor to moderate peaks eluting at approximately 29, 30, and 36 min and which contributed 2%, 0.5%, and 4% of the chromatogram, respectively, were absent from profiles that were developed from defatted four. Each extraction solvent resulted in qualitatively identical and quantitatively similar profiles. However, extracts made with 60% t-butanol, 1% 2-ME gave a slightly increased yield of eluted protein.

The percentage of common peaks that were resolved between pairs of chromatograms resulting from replicate injections ranged from 86% to 95% with a mean over 9 pairs of 91%. Following data analysis, the least percentage of common peaks were revealed following replicate injections of PH104, source 3. Visual inspection shows these two chromatograms to be very similar (Fig. 1 a). However, peaks 7 (0.53%), 51 (1.16%), and 84 (0.54%), (percent eluted proteins given in parentheses) were not always of sufficient magnitude to be revealed. Over all pairs of replicate injections, there were 9 such peaks, contributing from 0.5% to 1.16% of eluted proteins, that were present in chromatograms but not always recorded since they sometimes contributed $\leq 0.49\%$ of the profile. In addition, peak no. 47 (2.57%), that eluted at approximately 27.5 min following the initial injection of PH104 (Fig. 1 a) was replaced by a closely eluting peak that was recorded as no. 46 (2.67%) in the second chromatogram. Over all replicated pairs of chromatograms, there were 8 such instances and these involved peaks that contributed from 0.6% to 8.0% of eluted protein. Additional instances of recorded mis-matches between pairs of chromatograms that were developed from replicate injections were due to the sometimes incomplete resolution of peak 73. Chromatograms of replicated injcections of PH102, source 8 (not shown), and PH210, source 5 (Fig. 1 b) in which peak 73 contributed 4.5% and 7.55%, respectively, revealed this protein either as a unique peak or as a shoulder.

The percentage of peaks that were recorded as being common in all replicate extractions were 60%, 79%, and 93% for TX2737, source 20; PH113, source 12; and PH132, source 17, respectively. Even though replicate extractions of TX2737 resulted in the least percentage of peaks recorded as matching, visual inspection of the chromatograms (Fig. 2) shows them to be extremely similar. All peaks contributing $\geq 5\%$ of the profile were revealed and were recorded as matching across replicate extracts. Mis-matches occurred because, first, pairs of closely eluting peaks (81, 82; 66, 67; 32, 33) rather than three single peaks, were identified. Second, peaks 2, 13,

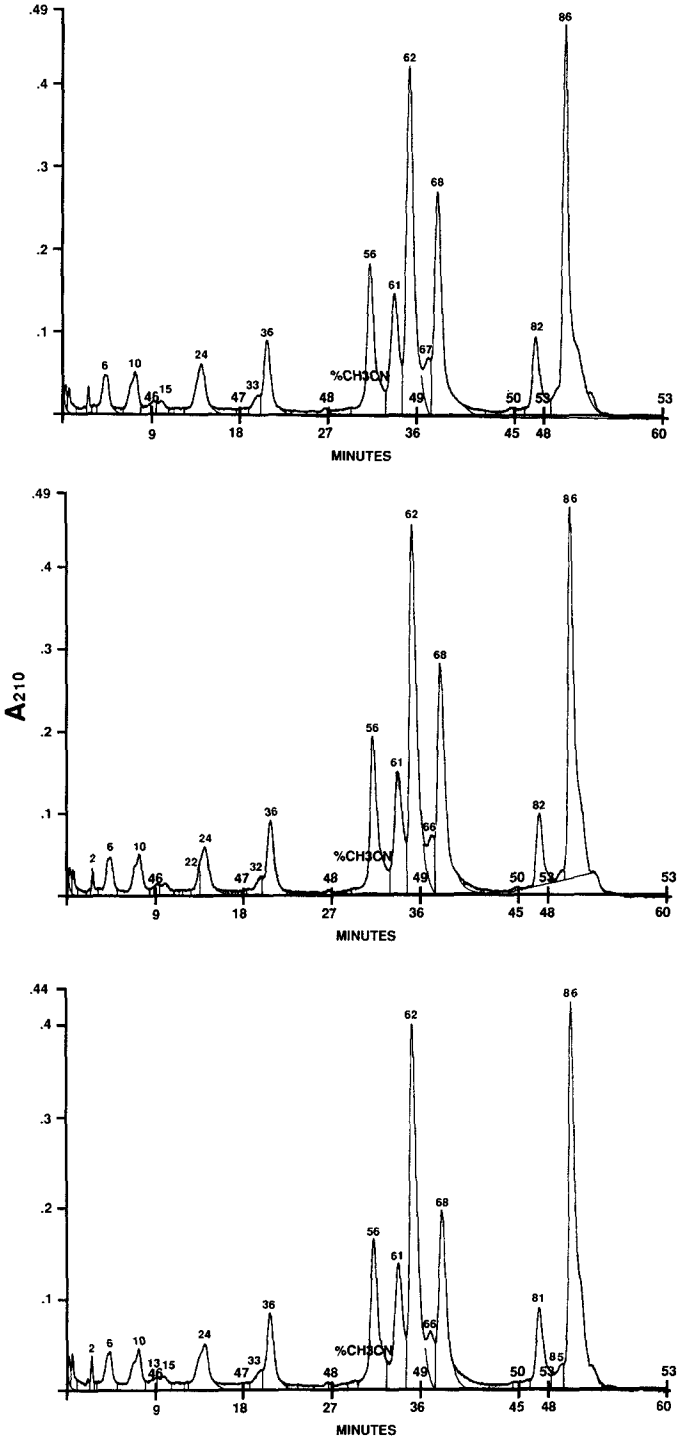


Fig. 2. Chromatograms resulting from replicate extractions of TX2737

15, and 22, which contributed from 0.55% to 0.73% of eluted protein, were not always recorded since they sometimes contributed $\leq 0.49\%$ of eluted protein. Third, peak 85 (0.93%) was recorded as a unique peak in only one extract but was otherwise present as a shoulder (Fig. 2). Over all of these replicate extractions, the percent variation attributable to genotype was 80.54%.

Differences between the chromatograms derived from alternate sources of four inbred lines were noticeable upon visual inspection (Fig. 3). No differences between sources were shown for inbred TX2737. For sources of PH104 (Fig. 3a) differences were only quantitative and were restricted to one peak alone, (no. 61), that was relatively abundant in source 4. For sources 10, 11, 12, and 13 of PH113 (Fig. 3b) the sole difference was the presence of peak 59 as a separate entity in sources 11 and 13 rather than as a shoulder in sources 10 and 12. In contrast, source 14 showed a quantitative difference for peak 60 and additional qualitative differences; peaks 52 (2.8%), 64 (4.14%), 70 (4.24%), and 79 (1.92%) were revealed in this source alone. For PH132, sources 15, 16, and 18, (not shown) were either identical to source 17 (Fig. 3c) or differed only in the revelation of peak 59 as a separate entity or as a shoulder. However, qualitative differences were found for source 19 where peaks 52 (2.19%), 64 (5.0%), 70 (3.84%), and 79 (1.78%) were additionally revealed. For PH210, sources 5, 6 and 8 (not shown) were qualitatively identical and quantitatively closely similar to source 7 (Fig. 3d). However, for source 9, peaks 40 (1.35%), 58 (2.93%), and 64 (5.0%) were additionally revealed.

Table 2. Analysis of variance table of chromatographic profiles pooled over peaks (percent area) for inbreds PH104, PH210 (Kafir cytoplasm), and PH210 (milo cytoplasm)

Source	df	MS	Variance component	% total
Genotype (peak)	45	67.37	18.77	92.7
Env (genotype peak)	109	2.41	1.00	4.9
Among replicate injections (peak)	172	0.46	0.46	2.3

Table 3. Analysis of variance of chromatographic profiles pooled over peaks (percent area) for 13 genotypes

Source	df	MS	Variance component	% total
Genotype (peak)	178	18.90	9.16	75
Env (genotype peak)	203	3.05	2.59	21
Among replicates/ extracts	172	0.46	0.46	4

The average difference between peak areas for replicate injections (Table 2) was 0.68% [$(0.46)^{1/2}$)], and the variation attributable to the environment in which the seed was produced was 1.0% or about 32% larger than the variation due to replicate injections. However, the variation between peak areas due to differences in inbreds accounted for 93% of the total variation in peak area percents. This is also true for the analysis where a larger sample of genotypes, but not always with replicate injections were examined (Table 3). Again the source of variation associated with genotypes accounted for 75% of the total variation. In this case the variation due to the environment accounted for 21% of the total variation. However, the mean square due for environments is only a little larger than that in Table 2, and the mean square for genotypes is smaller than that for Table 2. The estimate of the environmental variation from Table 3 is probably a better estimate since more genotypes were included in this analysis.

Malecot's coefficients of kinship (Kempthorne 1969; Delanney et al. 1983), calculated from pedigree records, for six of the inbred lines used in this study are presented in Table 4. Representative chromatograms of inbred PH104, PH210, PH113, PH132, and TX2737 are pre-

Table 4. Malecot's coeficient of kinship calculated from pedigree records; designations F and G refer to A (male sterile) and B (male fertile) cytoplasm lines, respectively

	Percentage relationship									
	PH104	PH113F	PH113G	PH132F	PH132G	PH210F	PH210G	RK1F	RK1G	TX2737
PH104	100	0	0	0	0	0	0	0	0	0
PH113F	0	100	97	19	19	25	25	0	0	0
PH113G	0	97	100	19	19	25	25	0	0	0
PH132F	0	19	19	100	90	9	9	0	0	0
PH132G	0	19	19	90	100	9	9	0	0	0
PH210F	0	25	25	9	9	100	98	0	0	0
PH210G	0	25	25	9	9	98	100	0	0	0
Redlan F	0	0	0	0	0	0	0	100	99	0
Redlan G	0	0	0	0	0	0	0	99	100	0
TX2737	0	0	0	0	0	0	0	0	0	100

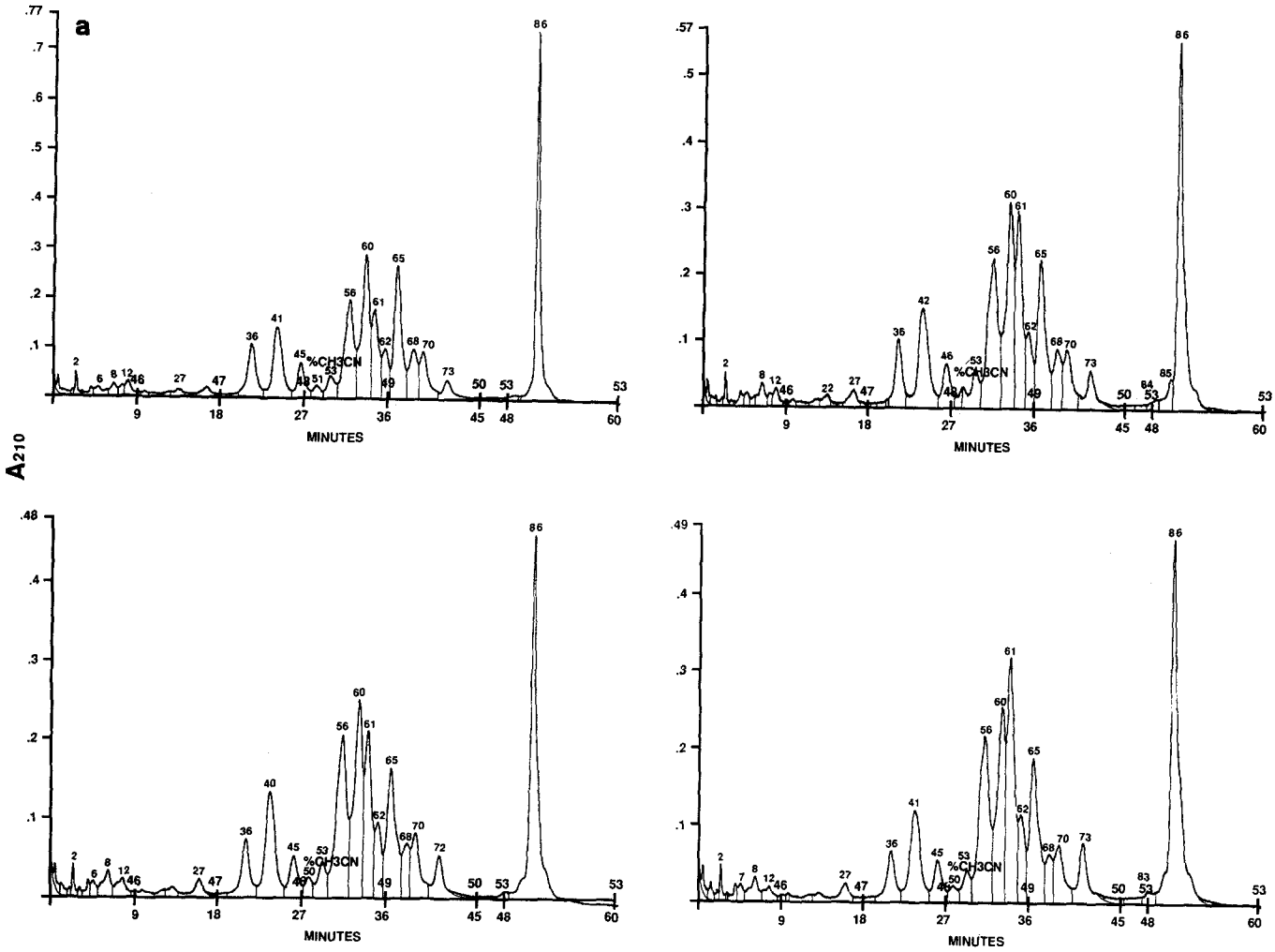


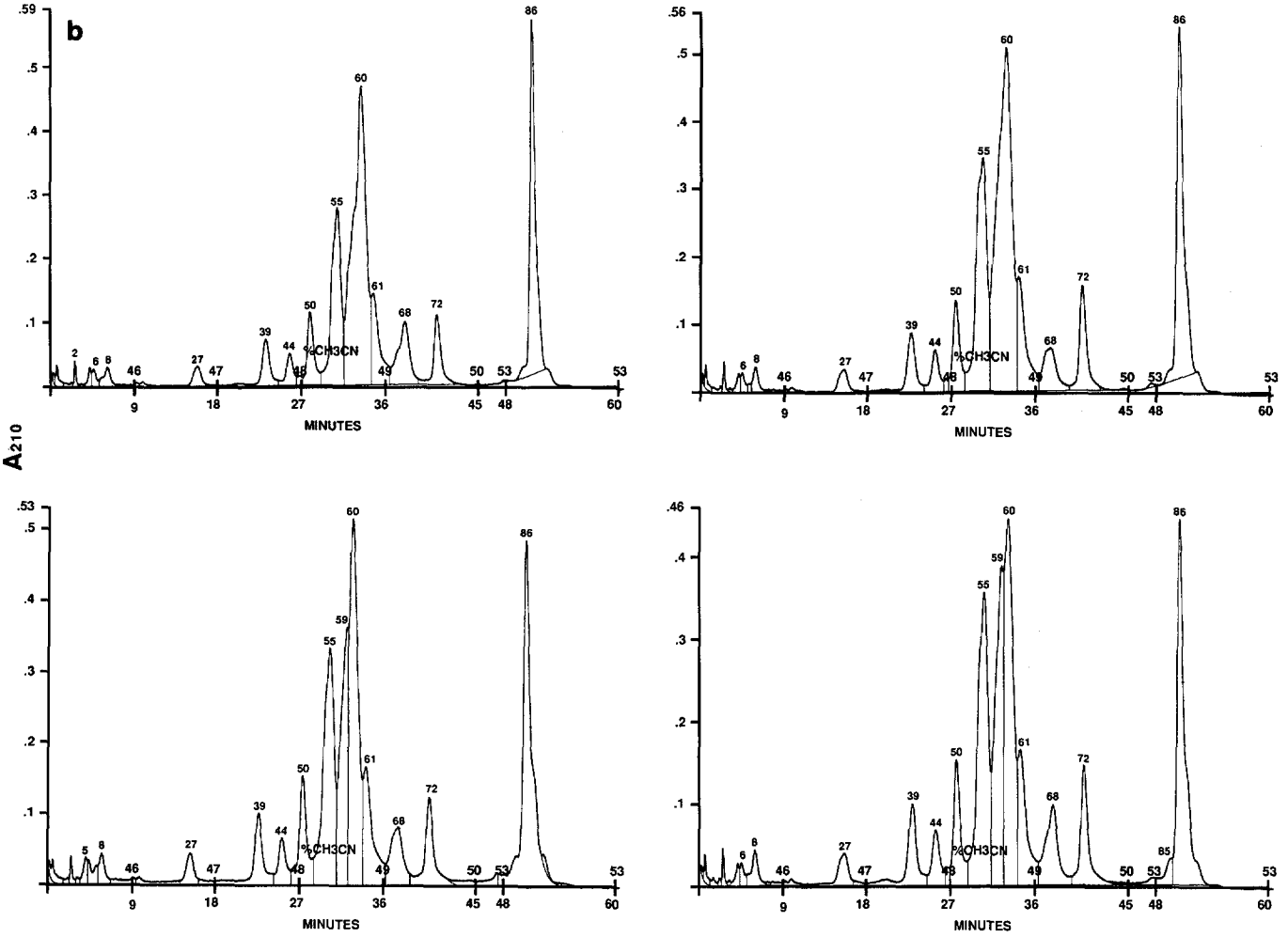
Fig. 3a–d. Chromatograms resulting from alternate sources **a** of PH104; sources 1, 2, 3, and 4 (*top to bottom*), **b** of PH113; sources 10, 11, 12, 13, and 14 (*top to bottom*), **c** of PH132; sources 17 (*top*) and 19 (*bottom*), and **d** of PH210; sources 7 (*top*) and 9 (*bottom*)

sented in Fig. 4. Multivariate analysis of chromatograms gave very similar patterns of association for both weighted (Figs. 5 and 6) and unweighted data sets (not shown). Generally, all extracts, injections, or sources of a given inbred were most closely associated with other sources of that same genotype.

All extracts of PH104, except one, clustered at ≤ 0.2. A third injection of PH104 was anomalous, clustering with 'Redlan'. Similarly, all sources of PH210 clustered at ≤ 0.3; two sources were identical. A third injection of PH210 clustered instead with 'Amber'. All extracts of TX2737 clustered together at ≤ 0.3. Individual sources of PH113 and PH132 clustered together at ≤ 0.7. Extracts of these 2 lines formed 2 sub-clusters at 0.3 and 0.15, the latter being comprised of only, but not all, lines that are either known, or suspected to be of kafir cytoplasm.

Associations among pedigrees were revealed by cluster analysis, for which the first two vectors accounted for 50% and 28% of the variation, respectively, (Fig. 5) which showed associations between sources of PH132 and PH113. The first two principal components, which encompassed 57% and 18% of the variation, respectively, (Fig. 6) also showed an association of PH132 and PH113 with a separate sub-grouping solely of lines with kafir cytoplasm according to the third and fourth principal components (not shown). Although PH210 and TX2737 were associated according to the first two principal components (Fig. 6), they were widely separated by the third principal component. PH104 was not closely associated with any of these inbreds.

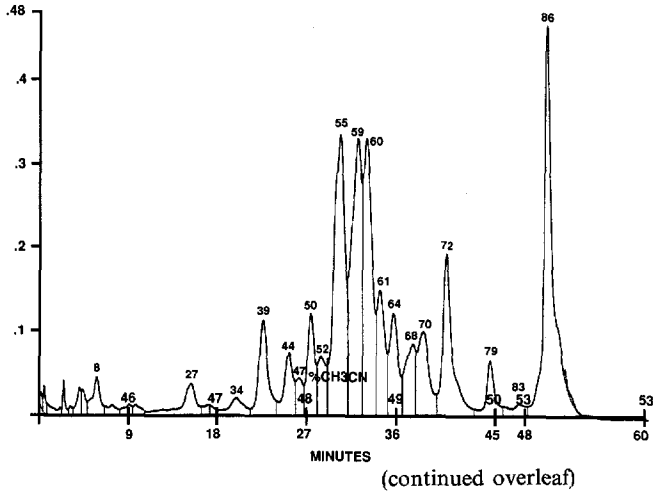
The chromatograms of TXS214 (source 26) closely matched that of the famale parent (Redlan, source 25) with additional peaks having been contributed by the male parent (Amber, source 27) (Fig. 7). A chi-square test showed no deviation ($P > 0.995$) from the expected 2:1 ratio of female:male peaks to the observed profile of 2



peaks that were revealed in the F_1 hybrid (Fig. 5). The chromatogram of Johnsongrass [*S. halapense* (L.) Pers.] (Fig. 8) differed most noticeably from all others in its lack of major eluent for peak 86 (Figs. 1–4). Johnsongrass was not closely associated with any source of *S. bicolor* according to cluster analysis (Fig. 5); an association, at least according to the first two principal components, was revealed between Johnsongrass and Amber, a weedy sorgo (Fig. 6).

Discussion

Initial experiments led to the adoption of defatting and extraction conditions that were similar to those employed by Sastry et al. (1986). However, kafirin and alcohol soluble glutelin proteins were co-extracted in the present study. Reproducible retention times were often difficult to achieve with non-defatted extracts and these extracts were also the least clearly resolved by RP-HPLC.



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Results from chromatographic separations of replicate injections and replicate extractions revealed that the majority of variation was between genotypes. Most instances of mis-matched peaks between chromatograms of the same genotype were due either to closely eluting

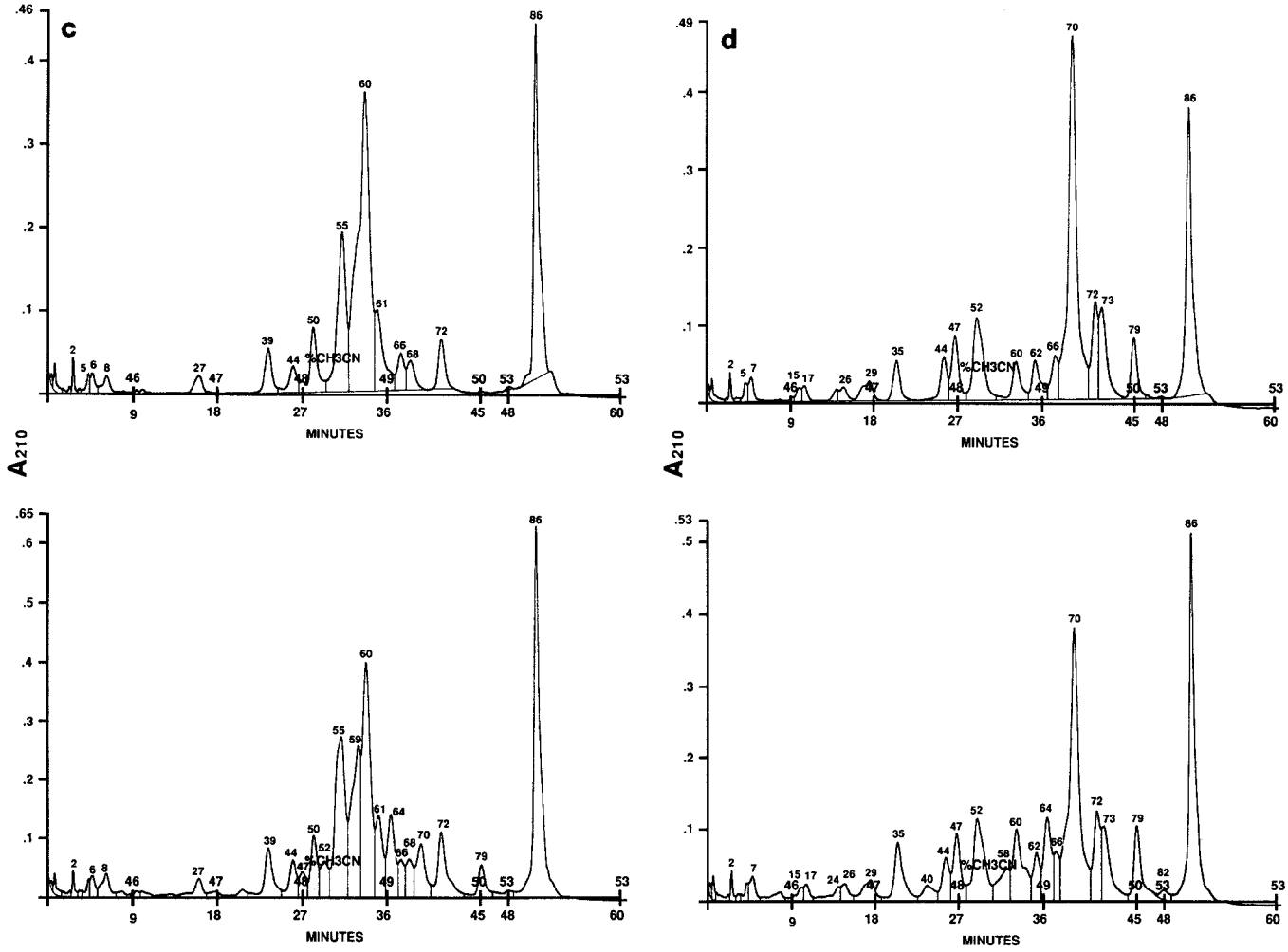

Fig. 3. (continued)

peaks that were ascribed adjacent, rather than the same, peak numbers or because the amount of eluted protein was around the threshold (0.5% of eluted profile) of recognition. There were a lesser number of instances (3) in which eluted protein was not always revealed as a separate peak, but rather as a shoulder of an adjacent peak.

All but one instance of peak mis-matches occurred for peaks contributing $\leq 5\%$ of the eluted profile; most occurred for peaks that contributed $\leq 1\%$ of the eluent. Thus, in data sets that are weighted for percentage contribution of the eluted profile, this level of mis-matched peaks will have minimal effect upon the associations that are revealed. However, further improvements could be made in the process whereby chromatographic peaks are assigned identities based on their relative elution times. First, adjustments of elution times based upon several reference peaks that occur throughout the chromatogram from periodic injections of a control sample could enable non-linear transformations to be made. These more complex transformations appear to be necessary with the separation of sorghum proteins. For example, simple linear transformations based on the elution of a single major common peak did not succeed in matching chromatograms from the third replicate injections of inbreds PH104 and PH210 that were made 33 h, or more apart. A similar procedure using 3 reference peaks provided significantly improved resolution and accuracy for computer-based comparison of electrophoretic profiles of wheat (Sapirstein and Bushuk 1986). Second, it would be extremely useful if a set of compounds could be found that would provide peaks and could thereby be used as a set of internal standards for each protein separation. These compounds would have to satisfy the stringent criteria of, 1) non-interaction with protein; 2) interaction with the column in a protein-like fashion; and 3) absorption at a wavelength other than that routinely used in RP-HPLC of protein (210 nm).

In spite of the improvements that could still be made in the transformation and resultant comparisons among

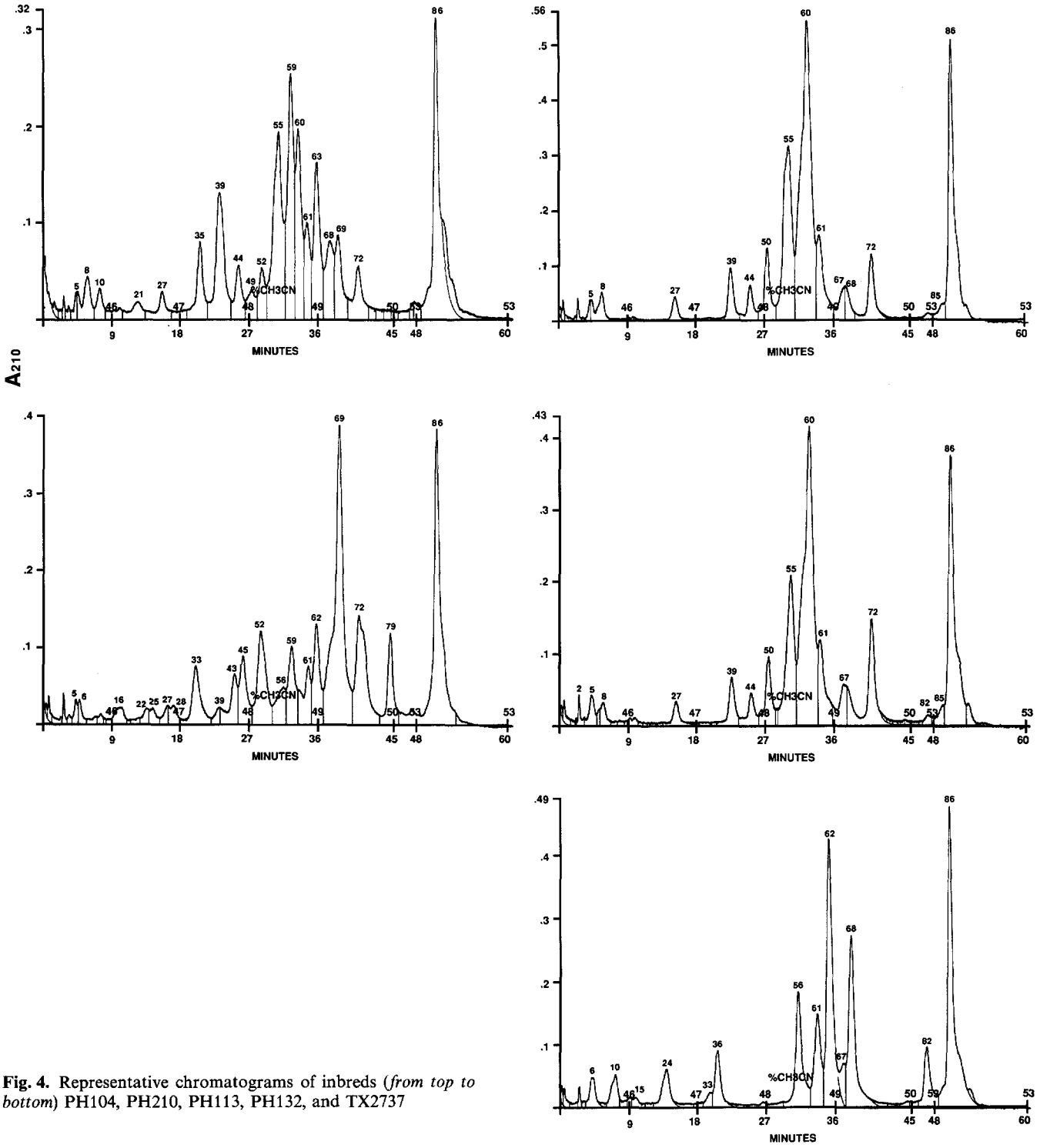

Fig. 4. Representative chromatograms of inbreds (*from top to bottom*) PH104, PH210, PH113, PH132, and TX2737

sorghum chromatographic profiles, the data presented herein demonstrate that location and environmental effects are minimal and they make no significant contribution to the observed profiles. Thus, RP-HPLC can provide profiles of sorghum that are reflective of genotype. The sources that were available for analysis did not give the opportunity to investigate the effects of cytoplasm on the chromatographic profile. However, cytoplasmic influence may be involved, at least in some cases, as was possibly indicated by the extremely similar chromatograms of some kafir and suspected kafir cytoplasm sources of inbreds PH113 and PH132. Furthermore,

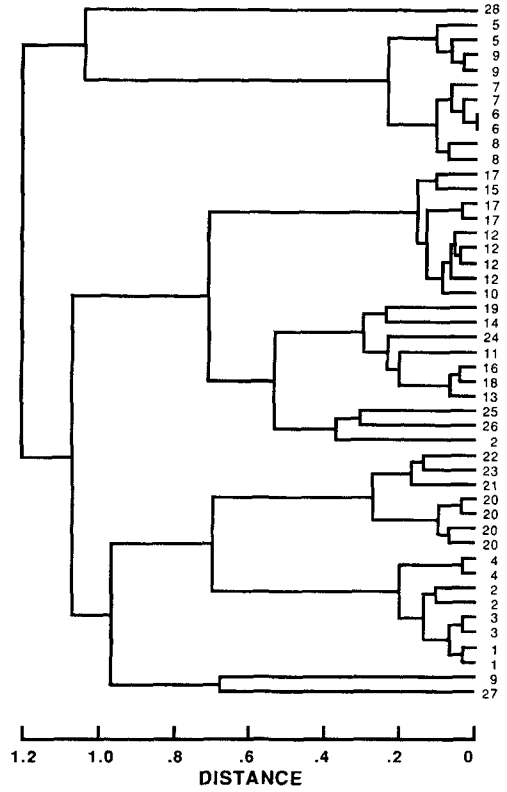

Fig. 5. Associations between individual sources of sorghum inbred lines and hybrids revealed by cluster analysis of chromatographic data

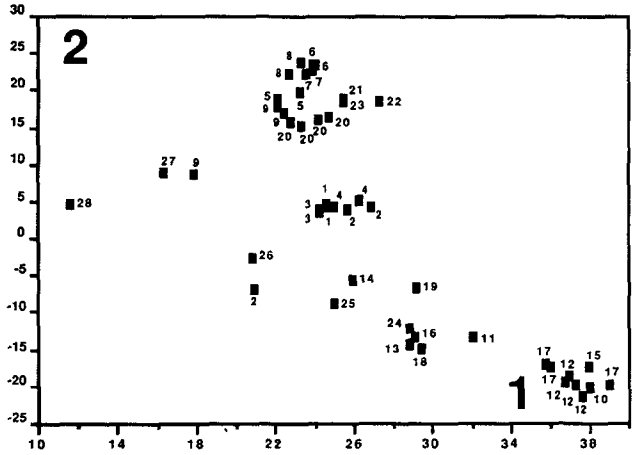

Fig. 6. Associations between individual sources of sorghum inbred lines and hybrids revealed by principal component analysis of chromatographic data

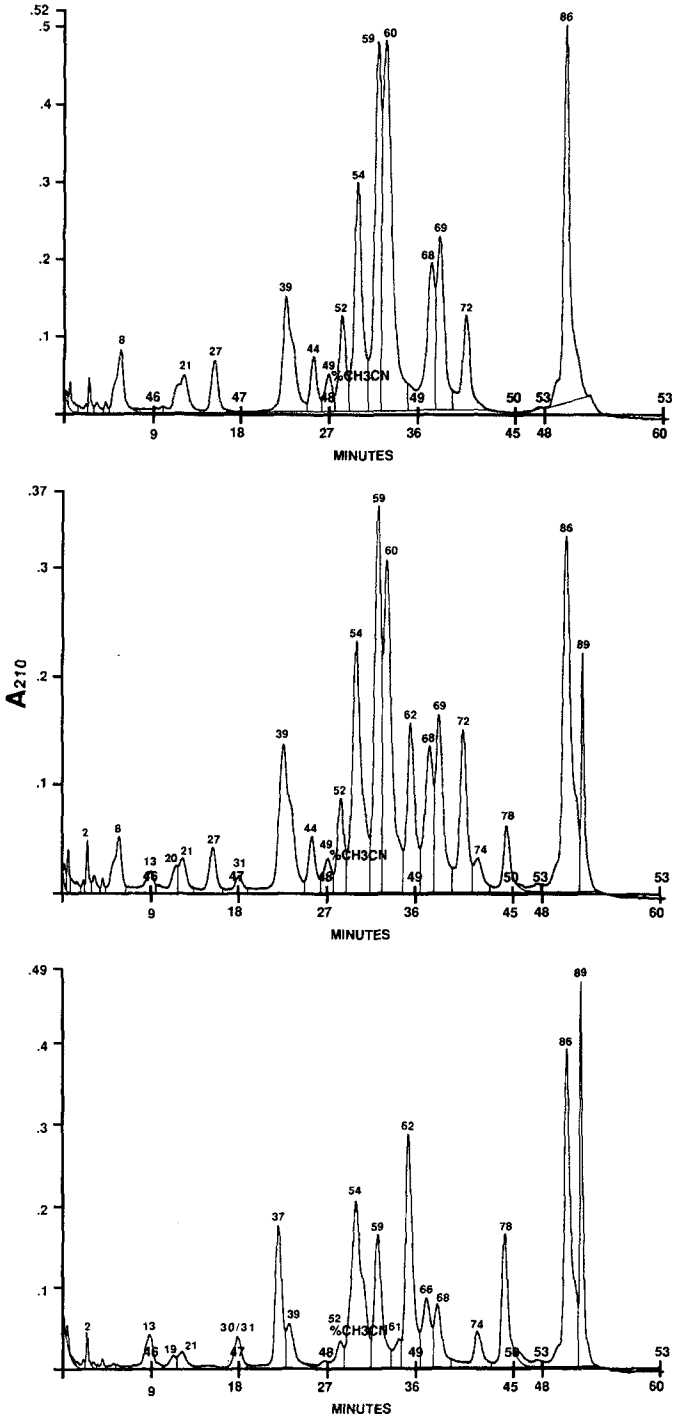

Fig. 7. Chromatograms of (*from top to bottom*) inbred Redlan, hybrid TXS214, and inbred Amber

some sources of different inbred lines (PH113, PH132, and PH210) with milo cytoplasms, revealed additional common proteins. Since their initial cross as males onto a female milo cytoplasm stock, PH113 and PH132 have been backcrossed at least 9 times with nuclear genes from the respective PH113 and PH132 lines (G. Dalton, personal communication). Thus, large residual nuclear gene differences between the milo and kafir cytoplasmic stocks of PH113 and PH132 would not be expected.

The data presented herein, together with those of Sastry et al. (1986), show that RP-HPLC may be used to reliably differentiate between at least some elite sorghum

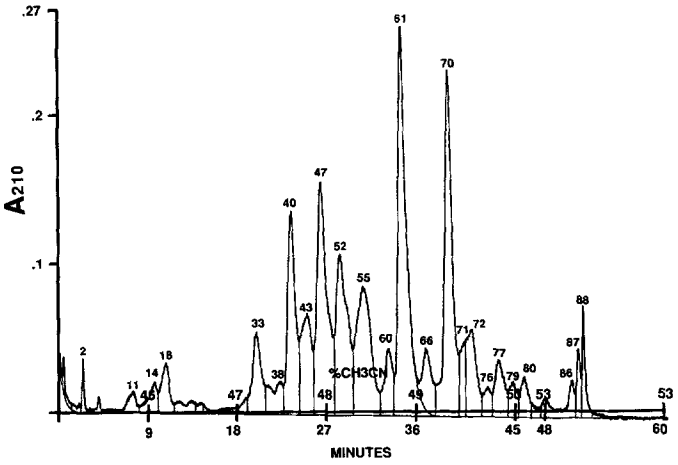

Fig. 8. Chromatogram of Johnsongrass

inbreds and hybrids that are used in the U.S. The F_1 profile of TXS214 showed the expected 2:1 addition of female:male chromatograms, in both qualitative and quantitative terms. Thus, there was no indication of the effects of differential protein extractability or gene expression which were cited as possible factors affecting the qualitative nature of chromatograms for two other F_1 hybrids (Sastry et al. 1986).

Sources of lines that were unrelated by pedigree (Table 4) grouped into separate clusters (Fig. 5) at or below a distance of 0.2. Cluster and principal components analysis both showed an association of the lines PH132 and PH113 and these lines are related by pedigree (Table 4). However, the relationship by pedigree of PH210 with PH113 was not reflected in the associations shown following analysis of biochemical data. The tetraploid Johnsongrass was not closely associated with any of the diploid sorghums, although a loose association was shown to a weedy sorgo which may have germplasm in common with Johnsongrass. The associations between lines that were shown by multivariate analysis of RP-HPLC data, therefore, mirrored the relationships that might be expected on the basis of pedigree. These, albeit limited, data provide an indication that RP-HPLC might offer a rapid and convenient means of investigating associations among both elite and exotic materials that may provide information useful to taxonomists, geneticists, evolutionists, and plant breeders. To date, this degree of discrimination has not been achieved, at least among elite cultivated U.S. germplasm, using isozymic data (Thomas-Compton and Gardner 1982; J. Doebley, personal communication).

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