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Investigation and Optimization of the Factors Influencing Sorghum Protein Extraction

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To optimize the extraction of sorghum proteins, several variables were examined: sample-to-solvent ratio, detergent type and concentration, reducing agent type and concentration, extraction time, and buffer pH and concentration. Samples were quantified and characterized by RP-HPLC, FZCE, and nitrogen analysis. These studies revealed that pH, detergent type, reducing agent type, and sample-to-solvent ratio all had significant effects on the levels of protein extracted. Increasing SDS concentration (2%) and solvent-to-flour ratio (20:1) with multiple 5 min extracts reduced extraction time by 35−80% while still extracting the same levels of total protein relative to the control methodology. Reproducibility using the multiple extractions was found to be excellent with relative standard deviations of <2% for consecutive extractions.

KEYWORDS: Sorghum; protein extraction; detergent

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is a drought-resistant, low-input cereal grain grown throughout the world. Sorghum ranks fifth in worldwide production of cereal grains (*1*), with annual worldwide production of ∼60 mmt (*2*). In the United States, sorghum is used primarily as animal feed, although interest in using sorghum as a renewable resource for bioindustrial applications, such as ethanol production, is growing. In other parts of the world, particularly Africa and India, sorghum is used as human food, where it is a basic food staple for millions of people (*1*, *3*).

Sorghum varies in protein content from 6 to 18%, with the storage proteins (kafirins) generally comprising 70−90% of the total protein (*4*, *5*). As with other cereals, the storage proteins of sorghum, the kafirins, have been divided into subclasses. In this paper, the nomenclature of Shull et al. (*6*) was followed for classification of the kafirin subclasses, which divided kafirins into α, β, and γ groups on the basis of solubility, molecular weight, and immunology. For sorghum, α-kafirin represents 66−84% of total kafirin; β-kafirin, 7−8%; and γ-kafirin, 9−12% (*7*, *8*).

Several studies have been conducted on the solubility of sorghum proteins (*6*, *8*−*13*). Hamaker et al. (*8*) reported that the use of SDS at alkaline pH values was more effective at extracting kafirins than other methods based primarily on the use of aqueous alcohols. The method of Hamaker et al. (*8*) was based upon work developed to extract maize proteins (*14*). Although effective, Wallace et al. (*14*) did not report any optimization of this methodology. Furthermore, despite its being simpler than previous multisolvent methods, this methodology required 1−2 h to complete the extractions (*8*, *14*, *15*).

Thus, the objectives of this project were to fully investigate the factors involved in extracting proteins from sorghum according to the method of Hamaker et al. (*8*) and to optimize these conditions to reduce the time required for extraction while maintaining good reproducibility. Furthermore, conditions were varied in such a way as to provide basic information on the biochemistry and solubility of sorghum proteins. Such information should benefit future studies on sorghum proteins and the development of new industrial and food uses of sorghum.

MATERIALS AND METHODS

Sample Preparation. Whole sorghum kernels were ground using either an Udy mill (Udy Corp., Fort Collins, CO) equipped with 0.25 or 1.0 mm screens or a commercial coffee grinder for 30 s. Samples ground with the coffee grinder were not sieved.

Protein Extraction. The protein extraction procedure used in this study was based on that reported by Hamaker et al. (*8*) and Wallace et al. (*14*). Total proteins were extracted with a 12.5 mM sodium borate buffer, pH 10.0, containing 1% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol (β-ME). Non-kafirin proteins were precipitated from the total protein extract by precipitation with *tert*-butyl alcohol (final concentration = 60%) (*8*). For free zone capillary electrophoresis (FZCE) analysis, after the precipitation of non-kafirin proteins, the kafirins were precipitated by adding acetone (8:1 ratio of acetone to sample). Precipitated kafirins were redissolved in 60% 1-propanol plus 2% β-ME before separation. Extraction times, detergents, reducing agents, and solvent-to-flour ratios were all varied over the course of this project to optimize each of these factors. When the effects of

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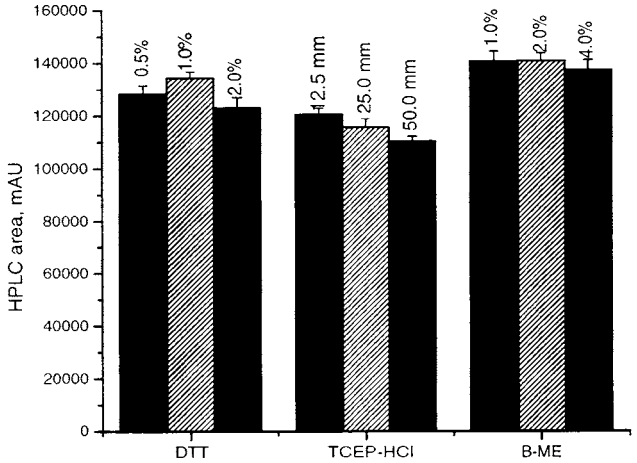

Figure 1. Effect of reducing agent type and concentration on extraction of sorghum proteins. Samples were extracted with different levels and types of reducing agents in a pH 10.0 buffer containing 1% SDS. Samples were analyzed using RP-HPLC, and total peak area was calculated. Error bars represent standard deviation, $n = 3$.

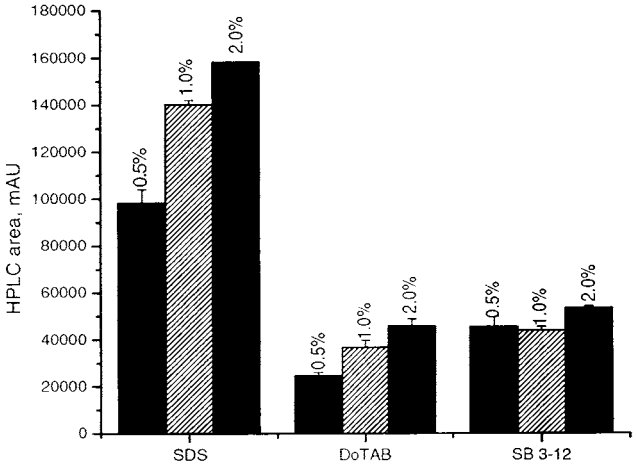

Figure 2. Effect of detergent type and concentration on extraction of sorghum proteins. Samples were extracted with different levels and types of detergents in a pH 10.0 buffer containing 2% β-ME. Samples were analyzed using RP-HPLC, and total peak area was calculated. Error bars represent standard deviation, $n = 3$.

altering the sample-to-solvent ratio were tested, extracted samples were diluted with extraction buffer to identical volumes. This was done so that levels of extracted protein could be directly compared when analyzed by RP-HPLC. All samples were extracted using a VortexGenie2 (Scientific Industries Inc., Bohemia, NY) equipped with a 30 place foam microfuge holder. Samples were vortexed continually during extraction.

RP-HPLC and FZCE. Separations were performed using an Agilent 1100 high-performance liquid chromatography (HPLC) system. Reversed phase (RP) HPLC separations were made using a C18 column as described in Bean et al. (*16*). FZCE separations were performed using either a Beckman MDQ or a Beckman PACE 2100 capillary electrophoresis system. Separation conditions were the same as described in Bean et al. (*16*).

Nitrogen Analysis. Nitrogen analysis on extracted samples was performed using a LECO FP-528 nitrogen determinator (St. Joseph, MI) according to AACC method 46-30. The amount of protein extracted was determined by subtracting initial protein values from protein values remaining after extractions.

Statistical Analysis. Data were analyzed using SAS software version 8.2 (*17*). Comparison among treatments was made using least significant difference (LSD).

RESULTS AND DISCUSSION

Reducing Agent Type and Concentration. The first factors investigated in this project were the type and concentration of reducing agents used. Using a 12.5 mM sodium borate, pH 10.0, buffer and 1% SDS (*8*), three different reducing agents, β-ME, dithiothreitol (DTT), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl), were tested for the effects on the amount of sorghum proteins solubilized from whole meal sorghum.

Differences in protein extraction were seen when the different types of reducing agents were compared (**Figure 1**). β-ME extracted the highest level of protein of the reducing agents tested, with all three concentrations of β-ME extracting roughly the same levels of protein (**Figure 1**). DTT extracted lower amounts of protein than β-ME, with 1% DTT extracting slightly more protein than the other concentrations tested (**Figure 1**). TCEP-HCl extracted the least amount of protein of all three reducing agents (**Figure 1**). In addition, as the amount of TCEP-HCl increased, the amount of protein extracted decreased. This may have been due to decreased pH of the extraction buffer when levels of TCEP-HCl increased (due to the HCl form of TCEP used). The amount of protein extracted with either 1 or 2% β-ME was statistically significantly different ($p = 0.05$) from the other reducing agent types and amounts tested (data not shown); 2% β-ME was arbitrarily chosen for use in all following experiments.

Effect of Detergent Type and Concentration. Different detergents were then tested for their effects on extracting sorghum proteins. The method of Hamaker et al. (*8*) utilized the anionic detergent SDS. For comparison, two other detergents with similar structures but different charges were selected. These were the cationic detergent dodecylammonium bromide (DoTAB) and the zwitterionic detergent SB 3-12, both of which contain 12 carbon tails similar to that of SDS.

Each detergent was tested at three levels in a 12.5 mM sodium borate, pH 10.0, buffer with 2% β-ME used as the reducing agent (**Figure 2**). Differences in extraction levels were observed between the types of detergents. SDS clearly extracted more protein than either DoTAB or SB 3-12 (**Figure 2**). As the SDS concentration was increased, the amount of protein extracted also increased (**Figure 2**). Because of this trend, additional concentrations of SDS were tested. The amount of protein extracted leveled off at 2%; no increases in protein extraction were seen at higher concentrations of SDS (data not shown).

SDS clearly extracted the most protein at alkaline pH values (**Figure 2**). However, the pH of the extraction buffer would influence the net charge on the proteins; at high pH the proteins would be expected to carry an overall net negative charge, which could influence the interaction, and thus solubility, of the proteins with detergents tested in this study. Therefore, each detergent was tested at acidic pH, at which the proteins would be expected to carry a net positive charge, and at neutral pH, at which the proteins could carry either a net negative or positive charge. Therefore, this experiment would determine if there was an interaction between the detergent charge and the net charge on the proteins in determining the amount of protein extracted.

At each pH, SDS still extracted much higher levels of protein than the other detergents (**Figure 3**). As the pH increased, the amount of protein extracted by SDS also increased (**Figure 3**). Increasing the buffer pH to 12.0, however, resulted in a decrease in the amount of protein extracted relative to pH 10.0 (data not shown). Thus, the optimum detergent for extracting sorghum proteins appears to be 2% SDS at pH 10.0. This is a higher level of SDS than used in previous methods (*8*, *14*).

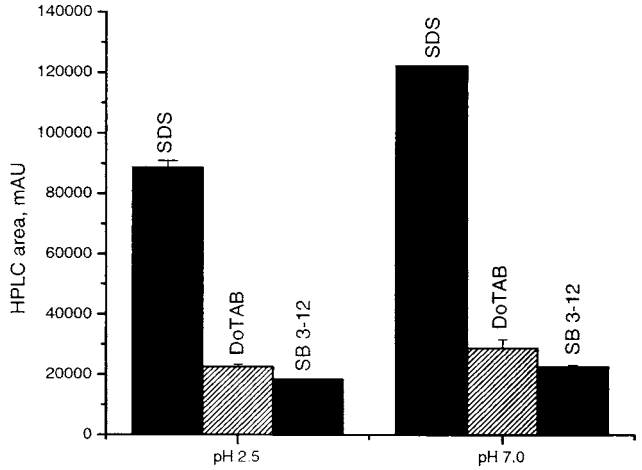

Figure 3. Impact of buffer pH and detergent type on extraction of sorghum proteins. Samples were extracted with three different detergents at pH 2.5 and 7.0 to investigate any possible interactions between detergent type and charge on the proteins. Samples were analyzed using RP-HPLC, and total peak area was calculated. Error bars represent standard deviation, $n = 3$.

Buffer Concentration. Next the effect of varying the concentration of the buffer used in the extraction solution was tested. Concentrations were varied from 12.5 to 625 mM. No significant differences ($p = 0.05$) were seen in the amount of protein extracted at any level (data not shown).

Solvent-to-Sample Ratio. The effect of varying the solvent-to-sample ratio was also tested. Increasing the ratio of solvent to sample can lead to higher protein extraction rates (e.g., ref *18*). Previous methods have used a ratio of 10:1 (*8*, *14*). Thus, for this project, three different solvent-to-sample ratios were tested, 4:1, 10:1, and 20:1. As the ratio was decreased, the amount of protein extracted also decreased (**Figure 4**). Thus, the largest ratio, 20:1, extracted the most protein, and levels at this ratio were statistically significantly ($p = 0.05$) different from the other ratios (data not shown). Thus, a 20:1 ratio (50 mg of sample to 1 mL of solvent) was chosen for future use.

Use of Multiple Extractions. One of the goals of this project was to reduce the time necessary to extract sorghum proteins. Existing methods used 1 h extractions (*8*, *14*); if duplicate extractions are used to improve reproducibility, total extraction time is then 2 h (*15*, *19*). Short multiple extractions have been used to rapidly extract cereal storage proteins (*20*). For this reason, the possibility of using multiple short extractions of different times was tested for their effectiveness in extracting sorghum proteins. Times of 5, 10, and 15 min were tested. For each extraction time tested, three consecutive extractions were made and the amount of protein extracted (in each extract) was measured by RP-HPLC. The cumulative amount of protein extracted was compared to that from a single 60 min extract. RP-HPLC and nitrogen analysis of the samples showed that three 5 min extractions solubilized 84% of the protein in the sample, whereas the single 60 min extract removed 83% (data not shown). Thus, by using short multiple extractions that were pooled together before analysis, the same amount of protein can be extracted as with a single longer extraction. However, the short multiple extractions require only ~21 min (counting centrifuge time) to complete. Thus, the overall time required for extraction was reduced by 35% compared to a single 1 h extraction [as used by Hamaker et al. (*8*)] and by >80% compared to two 1 h extractions [as used by Bean et al. (*15*) and Hicks et al. (*19*)].

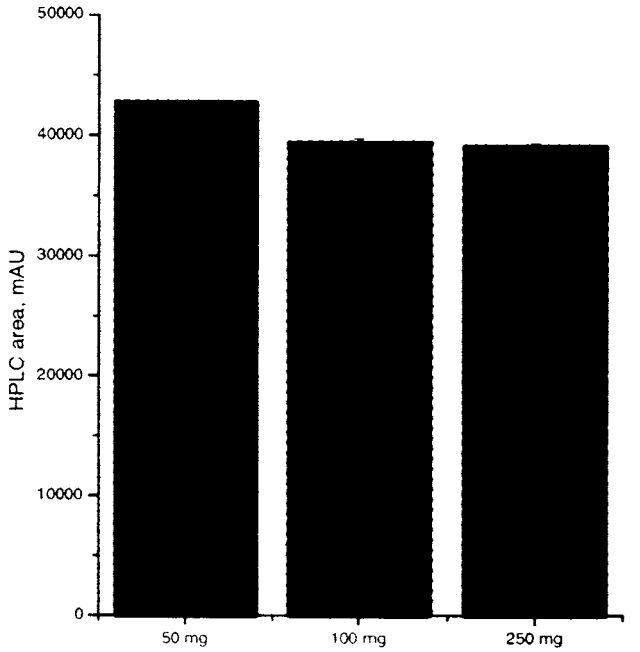

Figure 4. Impact of solvent-to-sample ratio on extraction of sorghum proteins. Samples were extracted at three different solvent-to-sample ratios. After extraction, samples at 10:1 and 4:1 were diluted for direct comparison to the sample extracted at 20:1. Samples were analyzed using RP-HPLC, and total peak area was calculated. Error bars represent standard deviation, $n = 3$.

Kafirin Precipitation. One final optimization step was carried out for this project. The method of Hamaker et al. (*8*) used 60% *tert*-butyl alcohol to precipitate the non-kafirin proteins away from the kafirins. Although effective, *tert*-butyl alcohol can be tedious to work with, as it is often solid at room temperature. Because of this, several solvents at various concentrations were tested for their effectiveness in precipitating non-kafirin proteins away from the kafirins. Total proteins were extracted, pooled, and divided into aliquots that were then mixed with different solvents at various concentrations. Blank extraction solvent was used to keep the volume of the protein sample the same in all treatments. After precipitation of the non-kafirin proteins, the supernatant (i.e., the kafirins) were analyzed by RP-HPLC and the amount of protein remaining in the supernatant was compared to the control, which was precipitated using 60% *tert*-butyl alcohol. RP-HPLC separations were compared both quantitatively (by measuring peak area) and qualitatively (visually comparing RP-HPLC patterns to look for differences in patterns). Three different solvent combinations showed approximately the same levels of protein (i.e., RP-HPLC peak area) remaining in the supernatant, 60% 1-propanol, 70% ethanol, and 70% ACN (**Figure 5**). All other combinations had either higher or lower amounts of protein in the supernatant, suggesting that these combinations were not precipitating the same set of proteins as the control conditions. Qualitative comparisons of the RP-HPLC separations showed that the patterns of kafirins left after precipitation of non-kafirins by 60% 1-propanol, 70% ethanol, and 70% ACN were essentially identical (data not shown).

To further investigate which proteins were being precipitated and which were left in the supernatant, the RP-HPLC patterns of the supernatant from 70% EtOH and 60% 1-propanol precipitations were qualitatively compared to the control (60% *tert*-butyl alcohol precipitations). In all cases the patterns were essentially identical to the control (**Figure 6a**). In addition to

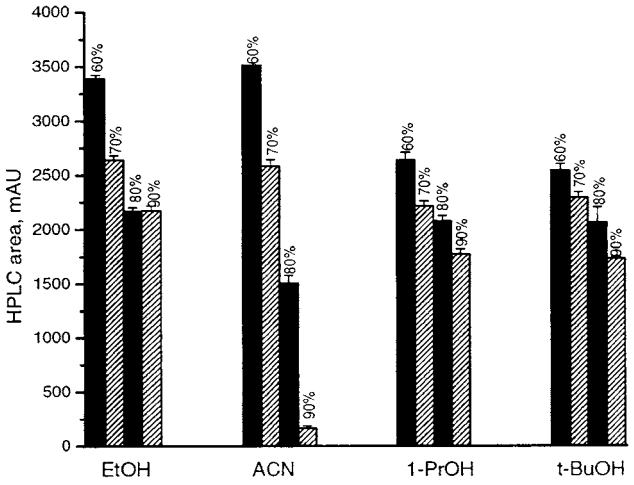

Figure 5. Amount of protein remaining after precipitation of non-kafirins with various solvents. Total proteins were extracted using three 5 min extracts. Aliquots were divided out, and non-kafirins were precipitated by the addition of various solvents at several concentrations. Blank extraction solvent was used to keep the sample volume the same in all cases to allow direct comparison of the protein remaining in the supernatant after precipitation of the non-kafirin proteins. Samples were analyzed via RP-HPLC, and total peak area was calculated. Error bars represent standard deviation, $n = 3$.

the RP-HPLC analysis, kafirins from these solvent combinations were compared to the control by FZCE. As with RP-HPLC, the FZCE patterns were the same among the various treatments and the control (**Figure 6b**). These results demonstrated that these solvent combinations were precipitating the same non-kafirins proteins as 60% *tert*-butyl alcohol (**Figure 6**). Thus, any of these solvents could be used interchangeably in preparing kafirin samples using this methodology. The use of 70% ethanol may have some benefit over *tert*-butyl alcohol in toxicity and expense. The use of ethanol may be helpful in preparing kafirins for potential use in bioindustrial applications, in much the same way that maize proteins are commercially utilized.

Optimum Extraction Conditions. Thus, the optimum conditions for extracting total proteins of sorghum were found to be the following: a 12.5 mM sodium borate buffer, pH 10.0, containing 2% SDS and 2% β-ME with the samples extracted using a 20:1 solvent-to-sample ratio (50 mg to 1 mL) using three 5 min extractions with the supernatant from each extract pooled 1:1:1 to produce the final extract. Sorghum grain should be ground to a fine particle size, and all samples should be ground to the same particle size.

Repeatability. To test the repeatability of this procedure, 10 individual extractions were made and analyzed by RP-HPLC. Repeatability was very good with relative standard deviations of $<2\%$ (data not shown).

Conclusions. Several factors were investigated to determine their effects on the extraction of sorghum proteins and to optimize the extraction protocol. The resulting method was 35−80% faster than current protocols and maintained excellent reproducibility. Furthermore, these studies provided basic information on the solubility of sorghum proteins. Sorghum proteins were found to be more soluble in the presence of SDS at all pH values than in zwitterionic or cationic detergents with structures similar to that of SDS. Sorghum proteins were more soluble at basic pH than at either neutral or acidic pH in the presence of detergents. It was also found that non-kafirin proteins could be precipitated away from kafirins with a variety of solvents, including 70% ethanol. This may be useful in the development of kafirins for bioindustrial applications.

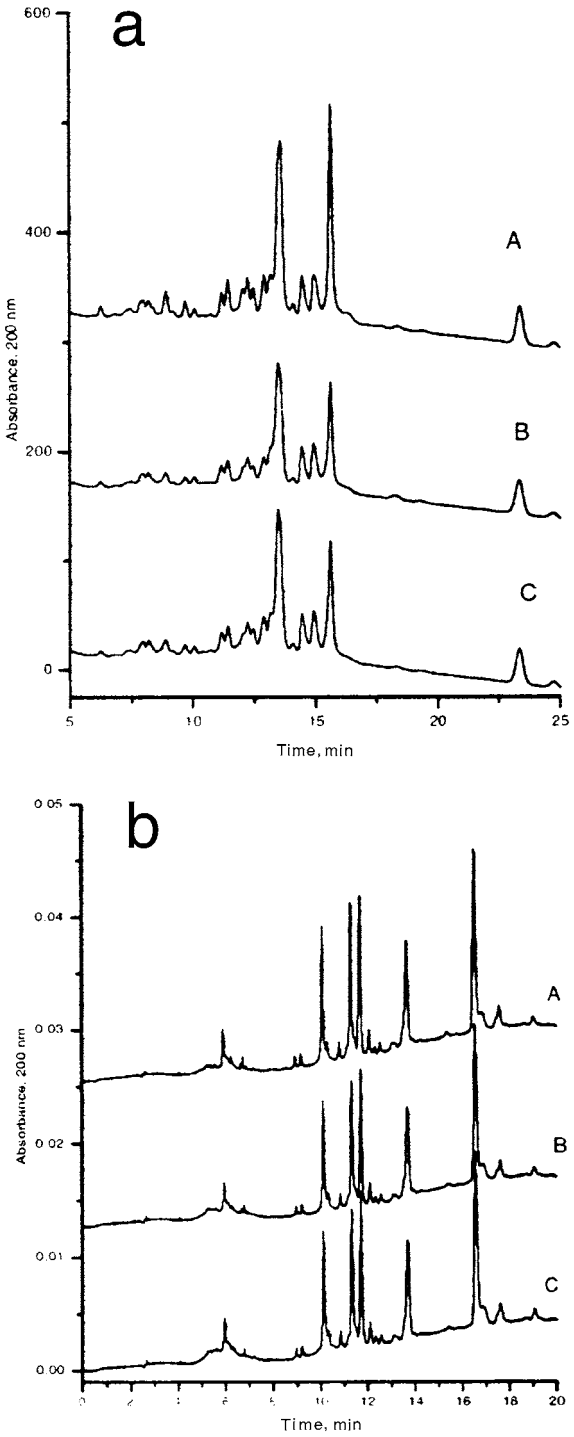

Figure 6. Comparison of the RP-HPLC and FZCE separations of kafirins after precipitation of kafirins. Kafirins remaining after precipitation of non-kafirins with 60% *tert*-butyl alcohol (control), 60% 1-propanol, or 70% ethanol were analyzed by (**a**) RP-HPLC and (**b**) FZCE to compare the patterns between the various treatments.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; β-ME, β-mercaptoethanol; DTT, dithiothreitol; DoTAB, dodecylammonium bromide; TCEP-HCl, tris(2-carboxyethyl) phosphine hydrochloride; FZCE, free zone capillary electrophoresis; RP-HPLC, reversed phase

high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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