JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2011, 59, 85–91 85
DOI:10.1021/jf1036195

Separation of Kafirins on Surface Porous Reversed-Phase High-Performance Liquid Chromatography Columns

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Surface porous high-performance liquid chromatography (HPLC) columns were investigated for the separation of kafirins, storage proteins of grain sorghum. Kafirins were successfully separated using C3, C8, and C18 surface porous stationary phases in less than 17 min. Separations using a monolithic C18 stationary phase were also developed and were slightly faster than those achieved on the surface porous C18 stationary phase. However, the resolution was higher on the latter column. Using an ammonium hydroxide/acetonitrile mobile phase, separations were performed on a novel, alkaline stable surface porous C18 stationary phase. The resolution at alkaline pH was not as high, however, as with the traditional acidic acetonitrile mobile phases. In comparison to fully porous stationary phases, the surface porous phases provided higher resolution with much lower separation times (17 versus 40 min). Total peak areas were correlated to total protein content of sorghum (r^2 = 0.96; n = 10), and a method to measure *in vitro* pepsin digestibility using reversed-phase (RP)-HPLC peak areas showed good correlation to the traditional nitrogen combustion method (r^2 = 0.82; n = 20). Thus, the surface porous stationary phases could be used not only for more rapid separations but also to provide simultaneous information on total protein content and digestibility.

KEYWORDS: Cereal proteins; HPCE; kafirins; reversed-phase high-performance liquid chromatography; RP-HPLC; SDS−PAGE; sorghum

INTRODUCTION

Cereal proteins are an important class of food proteins worldwide, not only for human and animal nutrition but also for their functional properties (*1*−*3*). Because of their wide ranging importance, a number of analytical methods have been employed to study cereal proteins, including high-performance liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE), high-performance capillary electrophoresis (HPCE), and microfluidics (*4*−*8*). Analytical separations of cereal proteins have been used in research projects in numerous ways, such as relating the properties, amount, or presence of specific proteins to functional properties, investigating how processing of cereals has altered the properties of the proteins, adulteration of cereal food products, studing protein expression, and differentiating cereal cultivars (*4*−*8*).

Arguably, the most widely used methods to separate cereal proteins are reversed-phase (RP)-HPLC and sodium dodecyl sulfate (SDS)−PAGE. For both of these techniques, a number of improvements have been made over time in terms of improving resolution, reducing separation time, and improving methods of sample preparation. For RP-HPLC, many of the improvements in methods have been realized with improvements in stationary phases. For example, the development of columns able to withstand elevated temperatures allowed for improved resolution of wheat proteins (*9*). More recently, researchers have taken advantage of new column materials to drastically reduce separation times when analyzing cereal proteins, often maintaining the resolution of previous methods. Both monolithic and perfusion stationary phases have been successfully used to separate maize proteins. Rodriquez et al. (*10*) optimized extraction conditions and gradients to separate proteins from maize whole meal and maize food products using a perfusion stationary phase. Optimized conditions led to samples resolved into ∼7−9 peaks in less than 4 min. These same authors (*11*) also optimized the separation of maize proteins using monolithic stationary phases and were able to resolve maize proteins from grain and food products into 9−12 peaks with separation times of 8−10 min. Both perfusion and monolithic stationary phases were used to separate maize proteins from a number of North American and European maize samples (*12*). Naeem and Sapirstein (*13*) used surface porous stationary phases for the separation of wheat proteins and were able to achieve high-resolution separations in less than 14 min (comparable to separations achieved in 60 min on fully porous columns). Separations of wheat proteins in under 4 min were also achieved with enough resolution to differentiate high-molecular-weight glutenin subunits, which would allow for the use of these columns in high-throughput screening of breeding lines (*13*).

In comparison to wheat and maize, very little research has been conducted to improve the separation of sorghum proteins. By far the most common method for separating sorghum proteins has been SDS−PAGE. Kafirins are typically divided into three major

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Published on Web 12/09/2010

subclasses, α, β, and γ (*14*). The α kafirins are the most abundant (80−85% of total kafirin) and are typically resolved into two bands on SDS−PAGE of approximate M_w of 23 and 25 kDa. The β kafirins make up 7−13% of the total kafirins and migrate at M_w of ∼19 kDa. The remaining 10−20% are γ kafirins that migrate at ∼28 kDa on SDS−PAGE. Interestingly, sequence data for the kafirin subclasses lists the α kafirins as the largest, with M_w of 26−27 kDa; γ kafirins, ∼21 kDa; and β kafirins, ∼19 kDa (*14*). However, despite differences between sequence M_w and SDS−PAGE migration order, SDS−PAGE easily resolves the three major subclasses of kafirins and much has been learned about kafirins from the use of SDS−PAGE. However, as can be seen from the data above, the kafirins have a relatively narrow M_w distribution of only ∼4−8 kDa. Note also that, while the majority of the kafirins are grouped into these three subclasses, there are a number of proteins within each class. There may be up to 20 genes in the α kafirin subclass and 3 genes in the γ kafirin subclass (*14*).

Additional analytical methods that separate proteins by differences other than M_w, such as RP-HPLC, HPCE, and isoelectric focusing (IEF), have been shown to resolve the kafirins into many more peaks/bands than SDS−PAGE because of the narrow M_w distribution of these proteins. For example, Taylor and Schussler (*15*) used an acidic urea PAGE method, where proteins were separated by differences in charge density to resolve kafirins into multiple bands. IEF was later used to resolve reduced and alkylated kafirins into ∼18−25 bands, with pI values between 6 and 8 (*16*). Likewise, these same authors successfully separated kafirins by RP-HPLC using a C18 stationary phase, and resolution was sufficient (∼6−8 major peaks resolved) to differentiate various sorghum lines and hybrids. The RP-HPLC run time was ∼50 min for these separations (*16*). Later, kafirins were separated using a C8 stationary phase into ∼10−12 major peaks, with run times of ∼60 min (*17*). A method for separating kafirins by HPCE using an acidic buffer containing high levels (60%) of acetonitrile was reported to resolve kafirins into ∼12 major peaks in less than 20 min (*18*). The HPCE separations were compared to RP-HPLC separations using a C18 stationary phase, and fractions were collected from the RP-HPLC separations and analyzed using SDS−PAGE to identify tentative locations of the kafirin subclasses in both the RP-HPLC and HPCE separations (*18*). RP-HPLC separation times in this work were ∼40 min.

Sorghum proteins have lower digestibility than those of other cereals and are known to cross-link when heated to the degree of which is unique to cereals (*19*). The reason for the lower digestibility of sorghum proteins is not completely known (*19*), and understanding the underlying reason for this is a major emphasis of sorghum protein research. It is also thought that sorghum proteins are a primary determinant of grain hardness in sorghum, which is an important grain quality factor (*20*). Several studies have shown the potential of sorghum proteins for the production of films and edible coatings (*21*). Thus, sorghum proteins play an important role in the chemistry, use, and structure of the sorghum grain. New methods for characterizing sorghum proteins are vital to researchers trying to understand the relationships between the properties of sorghum proteins and end-use quality of sorghum in a number of applications. Therefore, the goal of this research was to investigate the use of new stationary-phase technology to improve the separation of sorghum proteins, specifically the kafirins. A secondary objective was to evaluate how well rapid RP-HPLC separations could be used to predict total protein and protein digestibility of sorghum proteins.

MATERIALS AND METHODS

Chemicals and Samples. HPLC-grade acetonitrile was obtained from Honeywell Burdick and Jackson (Muskegon, MI). All other chemicals were obtained from Sigma (St. Louis, MO). Tannin-containing sorghum samples were provided by the Department of Agronomy, Kansas State University (Manhattan, KS), and the commercial hybrids were provided by Dr. Jeff Dahlberg, National Sorghum Producers Association (Lubbock, TX). A maize sample was used as a reference in the protein digestibility assay and was a commercial sample obtained in Marshall County, KS.

Sample Preparation. Prior to extraction of kafirins, albumins and globulins were first pre-extracted as described in ref *22*. Kafirins were then extracted from whole ground sorghum (100 mg) with 1 mL of 60% (v/v) *t*-butanol containing 0.5% sodium acetate (w/v) and 2% β-mercaptoethanol (β-ME) (v/v) using two 5 min extractions, which were pooled 1:1 as described for wheat protein extractions (*22*). Similar, short multiple extractions have been found to be effective for sorghum proteins previously (*23*). After extraction, extracts (1 mL) were alkylated by the addition of 66.7 μL of 100% 4-vinylpyridine (4-VP) and the sample was vortexed for 10 min. Wheat proteins have commonly been alkylated using 4-VP in a number of studies (e.g., see ref *24*). For some experiments, samples were not alkylated; in which case, samples were immediately injected after extraction was completed.

To develop standard curves for protein concentration versus HPLC peak area, kafirins were isolated as described in ref *25*. Briefly, 100 g of whole ground sorghum was extracted with 350 mL of 70% ethanol containing 2% β-ME (v/v) for 1 h at 50 °C. Co-extracted lipids were removed by lowering the ethanol content to 60% (v/v) and centrifuging at 4 °C. Kafirins were then precipitated by reducing the ethanol concentration of the remaining supernatant to 30%. After centrifugation, precipitated kafirins were lyophilized and stored frozen until needed. The purity of the isolated kafirins was 91.46% as determined by nitrogen combustion (see details below).

HPLC. RP-HPLC was performed using an Agilent 1100 series instrument (Agilent, Santa Clara, CA). Several different columns were tested including the following: surface porous Poroshell 300 SB columns (2.1 × 75 mm) with either C3, C8, C18, or Poroshell C18Ext stationary phases (Agilent, Santa Clara, CA), a monolithic Onyx C18 (4.6 × 100 mm, Phenomenex, Torrance, CA), and a Jupiter C18 (2.0 × 150 mm, Phenomenex, Torrance, CA). For all columns except the C18Ext, mobile phase A was water plus 0.1% trifluoroacetic acid (TFA) (w/v) and mobile phase B was acetonitrile plus 0.07% TFA (w/v). For the C18Ext column, an alkaline mobile phase system was used, where mobile phase A was water plus 20 mM ammonium hydroxide and mobile phase B was 80% acetonitrile plus 20 mM ammonium hydroxide.

Protein Content Analysis and Digestibility Assay. Protein digestibility was determined using the *in vitro* pepsin digestibility assay as described in ref *26*, using sorghum ground with a UDY cyclone mill (UDY Coproration, Fort Collins, CO) equipped with a 0.5 mm screen. After digestion, residues were lyophilized along with the original raw flour for each sample. The protein content of raw and digested flours was then determined by nitrogen combustion using a Leco FP-528 nitrogen determinator (Leco, St. Joseph, MI) according to American Association of Cereal Chemists (AACC) method 46-30 (*27*). Nitrogen values were converted to protein using a factor of 6.25. When digestibility values were determined by RP-HPLC, proteins were extracted from the lyophilized raw and digested flours as described above. Total peak areas from the RP-HPLC chromatograms were measured and converted to percent protein using a standard curve of peak area versus protein concentration. Digestibility was then calculated as percent protein digested sample/percent protein undigested flour.

RESULTS AND DISCUSSION

Sample Preparation and Stability. Cereal protein extracts are typically a complex mixture of various proteins. Often these proteins or subsets of them are high in cysteine and readily form disulfide cross-links, both inter- and intramolecularly. For example, the β and γ kafirins of sorghum both contain high levels of cysteine and are thought to be extensively cross-linked via disulfide bonds (*14*). Dependent upon the research being conducted, it is often desirable to break these disulfide bonds during extraction of the proteins, and as such, reducing agents are

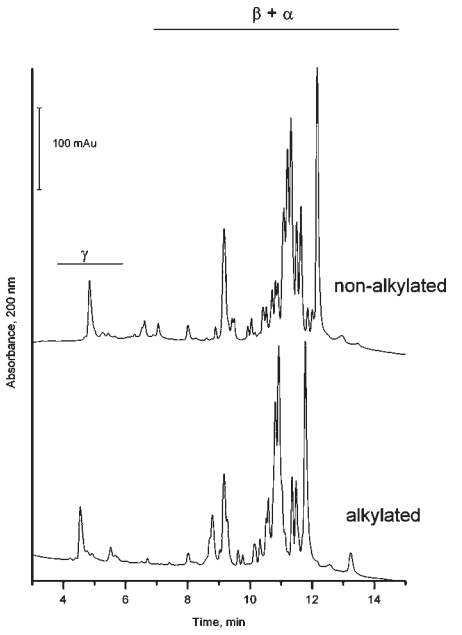

Figure 1. Comparison of alkylated and non-alkylated protein samples. Samples were analyzed on a Poroshell SB300 C18 column using water + 0.1% TFA (w/v) (solvent A) and acetonitrile + 0.07% (w/v) TFA (solvent B). The gradient was as follows: 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; and 17 min, 20% B. The column temperature was maintained at 55 °C and flow rate at 0.7 mL/min with a 5 μL injection.

common in cereal protein extraction procedures. As expected, it has long been common to alkylate cereal protein samples to block re-oxidation of reduced disulfide bonds (*28*). It was soon discovered that, at least for some proteins, especially those in wheat, alkylation also provided important increases in resolution in RP-HPLC separations, allowing proteins not fully separated without alkylation to be resolved by changing the surface hydrophobicity of the proteins when alkylated (*29*,*30*). New developments in RP-HPLC technology, however, led to the development of methods that did not require the alkylation of extracts to resolve these proteins (*6*), thus simplifying the extraction procedures.

As found with wheat proteins, the alkylated sorghum samples eluted earlier in the gradient compared to the non-alkylated sorghum samples, but overall, chromatograms between the two samples were similar (**Figure 1**). Slight improvements in resolution were noted in the alkylated sample early in the separation, where the γ and β kafirins elute (*18*) (between 4 and 10 min in **Figure 1**), although it appeared that resolution was decreased in the 10−12 min range, where the α kafirins elute (*18*) with the alkylated samples. As mentioned previously, both the β and γ kafirins are high in cysteine; therefore, alkylation would be expected to potentially impact these proteins more than the α kafirins. However, after repeated analysis over time, the chromatograms of the non-alkylated samples were not stable. This was most apparent in the early eluting γ kafirin (eluting at ~4.5 min). When areas for this peak were measured over a 650 min period (during which samples were left in clear glass vials in the HPLC instrument), it was readily apparent that these proteins were not stable without alkylation (**Figure 2**). Analysis of the extracts by size-exclusion chromatography showed that the non-alkylated samples were re-oxidizing into oligomers (data not shown). Thus, it is important when analyzing sorghum proteins by RP-HPLC and extracted in aqueous alcohols (or any technique where the samples are not analyzed immediately) that the samples be alkylated. On a side note, cereal protein extracts are also often heated to stabilize samples against enzyme activity (e.g., see

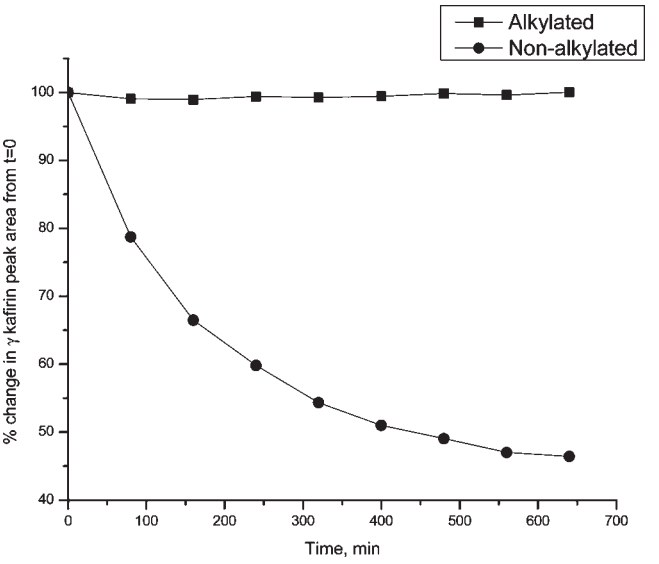

Figure 2. Comparison of changes in γ-kafirin peak area between alkylated and non-alkylated samples over time.

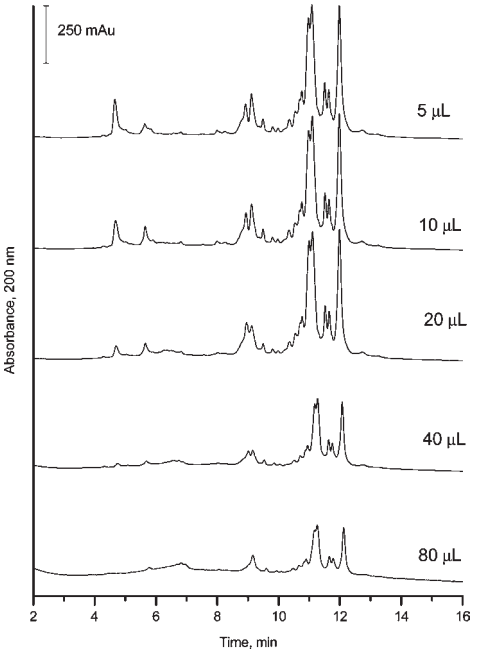

Figure 3. Effect of the injection volume on kafirin separations. Samples were analyzed on a Poroshell SB300 C18 column using water + 0.1% TFA (w/v) (solvent A) and acetonitrile + 0.07% (w/v) TFA (solvent B). The gradient was as follows: 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; and 17 min, 20% B. The column temperature was maintained at 55 °C and flow rate at 0.7 mL/min. The protein concentration was adjusted for each sample, so that the same amount of protein was loaded onto the column at each injection amount.

ref *31*). We observed that, when the aqueous *t*-butanol extracts used in this study were heated, the γ kafirin peak quickly disappeared from the chromatograms, whether the samples were alkylated or not (data not shown). Thus, it is not recommended that reduced samples be heated prior to RP-HPLC.

Effect of the Injection Volume. Cereal storage proteins, such as kafirins, are typically extracted using various aqueous alcohols and, as such, are in a relatively nonpolar sample matrix. This has been found to reduce the binding of cereal proteins to RP-HPLC columns, dependent upon injection volume, particularly of the

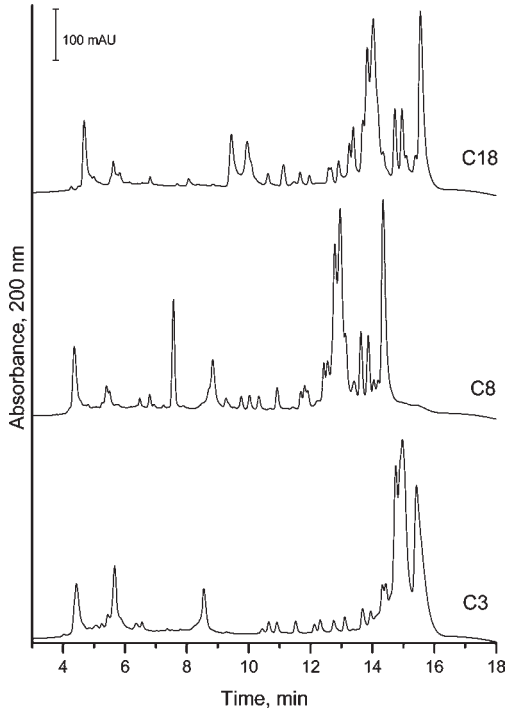

Figure 4. Chromatograms of kafirins separated on different stationary phases of surface porous HPLC columns under the same gradient conditions using water + 0.1% TFA (w/v) (solvent A) and acetonitrile + 0.07% (w/v) TFA (solvent B). The gradient was as follows: 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; and 17 min, 20% B. The column temperature was maintained at 55 °C and flow rate at 0.7 mL/min.

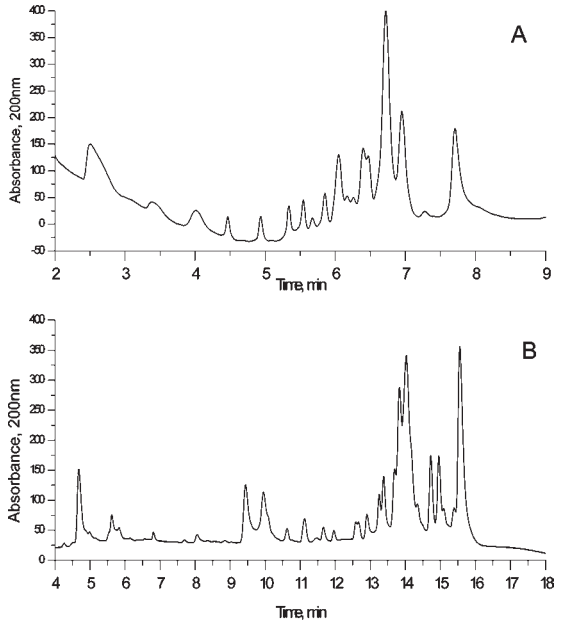

Figure 5. Chromatograms of kafirins separated with a (A) Poroshell SB300 C18Ext column and (B) Poroshell SB300 C18 column. Separations on the Poroshell SB300 C18Ext column was carried out using water plus 20 mM ammonium hydroxide (solvent A) and 80% acetonitrile plus 20 mM ammonium hydroxide (solvent B). The gradient was as follows: 0 min, 25% B; 5 min, 50% B; 15 min, 75% B; and 17 min, 25% B. The column temperature was maintained at 30 °C and flow rate at 0.7 mL/min. Separations on the Poroshell SB300 C18 column were carried out using water + 0.1% TFA (w/v) (solvent A) and acetonitrile + 0.07% (w/v) TFA (solvent B). The gradient was as follows: 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; and 17 min, 20% B. The column temperature was maintained at 55 °C and flow rate at 0.7 mL/min.

early eluting proteins (*32*). Because the γ kafirins elute much earlier than the β and α kafirins (*18*), it is possible that the injection volume could have a major impact on the quantitative analysis of kafirins (and likely maize zeins) by RP-HPLC. The protein concentration was adjusted for each injection volume by diluting the original extract as required by the injection volume, so that the same amount of protein was injected at each volume. As found for wheat proteins, at higher injection volumes, the amount of kafirin eluted during the gradient decreased (**Figure 3**). This was especially noticeable for the early eluting γ kafirin, which was substantially decreased at injection volumes over 5 μL. The later eluting α kafirins were not affected until injection volumes reached 20 μL, however (**Figure 3**). This was in agreement with similar results found with wheat proteins; i.e., the early eluting proteins were more affected than later eluting proteins. Therefore, with the columns used in this study, it is not recommended that injection volumes over 5 μL be used for quantitative research.

Effect of the Stationary Phase on Kafirin Separations. Three different stationary phases of the surface porous HPLC columns were used to evaluate their effect on the separation of kafirin proteins (**Figure 4**). Overall, the C18 column provided the best resolution of kafirins, especially the more hydrophobic α kafirins eluting last in the separations (between ~13 and 18 min for the C18 column). The C8 column provided similar resolution as the C18 column, with better resolution of the intermediate peaks eluting at ~10 min. Resolution of the α kafirins (between 14 and 16 min) on the C3 column was noticeably lower compared to the C8 and C18 columns. Optimization of separation gradients for each column individually did not noticeably change these results; i.e., the C3 column failed to adequately separate the more hydrophobic α kafirins (data not shown). Thus, either the C8 or C18 columns could be used to separate the complex mixture of proteins in the kafirin extracts into numerous peaks. While the C18 phase had perhaps the highest overall resolution, it was found that the C8 offered some advantages in reducing separation times while maintaining a high degree of resolution. The C8 stationary phase also showed improved separation in the earlier eluting proteins than the C18 stationary phase.

In addition to the stationary phases tested for use in acidic pH acetonitrile/TFA mobile phases, a C18 stationary phase that is stable at alkaline pH was also available with the same surface porous material. Kafirins are often extracted in alkaline buffers or aqueous organic solvent mixtures and, therefore, should have good solubility in alkaline mobile phases. Separation of kafirins in the alkaline mobile phase system, however, was lower than that found in the low pH acetonitrile/TFA mobile phase (**Figure 5**). Peaks eluting early in the chromatogram showed excess tailing as well in the alkaline mobile phase system, and baselines were often not stable. While individual kafirin subclasses were not identified in the alkaline pH separation, on the basis of peak heights, it appeared that the more hydrophobic α kafirins eluted last. However, there were sufficient differences between the chromatograms that these columns could possibly be used for two-dimensional RP-HPLC × RP-HPLC separations in a similar fashion as acidic and alkaline electrophoresis was performed for analyzing wheat proteins (*32*).

The surface porous columns used in this research were also compared to a monolithic C18 stationary phase. Monolithic columns have been successfully used for the separation of proteins from maize and were found to provide higher resolution than perfusion stationary phases (*12*). Chromatograms from both the surface porous C18 stationary phase and the monolithic C18 stationary phase were similar overall (**Figure 6**). For the kafirin

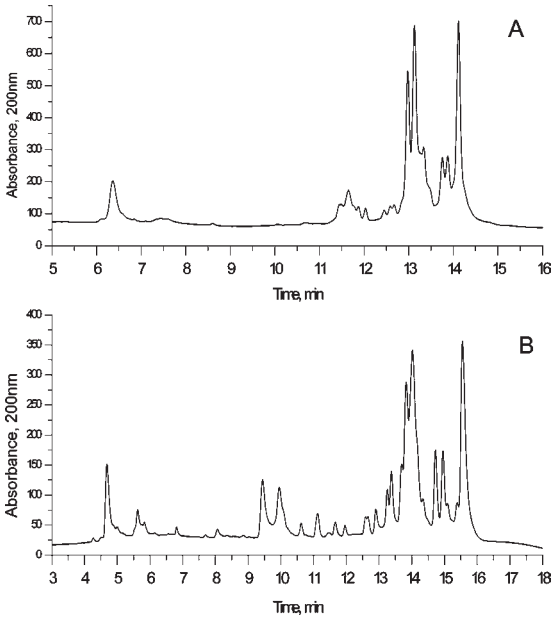

Figure 6. Comparison of the (A) monolithic C18 stationary phase and (B) surface porous C18 stationary phase. Separations were carried out using water + 0.1% TFA (w/v) (solvent A) and acetonitrile + 0.07% (w/v) TFA (solvent B). The gradient for the monolithic C18 stationary phase was as follows: 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; and 17 min, 20% B. The column temperature was maintained at 45 °C and flow rate at 0.7 mL/min. The gradient for the Poroshell SB300 C18 column was as follows: 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; and 17 min, 20% B. The column temperature was maintained at 55 °C and flow rate at 0.7 mL/min.

proteins, the surface porous column had higher resolution, especially in the early and mid-eluting (4−13 min) peaks of the chromatogram. The monolithic column had slightly faster separation times.

Because the surface porous C18 stationary phase had the best overall resolution, it was compared to a previously published method using a fully porous C18 stationary phase (**Figure 7**). Resolution between the two columns was again similar; however, the surface porous column resolved more peaks than the fully porous stationary phase, with separation times reduced from ~40 to ~17 min (**Figure 7**).

Application to the Prediction of the Protein Content and Digestibility. Because of the improved resolution and reduced analysis times found when using the surface porous columns, we next evaluated these columns for use in predicting total flour protein and protein digestibility in sorghum. Purified kafirins were injected at various concentrations designed to span the range of flour protein found in sorghum (~6−17%) (**Figure 8**). The peak area showed good linearity up to concentrations of 20 mg/mL (**Figure 8**). Note each concentration was performed at the same injection volume to avoid the loss of peaks at large injection volumes, as discussed previously. Next, proteins from 10 sorghum lines that ranged in total protein content from ~8 to 14% were extractedm and the total peak area was correlated to total flour protein. For these analyses, flour samples were extracted without pre-extraction to remove albumins and globulins, so that these proteins would be included in the prediction of total protein. Good correlation (r^2 = 0.97) was found between peak and total flour protein (**Figure 9**). Thus, when sorghum protein extracts were analyzed by RP-HPLC, it is possible to predict total flour protein from the total peak area of the chromatograms.

Aboubacar et al. (*33*) demonstrated that it was possible to predict protein digestibility of sorghum by analyzing the proteins

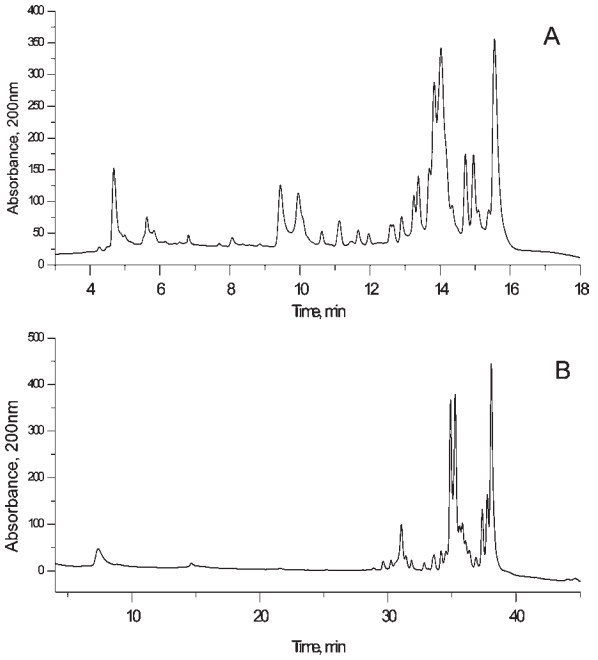

Figure 7. Comparison of kafirins separated on a (A) surface porous 2.1 × 75 mm C18 column and (B) 2.0 × 150 mm fully porous C18 column. Separations were carried out using water + 0.1% TFA (w/v) (solvent A) and acetonitrile + 0.07% (w/v) TFA (solvent B). The gradient for the Poroshell SB300 C18 column was as follows: 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; and 17 min, 20% B. The column temperature was maintained at 55 °C and flow rate at 0.7 mL/min. The gradient for the fully porous C18 stationary phase was as follows: 0 min, 28% B; 50 min, 60.5% B; and 60 min, 60.5% B. The column temperature was maintained at 50 °C and flow rate at 0.5 mL/min.

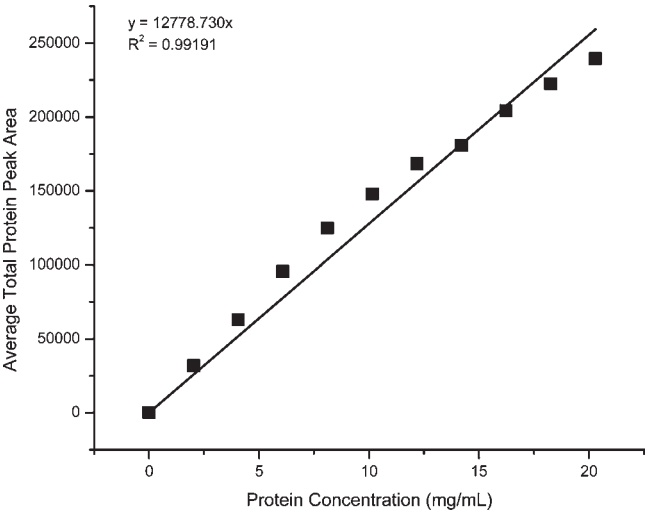

Figure 8. Correlation between the peak area and protein concentration for purified kafirins.

extracted from undigested flour and comparing to the amount of protein left after digestion using SDS−PAGE. Because of the ability to analyze samples via HPLC unattended and with automated data collection, we assessed the ability to use HPLC peak areas in a similar fashion to predict protein digestibility of sorghum. Protein digestibility values as determined from RP-HPLC peak areas were highly correlated (r^2 = 0.82) to those determined using nitrogen combustion (**Figure 10**). Thus, RP-HPLC could be used to measure digestibility in a similar fashion

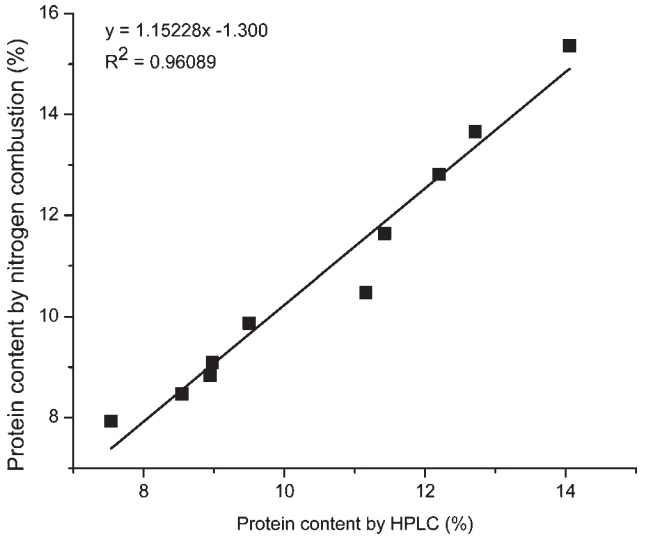

Figure 9. Correlation between the RP-HPLC peak area and total protein for 10 sorghum samples.

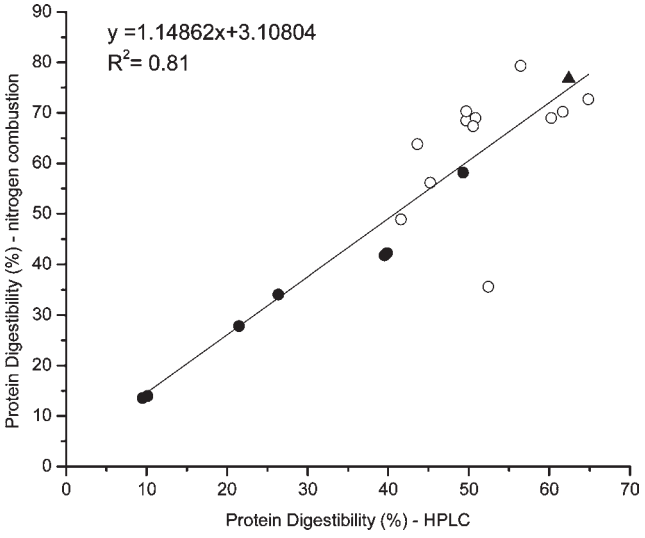

Figure 10. Correlation between *in vitro* protein digestibility as measured by nitrogen combustion and from RP-HPLC peak areas: (▲) corn sample used as a reference, (●) tannin-containing sorghum lines, and (○) non-tannin sorghum lines.

as SDS−PAGE (*33*) while at the same time providing information on protein composition of the samples being investigated.

Sorghum proteins were separated with high resolution using surface porous and monolithic RP-HPLC columns. Separation times were approximately 17 min using a surface porous C18 column, while previous separations using fully porous C18 columns were nearly 40 min. Separations using alkaline stable surface porous C18 stationary phases were also demonstrated. Peak areas from the separations were shown to be reliable to predict total protein and protein digestibility as well. Thus, the surface porous columns could be used to provide data on kafirin composition while simultaneously providing additional information on protein content and protein digestibility.

ABBREVIATIONS USED

4-VP, 4-vinylpyridine; β-ME, β-mercaptoethanol; HPCE, high-performance capillary electrophoresis; IEF, isoelectric focusing; M_w, molecular weight; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT

The authors thank Mr. Kevin Fay for providing nitrogen combustion analysis.

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Received for review September 17, 2010. Revised manuscript received November 22, 2010. Accepted November 23, 2010. Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and use of the name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.