Spectroscopic Analysis of Wheat Fractions and Reconstituted Whole Wheat Mixtures by ^{1}H-NMR and NIR

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ABSTRACT

Commercial success of whole wheat products has warranted development of new analytical approaches for differentiating whole grain products from conventional food products made from refined grains. Here, we have analyzed three different wheat fractions (namely, bran, germ, and refined flour) of two wheat varieties. In addition, a whole wheat sample containing all three fractions was also included in the study to investigate the application of two spectral fingerprinting methods—proton nuclear magnetic resonance (^{1}H-NMR) and near-infrared (NIR) spectroscopy—for differentiating the three fractions and the whole wheat. Results show that both these methods provide unique spectral fingerprints for the bran, germ, refined, and whole wheat flours. In addition, we were able to distinguish whole grain composed of different ratios of the germ, bran, and refined grain, exemplifying the potential applicability of both fingerprinting methods (NIR and NMR) for the differentiation of whole and refined wheat samples. Principal component analysis on ^{1}H-NMR data with four different bin sizes (0.02, 0.04, 0.08, and 0.16 ppm) did not have significant influence on differentiation of the four fractions.

Whole grain foods, especially whole grain wheat, have been touted as easily available and commonly used health foods (Shahidi 2009). They have also been classified as functional foods, which according the definition of the Food and Drug Administration is any food that provides additional health benefits beyond nutrition. Whole grain foods are defined as cereal grains that consist of the intact, ground, cracked, or flaked caryopsis, whose principal anatomical components (the starchy endosperm, germ, and bran) are present in the same relative proportions as they exist in the intact caryopsis (FDA 2016). Previous studies have indicated that consumption of whole grain foods can significantly reduce the risk of some chronic health conditions such as type 2 diabetes, cardiovascular disease, and cancer (Slavin 2004). Initially, the beneficial health effect of whole grains was primarily attributed to its high fiber content. However, recent research indicates that the beneficial effect of whole grain may arise from the combined action of several components such as fiber, vitamins, phenolics, carotenoids, alkylresorcinols, and other phytochemicals (Trowell 1972; Slavin 2004; Piironen et al. 2009).

The major grains include wheat, rice, corn, oats, rye, barley, sorghum, triticale, millet, amaranth, and teff. In Asia, nearly half of the annually consumed grain is rice, whereas the major grain consumed in Europe and the United States is wheat. The bioactive phytochemicals in wheat can be broadly subdivided into the following categories: phenolic acids, carotenoids, tocopherols, alkylresorcinols, fibers, and other miscellaneous compounds (sterols, steryl ferulates, benzoxazinoids, and lignans) (Slavin 2004; Piironen et al. 2009; Landberg et al. 2014). The wheat seed or kernel contains three edible parts: the bran, the germ, and the endosperm. These inner parts are protected by the outer inedible husk from sunlight, pests, water, and disease. The bran is the multilayered edible skin, rich in important antioxidants, B-vitamins, and fiber. The germ is the reproductive part of the plant. It contains many B-vitamins, some minerals, and healthy fats. The endosperm is the germ's food supply, which provides essential energy to the young plant. The endosperm is by far the largest portion of the kernel. It contains starchy carbohydrates, proteins, and small amounts of vitamins and minerals.

We have used three fractions (bran, germ, and refined wheat) and whole grain flour of two wheat varieties to serve as a model system to investigate whether two common spectral fingerprinting methods—near-infrared (NIR) spectroscopy and nuclear magnetic resonance (NMR)—can be used to differentiate samples by applying multivariate statistics analyses. NIR offers a rapid and nondestructive method of analyzing food samples and has been successfully used in the study of grains in several ways (Baker 1983; Williams and Norris 1983; Cattaneo and Holroyd 2013). The method has become one of the mainstays in the food industry and has been employed to study the moisture, protein, and oil contents of foods, milk, and milk products (Holroyd 2013); determination of amino acid content in germinated brown rice (Kaewsorn and Sirisomboon 2014); the swelling process in microcrystalline starch (Hattori and Otsuka 2014); green coffee biochemical phenotyping (Scholz et al. 2014); to detect adulteration of melamine in soybean products (Haughey et al. 2013); and to study antioxidant activity in mint (Dong et al. 2014).

NIR offers a unique possibility of studying both the physical characteristics of food and its chemical constituents at the same time. The method requires no additional chemical modifications of food, unlike other methods that may require lengthy extraction and derivatization steps. Different laboratories around the world have used infrared and NIR wavelengths in the study of microstructure and composition of foods. NIR, although a readily available technique, suffers from broad absorption bands having little structural information that can be readily inferred. However, the combination of NIR data with chemometrics multivariate statistical analysis offers a useful approach to extract valuable information about macro components such as protein, fat, fiber, and moisture contents (Munck et al. 2010).

NMR spectroscopy is another method that has been increasingly adapted for rapid identification of food composition over the last decade, because of advancements in electronics, instrumentation, and computing power (Mannina et al. 2012). High-resolution one-dimensional proton NMR (^{1}H-NMR) spectroscopy is a reproducible technique to provide structural information and quantitative data. NMR is a useful technique for the identification and quantification of plant metabolites either in plant extracts or in vivo (Consonni et al. 2011; Canlet et al. 2012). NMR fingerprinting and metabolomics approaches have been used for the quality assessment, authenticity, and classification of olive oils (Piccinonna et al. 2016). Recently, Dais and Hatzakis (2013) demonstrated the application of olive oil ^{1}H-NMR fingerprinting for cultivar classification.

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In this article, we have investigated the application of ^{1}H-NMR and NIR spectroscopy in conjunction with principal component analysis (PCA) for the differentiation of three different wheat fractions (bran, germ, and refined flour) in addition to the whole wheat flour from two wheat varieties. Furthermore, we have applied the same spectral fingerprinting approach to classify reconstituted whole grain composed with different proportions of bran, germ, and refined wheat and investigated the effect of ^{1}H-NMR spectral data binning. Finally, we have compared the two methods (^{1}H-NMR and NIR) with respect to their ability to discriminate between the wheat fractions.

MATERIALS AND METHODS

Sample Preparation. Authentic whole wheat flour samples and their respective three fractions (refined wheat flour, bran, and germ) from the two wheat cultivars, white hard wheat and red hard wheat, were provided by Reuben McLean of Pendleton Flour Mills in Blackfoot, Idaho. Bran and the germ fractions were ground in a coffee grinder before reconstituting the different whole grain mixtures containing various proportions (95:4:1, 90:8:2, and 85:12:3 [w/w/w]) of refined wheat flour, bran, and germ samples, respectively. In addition, only samples ground in the coffee grinder were used for extraction and analysis by NMR.

Extraction of Samples for ^{1}H-NMR Analysis. Each 500 mg sample (bran, germ, whole, refined wheat flour, and different mixtures) from two wheat varieties was individually extracted with two solvents, chloroform or methanol (5 mL), by using ultrasonic-assisted extraction (Advanced Sonic Processing Systems, Oxford, CT, U.S.A.) at 300 W, followed by centrifugation at 4,000 rpm for 10 min. The solvent was transferred to another tube, the residue resuspended in 5 mL of fresh solvent (chloroform or methanol), and the process repeated. The pooled extracts from the two extractions were passed through a 0.45 μm polyvinylidene diflouride syringe filter (VWR Scientific, Seattle, WA, U.S.A.) to remove any suspended insoluble material. The filtered extract was evaporated under nitrogen, and the residue and the chloroform extract were resuspended in 0.75 mL of $CDCl_3$ for NMR analysis. Similarly, the methanol extract was resuspended in CD_3OD (Cambridge Isotopes Labs, Cambridge, MA, U.S.A.) for NMR analysis. All extractions and analyses were carried out in four replicates.

NIR Spectroscopy. Different wheat fractions (5 g each) were taken in thin-walled glass vials (VWR Scientific), and NIR spectra were collected in absorbance mode averaging over 128 scans, in the wavelength region 4,000–10,000 cm^{-1} at ambient temperature, with a Nicolet 6700 spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.). The resolution factor was set at 4, and the spacing between data points was 1.928 cm^{-1}. The source white light was split with a potassium bromide beam splitter, and the gain was set to auto. The inbuilt OMNIC software (Thermo Scientific) was used for the collection and processing of data, with automatic background correction using a background spectrum collected with 64 scans. No zero filling was applied, phase correction was done in Mertz mode, and apodization was employed with the Happ-Genzel algorithm. Spectra were analyzed with Unscrambler X software (Camo Software AS, Oslo, Norway).

NMR Spectroscopy. Spectra were obtained with 32,000 time points in the direct dimension, with a sweep width of 14 ppm in the ^{1}H dimension. In total, 64 scans were measured with an acquisition time of approximately 1 s and a time delay of 2.5 s between the scans. Data were acquired with a 600 MHz Agilent spectrometer and processed with linear prediction by using VNMRJ software (Agilent, Santa Clara, CA, U.S.A.). Processed data were later analyzed with Mestrenova 10.0.1 software (Mestrelab Research, Santiago de Compostela, Spain). Phase correction and baseline correction (full splines) were carried out with an automated algorithm with Mestrenova software (Lourenço et al. 2012). All spectra were referenced with the standard reference chemical shift of 3.31 ppm for CD_3OD and 7.26 ppm for $CDCl_3$. The spectra were subjected to global spectral deconvolution. The peak tables were binned into different buckets of 0.020, 0.040, 0.080, and 0.160 ppm intervals.

RESULTS AND DISCUSSION

Prior to designing the experiments, we considered using phenolic acids (Boz 2015) as reference phytochemicals. Ferulic acid is the most predominant phenolic acid identified in wheat (Yu et al. 2014). In the case of cereals, phenolic acids are localized in the bran, the outer layer of grain. In wheat grains, it has been estimated that approximately 60–200 mg of ferulic acid is found in 1 kg (dry weight) of grain. Ferulic acid is present in three different forms in wheat grains: namely, the soluble free and conjugated forms and the insoluble bound form, which is found in the structural components of the plant (Zhao and Moghadasian 2008; Yu et al. 2014). Most of the ferulic acid in bran is present in the insoluble bound form, and only less than 10% is in soluble form. It was also reported in recent literature that the ferulic acid concentration in whole wheat grain is 10-fold greater than the refined grain, which is primarily composed of endosperm (Manach et al. 2004). Thus, in the present study, ferulic and other phenolic acids were considered to be chemical markers for the fingerprinting differentiation of various grain fractions and mixtures. Because commercial whole wheat products are commonly formed by mixing different proportions of endosperm, bran, and germ, a spectroscopic investigation of the food samples would allow differentiation of fractions. The ^{1}H-NMR of ferulic acid shows five aromatic protons and one methoxyl, hydroxyl, and carboxyl group. In the present study, we focused on the aromatic region (5–8 ppm) and the methoxyl (3–4 ppm) resonances for differentiation of the three fractions, whole wheat, and the reconstituted mixtures.

NMR samples were divided into two sets for the sake of comparison. One set of samples included bran, germ, whole wheat, and refined flour samples, in replicates of four, and the other set of samples included refined flour–bran–germ mixtures in proportions of 95:4:1, 90:8:2, and 85:12:3 (w/w/w) along with refined and whole wheat flours, also in four replicates each.

^{1}H-NMR of Red and White Hard Wheat. The ^{1}H-NMR spectra for three fractions and whole wheat from red hard wheat samples in two commonly used deuterated solvents ($CDCl_3$ and CD_3OD) are shown in Figure 1. Sample extractions were separately performed with chloroform (relative polarity 0.259) and methanol (relative polarity 0.791) to investigate variations in the extraction of phenolic acids and other phytochemicals in two solvents (https://sites.google.com/site/miller00828/in/solvent-polarity-table). PCA was performed on the ^{1}H-NMR data. Samples dissolved in deuterated chloroform and methanol were analyzed separately. The NMR data from each category (chloroform and methanol) were divided into three sets: 0–9 ppm (almost entire region), 5–8 ppm (aromatic region, excluding the $CDCl_3$ resonance at 7.26 ppm), and 3–4 ppm (methoxyl and sugar proton resonances, excluding the CD_3OD signal at 3.31 ppm). We intended to utilize the prominence of phenolic acids, sugars, and methoxyl resonances in samples as factors for differentiation based on our earlier published results on refined and whole grain LC-MS analysis (Geng et al. 2015).

PCA of ^{1}H-NMR data was compared between red hard wheat fractions (Fig. 2A, C, and E) and white hard wheat fractions (Fig. 2B, D, and F) in $CDCl_3$. The three grain fractions (germ, bran, and refined flour) along with whole grain flour of both wheat varieties distinguished well during the PCA of the ^{1}H-NMR spectral data (entire region, 0–9 ppm; Fig. 2E and F). However, when the data from the aromatic region (5–8 ppm; Fig. 2A and B) or the methoxyl and sugar proton region (3–4 ppm; Fig. 2C and D) were subjected to similar PCA analysis, the clustering in the case of fractions (germ, bran, refined, and whole wheat flours) was not as clear as obtained with the entire ^{1}H-NMR spectral data (0–9 ppm). This indicates that chloroform as a solvent could not extract all phenolic acids well enough and, therefore, did not resolve the differences in the three fractions and the whole grain. Limited or poor solubility of phenolic acids in chloroform and other solvents with low relative polarity has been documented in the previous literature (Mukhopadhyay et al. 2006; Stalikas 2007).

PCA of ^{1}H-NMR data of the three grain fractions (germ, bran, and refined flour) along with whole grain flour in deuterated methanol showed better differentiation with the entire region (0–9 ppm; Fig. 3E and F), whereas sugar and methoxyl proton resonances (3–4 ppm; Fig. 3C and D) and the aromatic region (5–8 ppm; Fig. 3A and B) were quite similar in both red and white hard wheat varieties. This can be attributed to greater solubility of phenolic acids in methanol.

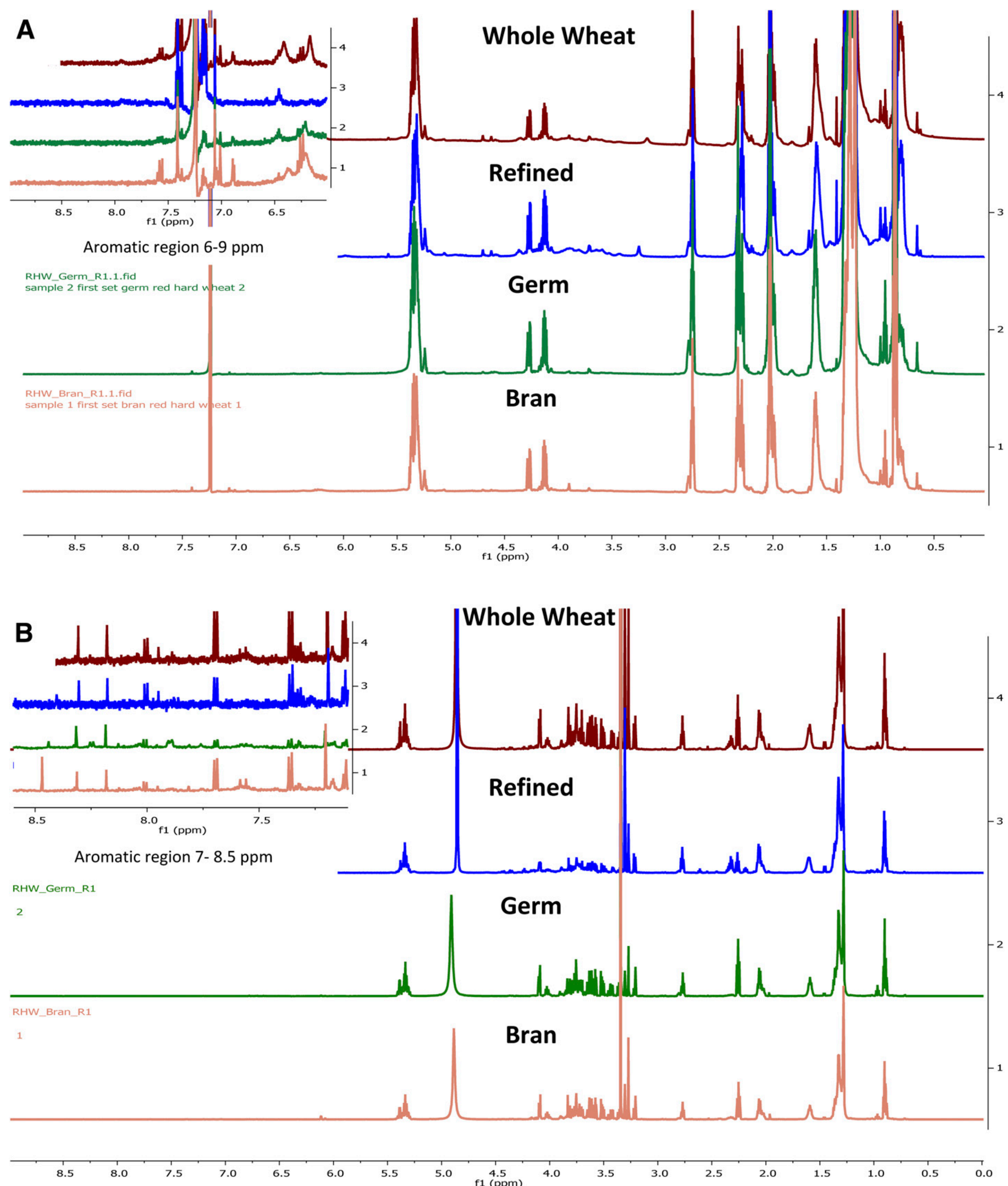

Fig. 1. One-dimensional proton nuclear magnetic resonance data of four fractions (whole wheat, refined, germ, and bran) in $CDCl_3$ (**A**) and deuterated methanol (**B**). The insert shows the magnification of resonances of 5–8 ppm.

From the above results, one can positively illustrate that total (0–9 ppm), aromatic (5–8 ppm), and methoxyl and sugar protons (3–4 ppm) contributed toward the differentiation of three fractions (germ, bran, and refined wheat) and whole wheat flours. Phenolic acids are the predominant phenolic phytochemicals present in wheat (Lu et al. 2015). Bran contains the highest amount of total phenolic acids, with ferulic acid being dominant. The content of total phenolic acids in whole wheat flour is approximately 10 times higher compared with refined wheat flour (Lu et al. 2015). This distinct differentiation between four samples (germ, bran, refined, and whole wheat flours) in CD_3OD may be attributed to the better solubility of the phenolic acids in deuterated methanol compared with chloroform. These results confirm previous findings that differentiated three grain fractions and whole wheat with fuzzy chromatography

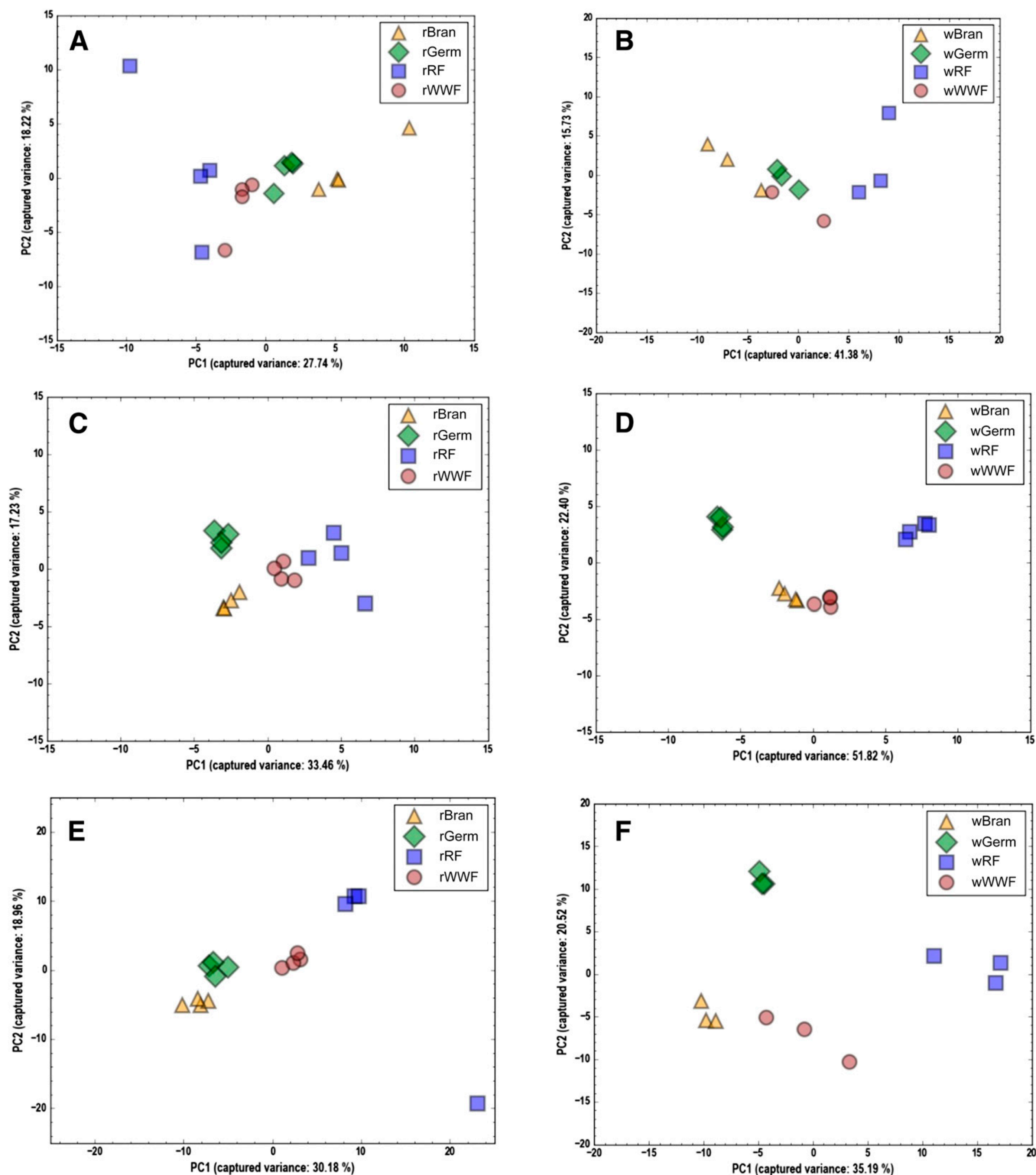

Fig. 2. Principal component (PC) analysis of the data from one-dimensional proton nuclear magnetic resonance data in $CDCl_3$: **A,** red hard wheat fractions (5–8 ppm); **B,** white hard wheat fractions (5–8 ppm); **C,** red hard wheat fractions (3–4 ppm); **D,** white hard wheat fractions (3–4 ppm); **E,** red hard wheat fractions (0–9 ppm); and **F,** white hard wheat fractions (0–9 ppm). RF = refined flour, and WWF = whole wheat flour.

mass spectrometry, which provides crude separation of mixtures on a short column into different categories (namely, saccharides, organic acids, flavonoids, lipids, and other classes) (Geng et al. 2015).

Data (0–9 ppm) from the three whole grain samples made with different proportions of refined wheat flour, bran, and germ samples (95:4:1, 90:8:2, and 85:12:3 [w/w/w]) along with the whole and refined wheat samples from both white and red hard wheat varieties are presented in Figure 4. The differentiation of refined and whole wheat flours was distinct. However, the three whole grain mixtures with different proportions of refined wheat flour, bran, and germ samples did not cluster as distinctly compared with the refined and whole wheat flours in the ^{1}H-NMR spectral data set of 0–9 ppm. The mixture containing a higher proportion of bran and germ (85:12:3 [w/w/w]) showed closer

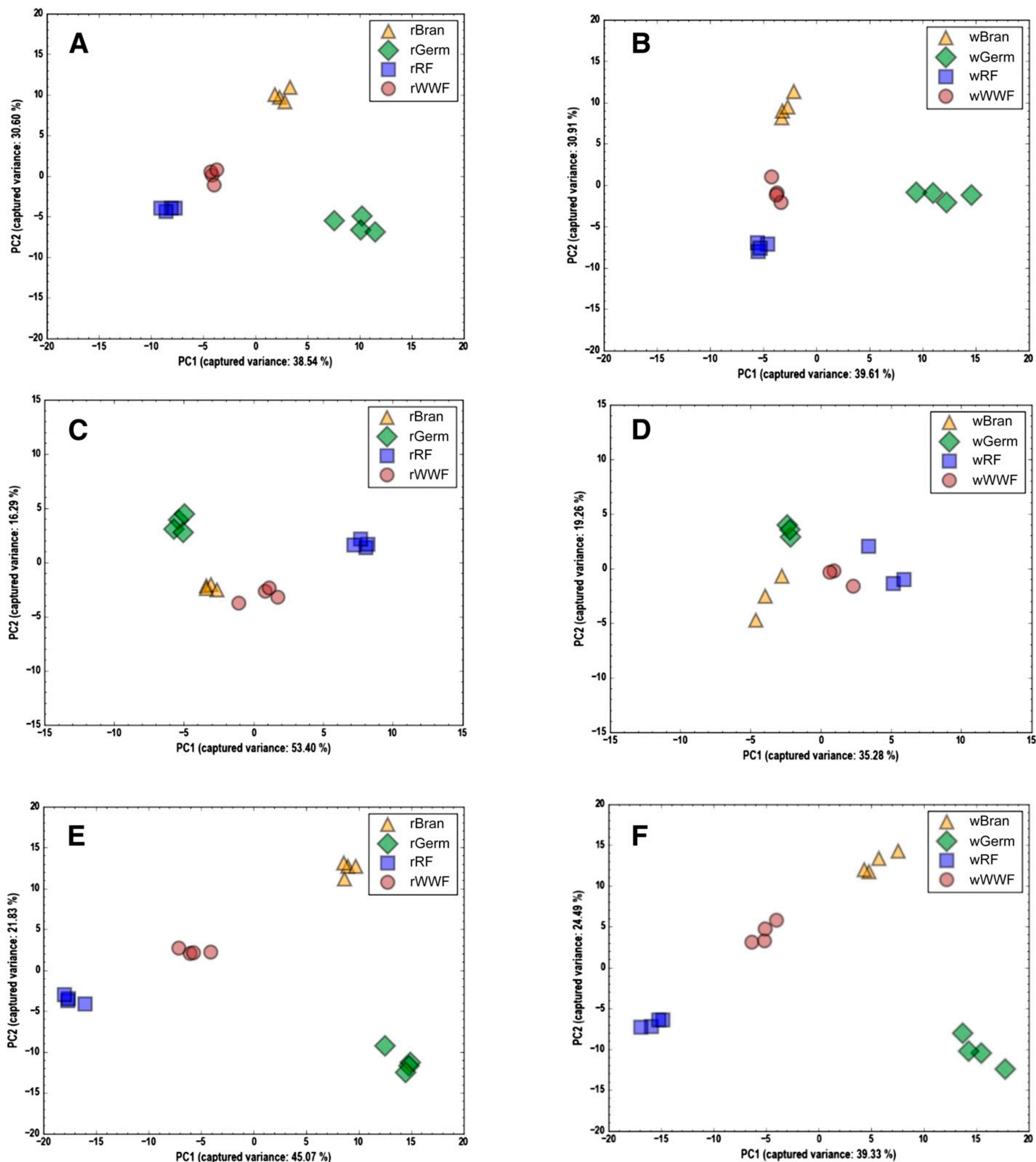

Fig. 3. Principal component (PC) analysis of the data from one-dimensional proton nuclear magnetic resonance data in CD_3OD: **A,** red hard wheat fractions (5–8 ppm); **B,** white hard wheat fractions (5–8 ppm); **C,** red hard wheat fractions (3–4 ppm); **D,** white hard wheat fractions (3–4 ppm); **E,** red hard wheat fractions (0–9 ppm); and **F,** white hard wheat fractions (0–9 ppm). RF = refined flour, and WWF = whole wheat flour.

proximity to whole grain, and the mixture containing a higher proportion of endosperm (95:4:1 [w/w/w]) showed closer proximity to the refined grain.

Effect of Binning of ¹H-NMR Spectral Data on Differentiation of Three Fractions and Whole Wheat. ¹H-NMR spectra are sensitive to variations in temperature, pH, ionic strength, viscosity of the solvent, and other factors that change the average electronic environment of the detected proton spins. This leads to small variations in the chemical shifts. In other words, the peak positions are not always uniform between spectra. Therefore, when data sets collected from multiple samples with several replicates are subjected to multivariate analysis, reproducibility of identical chemical shifts can be a problem. This problem severely hampers the process of multivariate statistical methods such as PCA.

This problem potentially can be resolved with uniform binning of spectral data with equal intervals of chemical shifts, so that the area of peaks that fall within a predefined chemical shift bucket is added up after integration. This produces a smaller set of variables, which is desirable, in addition to ensuring a uniform number of variables across all the spectra, thereby aiding multivariate analysis.

We investigated the effect of bin size in PCA of ¹H-NMR spectra. In the case of liquid-state NMR, it is well known that the magnetic

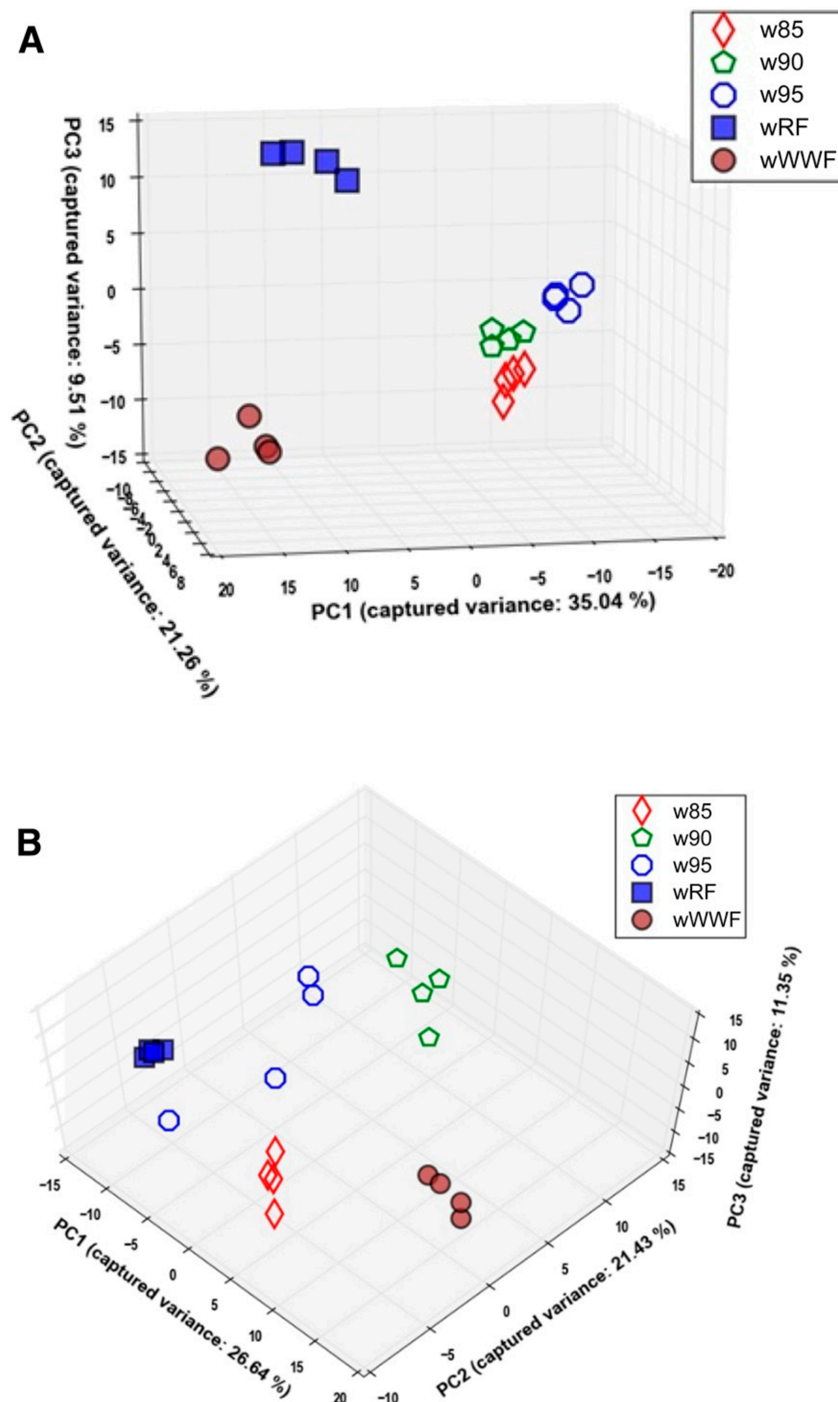

Fig. 4. Principal component (PC) analysis of the data from one-dimensional proton nuclear magnetic resonance spectra (0–9 ppm) of refined flour (RF) and whole wheat flour (WWF) along with three mixtures reconstituted with different proportions of germ, bran, and refined wheat fractions (1:4:95, 2:8:90, 3:12:85) in deuterated methanol: **A,** red hard wheat; and **B,** white hard wheat.

field strength is proportional to the resolution. Increasing the bin size is equivalent to lowering the resolution. Therefore, increasing the bin size mimics the resolution decrease seen in NMR at lower fields. That is to say, the larger one sets the bin size, the smaller is the spectral resemblance toward the lower field, and vice versa. There are other effects of a lower field that are not reproduced by increased bin size such as the scalar coupling and relaxation effects, but these are not considered here. For purposes of chemical fingerprinting, it may be possible that spectra measured at lower fields are sufficient. We wanted to test this assumption and, therefore, performed PCA analysis on data with four different bin sizes: 0.02, 0.04, 0.08, and 0.16 ppm chemical shift bins. It is also important to note that a chemical shift tolerance of 0.02–0.04 ppm for a proton spectrum is accepted as a limit, as far as its identification is concerned. This leads to the fact that, as the bin size exceeds the tolerance limit for a proton, any possible information for its identification is diminished. The PCA results for these four different bins showed very similar results (Fig. 5), and principal components 1 and 2 captured similar corresponding variances; at least qualitatively, the bin size did not affect the differentiation efficiency.

NIR of Red and White Hard Wheat. With solution-state NMR samples, only a part of the phytochemicals soluble in methanol contributed to the separation of different samples. Because there are many compounds with varying polarity present in free and insoluble bound states in wheat, we used NIR spectroscopy to study the same set of samples used in NMR experiments. The results from the NIR wavelength experiments, in the case of red and white hard wheat fractions, were similar to those from ^{1}H-NMR experiments, in which the three grain fractions (germ, bran, and refined flour) along with whole grain flour of both the wheat varieties differentiated well during the PCA (Fig. 6A and B). In the case of reconstituted mixtures (Fig. 6C and D), the whole and the refined grains were well distinguished and the mixture containing a higher proportion of bran and germ (85:12:3 [w/w/w]) showed closer proximity to whole grain and the mixture containing a higher proportion of endosperm (95:4:1 [w/w/w]) showed closer proximity to the refined grain. However, clustering of different fractions was not as clear compared with the ^{1}H-NMR experiments. This may be owing to the heterogeneous nature of reconstituted mixtures.

CONCLUSIONS

In this article, we have established the feasibility of using NIR and ^{1}H-NMR, two completely independent spectroscopy methods,

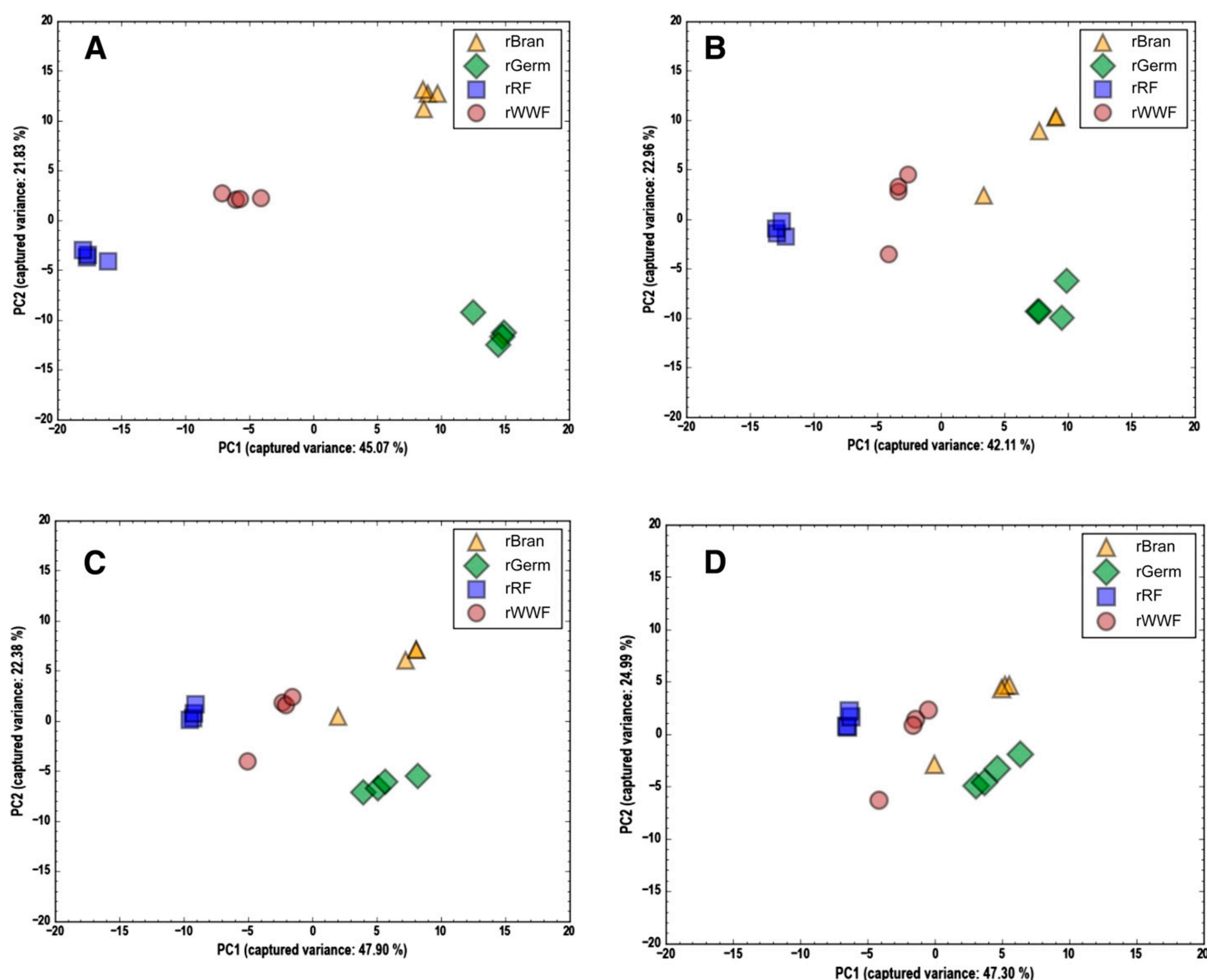

Fig. 5. Principal component (PC) analysis of one-dimensional proton nuclear magnetic resonance spectral data of red hard wheat samples (bran, germ, refined flour [RF], and whole wheat flour [WWF]) extracted in deuterated methanol. Spectral data were processed using Mestrenova software with four different bin sizes: **A,** 0.02 ppm; **B,** 0.04 ppm; **C,** 0.08 ppm; and **D,** 0.16 ppm.

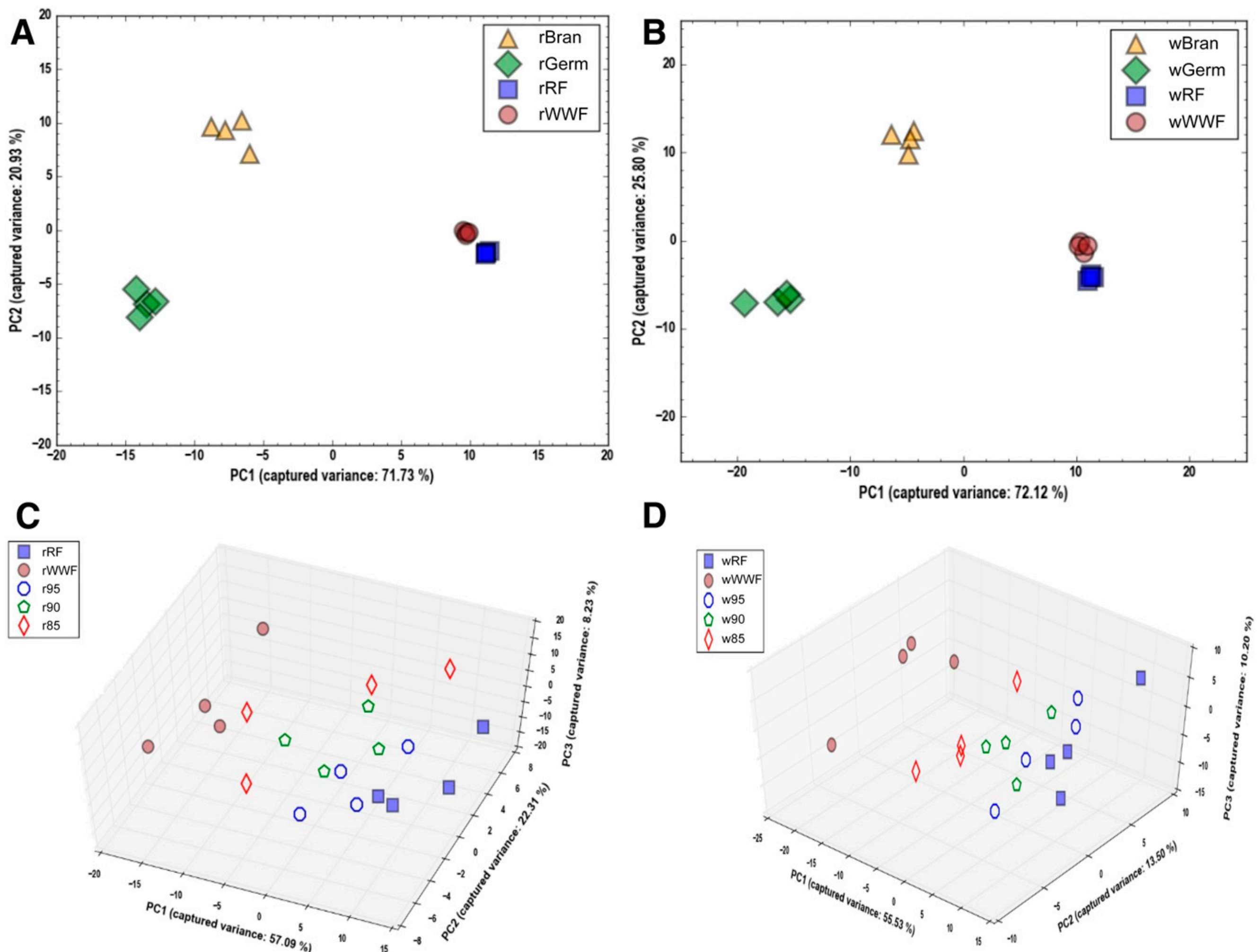

Fig. 6. Principal component (PC) analysis of the data from near-infrared spectra (after band selection): **A,** three red hard wheat fractions (bran, germ, and refined flour [RF]) and whole wheat flour (WWF); **B,** white hard wheat fractions (wheat bran, germ, and RF) and WWF; **C,** three red hard wheat reconstituted mixtures, RF, and WWF; and **D,** three white hard wheat reconstituted mixtures, RF, and WWF.

for distinguishing three grain fractions (germ, bran, and refined flour) along with whole grain flour of both wheat varieties. In addition, we have shown some distinction of whole grain mixtures reconstituted with different proportions of bran, germ, and endosperm. In solution-state NMR, the extracts are homogeneous but lack information from the nonsoluble part of the samples compared with heterogeneous issues associated with NIR caused by reconstitution of whole grain samples made by mixing three grain fractions (germ, bran, and refined flour) in different proportions. Both NIR and NMR techniques in conjunction with multivariate data analysis show promising potential for the classification of refined and whole grain samples.

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