13C ss-NMR Singular value decomposition and fitting for sorghum proteins conformation elucidation

Tatiana Santana Ribeiro1*, Juliana Aparecida Scramin2, José Avelino Santos Rodrigues3, Rubens Bernardes Filho4, Luiz Alberto Colnago4 and Lucimara Aparecida Forato4

1Departamento de Ciências da Natureza, Matemática e Educação, Universidade Federal de São Carlos – UFSCar, Araras, SP, Brasil
2Programa de Pós-graduação Inter Unidades em Biotecnologia, Universidade Federal de São Carlos – UFSCar, São Carlos, SP, Brasil
3Embrapa Milho e Sorgo, Sete Lagoas, MG, Brasil
4Embrapa Instrumentação, São Carlos, SP, Brasil
*tatianaribeiro@ufscar.br

Abstract

Kafirins, water-insoluble proteins from Sweet Sorghum BR501 grains, have been an alternative to prepare edible coatings for food due to their hydrophobic character. In this work, the secondary structures (SS) content of reduced (SSr) and unreduced (SSu) kafirins were determined by 13C solid-state-NMR spectroscopy using areas of carbonyl peak. The SS elucidate by fitting the signal with the Lorentzian function shows 56% and 59% of α-helix and 40% and 12% of β-sheet structures for SSr and SSu, respectively. Since SVD does not depend on the operator and has higher correlation coefficients for α-helix (0.96%) and β-sheet (0.91%), it is a reliable method to quantify the SS of insoluble proteins using the 13C NMR signal.

Keywords: kafirin, secondary structures proteins, sweet sorghum, insoluble protein, solid-state 13C NMR spectroscopy.

How to cite: Ribeiro, T. S., Scramin, J. A., Rodrigues, J. A. S., Bernardes Filho, R., Colnago, L. A., & Forato, L. A. (2022). 13C ss-NMR Singular value decomposition and fitting for sorghum proteins conformation elucidation. Polímeros: Ciência e Tecnologia, 32(1), e2022009. https://doi.org/10.1590/0104-1428.20210082

1. Introduction

The sweet sorghum has a sucrose-rich stem (like sugar cane). It can be used for ethanol production with advantages such as fast-growing and wide adaptability to different environments1-3. The grain is the by-product when sweet sorghum is used4-6. Although the grains can be used to feed livestock, it is preferable to find more technological applications that could increase the value of this by-product. Some food industries already use zeins, proteins extracted from the endosperm of corn kernels, to produce edible films and coatings. Kafirins are proteins extracted from sweet sorghum and can also be a good strategy for making these films and/or other technological applications6-7.

Kafirins are a group of proteins that respond for 68% to 73% of the total protein contents of the sorghum grain and between 77% and 82% of the endosperm. Kafirins are classified according to their solubility and kDa in dodecyl-sulfate polyacrylamide gel (SDS/PAGE). The electrophoresis analysis shows four kafirin bands known as α-, γ-, β- and δ-. The most abundant ones are the α-kafirins with values between 23 and 25 kDa, representing 66% to 84% of the total kafirins. The α-kafirins, in the same way as α-zeins, are insoluble in water and soluble in aqueous alcohol solutions of 70% ethanol (w/w). γ-kafirins are soluble in water after disulfide bonds reduction and present the monomer and dimer bands at 28 kDa and 49 kDa, respectively. The γ-kafirins represent 9% to 21% of the total kafirins. The β-kafirins represent 7% to 13% of the total kafirins, and there is some controversy about their kDa values. Some authors assigned the bands at 15, 17, and 18 kDa to these fractions, and others declare that it is only one band from 18 kDa to 19 kDa. β-kafirins are soluble in aqueous alcohol solutions after disulfide bonds reduction process8. The δ-kafirins possibly comprise less than 1% of the protein content of sorghum grain when in its mature stage8,9.

Some authors studied the kafirins’ conformation, vital information to understand their secondary structure. Gao et al.10 suggest that the secondary structure of these proteins is quite relevant to obtaining a good film formation. Wu et al.11 analyzed kafirins in 60% t-butanol solution by optical rotatory dispersion and circular dichroism (CD). The authors extracted kafirins from grain sorghum hybrids cultivars OK612, RS626, TE77, and Funk G-766, and they found 40% of α-helix content. Duodu et al.12 analyzed the sorghum protein bodies (PB) by ATR-FTIR and concluded that the kafirins in the PB have 54%, 55%, 58%, and 59% of α-helix for condensed-tannin-free sorghum P851171, P850029,
KAT 369, and NK 283 mutants respectively. Gao et al. analyzed kafirins from a mixture of two tannin-free cultivars (PANNAR 202 and 606) extracted with different solvents and drying methods using ATR-FTIR. They observed that kafirins extracted with 60% t-butyl alcohol + 0.5% DTT and freeze-dried had FTIR peak intensity ratio of helix/intermolecular β-sheet (1650 cm⁻¹/1620 cm⁻¹) of 1.39. Using 70% ethanol, 0.5% sodium metabisulfite, and 0.35% sodium hydroxide, they noticed a 1.10 ratio, and then using 70% ethanol plus 0.5% sodium metabisulfite as the solvent, they observed a 1.00 ratio. However, during all preparations, the ratio was 0.90 when samples were heated and dried at 40 °C. They concluded that the best conditions for film formation occurred when there were more α-helix contents since the intermolecular β-sheet can induce protein aggregation.

Wang et al. extracted kafirins from distillers dried grains with soluble (DDGS) using three different extraction procedures based on HCl/ethanol, acetic acid, and sodium hydroxide-ethanol. In all preparations, using the FTIR technique with samples prepared as KBr pellets, it was noticed that the α-helix is the predominant SS with a small portion of β-sheet.

Xiao et al. extracted kafirins using 60% t-butanol and ultrasoundation, and their SS were evaluated by ATR-FTIR of kafirin powder. The authors used high-resolution methods to calculate the amount of SS as second derivative and Gaussian fitting and found 49% of α-helix, 24% of β-sheet, and 27% of β-turns. They also analyzed the SS kafirins in solution by Circular Dichroism (CD). They found 68.4% of α-helix for kafirins dissolved in 85% ethanol: 57.9% in 60% t-butanol and 53.1% in 65% isopropanol. The authors concluded that these differences are due to different polarities of the solvents and that the lower polarity is related to the higher helical content. Dianda et al. prepared kafirins from pre-heated sorghum flour (70°C) using 70% ethanol at 68°C added by sodium metabisulfite and acetic acid in a water bath (70°C) as described by Olivera et al. By processing ATR-FTIR with Gaussian fitting of kafirin powder, Dianda et al. found 43.88%, 23.53%, and 19.30% for α-helix, β-sheet, and β-turns, respectively. They also evaluated the SS kafirins in ethanol at 70%, 80%, 85% and 90% and at 50%, 60%, 70% and 80% t-butanol using CD. In ethanol solutions, α-helix proportion varied from 40.47 to 47.12% and β-sheet from 8.76 to 15.12%. In t-butanol solutions, α-helix proportion varied from 26.89 to 41.72% and β-sheet from 16.54 to 11.626%. Compared to Xiao et al., these authors concluded that the differences are due to the different calculation methods for the solvent polarities. Dianda et al. also predicted a theoretical value of about 66% of α-helix based on amino acid sequences analysis of the kafirins.

High-resolution solid-state 13C NMR studies using cross-polarization magic angle spinning (CPMAS) sequence have been developed to determine insoluble proteins. It uses 13C chemical shift of carbonyl (C=O) peaks to determine α-helix and β-sheet conformation, usually observed between 172 ppm and 176 ppm, respectively. However, 13C solid-state NMR shows problems as the broad linewidth leads to low resolution, overlapped peaks, and inaccurate determination of secondary structures. The fitting methods have been used for analyzing them, though it depends on operators such as the number of peaks, specific line shape, and other parameters that are set by the operator.

In this research, SS kafirins were quantified using a pattern recognition method based on singular value decomposition (SVD) applied in areas of carbonyl peaks by solid-state 13C NMR spectra. The Lorentz fitting method was also used for comparison. The SVD method does not depend on analyst information, such as function fitting and peak numbers and positions, leading to 0.96% and 0.90% correlation coefficient for α-helix and β-sheet, respectively.

2. Materials and Methods

2.1 Protein extraction

Kafirins extraction was adapted according to the literature using the Sweet Sorghum grains (BR501-white) provided by Embrapa Maize and Sorghum, Minas Gerais, Brazil. Reduced protein fractions were extracted: Sorghum grains were ground and defatted with hexane in a Soxhlet apparatus for 24-hour. Defatted flour was mixed with NaCl 1.25 mol/L solution and maintained by agitation for 3 hours to solubilize the albumins and globulins. After vacuum filtration, a 100 mmol/L sodium bisulfite aqueous solution was added to the flour for 2 hours, and then the residue was added to 70% ethanol (w/w) for 24 hours under agitation (at room temperature). The solvent was evaporated, and the obtained kafirins were lyophilized. The unincorporated fractions were extracted with this mentioned methodology without only the step of adding sodium bisulfite.

2.2 SDS/PAGE

The SDS/PAGE was a 15% polyacrylamide gel, and the gel was stained with Coomassie Blue dye. The standard molecular weight employed was Benchmark Protein Ladder Cat. Nº. 10747-012 from Invitrogen.

2.3 13C solid-state Nuclear Magnetic Resonance (13C ss-NMR)

The solid-state 13C NMR spectra were obtained with a Bruker Advance III HD 400 MHz spectrometer equipped with a solid-state MAS probe, with two channels configured for H and 13C frequencies of 400 MHz and 100.5 MHz, respectively. 13C cross-polarization magic angle spinning (CP/MAS) was the sequence used for the analysis. The operational conditions were: 90° H pulse length of 2.3 us, contact time of 2 ms, a spectral width of 50 kHz, and a recycle delay of 3 s and 4096 scans. Spectra were filtered by an exponentially decaying function with 20 Hz of line broadening. Samples were packed in a zirconia rotor of 5 mm and rotated at the magic angle at 10 kHz. External hexamethyldibenzene standard chemical shift (at 17.3 ppm) was used as reference.

The carbonyl peak area in the 13C NMR spectra for reduced and unreduced kafirins has been used to calculate the SS with CP/MAS 13C NMR pulse sequence. The region from 180 to 160 ppm was obtained with a total of 859 data points from each ss-13C NMR spectrum, and its second derivative was used to find the three individual peaks: at 172 ppm that were assigned to β-sheet, 174 ppm to unordered ones, and 176 ppm to the α-helix structure.
These three peaks were used in the Lorentzian multiple peak fitting, and the area corresponded to each SS proportion as already indicated. The Lorentzian multiple peaks fitting Adjusted R-Square was 0.994 and 0.992 for unreduced and reduced kafirins, respectively, and both fittings reached the chi-square tolerance of $1 \times 10^{-9}$.

The calibration matrix to the SVD method was published elsewhere\cite{16}. Equation 1 shows the SVD method correlation with each NMR protein spectrum ($R$) and protein secondary structure concentration ($F$) by a calibration matrix ($X$). The $F$ and $R$ matrices with a medium square error are $1.5$ for α-helix and $1.8$ for β-sheet structures prediction were used by Andrade et al.\cite{16}. The $F$ matrix (15x4) consists of 15 proteins with four secondary structures proteins proportions obtained by X-ray crystallographic data\cite{17}. The $R$ matrix comprises the $^{13}$C NMR spectra (carbonyl area) of each protein in the F matrix.

$$F = XR$$

(1)

The SVD method reduces the rank of the $R$ matrix, making it consistent with the information, and calculates the generalized inverse as shown in Equation 2:

$$R = USV^T$$ and $R^{-1} = VS^{-1}U^T$

(2)

where: $S$: diagonal matrix with singular values on the diagonal and zeros out of the diagonal; $U$: is an orthogonal matrix of eigenvectors on the matrix rows; $V$: is an orthogonal matrix of coefficients.

The $X$ matrix can be calculated as shown in Equation 3:

$$X = F \left( VS^{-1}U^T \right)$$

(3)

It is possible to calculate the SS of a protein that was not determined (SS unknown protein) by its NMR spectrum ($N$) multiplied by the $X$ matrix as shown in Equation 4:

$$SS \text{ unknown} = XN$$

(4)

### 3. Results and Discussion

#### 3.1 Characterization by gel electrophoresis containing polyacrylamide sodium dodecyl sulfate (SDS/PAGE)

As described in the literature, kafirins are extracted with different solvents and reducing agents\cite{18,19}. We extracted the kafirins using the reduced and unreduced forms to compare the SS obtained in this work with that already published by other authors and therefore evaluate the SS calculation methods used here.

Reduced kafirin fraction showed four bands (Figure 1, second well) β-kafirin at 17 kDa and other three bands at 21, 25, and 26 kDa were assigned to α2-, α1- and γ-kafirins, respectively. This result is in good agreement with the description of Byaruhanga et al.\cite{20}. Unreduced kafirins fraction (Figure 1, first well) showed a more intense band at 17 kDa attributed to β-kafirin and a weak one at 25-26 kDa to the α→γ-kafirins agglomerate. El Nour et al.\cite{21} extracted kafirin with 60% t-butanol without reducing agent, and they also found the presence of the β-kafirin and weak bands for α2-, α1- and γ-kafirins. They concluded that in unreduced conditions, β-kafirin is extracted in the monomeric form.

#### 3.2 SS analysis using solid-state $^{13}C$ NMR spectroscopy

Figures 2A and 2B show the NMR spectra of unreduced and reduced kafirin fractions, respectively. The NMR spectra show typical protein signals: from 172 ppm to 176 ppm due to carbonyl peaks, from 140 ppm to 100 ppm due to amino acids with aromatic side chains; from 70 ppm to 45 ppm to α carbons, and from 45 ppm to 15 ppm to the aliphatic amino acid side chains\cite{22,23,24}. The signals at 73 ppm were assigned to starch in unreduced kafirin (2A), which are stronger than in the reduced kafirins (2B). Figure 2 spectra also showed an intense peak at 130 ppm. This peak has been assigned to unsaturated fatty acids, obtained using the single-pulse technique (Figure 3) used to detect the presence of mobile molecules\cite{25,26}. Figure 3 shows the unreduced kafirin spectrum by single-pulse sequence, similar for both reduced and unreduced kafirins. The carbonyl signal at 173 ppm was attributed to unsaturated fatty acids. The peak at 130 ppm was attributed to the double bond carbons. The intense peaks from 10 ppm to 40 ppm were assigned to the methyl and methylene carbons. Similar fatty acid contents have been noted in zeins extract\cite{27,28}, indicating that kafirins may also be fatty acid-binding proteins. The carboxyl peak at 173 ppm can be overlapped with other carbonyl signals from the aminocids. SVD method has the advantage over Lorentzian fitting because it does not need the assignment of peaks to calculate SS % proteins. The area of carbonyl peak was calculated by Equation 3 (section 2.3) using the $^{13}$C NMR of the 15 proteins $R$ matrix as well as the generalized inverse of $F$ ($F^{-1}$) calculated by the SVD method\cite{29}.

Figure 4 shows the expansion of carbonyl signals of unreduced kafirins (A) and reduced kafirins (B). The Lorentzian
fitting presents three signals, at 176 ppm, 174 ppm, and 172 ppm, attributed to the α-helix, unordered, and β-sheet structures, respectively\cite{19,20}.

The results of SS calculated for kafirins in this method and with the Lorentzian fitting and SVD method are in Table 1. Spectrum 4A (unreduced kafirins) peaks areas show 59% of α-helix, 12% of β-sheet, and 29% of unordered structures. The carbonyl signals of reduced kafirins (Figure 4B) show 56% of α-helix, 40% of β-sheet, and 4% of unordered structures. As a result, both extracted kafirins offer a high content of α-helix, regardless of the reducing agent utilized. The high content of α-helix and β-sheet is confirmed through the signals of α-carbon. Signals at 56 ppm and 59 ppm are typical of the α-helix, and signals at 53 ppm are typical of the β-sheet\cite{19,20}.

This fitting method depends on the following factors: the number of peaks and respective positions and the line shape function used in the fitting process, among other factors\cite{28,29}.

The SS was also calculated by the SVD method. This method multiplied the carbonyl area of kafirins ss-\textsuperscript{13}C NMR spectra by a calibration matrix\cite{37} as shown by Equation 3.

The SVD method also observed high helical content for reduced and unreduced kafirins, 55% and 49%, respectively, and 13% and 8% for β-sheet, respectively. The difference in the SS values may occur due to the different electrophoretic patterns, given that β-, α1-, α2- and γ-kafirin fractions were observed in the reduced kafirins, while in unreduced kafirins an intense signal for β-kafirin was observed, which may occur as a monomer\cite{23}, and a weak signal in the range of α1-, α2- and γ-kafirin that was named as α+γ-agglomerate.

The difference in absolute values of SS kafirins used to calculate Lorentzian fitting must be attributed because this fitting method depends on the operator, necessary to choose the number of peaks and positions assigned to each carbonyl area. The SVD method does not need the process described above, which is dependent of the operator. The α- and β- secondary structures content of reduced (SSr) and unreduced (SSu) kafirins has a high correlation coefficient for reduced and unreduced kafirins by the SVD method applied in CP/MAS \textsuperscript{13}C NMR. Table 1 shows the result of SS % calculate to reduced and unreduced kafirins using Lorentzian fitting and SVD methods. It is noteworthy that the CP/MAS \textsuperscript{13}C NMR is not a quantitative method. Its signal depends on cross-polarization (CP) efficiency and spin-lattice relaxation in the rotating frame\cite{17,20}. Despite that, when the carbons have similar chemical environments, such as the

| Applied method                  | α-helix (SS %) | β-sheet (SS %) |
|---------------------------------|----------------|----------------|
| Lorentzian fitting (unreduced)  | 59             | 12             |
| Lorentzian fitting (reduced)    | 56             | 40             |
| SVD (unreduced)                 | 49             | 8              |
| SVD (reduced)                   | 55             | 13             |

The CP/MAS \textsuperscript{13}C NMR is not a quantitative method. Its signal depends on cross-polarization (CP) efficiency and spin-lattice relaxation in the rotating frame\cite{17,20}. Despite that, when the carbons have similar chemical environments, such as the
carbonyl proteins, these two parameters are alike, giving quantitative results.

The high content of α-helix structure in kafirins from sweet sorghum grains agrees with the data published by Gao et al.\textsuperscript{[10]} Wu et al.\textsuperscript{[11]}, and Duodu et al.\textsuperscript{[12]}, who used another sorghum cultivar. According to these authors, the study of SS kafirins was quite relevant for obtaining good films to apply on foods once a higher proportion of β-sheet structures could induce protein agglomeration and reduce their solubility.

4. Conclusion

We applied the Lorentz fitting and a SVD method in carbonyl peaks area by CP/MAS \textsuperscript{13}C NMR of unreduced and reduced kafirins spectra to verify which mathematical method is more reliable to calculate their %SS. Kafirins reduced fraction extract from sweet sorghum grains are composed of four protein fractions assigned to β-, α1-, α2- and γ-kafirins and the unreduced by β-kafirins in the monomeric form and an α+γ agglomerate. Higher proportions of α-helix structure and smaller β-sheet structure were identified in the reduced and unreduced kafirins, confirmed by other spectroscopy techniques. Thus, high-resolution \textsuperscript{13}C solid-state NMR spectroscopy is promising to elucidate secondary structures of insoluble proteins. The main difference was in the absolute values for SS proportions since the SVD method does not depend on the operator, and it has high coefficient correlations for α-helix and β-sheet prediction\textsuperscript{[13]}. Our research indicates that the SVD method is more reliable than the Lorentzian fitting to calculate the % SS of proteins by CP/MAS \textsuperscript{13}C NMR spectroscopy.

5. Acknowledgements

We acknowledge CNPq (process 562470/2010-7) and CAPES for the assistance received and Rede Agronano/Embrapa.

6. References

1. Chen, H., Tian, X., Yu, Q., Hu, W., Chen, J., & Zhou, L. (2021). Sweet sorghum stalks extract has antimicrobial activity. \textit{Industrial Crops and Products}, 170, 113746. http://dx.doi.org/10.1016/j.indcrop.2021.113746.
2. Zegada-Lizarazu, W., & Monti, A. (2012). Are we ready to cultivate sweet sorghum as a bioenergy feedstock? A review on field management practices. \textit{Biomass and Bioenergy}, 40, 1-12. http://dx.doi.org/10.1016/j.biombioe.2012.01.048.
3. Ratnavathi, C. V., Sureshi, K., Kumar, B. S. V., Pallavi, M., Komala, V. V., & Seetharama, N. (2010). Study on genotypic variation for ethanol production from sweet sorghum juice. \textit{Biomass and Bioenergy}, 34(7), 947-952. http://dx.doi.org/10.1016/j.biombioe.2010.02.002.
4. Shah, U., Dwivedi, D., Hackett, M., Al-Salami, H., Utikar, R. P., Blanchard, C., Gani, A., Rowles, M. R., & Johnson, S. K. (2021). Physicochemical characterization of kafirins extracted from sorghum grain and dried distillers grain with solubles related to their biomaterial functionality. \textit{Scientific Reports}, 11(1), 15204. http://dx.doi.org/10.1038/s41598-021-94718-z. PMid:34312467.
5. Buchner, S., Kinnear, M., Crouch, I. J., Taylor, J., & Minnaar, A. (2011). Effect of the kafirin protein coating on sensory quality and shelf life of ‘Pacham Triumphant’ pears during ripening. \textit{Journal of the Science of Food and Agriculture}, 91(15), 2814-2820. http://dx.doi.org/10.1002/jsfa.4526. PMid:21725981.
6. Gao, C., Stading, M., Wellner, N., Parker, M. L., Noel, T. R., Mills, E. N. C., & Belton, P. S. (2006). Plasticization of a protein-based film by glycerol: a spectroscopic, mechanical and thermal study. \textit{Journal of Agricultural and Food Chemistry}, 54(13), 4611-4616. http://dx.doi.org/10.1021/jf060611w. PMid:16787005.
7. Wu, S., Myers, D. J., & Johnson, L. A. (1997). Factors affecting yield and composition of zein extracted from commercial corn gluten meal. \textit{Cereal Chemistry}, 74(3), 258-263. http://dx.doi.org/10.1094/CCHEM.1997.74.3.258.
8. Belton, P. S., Delgadillo, I., Halford, N. G., & Shewry, P. R. (2006). Kafirin structure and functionality. \textit{Journal of Cereal Science}, 44(3), 272-286. http://dx.doi.org/10.1016/j.csc.2006.05.004.
9. Laidlaw, H. K. C., Mace, E. S., Williams, S. B., Sakrewski, K., Mudge, A. M., Prents, P. J., Jordan, D. R., & Godwin, I. D. (2010). Allelic variation of the β-, γ- and δ-kafirin genes in diverse sorghum genotypes. *Theoretical and Applied Genetics*, 121(7), 1227-1237. http://dx.doi.org/10.1007/s00122-010-1383-9. PMid:20563549.

10. Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y. B., Parker, M. L., Mills, E. N. C., & Belton, P. S. (2005). Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. *Journal of Agricultural and Food Chemistry*, 53(2), 306-312. http://dx.doi.org/10.1021/jf049266e. PMid:15656666.

11. Wu, Y. V., Cluskey, J. E., & Jones, R. W. (1971). Sorghum prolamins: their optical rotatory dispersion, circular dichroism, and infrared spectra. *Journal of Agricultural and Food Chemistry*, 19(6), 1139-1143. http://dx.doi.org/10.1021/jf60178a008.

12. Duodu, K. G., Tang, H., Grant, A., Wellner, N., Belton, P. S., & Taylor, J. R. N. (2001). FTIR and solid state 13C NMR spectroscopy of proteins of wet cooked and popped sorghum and maize. *Journal of Cereal Science*, 33(3), 261-269. http://dx.doi.org/10.1016/s0926-6446(00)00352-9.

13. Wang, Y., Tilley, M., Bean, S., Sun, X. S., & Wang, D. (2009). Comparison of methods for extracting kafirin proteins from distillers dried grains with solubles. *Journal of Agricultural and Food Chemistry*, 57(18), 8366-8372. http://dx.doi.org/10.1021/jf901713w. PMid:19754169.

14. Xiao, J., Li, Y., Li, J., Gonzalez, A. P., Xia, Q., & Huang, Q. (2015). Structure, morphology, and assembly behavior of kafirin. *Journal of Agricultural and Food Chemistry*, 63(1), 216-224. http://dx.doi.org/10.1021/jf504674z. PMid:25510968.

15. Dianda, N., Rouf, T. B., Bonilla, J. C., Hedrick, V., & Kokini, J. (2019). Effect of solvent polarity on the secondary structure, surface, and mechanical properties of biodegradable kafirin films. *Journal of Cereal Science*, 90, 102856. http://dx.doi.org/10.1016/j.jcs.2019.102856.

16. Olivera, N., Rouf, T. B., Bonilla, J. C., Carriazo, J. G., Dianda, N., & Kokini, J. L. (2019). Effect of LAPONITE addition on the mechanical, barrier and surface properties of novel biodegradable kafirin nanocomposite films. *Journal of Food Engineering*, 245, 24-32. http://dx.doi.org/10.1016/j.jfoodeng.2018.10.002.

17. Andrade, F. D., Forato, L. A., Bernardes, R., Fo, & Colnago, L. A. (2016). Quantification of protein secondary structure by 13C solid-state NMR. *Analytical and Bioanalytical Chemistry*, 408(14), 3875-3879. http://dx.doi.org/10.1007/s00216-016-9484-1. PMid:27066964.

18. Park, S.-H., & Bean, S. B. (2003). Investigation and optimization of the factors influencing sorghum protein extraction. *Journal of Agricultural and Food Chemistry*, 51(24), 7050-7054. http://dx.doi.org/10.1021/jf034533d. PMid:14611170.

19. Kricheldorff, H. R., & Müller, D. (1984). Secondary structure of peptides 16th. Characterization of protein by means of 13C NMR CPMAS spectroscopy. *Colloid & Polymer Science*, 262(11), 856-861. http://dx.doi.org/10.1007/BF01452215.

20. Wishart, D. S., & Sykes, B. D. (1994). Chemical shifts as a tool for structure determination. *Methods in Enzymology*, 239, 363-392. http://dx.doi.org/10.1016/s0076-6879(94)39014-2. PMid:7830591.

21. Bicudo, T. C., Forato, L. A., Batista, L. A. R., & Colnago, L. A. (2005). Study of the conformation of γ-zeins in purified maize protein bodies by FTIR and NMR spectroscopy. *Analytical and Bioanalytical Chemistry*, 383(2), 291-296. http://dx.doi.org/10.1007/s00216-005-0003-z. PMid:16132146.

22. Byaruhanga, Y. B., Emambux, M. N., Belton, P. S., Wellner, N., Ng, K. G., & Taylor, J. R. N. (2006). Alteration of kafirin and kafirin film structure by heating with microwave energy and tannin complexation. *Journal of Agricultural and Food Chemistry*, 54(12), 4198-4207. http://dx.doi.org/10.1021/jf052942z. PMid:16756347.

23. El Nour, I. N. A., Peruffo, A. D., & Curioni, A. (1998). Characterization of sorghum kafirins in relation to their cross-linking behavior. *Journal of Cereal Science*, 28(2), 197-207. http://dx.doi.org/10.1016/j.jcs.1998.0185.

24. Kamashiro, K. K., Kurano, T. L., Niemczura, W. P., Martino, M., & Tamburro, A. M. (2003). 13C CPMAS NMR Studies of the Elastin-Like Polypeptide (LLGVG)n. *Biopolymers*, 70(2), 221-226. http://dx.doi.org/10.1002/bip.10470. PMid:14517910.

25. Forato, L. A., Colnago, L. A., Garratt, R. C., & Lopes, M. A. (2000). Identification of free fatty acids in maize protein bodies and purified alpha zeins by 13C and 1H nuclear magnetic resonance. *Biochimica et Biophysica Acta*, 1543(1), 106-114. http://dx.doi.org/10.1016/S0167-4838(00)01904-4. PMid:11087946.

26. Bardet, M., Foray, M. F., Bourguignon, J., & Krajewski, P. (2001). Investigation of seeds with high-resolution solid-state 13C NMR. *Magnetic Resonance in Chemistry*, 39(12), 733-738. http://dx.doi.org/10.1002/mrc.958.

27. Forato, L. A., Yushmanov, V. E., & Colnago, L. A. (2004). Interaction of two prolamins with 1-13C Oleic Acid by 13C NMR. *Biochemistry*, 43(22), 7112-7126. http://dx.doi.org/10.1021/bi035562k. PMid:15170349.

28. Forato, L. A., Bernardes-Filho, R., & Colnago, L. A. (1998). Protein structure in KBr pellets by infrared spectroscopy. *Analytical Biochemistry*, 259(1), 136-141. http://dx.doi.org/10.1006/abio.1998.2599. PMid:9606154.

29. Hegland, M., & Anderssen, R. S. (2005). Resolution enhancement of spectra using. *Inverse Problems*, 21(3), 915-934. http://dx.doi.org/10.1088/0266-5611/21/3/008.

30. Koldziejski, W., & Klinojczy, J. (2002). Kinetics of cross-polarization in solid-state NMR: a guide for chemists. *Chemical Reviews*, 102(3), 613-628. http://dx.doi.org/10.1021/cr000060n. PMid:11890752.