UNIVERSIDADE ESTADUAL DE MARINGÁ
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Desenvolvimento e caracterização de alimentos isentos de glúten contendo farinha de grãos integrais como fonte de nutrientes essenciais

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá como critério para obtenção do grau de Doutor em Ciência de Alimentos.

Orientador: Prof. Dr. Makoto Matsushita

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BIOGRAFIA

Aline Kirie Gohara, natural de Bastos, no estado de São Paulo, Brasil, possui graduação em Engenharia de Alimentos pela Universidade Estadual de Maringá (2010). Mestre em Ciência de Alimentos pela Universidade Estadual de Maringá em 2013. Tem experiência em análises físico-químicas, microbiológicas, sensoriais e instrumentais de alimentos atuando principalmente nos seguintes temas: desenvolvimento e caracterização de alimentos com propriedade funcionais; química analítica de alimentos; métodos cromatográficos de separação; quimiometria aplicada ao desenvolvimento e caracterização de alimentos.

DEDICO

À minha amada mãe, amiga para todas as horas,
Ao meu pai (in memorian),
Ao meu tio Ricardo, um verdadeiro pai e amigo,
À minha querida avó Helena,
Aos meus irmãos e companheiros Alex e Anderson,
que compartilho os meus momentos de alegria e de dificuldade.
Obrigada pelo carinho, confiança, paciência, compreensão e apoio nesta fase.
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APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de dois artigos científicos publicados em revistas com Qualis-Capes B2 para Ciência de Alimentos.

ARTIGO 1: GOHARA, ALINE K.; SOUZA, ALOISIO H. P.; ROTTA, ELIZA M.; STROHER, GISELY L.; GOMES, SANDRA T. M.; VISENTAINER, JESUI V.; Souza, Nilson E.; Matsushita, Makoto. Application of Multivariate Analysis to Assess the Incorporation of Omega-3 Fatty Acid in Gluten-Free Cakes. Journal of the Brazilian Chemical Society (Impresso), v. 27, p. 62-69, 2015. DOI: 10.5935/0103-5053.20150241.

ARTIGO 2: GOHARA, A. K. ; SOUZA, A. H. P. ; GOMES, S. T. M. ; SOUZA, N. E. ; Visentainer, J.V. ; MATSUSHITA, M. Nutritional and bioactive compounds of adzuki beans cultivars using chemometric approach. Ciência e Agrotecnologia (UFLA), v. 40(1), p. 104-113, 2016. DOI: 10.1590/S1413-70542016000100010.

Além dos artigos publicados, outros produtos foram elaborados durante o período, gerando mais 3 trabalhos, os quais estão em fase de redação:

ARTIGO A: Mixture design applied in the development of gluten free pancakes formulations containing chia and flaxseed. LWT – Food Science and Technology. (Qualis B1)

ARTIGO B: Development and characterization of gluten free pasta formulations with grains source of essential nutrients. Journal of the Science of Food and Agriculture. (Qualis B1)

ARTIGO C: Incorporation of Omega-3 in pizza dough using chia, flaxseed and perilla. Food Chemistry. (Qualis A2)

Crom Alimentos
Grupo de Pesquisa

RESUMO GERAL

INTRODUÇÃO. O bolo é o segundo produto de panificação mais consumido mundialmente, perdendo apenas para o pão. Essa popularidade deve-se principalmente a praticidade e boa aceitação sensorial por parte dos consumidores. No entanto, uma das principais problemáticas apresentada pelo produto bolo é a carência de nutrientes essenciais à saúde humana, principalmente quando se trata de versão sem glúten, cujo principal ingrediente utilizado pela maioria dos fabricantes é a farinha de arroz, constituída basicamente de carboidratos. Azuki (*Vigna angularis*) é uma leguminosa amplamente produzida e consumida na Ásia, usado na fabricação de vários produtos, especialmente em doces típicos. Chia (*Salvia hispanica* L.) é uma planta angiosperma da família da menta (*Lamiaceae*), caracterizada como um grão de clima tropical e subtropical, largamente consumida no América pré-colombiana pelos astecas, na região que inclui México e Guatemala. Ambos os grãos, azuki e chia, são considerados fontes de muitos nutrientes essenciais para a manutenção da boa saúde. Um dos aspectos mais importantes diz respeito aos nutrientes é a composição lipídica. Diversos ácidos graxos são essenciais para a manutenção dos sistemas biológicos, especialmente os da série n-3. O planejamento fatorial permite um menor número de experimentos e conclusões a partir de resultados qualitativos. Análise de componentes principais (ACP) permite o reconhecimento de padrões, viabilização de informações e redução na dimensionalidade dos dados. A análise de desejabilidade permite a detecção da região ótima.

OBJETIVOS.
ARTIGO 1: O objetivo deste trabalho foi à aplicação de métodos quimiométricos para investigar a influência dos fatores: percentuais de farinha de chia e azuki adicionados a formulações de bolo de chocolate sem glúten na determinação da composição em ácidos graxos e aspectos nutricionais.
ARTIGO 2: Este trabalho teve como objetivo avaliar as cultivares angularis e niponensis de feijão azuki produzidos na região sul do Brasil.

MATERIAL E MÉTODOS.
ARTIGO 1: Um planejamento fatorial 2^2 completo (dois fatores em dois níveis) com ponto central em quintuplicata foi realizado para investigar a influência dos fatores: % de farinha de chia e farinha de azuki na composição de ácidos graxos de bolo de chocolate sem glúten. Foram propostos modelos matemáticos com as respostas, bem como análise de componentes principais e avaliação da função de desejabilidade.
ARTIGO 2: As cultivares de feijão Azuki (Vigna angularis): angularis e niponensis, foram adquiridas diretamente do produtor na região de Maringá, estado do Paraná, Brasil. Ambas as cultivares foram avaliadas considerando composição proximal e energia bruta, composição em ácidos graxos, índices da qualidade nutricional de lipídios, isômeros de tocoferol, atividade da vitamina E, minerais, valores de referência para ingestão diária, propriedades bioativas, e análise multivariada dos dados.

RESULTADOS E DISCUSSÃO.
ARTIGO 1: Os fatores % de farinha de chia e azuki foram significativos, sendo que a farinha de chia apresentou maior influência nos resultados. Os maiores valores de ambos os fatores contribuíram para melhorar as concentrações de ácidos graxos poli-insaturados, principalmente os da série n-3, e proporcionou índices nutricionais mais adequados. A análise de componentes principais distinguiu as amostras com teores mais elevados de farinha de

chia, e a análise de desejabilidade indicou a amostra com maior concentração de farinha de chia como a região ótima.
ARTIGO 2: Ambas as cultivares apresentaram composição proximal similares com a literatura e os ácidos graxos majoritários foram 18:2n-6, 16:0 e 18:3n-3. Todas as amostras mostraram prevalência de ácidos graxos poli-insaturados e valores de razões e índices nutricionais adequados para a manutenção do sistema biológico de um organismo saudável. Os grãos apresentaram teores significativos de tocoferol e atividade da vitamina E, resultando em uma alta contribuição para a ingestão de referência diária. Quantidades significativas de ferro, manganês e zinco também foram encontrados nos grãos de azuki, e eles são considerados importantes devido a sua função de cofator em reações metabólicas. Compostos fenólicos e flavonoides corroboraram com outros estudos e contribuíram para a atividade antioxidante. A análise de componentes principais permitiu distinguir as cultivares, e as duas componentes principais puderam explicar 92,28% da variância de dados.

CONCLUSÕES.
ARTIGO 1: Os métodos quimiométricos foram eficientes para melhorar a composição de ácidos graxos de bolo de chocolate isento de glúten. Os modelos estatísticos foram significativos e permitiram a obtenção das superfícies de resposta. O ponto de maior desejabilidade foi a formulação com maior teor de farinha de chia. A substituição parcial da farinha de arroz por farinha de chia e azuki em produtos isentos de glúten se mostrou uma boa alternativa para melhorar as características nutricionais de alimentos, especialmente os produtos de panificação que podem ser consumidos inclusive por pacientes celíacos.
ARTIGO 2: As cultivares de feijão azuki são excelentes fontes de vários compostos essenciais, tais como ácidos graxos, tocoferóis, minerais e propriedades antioxidantes. A análise multivariada permitiu distinguir lotes de cultivares angularis e niponensis, bem como avaliar a importância dos parâmetros. O uso de feijão azuki na dieta oriental é promissora devido as suas características intrínsecas.

Palavras-chave: *Salvia hispanica*; *Linus usitiassimum*; *vigna angularis*; *Perilla frutescens*; quimiometria; celíacos.

GENERAL ABSTRACT

INTRODUCTION. Cake is the second most consumed food among the bakery ones, the first product is bread. This popularity is mainly due to convenience and sensory acceptance. However, one negative point of this food is the lack of essential nutrients for human beings. This problem is worse in gluten-free products because the main ingredient used by bakery industries is rice flour, which consists basically of carbohydrates. Azuki (*Vigna angularis*) is a legume widely produced and consumed in Asia, used in the various products manufacturing, especially in typical sweets. Chia (Salvia hispanica L.) is an angiosperm plant from the mint family (Lamiaceae) characterized as a grain from tropical and subtropical climate, widely consumed in pre-Columbian America by the Aztecs, in the region that includes Mexico and Guatemala. Both grains, azuki and chia, are considered rich source of many essential nutrients to good health maintenance. One of the most relevant aspects regard to nutrients is the lipid composition. Several fatty acids are essential for the maintenance of biological systems, mainly the ones from n-3 series. Factorial design allows a smaller number of experiments and findings from qualitative results. Principal Component Analysis (PCA) allows the standard recognition, information is feasible and there is a reduction in the dimensionality of the data. Desirability analysis allows the detection of the optimum region.

AIMS.
ARTICLE 1: The objective of this work was the application of chemometric methods to investigate the influence of the factors: percentage of chia and azuki flours on lipid composition and nutritional aspects of gluten-free chocolate cakes.
ARTICLE 2: This work aimed at evaluating angularis and niponensis cultivars of azuki beans from south region of Brazil.

MATERIALS AND METHODS.
ARTICLE 1: A 2^2 full factorial design (two factors at two levels) with center point in quintuplicate was conducted to investigate the influence of factors: % of chia and azuki flours on the lipid composition of gluten-free chocolate cake. Mathematical models were proposed to the responses, as well as principal component analysis and evaluation of the desirability function.
ARTICLE 1: Adzuki beans (Vigna angularis) cultivars: angularis and niponensis, were acquired directly from the producer in the region of Maringa city, Parana state, Brazil. Both cultivars were evaluated concerning proximal composition and crude energy, fatty acid composition, indices of the nutritional quality of lipids, tocopherol isomers, vitamin E activity, minerals, dietary reference intake values, bioactive properties, and multivariate analysis.

RESULTS AND DISCUSSION.
ARTICLE 1: The factors % of chia and azuki flours were significant, but chia flour presented higher influence on the results. The increasing of these values contributed to improve the fatty acid composition, Increased values of both factors contributed to improve contents of polyunsaturated fatty acids, mainly n-3 series, and provided more adequate nutritional indexes. The principal component analysis distinguished the samples with higher contents of chia and the desirability analysis indicated the sample with the highest level of chia as the optimal region.
ARTICLE 2: Both cultivars presented proximal composition similar to literature and the majority fatty acids were 18:2n-6, 16:0 and 18:3n-3. All samples showed polyunsatured fatty

acids prevalence and nutritional indices and ratios considered adequate for biological system maintenance of a healthy organism. The grains presented significant contents of tocopherols and vitamin E activity, resulting in a high contribution to the dietary reference intake. Significant contents of iron, manganese and zinc were also found in the azuki beans, and they are very important mainly due to their function as cofactors in metabolic reactions. Phenolic compounds and flavonoids corroborated with other studies and contributed to the antioxidant activity. The principal components multivariate analysis allowed distinguishing the cultivars, and the two principal components could explain 92.28% of data variance.

CONCLUSIONS.

ARTICLE 1: Chemometric methods were very useful for improvement of fatty acids composition of gluten-free chocolate cake. The models analyzed were significant and allowed obtaining the response surfaces. The formulation with higher contents of chia and azuki flour was considered as a point of greatest desirability. The addition of chia and azuki flours in gluten-free products is a great alternative to improve nutritional characteristics of foods, mainly bakery ones that can be consumed even by celiac patients.

ARTICLE 2: Cultivars of adzuki beans are excellent sources of many essential compounds, such as essential fatty acids, tocopherols, minerals and antioxidant properties The multivariate analysis allowed distinguishing batches of angularis and niponensis cultivars, as well as evaluating the importance of the parameters. The traditional use of adzuki beans in the eastern dietary is promising due to their intrinsic characteristics.

Keywords: *Salvia hispanica*; *Linus usitiassimum*; *vigna angularis*; *Perilla frutescens*; chemometry; celiac.

TRABALHOS GERADOS NA TESE

Artigos publicados

ARTIGO 1: Application of Multivariate Analysis to Assess the Incorporation of Omega-3 Fatty Acid in Gluten-Free Cakes. Journal of the Brazilian Chemical Society.
DOI: 10.5935/0103-5053.20150241.
Anexo 1

ARTIGO 2: Nutritional and bioactive compounds of adzuki beans cultivars using chemonetric approach. Ciência e Agrotecnologia (UFLA).
DOI: 10.1590/S1413-70542016000100010.
Anexo 2

Artigos em redação

ARTIGO A: Mixture design applied in the development of gluten free pancakes formulations containing chia, flaxseed and perilla. LWT – Food Science and Technology. (Qualis B1)
Descrição: As formulações das massas de panqueca foram desenvolvidas utilizando-se metodologia de planejamento de misturas. A base para a massa constituiu-se farinha de arroz e fécula de batata, sendo que parte dela foi substituída por farinhas de chia, linhaça e/ou perilla.

ARTIGO B: Chemometric tools applied to the development and characterization of gluten free pasta formulations with grains source of essential nutrients. Journal of the Science of Food and Agriculture. (Qualis B1)
Descrição: Foram elaboradas massas de macarrão tipo espaguete, utilizando farinha de arroz e amido de milho como base. Foram utilizadas farinha de chia, linhaça e/ou perilla para substituição parcial da farinha base, seguindo delineamento experimental.

ARTIGO C: Multivariate analysis of the incorporation of Omega-3 in pizza dough using chia, flaxseed and perilla. Food Chemistry. (Qualis A2)

Descrição: Utilizando-se planejamento experimental, foram desenvolvidas massas de pizza com farinhas de linhaça, chia e/ou perilla para substituição parcial da farinha base constituída de arroz e fécula de mandioca.

ANEXO 1

ARTIGO 1: Application of Multivariate Analysis to Assess the Incorporation of Omega-3 Fatty Acid in Gluten-Free Cakes.

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Article

Application of Multivariate Analysis to Assess the Incorporation of Omega-3 Fatty Acid in Gluten-Free Cakes

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A complete 2^2 experimental planning (two factors at two levels) with central point in quintuplicate was used to investigate the influence of the factors: chia and azuki flours on fatty acids composition of gluten-free chocolate cake. Both factors were significant, but chia flour presented greater influence on the results. Increased values of both factors contributed to improve contents of polyunsaturated fatty acids (380.96 g kg^{-1} of product), mainly n-3 series (70.25 g kg^{-1} of product), and provided more adequate nutritional indices. The principal component analysis and desirability function indicated the sample with higher level of both factors as the optimal region. This sample showed an increase in contents of alpha-linolenic acid (188.03%) and polyunsaturated fatty acids (18.16%) when compared to control formulation. The addition of chia flour can improve nutritional characteristics of food stuffs such as bakery products, especially their lipid composition.

Keywords: *Salvia hispanica* L., alpha-linolenic, response surface methodology, principal components analysis, desirability function

Introduction

Mankind has suffered lots of changes in all fields of life, such as education and job, and it has a deep reflection in eating habits. People search for products that do not need much time to prepare before eating. However, most of these foods are made of poor ingredients and they cannot offer essential nutrients for human body. In this context, food industries started developing enriched products using potential ingredients with nutritional properties.[1-3]

The bakery products are the most consumed by population due to their practicality and good sensory acceptance. After bread, the cakes ready for consumption are considered the preferred products in this category.[4] Nowadays, there are gluten free versions of these foods and they can be consumed by celiac patients, however, almost all of these products are totally made of rice flour, which is composed basically only of carbohydrates.[5]

On the other hand, there are several kinds of potential ingredients which may be used to replace rice flour in gluten-free products, such as azuki and chia. Azuki (*Vigna angularis*) is a legume widely produced in Asia and generally used to make some kinds of sweets appreciated by Asiatic people.[6] Chia (*Salvia hispanica*, L.) is an angiosperm plant from the mint family (*Lamiaceae*) characterized as a grain from tropical and subtropical climate, widely consumed in pre-Columbian America by the Aztecs, in the region that includes Mexico and Guatemala.[7] Both grains, azuki and chia, are considered rich source of many essential nutrients. According to

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Zanqui *et al.*,[8] chia presents high levels of total lipids, and the lipid fraction of this grain is considered a great source of polyunsaturated fatty acids, especially alpha-linolenic acid (LNA, 18:3n-3), and other lipophilic compounds such as tocopherols and phytosterols. According to Ratnayake and Galli,[9] LNA is considered essential because it cannot be metabolized by the human body and must be consumed through the diet.

Factorial design is an important chemometric tool which enables evaluating the contribution of each specific ingredient to several characteristics of the final product, using a smaller number of experiments, simultaneous analysis of many variables and their effects, reliability of the results, performance of the research in stages, an interactive process of inclusion of tests to the model, main variables selection, presentation of the process through mathematical models, and conclusions from qualitative results.[10] Multivariate analysis enables extracting additional information when compared to the univariate analysis. The principal components analysis (PCA) allows the pattern recognition, the gathering of information, reduction of data dimensionality and also the organization of data into a simpler structure, easier to understand. This analysis is based on performing linear comparisons of the original variables. The principal components (PC) are orthogonal among each other and the explained variance decreases with increased PC number.[11]

This study aimed at applying chemometric methods to investigate the influence of the factors: percentages of chia and azuki flour, added in gluten-free chocolate cake for the determination of fatty acid composition and nutritional aspects.

Experimental

Sampling

The grains of azuki used in this study were cultivated in the region of Maringá-PR and purchased at the local market. Approximately 6 kg of grains were ground in a hammer mill to obtain homogeneous flour that was sieved using a 20-mesh sieve. Chia flour (*Salvia hispanica*, L.) used in this research was partially defatted, it is a byproduct of the cold-pressing process for lipid extraction. This ingredient was provided by the company Giroil Agroindústria Ltda. (Santo Angelo, Rio Grande do Sul, Brazil). The other ingredients were obtained in retail stores in Maringá.

Experimental design

A complete 2^2 experimental planning (two factors at two levels) with central point in quintuplicate was used to investigate the influence of both factors on the fatty acids composition of chocolate cake. The factors were: concentrations of azuki and chia flour, as shown in Table 1. The total of 100% of flour for each formulation was achieved with rice flour addition. The responses analyzed in this study were: sums of fatty acids from n-3 series, polyunsaturated fatty acids (PUFA), ratio of polyunsaturated by saturated fatty acids (PUFA / SFA), atherogenicity (IA) and thrombogenicity indices (IT) of fatty acids. A control cake (100% rice flour) was produced to be compared to experimental optimum point. The cakes were processed according to the recommendations of Gohara *et al.*[12]

Formulation processing

All the ingredients were previously weighed individually. The rice flour, azuki and chia, at the respective percentage for each formulation, were mixed to obtain a homogeneous fraction (288.00 g kg^{-1} of the whole formulation); and egg white (87.00 g kg^{-1}) was mixed to form a solid phase. The egg yolk (58.00 g kg^{-1}), butter (58.00 g kg^{-1}) and sugar (169.00 g kg^{-1}) were homogenized in a bowl to form a cream. The mixture of flours, chocolate powder (80.00 g kg^{-1}), cocoa powder (38.00 g kg^{-1}), egg white, water (190.80 g kg^{-1}), milk powder (21.20 g kg^{-1}) and baking powder (10.00 g kg^{-1}) were added slowly to the cream to form a homogeneous mass. The cake mass was transferred to a rectangular baking dish and baked in a conventional oven for 30 min at 200 °C, with subsequent cooling to room temperature (25 °C). The sequence of ingredients mixture and temperature conditions described above followed the process performed in previous study.[13]

Table 1. Factors and levels investigated in the experimental design for the development of a 2^2 full factorial design

Factor	Unit	Symbol	Type	Level		
				–1	0	1
Chia flour	g Kg^{-1}	C	numeric	100	150	200
Azuki flour	g Kg^{-1}	A	numeric	100	150	200

Total lipid extraction

The total lipids were extracted according to Bligh and Dyer,[14] using a mixture of the solvents: methanol, chloroform and water (final proportion 2:2:1.8, v/v/v).

Fatty acid composition

The first step to determine the fatty acid composition was the conversion of the lipids into fatty acid methyl esters (FAME), according to Hartman and Lago.[15] The FAME were separated using a CP-3380 gas chromatograph (Varian, Walnut Creek, USA) fitted with a flame ionization detector and a CP 7420-select FAME fused-silica capillary column (100 m × 0.25 mm × 0.25 µm, cyanopropyl). The carrier gas was hydrogen at 1.4 mL min^{-1}, the make-up gases were nitrogen at 30 mL min^{-1} and synthetic air at 300 mL min^{-1}, and the flame gas was hydrogen at 30 mL min^{-1}. The sample was injected in a split ratio of 1:100. The injector and detector temperatures were 235 °C. The column temperature was maintained at 165 °C for 4 min, increased to 185 °C at 4 °C min^{-1} and maintained for 5 min, and then increased from 185 to 225 ºC at 10 °C min^{-1} and maintained for 10 min. The retention times were compared with those of standard methyl esters (Sigma, St. Louis, USA). The fatty acids were quantified using tricosanoic acid methyl ester (Sigma) as an internal standard.[16] The peak areas were determined with Star 5.0 software (Sigma, Santa Clara, USA). According to Joseph and Ackman[16] (equation 1), correction factors of FAME for flame ionization detectors in individual fatty acids (FA) were used and their concentrations expressed in mg FA *per* kg^{-1} of food:

$$M_x = \frac{A_X \times Mp \times F_{CT}}{A_P \times M_A \times F_{CEA}} \quad (1)$$

where M_x: X fatty acid mass in mg g^{-1} of sample; Mp: internal standard mass in milligrams; M_A: sample mass in g; A_X: X fatty acid area; A_p: internal standard area; F_{CT}: theoretical correction factor; F_{CEA}: methyl ester correction factor to fatty acid.

The limits of detection (LOD) and quantification (LOQ) were estimated by triplicate analysis of diluted methyl arachidate standard solution (1.0 mg mL^{-1}), considering the signal-noise ratio relative to the background signal as 3 and 10, respectively.[17]

Nutritional quality indices of lipid fraction

The atherogenicity index (IA) and thrombogenicity index (IT) were determined as IA = [(12:0 + (4 × 14:0) + 16:0)] / (MUFA + n-6 + n-3); and IT = (14:0 + 16:0 + 18:0) / [(0.5 × MUFA) + (0.5 × n-6) + (3 × n-3) + (n-3 / n-6)], according to Ulbricht and Southgate.[18]

Statistical analysis

All the analyses were done in triplicate. Fatty acid composition was demonstrated by the general average of the experiments repetitions (A: test 1; B: test 2; C: test 3; D: test 4 and E: tests 5, 6, 7, 8 and 9). Initially, the values of effects, interaction, and analysis of variance (ANOVA) were obtained. Afterwards, the whole variables had their normal and homogeneity of variance assessed through the combings. Then, the ANOVA among the groups was done for all the responses. Considering the independent variables effect on the responses, the response surface methodology was applied. The basic mathematic model used to adjust the data was (equation 2):

$$\hat{Y}_i = b_0 + b_1 x_1 + b_2 x_2 \quad (2)$$

where $\hat{Y}_i$ is the expected response; b_0 is the constant term; b_1 and b_2 are the terms of the regression model and x_1 and x_2 are the levels of the independent variables.[19]

The model's equations were arranged to a global response using a desirability function. The results obtained for the sums of fatty acids from n-3 series, polyunsaturated fatty acids, ratio of polyunsaturated by saturated fatty acids, atherogenicity and thrombogenicity indices were used to estimate the global response. This procedure involved a transformation of each response ($\hat{Y}_i$) estimated for an individual value of desirability (d_i), in which $0 \le d_i \le 1$, according to Derringer and Suich.[20]

If the objective or target T to the response $\hat{Y}_i$ is a maximum value, then the equation 3 should be used:

$$d_i = \begin{cases} 0 & \hat{Y}_i < L \\ \left(\frac{\hat{Y}_i - L}{T - L}\right)^r & L \le \hat{Y}_i \le T \\ 1 & \hat{Y}_i > T \end{cases} \quad (3)$$

If the objective or target to the response $\hat{Y}_i$ is a minimum value then the equation 4 should be used:

$$d_i = \begin{cases} 1 & \hat{Y}_i < T \\ \left(\frac{U - \hat{Y}_i}{U - T}\right)^r & T \le \hat{Y}_i \le U \\ 0 & \hat{Y}_i > U \end{cases} \quad (4)$$

where L and U are minimum and maximum limits, respectively.

The convenience function is linear when the weight ‘r’ is equal to 1. If $r > 1$ there is more emphasis on targeting the closest value. Using $0 < r < 1$ makes this less important.

Individual values of desirability (d_i) were arranged through a geometric average to form a global desirability value (D), which will attend to satisfy all response simultaneously. This single value of D [0,1] gives a global assessment of convenience and the arranged response levels, and D will increase at the same time that the properties balance becomes more favorable.

PCA consisted of the sums of fatty acids from n-3 series, PUFA, PUFA / SFA, IA and IT of fatty acids - loadings. For this analysis, the average values of the five different formulations (A: test 1; B: test 2; C: test 3; D: test 4; E: tests 5, 6, 7, 8 and 9) were used - scores. Averages were autoscaled, so that the whole variables showed the same weight. In this way, PCA bidimensional graphics were obtained.

The formulation considered the best one by desirability assay was compared to the control cake. The Student’s *t*-test was applied to analyze differences between the two different samples. All the statistical analyses were done using the software Statistica, version 8.0,[21] adopting the 5% significance level for rejection of the null hypothesis ($p < 0.05$).

Results and Discussion

The total lipids contents for samples A, B, C, D, E and control were 73.50, 76.64, 75.57, 77.65, 76.43 and 59.50 g kg^{-1}, respectively, according to previous study.[12] This research showed that enriched gluten free cake presented good sensory acceptance, as well as great contents of nutrients, such as protein and lipids, mainly the formulation with higher contents of chia flour.[12] Another previous study showed the great levels of essential minerals in all the formulations of cake, the minerals were Ca, Cu, Fe, K, Mg, Mn and Zn. The highest mineral contents were found in the formulations with the highest concentration of both flour (chia and azuki).[13] In this present study, similar results were found for essential fatty acids, as shown below.

Under the selected operation system, LOD and LOQ were estimated at 0.15 and 0.50 mg g^{-1} of total lipids, respectively.

Gas chromatography allowed identifying and quantifying eighteen fatty acids and the major ones were: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9), linoleic acid (18:2n-6) and alpha-linolenic acid (LNA, 18:3n-3). All the formulations, mainly formulation with higher contents of chia, showed the prevalence of polyunsaturated fatty acids, which are involved with the smaller risk of cardiovascular diseases.[9]

LNA presented the highest content value in all the formulations. This fatty acid plays important roles in metabolic processes. Through desaturases and elongases enzymes, LNA can be converted into other eicosanoid fatty acids from n-3 series (omega-3), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:5n-3), which are very important for human health due to their anti-inflammatory properties.[22,23]

These results were similar to those found for food products enriched with omega-3 using flaxseed grain, such as panettone,[24] granola,[25] food bars[26] and cookies.[27]

Table 2 presents the conditions of the complete 2^2 factorial model with central point in quintuplicate, applied to the tests, as well as the values obtained for the sums, ratio, and nutritional indices of fatty acids.

Table 2. Complete 2^2 design, the obtained responses to the sums, ratios and fatty acids indices

Test	Independent variable[a]		Sums and ratios of fatty acids / (mg FA g^{-1} TL)				
	x_1	x_2	n-3[b]	PUFA[b]	PUFA / SFA[b]	IA	IT
1	–1	–1	45.83	343.55	1.06	0.47	0.66
2	–1	1	48.50	354.88	1.17	0.45	0.61
3	1	–1	67.31	366.41	1.24	0.43	0.54
4	1	1	70.25	380.96	1.35	0.39	0.49
5	0	0	59.04	367.78	1.26	0.41	0.55
6	0	0	58.30	353.71	1.13	0.44	0.62
7	0	0	57.16	349.23	1.10	0.45	0.63
8	0	0	57.18	350.18	1.11	0.45	0.63
9	0	0	59.55	363.68	1.22	0.41	0.57

[a]Expressed according to Table 1; [b]mg fatty acid *per* g^{-1} of total lipid. x_1: chia flour; x_2: azuki flour; n-3: total omega-3 fatty acids; PUFA: total of polyunsaturated fatty acids; SFA: total of saturated fatty acids; IA: index of atherogenicity; IT: index of thrombogenicity.

Table 3. Regression coefficients, confidence intervals and coefficients of determination of the responses applied to the response surface methodology

Parameter	n-3	PUFA	PUFA / SFA	IA	IT
Mean	58.13 (57.393, 58.858)[a]	358.93 (351.208, 366.653)[a]	1.18 (1.129, 1.232)[a]	0.43 (0.419, 0.447)[a]	0.59 (0.563, 0.617)[a]
x_1	10.81 (9.709, 11.905)[a]	12.23 (0.650, 23.819)[a]	0.09 (0.014, 0.167)[a]	–0.03 (–0.046, –0.003)[a]	–0.06 (–0.112, –0.008)[a]
x_2	1.40 (0.303, 2.500)[a]	6.47 (–5.116, 18,053)[a]	0.06 (–0.021, 0.133)[a]	–0.02 (–0.036, 0.006)	–0.06 (–0.064, 0.017)[a]
R^2	0.990	0.701	0.657	0.654	0.707

[a]Confidence interval for 95% level. n-3: total fatty acids from omega-3 series; PUFA: total polyunsaturated fatty acids; SFA: total of saturated fatty acids; IA: index of atherogenicity; IT: index of thrombogenicity; x_1: chia flour; x_2: azuki flour; R^2: coefficient of determination.

The regression coefficients for each one of the models, their confidence intervals and coefficients of determination (R^2) of the responses applied to the response surface methodology are presented in the Table 3. Values close to 1 indicate a good correlation between experimental and predicted data.[28] Pagamunici *et al.*[29] found coefficients of determination of 0.85 for instrumental data *vs.* sensory analysis as a function of time, and the models were well fitted. In this study, the values were greater than 0.60, which means that the linear model explained more than 60% of the data variability. The residual plots for each response showed normality, and homogeneity of variance was explained satisfactorily.

The limits of confidence intervals for the first-order term (x_2) for the sum of polyunsaturated fatty acids, PUFA / SFA ratio, IA and IT showed values with opposite signs (Table 3). All the values are possibly within a confidence interval, therefore it is possible that this value is zero. This fact demonstrates that there was a linear correlation between the variables, so there is no statistical evidence to keep this term in the model. Nevertheless, its permanence was preferred to preserve the mathematical hierarchy.[10]

The effect values can be obtained by regression coefficients values (effects are twice the coefficients). Data from Table 3 show that the main effects of the two factors (chia and azuki) were positive for fatty acids from n-3 series, PUFA and the PUFA / SFA ratio, and they were negative for IA and IT. These data enable us to estimate that greater concentration of chia and azuki may increase contents of n-3 series fatty acids and PUFA. This increase was also found for hamburgers in studies performed by Souza *et al.*[30] All the experimental models did not present interaction effects for the responses analyzed and were removed.

Tables 4 and 5 show the results obtained by ANOVA for each factor studied in the model response. The factor chia most influenced the increased levels of fatty acids from n-3 series, PUFA and PUFA / SFA, with contributions of 99.43, 54.78 and 47.83%, respectively (Table 4).

The values of F-test (Table 5) demonstrate the significance of coefficients of regression and lack of fit. According to data from Table 4, the lack of fit of the model was not significant for the models.

The effects may be better observed in the response surface models (Figure 1). Surfaces of n-3 series fatty acids, PUFA and PUFA / SFA ratio clearly show the positive effect caused by increasing the factors, mainly the factor chia. On the other hand, surfaces of IA and IT show a negative effect. These nutritional indices are associated with the presence of lauric (12:0), myristic (14:0), palmitic (16:0)

Table 4. Results of ANOVA, sum of squares of the responses obtained in full 2^2 factorial design with central point

Source	Sum of squares					
	DF	n-3	PUFA	PUFA / SFA	IA	IT
x_1	1	467.169	598.727	0.033	0.002	0.014
x_2	1	7.853	167.364	0.013	0.001	0.002
Lack of fit	2	0.181	48.346	0.004	0.000	0.001
Pure error	4	4.655	278.524	0.020	0.002	0.006
Total SS	8	469.859	1092.961	0.069	0.005	0.023

DF: degree of freedom; n-3: total fatty acids from omega-3 series; PUFA: total polyunsaturated fatty acids; SFA: total of saturated fatty acids; IA: index of atherogenicity; IT: index of thrombogenicity; x_1: chia flour; x_2: azuki flour; SS: sum of squares.

Table 5. Results of ANOVA and F-test of the responses obtained in full 2^2 factorial design with central point

Source	F-test					
	DF	n-3	PUFA	PUFA / SFA	IA	IT
x_1	1	579.584	10.990	8.304	7.436	12.003
x_2	1	9.742	3.072	3.176	2.694	1.948
Lack of fit	2	0.078	0.347	0.370	0.070	0.462
Pure error	4	–	–	–	–	–

DF: degree of freedom; n-3: total fatty acids from omega-3 series; PUFA: total polyunsaturated fatty acids; SFA: total of saturated fatty acids; IA: index of atherogenicity; IT: index of thrombogenicity; x_1: chia flour; x_2: azuki flour.

and stearic (18:0) fatty acids which are more related to the increased incidence of coronary diseases when compared to monounsaturated fatty acids, especially oleic (18:1n-9) and the series omega 3 and 6. Souza *et al.*[31] and Silva *et al.*[32] found low values of IA and IT for sacha inchi nut and perilla oil, respectively, emphasizing the direct correlation between the lowest ratio and an attenuated risk of coronary disease.

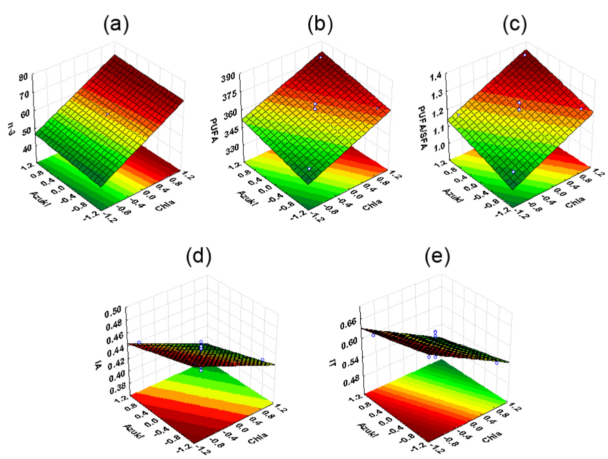

Figure 1. Response surfaces to sum, proportion and indices of fatty acids. n-3: sum of fatty acid from omega-3 series; PUFA: polyunsaturated fatty acids; SFA: total of saturated fatty acids; IA: atherogenicity index; IT: thrombogenicity index.

Figure 2 presents PCA. The principal components 1 and 2 (PC1 and PC2) were selected due to their statistical significance ($p < 0.05$). PC1 explained 55.51% of data variance, and loadings indicated a positive contribution of the contents of n-3 series fatty acids (0.9012), total PUFA (0.9865) and PUFA / SFA ratio (0.9724), which characterized samples C and D (formulations with higher contents of chia). A negative contribution of IA (–0.9377) and IT (–0.9866) was observed on PC1, which characterized formulations A and B (formulations with lower contents of chia).

PC2 was responsible for 17.11% of the data variance, and there was the separation of sample E on the upper region of the graph, which involves the 5 replicates of the central point (tests 5, 6, 7, 8 and 9). These formulations

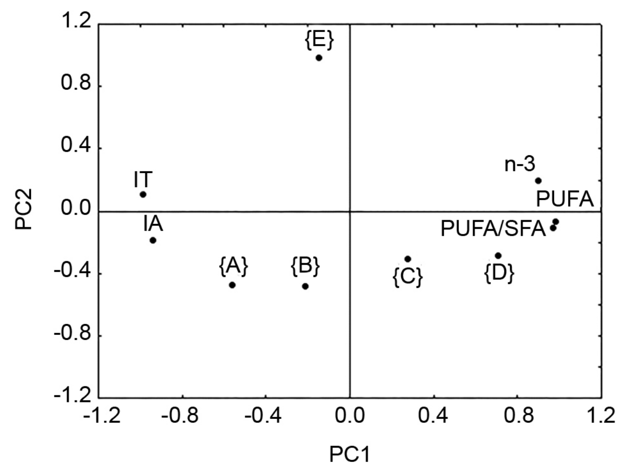

Figure 2. Principal components analysis for complete 2^2 experimental design with central point in quintuplicate. PC: principal component; A: test 1; B: test 2; C: test 3; D: test 4; E: tests 5, 6, 7, 8 and 9; n-3: sum of fatty acid from omega-3 series; PUFA: polyunsaturated fatty acids; SFA; total saturated fatty acids; IA: atherogenicity index; IT: thrombogenicity index.

present the intermediate values for n-3 series fatty acids, PUFA and PUFA / SFA ratio. Increased PUFA and n-3 in the cake formulations are associated with the high contents of alpha-linolenic fatty acid in the chia.[8]

Figure 3 shows the desirability function for the following constrictions: maximum value of fatty acids from n-3 series, total PUFA and PUFA / SFA ratio (equation 3); and minimum value of IA and IT indices (equation 4). The highest levels of chia and azuki flours were described as a point of major desirability (test 4). The higher concentrations of the factors studied were determinant for greater composition of fatty acid, such as higher contents of PUFA and fatty acids from n-3 series. The nutrient with the greatest increase was the alpha-linolenic fatty acid whose content in test 4 was 153.28% bigger than in test 1, corroborating with results found by Souza *et al.*[30]

According to the Institute of Medicine,[33] the contribution of alpha-linolenic fatty acid to the dietary reference intakes were determined for adults (1.6 g day^{-1}), using a portion of 60 g of cake (equivalent to one slice). The values were 12.63, 13.94, 19.07, 20.46 and 16.70%, for formulations A, B, C, D and E, respectively. The greatest contribution was

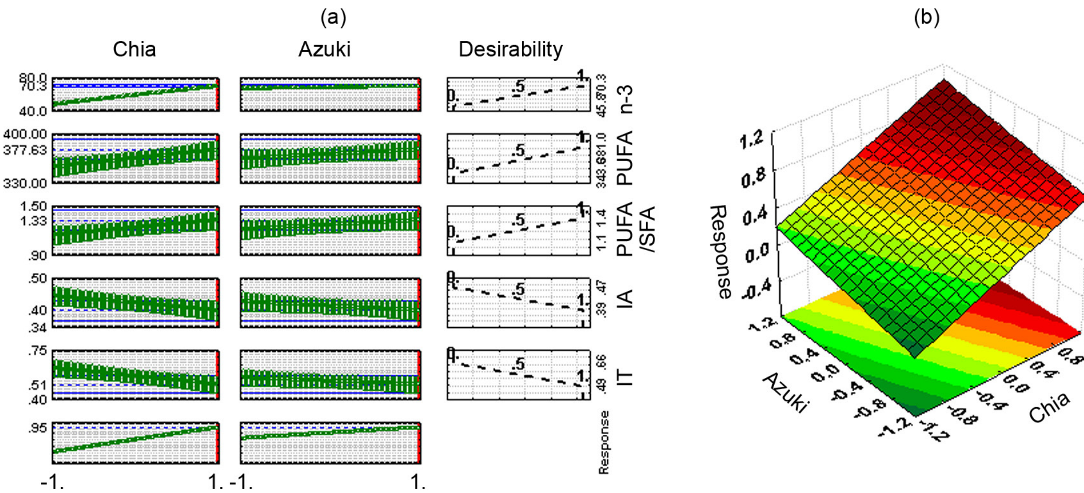

Figure 3. (a) Conditions of restrictions and responses of the desirability function for complete 2^2 experimental design with central point in quintuplicate. n-3: sum of fatty acid from omega-3 series; PUFA: polyunsaturated fatty acids; SFA; total saturated fatty acids; IA: atherogenicity index; IT: thrombogenicity index; (b) response surface for the optimum region using the application of desirability functions.

presented by formulation D, which has the highest level of both factors chia and azuki flour.

Table 6 shows fatty acid composition of optimum experimental point by desirability function (formulation D) and of control product. Both samples presented the same fatty acids, however, there were some significant differences in contents of butyric acid (4:0), capric acid (10:0), lauric acid (12:0), heptadecanoic acid (17:0), oleic acid (18:1n-9), linoleic acid (18:2n-6) and arachidic acid (20:0). The main difference was observed for alpha-linolenic acid, which increased 188.03% in optimum formulation when compared to control cake. As a result, PUFA contents also showed an increase in the optimum formulation (18.16%). Furthermore, atherogenicity index and thrombogenicity index showed lower values for optimum point when compared to control cake (21.57% IA and 41.18% IT, respectively).

Conclusions

Chemometric methods were very useful for improvement of fatty acids composition of gluten-free chocolate cake. Factor chia flour presented higher contribution for increasing n-3 series and polyunsaturated fatty acids, as well as nutritional indices. The models analyzed were significant and allowed obtaining the response surfaces. Principal components analyses distinguished samples with higher contents of chia flour. The formulation with higher contents of chia and azuki flour was considered as a point of greatest desirability. This formulation presented higher levels of n-3 fatty acid and PUFA, and lower indices of atherogenicity and trombogenicity than control cake. The addition of grains with high nutritional value is a great

Table 6. Fatty acid quantification and *t*-test for comparison between optimum experimental point and control cake

Fatty acid	Composition / (mg kg^{-1} of product)		*p*-Value
	Optimum experimental point	Control	
4:0	6.61 ± 0.17	4.94 ± 0.81	0.0515
6:0	1.52 ± 0.07	1.12 ± 0.11	0.0413
8:0	1.38 ± 0.06	1.06 ± 0.06	0.0139
10:0	2.53 ± 0.26	2.49 ± 0.13	0.8192
12:0	3.57 ± 0.40	3.71 ± 0.14	0.5734
14:0	22.88 ± 2.81	30.83 ± 1.38	0.0146
15:0	13.79 ± 1.16	18.93 ± 0.90	0.0013
16:0	156.20 ± 3.53	172.61 ± 3.24	0.0006
16:1n-9	5.78 ± 0.38	6.50 ± 0.17	0.0315
17:0	1.38 ± 0.13	1.28 ± 0.06	0.3798
18:0	65.16 ± 0.90	91.30 ± 3.82	0.0099
18:1n-9	239.69 ± 3.18	240.58 ± 2.88	0.3101
18:1n-7	9.90 ± 0.13	9.73 ± 0.08	0.2893
18:2n-6	307.26 ± 3.47	299.13 ± 7.48	0.0844
18:3n-6	3.45 ± 0.02	3.25 ± 0.14	0.0005
18:3n-3	70.25 ± 0.93	24.39 ± 1.38	0.0005
20:0	1.49 ± 0.05	1.42 ± 0.02	0.1892
22:0	5.15 ± 0.01	4.74 ± 0.07	0.0061
Sums and ratios of fatty acid			
n-3	70.25 ± 0.93	24.39 ± 1.38	0.0005
PUFA	386.11 ± 3.59	326.76 ± 7.60	0.0006
PUFA / SFA	1.37 ± 0.03	0.98 ± 0.03	0.0003
Indices of the quality of the lipid fraction			
IA	0.40 ± 0.03	0.51 ± 0.02	0.0017
IT	0.49 ± 0.02	0.84 ± 0.03	0.0007

Mean and standard deviation for optimum experimental point and control cakes; n-3: total fatty acids from omega-3 series; PUFA: total polyunsaturated fatty acids; SFA: total of saturated fatty acids; IA: index of atherogenicity; IT: index of thrombogenicity.

alternative to increase nutrients contents in food products, especially bakery ones.

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ANEXO 2

ARTIGO 2: Nutritional and bioactive compounds of adzuki beans cultivars using chemometric approach.

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Nutritional and bioactive compounds of adzuki bean cultivars using chemometric approach

Compostos nutricionais e bioativos de duas cultivares de feijão adzuki utilizando abordagem quimiométrica

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ABSTRACT

Azuki beans are small red grains rich in several essential nutrients used in traditional dishes in Asia that, nowadays present many applications around the world. This work aimed at evaluating angularis and niponensis cultivars from south region of Brazil. Both cultivars presented proximal composition similar to literature and the majority fatty acids were 18:2n-6, 16:0 and 18:3n-3. All samples showed polyunsatured fatty acids prevalence and nutritional indices and ratios considered adequate for biological system maintenance of a healthy organism. The grains presented significant contents of tocopherols and vitamin E activity, resulting in a high contribution to the dietary reference intake. Significant contents of iron, manganese and zinc were also found in the azuki beans, and they are very important mainly due to their function as cofactors in metabolic reactions. Phenolic compounds and flavonoids corroborated with other studies and contributed to the antioxidant activity. The principal components multivariate analysis allowed distinguishing the cultivars, and the two principal components could explain 92.28% of data variance.

Index terms: *Vigna angularis*; alpha-linolenic; principal component analysis; vitamin E; antioxidant activity.

RESUMO

O feijão adzuki consiste de pequenos grãos de cor vermelha, ricos em diversos nutrientes essenciais e que são utilizados em pratos tradicionais na Ásia e, atualmente têm várias aplicações ao redor do mundo. Neste trabalho, objetivou-se avaliar os cultivares angularis e niponensis produzidas na região sul do Brasil. Ambas os cultivares apresentaram valores de composição proximal similares aos encontrados na literatura e os ácidos graxos majoritários foram 18:2n-6, 16:0 e 18:3n-3. Todas as amostras apresentaram prevalência de ácidos graxos poli-insaturados, bem como razões e índices nutricionais adequados. Os grãos apresentaram teores significativos de tocoferóis e atividade de vitamina E, resultando em alta contribuição para a ingestão de referência diária. Concentrações de ferro, manganês e zinco também foram importantes, em razão das suas funções como cofatores em reações metabólicas. Compostos fenólicos e flavonoides corroboraram com outros estudos e contribuíram para atividade antioxidante. A análise multivariada de componentes principais permitiu distinguir as cultivares, e os dois componentes principais selecionados puderam explicar 92,28% da variância total dos dados.

Termos para indexação: *Vigna angularis*; alfa-linolênico; análise de componentes principais; vitamina E; atividade antioxidante.

INTRODUCTION

Adzuki, azuki and small red beans are the common names for several cultivars of *Vigna angularis* (Willd.) Ohwi & Ohashi spice. There is a large number of varieties of adzuki beans around the world, mainly in Asiatic Countries, and their characteristics may depend on grain size and color, genetic factors, cultivar type, cultivation and harvest time, climate and region where it was cultivated. This bean has been used in traditional Japanese confections, such as *wagashi*, *youkan*, *manju* and *amanatto*. In Chinese Medicine, adzuki beans have commonly been used to treat diuretic functions, and other disease such as dropsy and beriberi. Its medical application has been reported also in Korea (Yousif et al., 2003; Yoshida et al., 2009). Furthermore, adzuki beans, as well as many other types of beans, are considered source of carbohydrates, protein, fiber, vitamins and minerals (Palombini et al., 2013; Gohara et al., 2014a; Lam-Sanchez, 1990).

The lipid fraction of adzuki beans is composed mostly of unsaturated fatty acids (Yoshida et al., 2008) and presents low contents of saturated fatty acids. This is considered a positive point, according to the Institute of Medicine (2002/2005), who established that the consumption of saturated fatty acids should be avoided in a balanced diet. Important unsaturated fatty acids were found by Yoshida et al. (2010), their results ranged from 31.9 to 32.7% of linoleic fatty acid (18:2n-6) and from 25.4 to 26.8% of alfa-linolenic (18:3n-3). These fatty acids are considered essentials because they cannot be metabolized by the human body and must be obtained from the diet (Ratnayake; Galli, 2009).

The bioactive compounds in the adzuki bean seed coat have received significant interest because of their health-promoting antioxidant properties (Lin; Lai, 2006). The presence of antioxidant compounds such as flavonoids and tocopherols in foods is very important; the latter may reduce the risk of heart diseases, type 2 diabetes and cancer (Carocho; Ferreira, 2013). Maruyama et al. (2008) suggested that the consumption of the adzuki bean is linked to a reduced risk of lifestyle related diseases in humans. The isomers of tocopherols show different activities of vitamin E, and the isomer α-tocopherol is the most biologically active. These compounds are found only in plant foods (Yada; Lapsley; Huang, 2011).

Principal component analysis (PCA) is a very useful chemometric tool. This technique allows organization of data in a simpler and easier way to understand structure, and it's possible to extract additional information when compared to univariate analysis. The principal components (PC) are mutually orthogonal, and the explained variance decreases with the increase in the number of PC (Correia; Ferreira, 2007). This method has been applied in many studies to characterize foodstuffs and natural products (Souza et al., 2015; Pagamunici et al., 2014; Silva et al., 2015; Fuchs et al., 2013; Nishiyama et al., 2014; Souza et al., 2014).

This study investigated the chemical characterization of the two principal cultivars of adzuki bean most consumed in Brazil, both produced in Maringa city, in Parana State. The grains were analyzed concerning their proximal and fatty acids composition, isomers of tocopherols, minerals, bioactive compounds and nutritional aspects. The PCA was applied to extract more information from the data.

MATERIAL AND METHODS

Sampling

Adzuki beans (*Vigna angularis*) cultivars: angularis and niponensis, were acquired directly from the producer. The grains were cultivated in the region of Maringa city, Parana State, Brazil (23.4000º S, 51.9167º W); on September/October 2013. The mean altitude of the region is 596 m and September is the beginning of Spring Season. The harvest of azuki beans was performed on December 2013, when the levels of rainfall ranged from 50 to 100 mm (Instituto Nacional de Meteorologia - INMET, 2012). Sampling consisted of 3 batches of 5 kg, harvested at intervals of 10 days. All samples of adzuki beans were ground separately in a hammer mill to obtain a flour which was sieved to obtain a particle-size distribution between 14 and 16 mesh. Each sample of the two varieties: angularis and niponensis, were vacuum packed, protected from light and stored in a freezer at -18 ºC until analysis.

Proximal composition and crude energy

The moisture, ash and crude protein contents were determined according to Cunniff (1998) using factor 6.25 to convert the percentage of nitrogen into crude protein content (Gohara et al., 2014b). The total lipids were extracted and determined according to Bligh and Dyer (1959). The total carbohydrates were calculated by difference.

The caloric value was determined using the indirect method, which is based on the application of conversion factors for the main nutrients of the product (carbohydrates, crude protein and lipids), according to Holands et al. (1994). The results were obtained in cal g^{-1} of food and converted to kJ kg^{-1} product.

Fatty acid composition

The determination of fatty acid composition was performed using the lipid fraction extracted from the grain; these lipids were derivatized and converted into fatty acid methyl esters (FAME) according to Hartman and Lago (1973). The FAME were separated in gas chromatograph CP-3380 (Varian, USA) fitted with a flame ionization detector and a CP 7420-select Fame fused-silica capillary column (100 m x 0.25 mm x 0.25 µm cyanopropyl). The gas flows were: carrier gas hydrogen 1.4 mL min^{-1}, make-up gas nitrogen 30 mL min^{-1}, synthetic air 300 mL min^{-1} and flame gas hydrogen 30 mL min^{-1}; the sample was injected in split ratio of 1:100. The injector and detector temperature was 235 °C. The column temperature was maintained at 165 °C for 4 min, increased to 185 °C at 4 °C min^{-1} and maintained for 5 min, then increased from 185 °C to 225 ºC at 10 °C min^{-1} and maintained for 10 min. The same chromatographic conditions were used previously to analyze other plant foods (Souza et al., 2013).

The retention times were compared to those of standard methyl esters (Sigma, USA) for identification of fatty acids. The quantification of fatty acids was performed using tricosanoic acid methyl ester (Sigma, USA) as an internal standard, following Joseph and Ackman (1992). The peak areas were determined with software Star 5.0 (Varian, USA) and the concentrations were expressed in mg FA per g of total lipid. The limits of detection (LOD) and quantification (LOQ) were estimated by triplicate analysis of diluted methyl arachidate standard solution (1.0 mg mL^{-1}), considering the signal-noise rate relative to the background signal as 3 and 10, respectively (Analytical Methods Committee, 1987). The sums of some fatty acids (FA) classes were also determined: total fatty acids from n-6 and n-3 series (called as n-6 and n-3, respectively); total saturated fatty acids (SFA); total monounsaturated fatty acids (MUFA); and total polyunsaturated fatty acids (PUFA).

Indices of the nutritional quality of lipids

A better approach to the nutritional evaluation of fat is the utilization of some indices based on the functional effects of fatty acid composition. The fatty acids requested to calculate the indices were: 12:0 (lauric acid), 14:0 (myristic acid), 16:0 (palmitic acid), 18:0 (stearic acid), 18:1n-9 (elaidic acid), 18:2n-6 (linoleic acid), 20:4n-6 (arachidonic acid), 18:3n-3 (alpha-linolenic acid), 20:5n-3 (eicosapentaenoic acid), 22:5n-3 (docosapentaenoic acid), 22:6n-3 (docosahexaenoic acid), and the sums n-6 (total omega-6 fatty acids), n-3 (total omega-3 fatty acids) and MUFA (total monounsaturated fatty acids). The indices determined in this study were: Index of Atherogenicity (IA) = [(12:0 + (4 x 14:0) + 16:0)] / (MUFA + n-6 + n-3), and Index of Thrombogenicity (IT) = (14:0 + 16:0 + 18:0) / [(0.5 x MUFA) + (0.5 x n-6) + (3 x n-3) + (n-3:n-6)], according to Ulbricht and Southgate (1991); and ratio between Hypocholesterolemic and Hypercholesterolemic fatty acids ratio (HH) = (18:1n-9 + 18:2n-6 + 20:4n-6 + 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3) / (14:0 +16:0), according to Santos-Silva, Bessa and Santos-Silva (2002).

Tocopherols isomers analysis

All samples were saponified and the isomers of vitamin E were extracted according to the methodology described by Souza et al. (2014). Under stirring and protected from light, 50.0 mL of ethanol, 5.0 mL of aqueous solution of ascorbic acid 10% (w/v), 10 mL of aqueous solution of potassium hydroxide 60% (w/v) and 25 mL of water were added to 2.00 g of the ground sample. The unsaponifiable material extraction was performed with hexane and water. The organic phase containing the tocopherol fraction was collected and the solvent was evaporated under vacuum at 50 °C. The residue was dissolved in methanol to obtain an extract solution.

The tocopherol isomers (δ-tocopherol, (β+γ)-tocopherol and α-tocopherol) were determined using high efficiency liquid chromatography (Varian) with a C18 column (Microsorb, 250 mm × 4.6 mm, 5 μm particles) fitted with a scanning UV/Vis detector. The mobile phase used was methanol/dichloromethane in the ratio 85:15 (v/v); and the flow rate was 0.8 mL min^{-1} (Kornsteiner; Wagner; Elmadfa, 2006). The tocopherols were quantified using external standard method, according to Instituto Adolfo Lutz (1985). The sum of isomers β-tocopherol and γ-tocopherol was determined, since the separation of these is not possible by this methodology (Kornsteiner; Wagner; Elmadfa, 2006). The LOD and LOQ were estimated by triplicate analysis of standards calibration curve for each isomer of tocopherols, considering the signal-to-noise ratio relative to the background signal as 3 and 10, respectively (Analytical Methods Committee, 1987).

Vitamin E activity

The activity of vitamin E in the samples was performed according to Kornsteiner, Wagner and Elmadfa (2006): the value found for each isomer, in milligrams, was multiplied by the equivalent factor for α-tocopherol (α-TE). For α-tocopherol, α-TE = mg x 1.0; for (β + γ)-tocopherol, α-TE = mg x 0.25; and for δ-tocopherol, α-TE = mg x 0.01.

Mineral quantification

For the mineral composition analysis, all the azuki beans samples were digested by the dry method (Association Of Analytical Chemists-AOAC, 1995) and Ca, Cu, Fe, Mn, and Zn were quantified in an atomic absorption spectrophotometer AA240FS (Varian, USA) as mg of mineral per 100 g of sample using technical parameters of calibration according to Table 1. The LOD and LOQ were estimated by triplicate analysis of standards calibration curve for each mineral, considering the signal-to-noise ratio relative to the background signal as 3 and 10, respectively (Analytical Methods Committee, 1987).

Calculation of the dietary reference intake

The Dietary Reference Intake (DRI) is a percentage estimate of the daily nutrient requirements per age and gender, established by the Institute of Medicine (2000) for individuals aged over 12 months. The DRI of vitamin E was determined as the mean amount in 100 g portion of azuki beans.

Table 1: Technical parameters of calibration for atomic absorption spectrophotometer.

Element	Wavelength/nm	Slit width/nm	Gas - Flow (L min^{-1})	Burner height (mm)
Ca	239.9	0.2	Nitrous oxide – 11.0; Acetylene – 6.35	13.5
Cu	324.8	0.5	Air – 13.5; Acetylene – 2.0	13.5
Fe	248.3	0.2	Air – 13.5; Acetylene – 2.0	13.5
Mn	279.5	0.2	Air – 13.5; Acetylene – 2.0	13.5
Zn	213.9	1.0	Air – 13.5; Acetylene – 2.0	13.5

Extract preparation and evaluation of bioactive properties

The methanolic extracts were obtained from the varieties of adzuki beans. Each sample (10 g) was submitted to stirring process with 100 mL of methanol (m/v) at 25 °C and 150 rpm for 4 h and subsequently filtered through a filter paper (Whatman No. 4). Each extract was evaporated at 45 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove the methanol. The dried extract was maintained in amber vials with nitrogen atmosphere (N_2). After, the extracts were redissolved in methanol solvent (final concentration 5 mg mL^{-1}) for antioxidant activity evaluation. The final methanolic solutions obtained were further diluted to different concentrations to be submitted to distinct bioactivity evaluation in vitro assays.

The results for 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assays were expressed in IC_{50} values (sample concentration providing 50% of antioxidant activity) for DPPH radical-scavenging. These assays were evaluated using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and the results were calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution.

Total phenolic compounds were determined in triplicate according to the method proposed by Shahidi and Naczk (1995). A 0.25 mL aliquot of extract solution (2.5 mg mL^{-1} in methanol) was mixed with 0.25 mL of Folin–Ciocalteu's reagent previously diluted with water 1:1 (v/v), 0.5 mL of a saturated sodium carbonate solution and 4 mL of water. The reaction tubes, in triplicates, were wrapped in aluminum foil and kept at 25 ºC for 25 min in the dark. The tubes were centrifuged for 10 min and the absorbance of the supernatant fraction was measured at 725 nm using a spectrophotometer (Cary Win UV 50, Varian). Gallic acid (GA) was used as a standard and the results were expressed as gallic acid equivalents (mg GAE 100 g^{-1} sample).

Flavonoids analyses were performed according to the method proposed by Buriol et al. (2009). The same solution used in total phenolic assay was utilized for total flavonoids assay, with addition of aluminum chlorate solution at 5% (m/v). The yellow complex formed was measured at 425 nm. The results were expressed in quercetin equivalents (mg QE 100 g^{-1} sample).

Statistical and multivariate analysis

Proximal composition, crude energy, fatty acid composition, indices of the nutritional quality of lipids, tocopherols isomers, vitamin E activity, mineral quantification and antioxidant proprieties were carried out in triplicate for the three different batches. The results were compared using the t Student's test with 5% ($p<0.05$) significance level for rejection of the null hypothesis.

In multivariate analysis, the individual values of each replicate of the three batches analyzed (n = 9) were divided into arrays of data. The samples were arranged in rows (n = 18) and the results of the analyses were selected for principal component analysis (PCA; n = 19) in columns. These data used for PCA were: PUFA:SFA (ratio between polyunsaturated fatty acids and saturated fatty acids), n-6:n-3 (ratio between total omega-6 fatty acids and omega-3 fatty acids), and HH (ratio between Hypocholesterolemic and Hypercholesterolemic fatty acids) ratios; nutritional indices of lipids (index of atherogenicity and thrombogenicity – IA and IT); mineral composition (Ca, Cu, Fe, Mn and Zn); isomers of tocopherols (α-tocopherol, (β + γ)-tocopherol and δ-tocopherol); vitamin E activity; and antioxidant proprieties (DPPH radical-scavenging activity, total phenolic and total flavonoids compounds). The data were pre-processed by auto-scaling. This process was subsequently applied to the principal components analysis using the algorithm NIPALS. It was decomposed into a two-dimensional graph of scores (samples) and loadings (variables). The statistical software Statistica, version 7.0 (Statsoft, 2007), was used with a 5% ($p<0.05$) significance level for rejection of the null hypothesis. This same significance level was used to select principal components for the Principal Components Analysis.

RESULTS AND DISCUSSION

The results of the proximal composition and crude energy analyses (Table 2) were similar to those reported by Palombini et al. (2013) and Lam-Sanchez et al. (1990) for different cultivars of beans. Durak et al. (2013) found similar contents of protein, carbohydrates and total lipids (25.00, 55.00% and 0.45%, respectively) for adzuki beans. Angularis cultivar presented lower contents ($p<0.05$) of crude protein and moisture than niponensis cultivar. Yousif et al. (2003) studied the effect of storage of adzuki beans and their results suggested that detrimental changes occur in starch and protein of adzuki beans stored under unfavorable conditions; therefore, the proximal composition can influence the characteristics of beans and increase the shelf life of angularis cultivar. Nutritional composition of beans is also important to make some enriched food products. A recent study using adzuki flour in chocolate cakes showed a great contribution of these beans to increase contents of ash and crude protein in the final product (Gohara et al., 2014b).

Table 3 shows the conditions used to gas chromatography, high performance liquid chromatography and atomic absorption spectrophotometer. These analytical

Table 2: Proximal composition and crude energy in adzuki beans cultivars.

Parameters	Adzuki beans cultivars	
	Angularis	Niponensis
Moisture (g $100g^{-1}$)	13.07b±0.13	14.48a±0.08
Ash (g $100g^{-1}$)	3.85a±0.03	3.59b±0.04
Crude protein (g $100g^{-1}$)	20.36b±0.30	22.94a±0.22
Total lipids (g $100g^{-1}$)	0.45a±0.02	0.44a±0.03
Carbohydrates (g $100g^{-1}$)	62.26a±0.33	58.55b±0.24
Crude energy (kJ Kg^{-1})	1400.82a±0.04	1381.37b±0.06

Results expressed as mean ± standard deviation for analysis in triplicate of three batches. Means followed by the same letters in rows do not differ by t Student's test ($p < 0.05$). n = 9 replicates.

Table 3: Conditions of the selecting operation system for gas chromatography, high performance liquid chromatography and atomic absorption spectrophotometer.

Parameters	LOD	LOQ
	Fatty acid (mg g^{-1})	
	0.15	0.50
	Isomers of tocopherols (mg g^{-1})	
δ-Tocopherol	$4.55x10^{-5}$	$1.52x10^{-4}$
β+γ-Tocopherol	$3.46x10^{-5}$	$1.15x10^{-4}$
α-Tocopherol	$1.03x10^{-4}$	$3.44x10^{-4}$
	Minerals (mg g^{-1})	
Ca	$3.47x10^{-5}$	$1.15x10^{-4}$
Cu	$1.16x10^{-4}$	$7.31x10^{-2}$
Fe	$1.44x10^{-2}$	$4.79x10^{-4}$
Mn	$2.33x10^{-4}$	$7.76x10^{-4}$
Zn	$1.00x10^{-3}$	$3.33x10^{-3}$

LOD: limits of detection; LOQ: limits of quantification.

parameters allowed the detection and quantification of all the compounds showed in Tables 4, 5 and 6.

Table 4: Fatty acid absolute quantification, sums, ratios and indices of the nutritional quality of lipids in adzuki beans cultivars.

Fatty acid	Adzuki beans cultivars	
(mg g^{-1})	Angularis	Niponensis
14:0	2.21a±0.02	1.30b±0.03
14:1n-7	1.31a±0.02	0.88b±0.05
16:0	225.55a±2.00	226.84a±3.40
16:1n-7	2.84a±0.03	1.77b±0.06
17:0	2.88a±0.09	2.56b±0.04
18:0	56.67a±0.63	27.01b±0.37
18:1n-9	79.60a±0.98	40.88b±0.47
18:1n-7	18.29a±0.31	18.19a±0.21
18:2n-6	351.81a±3.36	390.91a±31.09
18:3n-3	160.58b±1.92	173.32a±3.09
20:0	7.97a±0.24	3.09b±0.02
18:3n-6	0.98a±0.04	0.77b±0.03
22:0	7.49b±0.29	11.55a±0.34
24:0	2.55b±0.04	4.99a±0.26
Sums and ratios of fatty acids		
SFA	305.33a±2.14	277.33b±3.44
MUFA	102.04a±1.03	61.72b±0.52
PUFA	513.37b±3.87	584.13a±31.14
n-6	352.78b±3.36	410.81a±31.09
n-3	160.58b±1.92	173.32a±1.75
n-6/n-3	1.46a±0.02	1.42b±0.08
PUFA/SFA	1.68b±0.01	2.11a±0.05
Index of the nutritional quality of lipid		
IA	0.38a±0.01	0.36b±0.01
IT	0.40a±0.01	0.34b±0.01
HH	2.60a±0.01	2.65a±0.01

Results expressed as mean ± standard deviation for analysis in triplicate of three batches. Means followed by the same letters in rows do not differ by t Student's test ($p < 0.05$). SFA: total saturated fatty acids, MUFA: total monounsaturated fatty acids, PUFA: total polyunsaturated fatty acids, n-6: total omega-6 fatty acids and n-3: total omega-3 fatty acids. IA: Index of atherogenicity. IT: Index of thrombogenicity. HH: ratio between Hypocholesterolemic and hypercholesterolemic fatty acids. n = 9 replicates.

The fatty acid composition of both cultivars of adzuki beans was similar, as shown in Table 4.

The majority fatty acids were 18:2n-6, 16:0 and 18:3n-3. Fatty acids classes have been largely studied by many researchers, as well as the effect of these compounds to the human body; the relationship between fatty acids quality and the body function may be verified using some nutritional indices and ratios (Ratnayake; Galli, 2009; Ulbricht; Southgate, 1991; Santos-Silva; Bessa; Santos-Silva, 2002; Durak et al., 2013). The nutritional indices calculated in this study with adzuki beans, IA and IT, presented better values than new formulations of some foodstuffs (hamburguer, food bar and cookies) containing whole flours of promising vegetables, such as chia and flaxseed (Yoshida et al., 2009; Souza et al., 2015; Pagamunici et al., 2014a; Pagamunici et al., 1024b). According to PCA analysis (Figure 1), IA and IT presented great contribution (0.9853 and 0.9929, respectively) to PC1 and influenced the separation of adzuki cultivars. Niponensis cultivar showed lower indices than angularis and its lipid fraction can be considered healthier (Ulbricht; Southgate, 1991).

The PUFA:SFA and n-6:n-3 ratios also presented high contribution (0.9923 and -0.9947) on PC1 to separate the two cultivars. Niponensis cultivar presented higher PUFA:SFA ratio and n-6:n-3 ratio closer to 1:1. According to Simopoulos (2011), the prevalence of polyunsaturated fatty acids is associated with a lower risk of cardiovascular disease, which makes the lipid quality of niponensis cultivar better than angularis.

According to Table 4 it was not verified statistical difference ($p<0.05$) between the values of HH ratios of the two samples. HH ratios presented a great value and significant contribution on PC2 (0.7322, Figure 1) for both cultivars of adzuki beans. Higher values for HH ratio are important to human health due to the hypocholesterolemic effects (Simopoulos, 2011).

As shown in Table 5, higher contents of tocopherol isomers and vitamin E activity were found in angularis cultivar. PCA presented positive contribution of δ-tocopherol (0.8932), (β+γ)-tocopherol (0.9859), α-tocopherol (0.7401) and vitamin E activity (0.7368) on PC1, causing the separation of angularis cultivar (Figure 1). Yoshida et al., (2009) found slightly lower results for δ-tocopherol in adzuki beans. Besides, other studies analyzing common beans (*Phaseolus vulgaris*) found lower contents of total tocopherols and lower activity of vitamin E than adzuki beans cultivars (Boschin; Arnold, 2011). The isomer α-tocopherol is the most biologically active form of vitamin E (Yada; Lapsley;

Huang, 2011), and it is related to the protection of unsaturated lipids present in biological systems, due to its lipophilic characteristic (Taipina et al., 2009). The DRI showed that adzuki beans present a high contribution of vitamin E for all populations analyzed. These levels ranged from 31% for women in lactation until upper 100% for children (1-3 years) (Institute of Medicine, 2000).

Table 6 showed greater contents of minerals Ca, Cu, Mn and Zn in angularis cultivar. This fact can be clearly observed in Figure 1, where the high contributions of the minerals Ca (0.9832), Cu (0.9721), Mn (0.9824) and Zn (0.8562) in PC1 could distinguish angularis cultivar. Niponensis cultivar presented high contribution of Fe (-0.9824, Figure 1 and Table 6). However, all samples presented great levels of iron, manganese and zinc, which are classified as trace minerals. These minerals are extremely important for the maintenance of biological systems because they act as cofactors in metabolic reactions (Hathcock, 2004).

The *in vitro* antioxidant properties of adzuki cultivars beans were evaluated and presented in Table 7. The antioxidant potential of extracts obtained from the two adzuki beans cultivars was similar to the results found for eight Brazilian bean cultivars (Palombini et al., 2013a). The total phenolic compounds of the adzuki beans cultivars were lower than the results found by Zhao et al. (2014) for some common legumes, but very similar to results obtained by Palomini et al. (2013b) for seven Brazilian rice cultivars; and for new cultivars of pseudocereals (Palombini et al., 2013c). Total phenolic compounds presented high contribution on PC1 (0.9823, Figure 1) to Angularis cultivar. On the other hand, Table 7 showed higher contents of total flavonoids in Niponensis than Angularis cultivar, and this antioxidant sort presented high contribution on PC1 (-0.8390) to separate Niponensis cultivar. The lowest concentration necessary for 50% inhibition of DPPH (Table 7) was obtained for Angularis cultivar, while Nioponensis cultivar presented the worst results.

Principal component analysis allowed the selection of PC1 and PC2, which explained 92.28% of the data variance of the parameters analyzed (Figure 1). PC1 showed the greatest data explanation (81.59%) and could make a clear separation of adzuki cultivars.

Table 5: Tocopherol composition of azuki beans cultivars analyzed.

Cultivars	δ-Tocopherol (mg 100 g^{-1})	β+γ-Tocopherol (mg 100 g^{-1})	α-Tocopherol (mg 100 g^{-1})	Total tocopherol content (mg 100 g^{-1})	α-TE
Angularis	4.28a±0.02	1.44a±0.06	0.84a±0.03	6.57a±0.07	1.22a±0.06
Niponensis	4.00b±0.15	1.05b±0.07	0.83a±0.01	5.88b±0.17	1.14b±0.08

Results expressed as mean ± standard deviation for analysis in triplicate of three batches. Means followed by the same letters in columns do not differ by t Student's test ($p < 0.05$). α-TE: α-tocopherol equivalents. n = 9 replicates.

Table 6: Mineral composition of adzuki beans cultivars.

Adzuki beans cultivars	Mineral (mg 100g^{-1})				
	Ca	Cu	Fe	Mn	Zn
Angularis	276.45a±20.77	1.11a±0.11	3.98b±0.37	2.39a±0.22	2.96a±0.44
Niponensis	95.60b±1.86	0.47b±0.01	6.05a±0.23	0.49b±0.02	2.05b±0.05

Results expressed as mean ± standard deviation for analysis in triplicate of three batches. Means followed by the same letters in columns do not differ by t Student's test ($p < 0.05$).

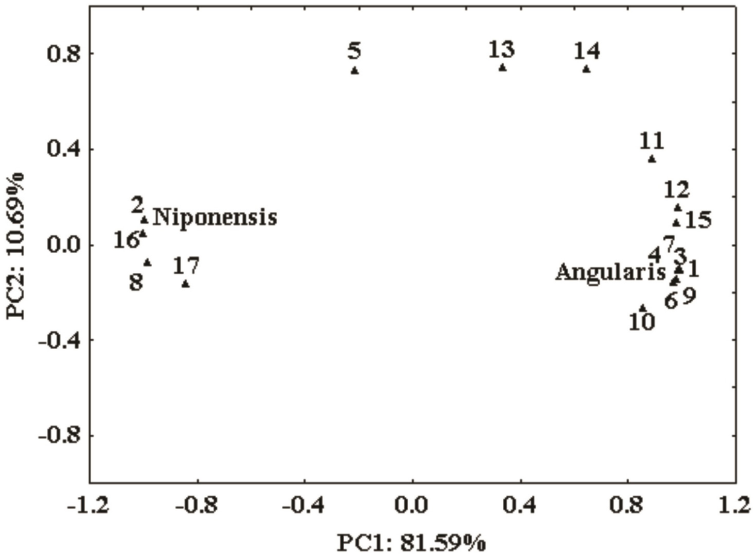

Figure 1: Principal components analysis of select data to characterize the adzuki beans cultivars. PC: Principal component. Scores (samples): var. Angularis and var. Niponensis. Loadings (analysis): 1 – PUFA:SFA (ratio between total polyunsaturated fatty acids and total saturated fatty acids); 2 – n-6:n-3 (ratio between total omega-6 fatty acids and total omega-3 fatty acids); 3 – IA (index of atherogenicity); 4 – IT (index of thrombogenicity); 5 – HH (ratio between hypocholesterolemic and hypercholesterolemic fatty acids); 6 – Calcium; 7 – Copper; 8 – Iron; 9 – Magnesium; 10 – Zinc; 11 – δ-tocopherol; 12 – (β + γ)-tocopherol; 13 – α-tocopherol; 14 – Vitamin E activity; 15 – Total phenolic compounds; 16 – DPPH radical-scavenging activity; 17 – Total flavonoid compounds.

Table 7: Antioxidant properties of the adzuki beans cultivars.

Adzuki beans	Folin-Ciocalteu assay (mg GAE 100 g^{-1} sample)	Flavonoids (mg QE 100g^{-1} sample)	EC_{50} values (mg mL^{-1}) DPPH radical-scavenging activity
Angularis	81.87a±4.39	7.21b±0.83	148.83b±4.83
Niponensis	59.94b±1.57	9.03a±0.70	243.41a±3.01

Results expressed as mean ± standard deviation for analysis in triplicate of three batches. Means followed by the same letters in columns do not differ by t Student's test ($p < 0.05$). GAE: Gallic acid equivalents; QE: quercetin equivalents. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity. n = 9 replicates.

CONCLUSIONS

Cultivars of adzuki beans are excellent sources of many essential compounds. The nutritional indices of the lipid fraction showed that these grains present anti-atherogenic, anti-thrombogenic and hypocholesterolemic effects, and the ratios PUFA: SFA and n-6:n-3 were considered appropriate for biological system maintenance of a healthy organism. The content of tocopherols was higher than amounts found in common beans. The values of DRI enable the evaluation of the nutritional potential of adzuki beans. Both cultivars presented good levels of iron, manganese and zinc, which are classified as trace minerals and responsible for the maintenance of biological systems. The antioxidant properties were very similar to the other kinds of beans, rice and pseudo-cereals cultivars from the literature. The multivariate analysis allowed distinguishing batches of angularis and niponensis cultivars, as well as evaluating the importance of the parameters. The traditional use of adzuki beans in the eastern dietary is promising due to their intrinsic characteristics.

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