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Full Length Research Paper

Significance of fluorescent inter simple sequence repeat technique for distinguishing domestic animal breeds

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Conservation of farm animal genetic resources is of fundamental importance for the study of the relationships among breeds. The aim of this study was to evaluate the usefulness of the nuclear fluorescence inter simple sequence repeat (FISSR) markers in order to shed light on the genetic biodiversity of domestic animals. Two modifications of the original technique were made so as to make it more suitable for routine needs. The modified FISSR protocol was tested on different breeds of goat and donkey from Sardinia, a Mediteranean island known for its biodiversity. The two species are affected by different management problems in Sardinia: goats need a traceability of local products from different breeds, whereas donkeys are drastically reduced in number. The primers used were found to be very informative suggesting that the modified FISSR can be successfully applied in studies on different breeds of animal species without expensive experimentations. This method could be of interest in many geographic regions where there are more breeds of the same species with similar morphological features and different genetic pattern. The strongest point of this method is its low cost.

Key words: Fluorescence inter simple sequence repeat (FISSR), biodiversity, genetic variability, goat, donkey.

INTRODUCTION

The nuclear fluorescence inter simple sequence repeat (FISSR) may achieve new insights on the genetic variability of populations of different species, in particular those involved in animal production (Nagaraja et al., 2005). This method has been termed FISSR-PCR by the inventors, who tested its usefulness in plants (a large number of rice varieties) and insects (*Bombyx mori*). The results clearly showed that this method is an important

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tool for large scale screening of varieties/cultivars and high throughput genotyping in mapping genomes where genomic information is scanty or absent (Nagaraju et al., 2002).

The FISSR technique is less laborious when compared with other finger-printing methods and produces highly reproducible bands and results with low statistical error, leading to multilocus and highly polymorphic banding patterns. In addition, it does not require prior knowledge of DNA sequences and could be introduced as an effective tool to gain helpful genetic information from wild, reared and natural populations (Leighton, 2002; Luikart et al., 2003; Casu et al., 2006).

In the present study, two changes in the technique presented by Nagaraju et al. (2002) were introduced to make it more applicable to routine needs, reducing the number of primers and changing the kind of label for analysis by sequencer. Such modified technique was tested in goats (*Capra hircus*) and donkeys (*Equus asinus*), two species with different management problems (Dossa et al., 2007; Colli et al., 2013). All the samples came from Sardinia, an island rich in animal biodiversity. In the analysis of goat genotype, the finding of high FISSR polymorphism may allow the molecular traceability of the different breeds, and this may be important for the control of local products and constitutes a quick tool for the economic development of the different breeds. Donkeys were used in agriculture until the middle of the last century. From this period, there was a drastic reduction in their number due to agricultural mechanization. Autochthonous donkeys belong to two different breeds in Sardinia: the Sardinian and the Asinara donkeys. The Sardinian donkey shows a grey coat with a black cross on its back and is spread all over Sardinia. In contrast, Asinara donkey is an albino variant originating from Asinara island, north-western Sardinia. Both breeds are numerically reduced and considered in danger of extinction. According to the FAO risk status classification of animal genetic resources (www.fao.org), Sardinian donkey is an “endangered breed” and Asinara donkey is a “critical breed”. Differentiation of the two donkey breeds is commonly performed on the basis of the morphological traits. Consequently, a genetically characterization of these breeds could be very useful in the studies on the relationships among local farms for the estimation of their relative importance and the correct application of preservation programs, in order to conserve the total genetic variance for this species, as made for other species in the past (Allendorf et al., 2001; Bennewitz and Meuwissen, 2005; Safari et al., 2005; Usai et al., 2006; Kefena et al., 2011; Iquebal et al., 2013).

MATERIALS AND METHODS

All specimens (seven goats and eight donkeys) and sampling sites are shown in Figure 1. In contrast to the protocol of Nagaraju et al. (2002), where a set of eight degenerate primers (non-dyed) and one fluorescent dye-labeled nucleotide (dUTP TAMARA) were employed, in the present work, only two non-anchored labeled primers were used for FISSR-PCR reaction. A preliminary screening of 29 primers allowed identifying 2 primers that were used to genotype all the individuals: UBC864 and UBC868 (Table 1) labeled respectively with 6-FAM and HEX fluorescent dyes (Sigma-Aldrich, St. Louis, MO, USA). These were used for both goat and donkey. DNA was extracted from 600 µl frozen blood with a standard Salting Out protocol after prewashing for 10 min at room temperature with 600 µl Triton Blood Buffer (0.32 M sucrose, 10 mM Tris HCl, 5 mM $MgCl_2$, 1% Triton X 100). Then, the aliquot was centrifuged at 9,000 g for 8 min, the suspension discarded and the pellet resuspended in a standard lysis buffer suitable for blood extraction. Each 25 µl PCR mixture contained about 100 ng total genomic DNA, PCR buffer, 3.5 mM $MgCl_2$, 0.5 µM primer and 0.2 mM each dNTP, with 0.05 U/µl of Taq DNA polymerase (Sigma-Aldrich). Both positive and negative controls were used to assess the effectiveness of the PCR reagents. The thermal cycling conditions were set as follows: initial denaturation of 3 min at 94° C followed by 35 cycles of: 1 min at 94°C, 2 min at 45°C, 1 min at 72°C and a final extension of 5 min at 72°C. A visual checking was carried out by electrophoresis on 1.5% agarose/TBE gel stained with ethidium bromide (10 mg/ml) at 4 V/cm for 240 min, and all samples were shown to give reproducible band patterns. The samples were genotyped with ROX100 BV labeled molecular weight standard onto an ABI377 automated sequencer, carried out by an external sequencing core service (BMR Genomics, Padua, Italy). Each FISSR electropherogram peak pattern was converted into a binary matrix (1 for band presence and 0 for absence) assuming that each peak represents a single diallelic locus.

In order to overcome potential problems due to the small sampling plan, the underlying genetic population structure in our preliminary analysis was inferred using an individual-based approach (Luikart et al., 2003) using the Bayesian model-based clustering algorithms implemented in the software structure 2.2.3 (Pritchard et al., 2000). Ten independent analyses were performed to assess the reliability of the results. In each analysis, the number of clusters (K) was estimated by a range of possible values (K=2 up to 8). Structure analysis, which assumes that K is known in advance, was used to assess the occurrence of hierarchical levels of genetic structure. In a given dataset, structure identifies the uppermost hierarchical structure that corresponds to the minimum number of clusters that captures the major structure in the data (Pritchard and Wen, 2004). After this first round, data are partitioned into smaller datasets according to the best clustering solution, and subsequent rounds are performed on each subset of data. The procedure was reiterated until it was not possible to partition the data any further. The results of the model-based clustering was then compared with a principal coordinate analysis (PCA) performed by the program genalex 6.3 (Peakall and Smouse, 2006) on a matrix of inter-individual distances via a covariance matrix with a data standardisation method, in order to avoid artifacts due to violation of the model assumptions (Hardy-Weinberg and linkage equilibrium) or isolation by distance (Guillot et al., 2009). Genetic structuring was not investigated by a hierarchical analysis of molecular variance (AMOVA) because both goats and donkeys had populations of a single individual.

RESULTS

Based on a total of 15 sequences, clearly reproducible patterns were found in the two species that yielded a total of 54 peaks in goats, indicating polymorphic sites, (25 of which for UBC864 and 29 for UBC868) and a total of 79

Table 1. Primer names and sequences used in the FISSR analysis, number of polymorphic bands per primer and range of molecular weight in base pairs (bp) amplified by FISSR-PCR.

Primer	Sequence (5'-3')	Goats		Donkeys	
		Number of bands	Size range of bands (bp)	Number of bands	Size range of bands (bp)
UBC864	ATGATGATGATGATGATG	25	500-1100	31	450-1060
UBC868	GAAGAAGAAGAAGAAGAA	29	350-1078	48	320-1080

Figure 1. Sampling locations of *Capra hircus* and *Equus asinus*. The sampling site of each specimen is reported. For further details see Table 1.

peaks in donkeys (31 of which for UBC864 and 48 for UBC868). Private bands were detected both in goats and donkeys, and interestingly, the donkey AST12 shows four private bands.

In goats, the size of bands ranged from 500 to 1100 bp for the primer UBC864 and 350 to 1078 bp for the primer UBC868 (Table 1). The uppermost hierarchical level of genetic structuring, as estimated from the first round of structure analyses (Figure 2a), resulted in a clustering solution of K=2. The green cluster corresponded to samples of Sarda (GOT18, GOT34), Maltese (GOT32) and their crossbreeds (GOT04, GOT10), the red cluster corresponded to samples of Saanen (SAA01) and an "alleged" Sarda (GOT35).

The successive round of structure analyses (overall K=4 and K=5, data not shown) did not show a further substructuring, according to the first clustering solution (K=2), which identified two main genetic clusters. The first two principal coordinates resulting from PCA carried out on the entire dataset, accounted for 67.29% of the total

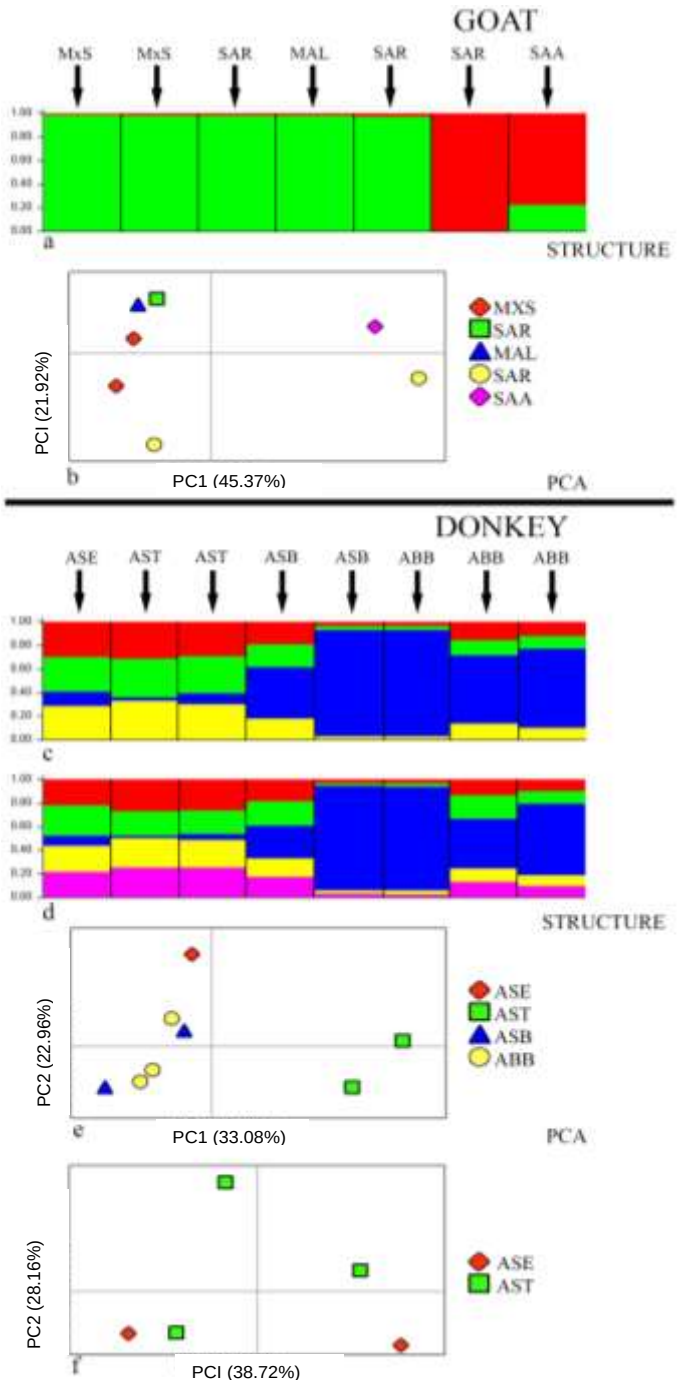

Figure 2. Structure and principal coordinate analysis (PCA) of *Capra hircus* and *Equus asinus*. a, c and d show the estimated genetic structure in both species, inferred using the Bayesian model-based clustering analysis. Each individual is represented by a thick vertical line, which is partitioned into K coloured segments (K = number of clusters). The height of each segment is proportional to the individual estimated membership in the corresponding cluster. Black lines separate the individuals. b, e and f represent the principal coordinate analysis (PCA). The ordination plots display the relationships among individuals according to the first two axes of variation. The first and second principal coordinates are plotted on the x and y axes, respectively. PCA plots include: b, all goats; e, all donkeys; f, Sardinian and Asinara donkeys from Burgos. Abbreviations are given in Table 1.

variation, and identified two groups of individuals (Figure 2b). These groups were consistent with the structuring found by structure analyses (Figure 2a). The sample GOT35 from Sorradile (Oristano), was previously morphologically classified as Sarda, grouped with Saanen (SAA01), as it showed a FISSR genetic profile almost perfectly super-imposable, so revealing a probable mistake in taxonomic attribution on morphological bases. Further PCA analysis on a subset of data corresponding to the breeds of Sarda, Maltese and their crossbreeds (data not shown) gave no additional information.

In donkeys, the banding pattern ranged from 450 to 1060 bp for the primer UBC864 and 320 to 1080 bp for the primer UBC868 (Table 1). For this species, the uppermost hierarchical level of genetic structuring resulted in a clustering solution of K=4 (Figure 2c). The genetic structure did not remark the distribution of individuals (despite the numerical evenness) because the two breeds from Burgos (Sardinian and Asinara donkeys) showed one individual (ASB12 and ABB28, respectively) completely characterized by the blue cluster. The successive round of structure analyses (K=5, Figure 2d) showed that the blue cluster roughly corresponding to the samples from Burgos was inconsistent with the samples from Tertenia. Conversely, the donkey from Senorbì had the same probability to belong to one of the five clusters. These results were consistent across replicate runs, which retrieved nearly the same clustering solution. The first two principal coordinates that resulted from PCA carried out on the entire dataset, accounted for 56.03% of the total variation, and identified three groups of individuals (Figure 2e). The four populations are separated according to their geographic origin in three groups, with the sample from Senorbì more distant from those from Tertenia than from Burgos, along the axis 1, which explains most of the variability (33.08%). Further PCA analysis on a subset of data corresponding to the donkeys from Burgos showed that ASB12 and ABB28 grouping is far from the other samples (Figure 2f).

DISCUSSION

The results shown here demonstrate that modified FISSRs can be successfully used in the studies on population genetics of *Capra hircus* and *Equus asinus* and it is conceivable that this technique could be a valid tool in a large number of species, especially those where there are no other genetic markers and not requiring DNA sequencing. In contrast to the classical protocol where a set of degenerate primers (non-dyed) and one fluorescent dye-labeled nucleotide (dUTP TAMARA) were employed, in the present work only two non-anchored labeled primers were used for FISSR-PCR reaction. This method is very useful both in the traceability of animal products and in the management of local breeds that are numerically vulnerable, as is the case of the different breeds belonging to the two species here examined (donkeys and goats). Indeed, both breeds are numerically reduced and considered in danger of extinction. Thus, the genetic characterization of such breeds could be very useful in the studies on the relationships among local farms for the estimation of their relative importance and the correct application of preservation programs. This method can provide a tool for the preservation of local genetic breeds widespread all over the world, giving a contribution to the safeguard of animal biodiversity. Moreover, this method is not expensive as compared to other genetic analyses. It could be of interest in many geographic regions where there are more breeds of the same species with similar morphological features and different genetic patterns. The strongest point of this method is its low cost.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Tommy Atkins mango (*Mangifera indica* L.) postharvest quality with cassava starch, chitosan and pectin based coatings

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Bearing in mind the use of natural and biodegradable products, this study aimed to evaluate physicochemical quality of 'Tommy Atkins' mangoes coated with a cassava starch, chitosan and pectin based edible coatings. The experiment was conducted using simplex-centroid mixture design. Compounds concentration corresponded to the independent variables studied, encoded as factors x1, x2 and x3: 1– cassava starch (CS) 2%; 2– chitosan (CH) 2%; and 3– pectin (PE) with 2% of water polymers. Seven coating compositions (100% CS; 100% CH; 100% PE; 50% CS + 50% CH; 50% CS + 50% PE; 50% CH + 50% PE and 33.33% CS + 33.33% CH 33.33% PE (center point)), and uncoated control treatment with five replications per treatment, with four replications having five fruits treated in the center point were used. Fruits were stored for 28 days at 13°C and 90% relative humidity (RH). Physiological loss, appearance, pulp color: lightness, chroma and hue angle, fruit firmness, soluble solids content, pH, titratable acidity, and ratio were evaluated as dependent variables. Significant difference occurred with Scott-Knott tests and response surface for all the analyzed variables. Increase in physiological loss, soluble solids content were observed from fruits of all treatments. Decrease in appearance, fruit firmness, lightness in pulp color were observed. Fruits coated with mixtures of chitosan at 50 and 33.33% showed lower physiological loss. Coated fruits from all treatments presented lower fruit firmness than the control, except for SC + PE (1:1) treatment which provided similar result with the control group. Coating based in cassava starch and chitosan at 50% delayed the buildup of soluble solids and ensured reduction and/or maintenance of the variables of quality analyzed.

Key words: Coatings, conservation, *Mangifera indica* L., postharvest, storage.

INTRODUCTION

Mango (*Mangifera indica* L.) is a tropical fruit, of climacteric behavior, with big economic importance in Brazilian national market and worldwide, due to its attractive color, nutritional value, taste and specific aroma (Singh et al., 2013). As visual quality, regular size, and firmness are the characteristics with most commercial importance, "Tommy Atkins" mango, in this context, is the most cultivated and exported cultivar in Brazil, because of its yield, good capacity in adapting to different environment and postharvest conservation (Carvalho et

al., 2004; Cohen et al., 2001). In 2012, Brazil was in the seventh position among major mango producing countries, with an area of 73.3 hectares and 1,575,735 tons produced (FAO, 2016). Out of that, 127,002,229 tons were exported, generating 138 millions of dollars in Brazilian exportations (MDIC, 2016).

Having in mind that mango trade to distant markets requires studies to extend its storage life, it is necessary to focus on: maturation stage at harvest, correct harvest procedures, and postharvest handling, as well as proper storage conditions (Osorio and Fernie, 2013; Razzaq et al., 2013). As a tropical product, mango shows sensibility to low temperatures, causing injuries when kept under temperatures below 12°C (Nair and Singh, 2003; Narayana et al., 2012).

The association of refrigeration and modified atmosphere packaging (the use of plastic films, wax or edible coatings) or controlled atmosphere packaging has been used to reduce deterioration, extend shelf life and sustain mango's quality (Singh and Singh, 2012). Edible coating works as a barrier, reducing gas exchange between fruit and atmosphere, results in a modified intern atmosphere (high CO_2 concentrations, and low O_2), and so decrease in water loss (Terry et al., 2011; Oliveira, 2014; Aquino et al., 2015; Petriccione et al., 2015).

The use of edible packages is important due to modern consumer being more responsible, preferring more natural, renewable and biodegradable products (Jiménez et al., 2012). Edible coatings used in postharvest are biodegradable, created from renewable sources, perfectly adjusting itself in the ecosystem and avoiding environmental pollution (Campos et al., 2011; Pascall and Lin, 2013).

In these terms, cassava starch, chitosan and pectin are polysaccharides being studied as feedstock in edible coatings production (Wills and Goulding, 2015), building a resistant and transparent layer, giving a nice and bright look to the fruit, and making them commercially attractive (Jiménez et al., 2012). The use of chitosan coatings reduced physiological loss and delayed fruit firmness loss in mangoes during storage (Cissé et al., 2015), such as the use of polysaccharides (methylcellulose, hydroxypropyl cellulose, carboxymethyl cellulose and chitosan) on sustaining citrus fruits quality (Arnon et al., 2014). Another positive effect of polysaccharide coatings (pectin and chitosan) was pointed by the decrease in respiration rate and ethylene production and, as a consequence, extended mango's shelf life (Medeiros et al., 2012).

Mangoes stored only in refrigerated environment reached the stage of consumption 20 days after refrigeration and four days at room temperature. For 'Tommy Atkins' mangoes, 12°C combined with a 5% of CO_2 + 5% of O_2 kept fruits in good marketable conditions for 31 days. These results supported the possibility of long sea shipping (21 to 23 days), and reducing costs.

Mango's postharvest life under refrigeration at 10 and 13°C and relative humidity (RH) of 85% varies from 2 to 3 weeks depending on maturation stage (Neves, 2009), while when it was wax coated or plastic packed, it increased its life between 28 and 35 days. However, Santos et al. (2012) verified postharvest life in 'Tommy Atkins' mangoes of 28 days after coating with a 2% cassava starch coating. This study's purpose was to evaluate effects of cassava starch (CS), chitosan (CH), and pectin (PE) isolated and mixtures of edible coatings on sustaining "Tommy Atkins" mangoes postharvest quality.

MATERIALS AND METHODS

The present study was carried out at Laboratório de Tecnologia de Alimentos (Laboratory of Food Technology), Universidade Federal Rural do Semi-Árido – UFERSA. Materials used for films production are cassava starch, chitosan, citrus pectin, and distilled white glycerin.

Mangoes from 'Tommy Atkins' cultivar were collected for this experiment, harvested at physiological maturity, at Fazenda Finobrasa Agroindustrial S/A, located at Ipanguaçu-RN. After transportation to the Laboratory of Food Technology at Universidade Federal Rural do Semi-Árido, fruits were selected, washed with chlorinated water (100 ppm) and dried at room temperature.

Fruits were identified and sorted according to the experimental design used (response surface in simplex-centroid mixture design), with five replications in each trial and seven coating types, and the uncoated control treatment.

Response surface methodology describes dependent variable (Y) behavior when there are changes in the independent variables, within the studied interval (Rodrigues and Iemma, 2005). Results for mixtures design are represented by a response surface ternary triangle, where basal vertices correspond to measured responses to pure components. Mean points in the edges represent responses for binary mixtures, and points within the triangle correspond to ternary mixtures (Bruns et al., 2001).

Cassava starch (CS), chitosan (CH) and pectin (PE) coatings were made with 20 g of polymer, 2 g of plasticizer (glycerol), in 978 g of distilled water (CS and PE), and 978 g of acetic acid (1) in a pH of 3. Cassava starch solution was stirred and heated to a temperature of 70°C, during 15 min, using a stirrer with a heating plate while chitosan and pectin solutions were homogenized with the stirrer for 45 min. After preparation of each solution, separately, mixtures were set and refrigerated at 25°C. Fruits were individually immersed for one minute in its respective treatment (coating) solution and dried at room temperature for one hour, just followed by storage at a 13±1°C and 90±5% RH for 28 days. The following physical and physicochemical analysis were evaluated at the end of storage period.

External appearance (EA)

Six trained people evaluated fruits using a visual and subjective

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scale, grading from 1 to 9, according to severity of damages (sunken lesions, wilt, fungal injuries, darker spots). Grading scale is from 1 to 9, where 9– extremely good – free from injuries; 8- very good – free from dark spots but loss of turgidity; 7– good – few dark spots and loss of turgidity (5% of the fruit); 6– regular – dark spots (5%) and shrinkage; 5– acceptable – 10% of darker spots (limit); 4– bad – 25% with darker spots; 3– very bad – 50% spots and/or shrinkage; 2– extremely bad – 75% of spots, injuries or shrinkage, and apparent softening; 1– awful - 75% unacceptable damage. Fruits grade 4 or less were considered unsuitable to the market (Lima et al., 2012).

Physiological loss (PL)

This is measured by the difference between mass at the start and each measurement period, expressed in percentage (%).

Pulp color

This was determined with reflectometry, using a Konica Minolta® CR-10 colorimeter, calibrated in an illuminated white porcelain surface. Readings were expressed in L, C and °h, which, according to the Commission Internacionale de L'Eclaraige (CIE), define color: L, corresponding to lightness (brightness, degree of lightness; 0 = dark/opaque and 100 = white); C, the chroma (saturation or color intensity; 0 = gray/dull and 60 = vivid); and °h, the hue angle (tonality; 0° = red; 90° = yellow; 180° = green; 270° = blue) (Minolta Corp, 2007). Measures were made in two equidistant spots, and considered the average of both.

Fruit firmness (FF)

Measurement was made based on penetration, using a McCormick FT 327 penetrometer (8 mm-diameter tip) in equatorial regions of the fruit, two per fruit. Some of the epicarp was removed. Results are expressed in Newton.

Total soluble solids content (SS)

This was determined after passing the whole fruit through a blender and measured with a digital Pallete PR-100 refractometer (AttagoCo. Ltd., Japan), with automatic temperature correction and reading range from 0 to 32 °Brix. Results are expressed in percentage (%) (AOAC, 1992).

Titratable acidity (TA)

This was determined by titration of an aliquot of 10 g of juice, in duplicate, which were added 40 mL of distilled water and titrated with NaOH (0.02 N) solution until pH equals 8.1 (using a digital pHmeter). Results were expressed in percentage of citric acid, according to IAL (1985) methodology.

pH (potential hydrogen)

This was measured in the fruit's juice in duplicate, using a digital pHmeter (AOAC, 1992).

SS/TA ratio

Result of division between average values of soluble solids content and titratable acidity average. Response surface statistical analysis of individual effects on experimental factors and their interactions on mangos' attributes of quality through 28 storage days, coated with cassava starch, chitosan and pectin isolated or in mixtures, were ran with Programa Statistica 7.0 software (STATSOFT, 2004). Adjustments of regression models in response surface were made. The model choice followed the significance criteria and its parameters, errors regression significance and coefficient determination (R^2). Data was subjectted to variance analysis using SISVAR software (Ferreira, 2003). Level of treatment factors were compared by Scott-Knott at 5% of probability.

RESULTS AND DISCUSSION

Table 1 shows the experimental arrangement used; it was verified that trials from 7 to 10 originated from four replications made at central point to determine experimental error. It was considered that the response shows homocedasticity within the experimental domain studied. Table 2 shows model's coefficients, standard errors estimate, Student test (t test) and p-value to main effects, and interactions of quadratic and cubic models used to analyze variables' description in mangos coated with different biopolymers compositions. Figure 1 shows a Pareto graph of standardized effects for comparison of factors significance and their interactions with variables.

Main effects are statistically significant at 95% of confidence: x1*x2 double interaction in physiological loss, appearance, and pulp lightness, x1*x3 interaction in chroma and hue angle of pulp color, and x1*x2*x3 cubic interaction in appearance, may be considered significant at 95% of confidence. Other interactions between factors are not too significant.

Best subset selection method was used to evaluate factors and interactions that must be disregarded from the models. Used as criteria of factors and interactions selection to set the mathematical model, was adjusted R-squared value maximization. Adjusted R-squared is calculated from R-squared adjustment of each subset from the model considering the amount of variables in the model and sample size.

Variance analysis (Table 3) shows that the regressions are significant at 5% of probability for analyzed variables. The models may explain 96.11; 99.80; 98.29; 82.10 and 91.77% of R-squared. During the period of storage, a significant difference of physiological loss occurred within treatments. Figure 2 presents response surface contour curves (a) obtained with application of mathematical model and Scott-Knott test (b) at 5% of probability to physiological loss.

Significant differences were observed in physiological loss of fruits within treatments, as shown in Figure 2. Physiological loss occurs due to the transpiration process, which may be increased with increase in temperature and decrease in relative humidity (Chitarra and Chitarra, 2005). Physiological loss of coated fruits is related to an attribute of water vapor barrier. Polysaccharides such as cassava starch, chitosan and pectin are excellent barriers to oxygen and carbon

Table 1. Experimental arrangements and results for physiological loss (%) – Y1, external appearance (Y2), pulp lightness color (L) (Y3), fruit firmness (Newton) (Y4), soluble solids content (%) (Y5), pH (Y6), titratable acidity (% of citric acid) (Y7), and SS/TA ratio (Y8) in 'Tommy Atkins' mangoes coated with chitosan, cassava starch and pectin coatings.

Trial number	Factor (encoded values)			Response variable							
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8
1	1	0	0	3.71	6.0	72.65	34.92	13.17	3.40	0.786	17.99
2	½	½	0	2.80	8.0	68.18	47.15	9.96	3.63	0.602	16.71
3	0	1	0	3.29	8.0	69.47	46.26	11.98	3.64	0.543	22.18
4	½	0	½	3.57	7.0	73.28	66.72	11.05	3.36	0.906	12.33
5	0	0	1	3.13	8.0	70.35	31.14	13.09	3.64	0.691	19.53
6	0	½	½	2.98	7.7	70.67	39.14	12.82	3.66	0.664	19.78
7	⅓	⅓	⅓	2.91	7.0	71.97	29.80	12.75	3.61	0.629	20.89
8	⅓	⅓	⅓	3.14	7.0	71.46	30.69	13.02	3.56	0.647	20.57
9	⅓	⅓	⅓	3.08	7.0	72.19	34.69	13.17	3.56	0.609	22.30
10	⅓	⅓	⅓	3.04	7.1	71.54	29.58	13.25	3.66	0.581	23.55
Lack of fit	-	-	-	0.000132	0.342857	0.00	0.00	0.00	0.002656	0.00	0.00
Adj R^2	-	-	-	0.9125	0.9939	0.9488	0.9583	0.9604	0.7927	0.9287	0.8225

X1, % cassava starch; X_2, % chitosan; X_3, % pectin; % biopolymers = 2.0%; plasticizer = 0.2% of glycerol.

dioxide; however, in isolated forms, they are hydrophilic and have high permeability to water vapor, which differ from each polymer matrix (Medeiros et al., 2012; Cissé et al., 2015). The coating may be optimized by combination with hydrocolloids turns the coatings formation more homogenous (Gao et al., 2013; Castañeda, 2013; Silva, 2015) or with lipidic materials (Xu et al., 2005; Oliveira, 2014).

When response surface quadratic model is analyzed for weigh loss on mango fruits during 28 storage days, it is seen that the model is adequate to describe the mixture of all three constituents, because the graph for ternary mixtures with quadratic model already have a higher synergic interaction in binary mixtures with cassava starch and chitosan.

Fruits coated with film-forming solution composed of 5 to 33.33 % of chitosan, and having cassava starch or pectin as the others components, provided lower physiological loss within treatments, under the experimental conditions. Notwithstanding, mangoes coated with mixtures of equal parts (1:1) of chitosan and cassava starch showed decrease of physiological loss (27.27%). According to Castañeda (2013), in refrigerated storage of apple postharvest conservation, mixture of cassava starch and chitosan at equal proportions made better coatings, due to better rearrangement of polymeric chain, proved by the scanning electron microscopy, which ensures a more efficient water vapor barrier.

Coatings with composition of CS, and CS+PE provided similar effect of physiological loss to control treatment fruits. On the other side, the other compositions resulted in positive effect in reducing fruit physiological loss as compared to the control treatment. Mixture coatings of CS+CH, CH+PE, and CS+CH+PE had less physiological loss than mixtures with PE and CH.

The differences of physiological loss between coated and uncoated mangoes are primarily related to water vapor barrier provided by the coatings, where each polymer has different properties and structures which gives different structural arrangements and different formation of polymeric chain (Wills and Goulding, 2015).

Tommy Atkins and Supresa cultivars of mangos, coated with cassava starch at 3 and 2%, respectively, presented lower physiological loss as compared to lower concentrations and to control treatment (Santos et al., 2011; Scanavaca Júnior et al., 2007).

Medeiros et al. (2012) coated 'Tommy Atkins' mangos with chitosan and pectin coating, kept for 45 days at 4°C and 90% of RH, and observed less physiological loss at 28 days to coated fruits. Cissé et al. (2015) also had lower physiological loss as compared to control treatment fruits in mango fruits from Kent cultivar stored for 8 days coated with 1 and 1.5% chitosan.

Reduction in external appearance scores was observed in fruits from all treatments at day 28 (Figure 3a and b). Fruits coated with CH, PE and CS+CH sustained higher external appearance scores when compared with the control treatment, with statistically equal scores, differing from fruits from other treatments, which had lower external appearance scores. The positive effect of coatings with CH, PE and CS+CH is due to a brighter mango skin resulting in a good-looking fruit and also having important effect on retarding senescence processes which is caused by the barrier layer that blocks gases. Ethylene, the main hormone associated with ripening process, has its effect reduced when CO_2 concentration within the cell is higher than 5% (Cissé et al., 2015).

Table 2. Coefficients, standard error, student t-test and p-value of physiological loss (%), appearance, lightness of pulp color (L), chroma (C), and hue angle (H) in 'Tommy Atkins' mangoes coated with chitosan, cassava starch, and pectin coatings.

Factor	Coefficients
Physiological loss	
(X_1) Cassava starch (%)	3.70808*
(X_2) Chitosan (%)	3.28920*
(X_3) Pectin (%)	3.12848*
$X_1.X_2$	-2.76832*
$X_1.X_3$	0.65328ns
$X_2.X_3$	-0.89008ns
External appearance	
(X_1) Cassava starch (%)	6.0000*
(X_2) Chitosan (%)	8.0000*
(X_3) Pectin (%)	8.0000*
$X_1.X_2$	4.0000*
$X_1.X_3$	0.0000ns
$X_2.X_3$	-1.2000*
$X_1.X_2.X_3$	-16.7250*
Lightness	
(X_1) Cassava starch (%)	72.6500*
(X_2) Chitosan (%)	69.4700*
(X_3) Pectin (%)	70.3500*
$X_1.X_2$	-11.5200*
$X_1.X_3$	7.1200*
$X_2.X_3$	3.0400ns
$X_1.X_2.X_3$	30.1800*
Firmness	
(X_1) Cassava starch (%)	34.917*
(X_2) Chitosan (%)	46.259*
(X_3) Pectin (%)	31.136*
$X_1.X_2$	26.243ns
$X_1.X_3$	134.774*
$X_2.X_3$	1.779ns
$X_1.X_2.X_3$	-657.025*
Soluble solids	
(X_1) Cassava starch (%)	13.1700*
(X_2) Chitosan (%)	11.9800*
(X_3) Pectin (%)	13.0900*
X1.X2	-10.4600*
X1.X3	-8.3200*
X2.X3	1.1400ns
X1.X2.X3	61.0425*
pH	
(X_1) Cassava starch (%)	3.393895*
(X_2) Chitosan (%)	3.631895*
(X_3) Pectin (%)	3.633895*
X1.X2	0.582105*
X1.X3	-0.509895ns
X2.X3	0.198105ns
Titratable acidity	
(X_1) Cassava starch (%)	0.7861*
(X_2) Chitosan (%)	0.5435*
(X_3) Pectin (%)	0.6910*
X1.X2	-0.2498
X1.X3	0.6715*
X2.X3	0.1885ns
X1.X2.X3	-3.3760*
SS/TA ratio	
(X_1) Cassava starch (%)	17.9921*
(X_2) Chitosan (%)	22.1798*
(X_3) Pectin (%)	19.5291*
X1.X2	-13.4892ns
X1.X3	-25.7110*
X2.X3	-4.2816ns
X1.X2.X3	182.4960*

Table 2. Contd.

*Effects considered statistically significant ($p < 0.05$) at 95% of confidence.

For each polymer of isolated form or in mixture, there are three elements in their formation or in the molecular structure of aqueous gel: (a) junction zones, where polymeric molecules are together; (b) polymer inter-junction segments that are relatively mobile; and (c) water bonded to polymeric net. A junction zone may involve covalent bonds, electrostatic, of hydrogen or hydrophobic interactions (Thakur et al., 1997).

Supporting this study results, Medeiros et al. (2012) indicated that mangos coated with pectin and chitosan had better appearance and lower physiological loss than fruits from control group. One of the reasons for appearance change after harvest is the physiological loss due to transpiration, which may cause fruits to wilt, making them unattractive in market.

Another factor that may affect fruits appearance is chilling injuries, just as mechanic injuries, microorganisms attack, physiological disturbs, and also wilt caused by some coatings (Lima et al., 2012). One way to avoid this event is reducing ethylene production (Wills and Goulding, 2015). Chien et al. (2007) verified reduction on external appearance of nine mango fruits when stored for seven days at 6°C and 80% RH. Fruits coated with chitosan at concentrations of 0.5, 1 and 1.5% obtained values of 5.79, 6.42 and 6.02, while uncoated fruits had value of 3.85. Similar results for appearance with the use of coatings were detected in eggplant coated with cassava starch by Souza et al. (2010); Formosa papaya coated with carnaúba palm wax by Fernandes et al. (2012), showed better scores for external appearance at the end of storage period as compared to fruits from the control treatment.

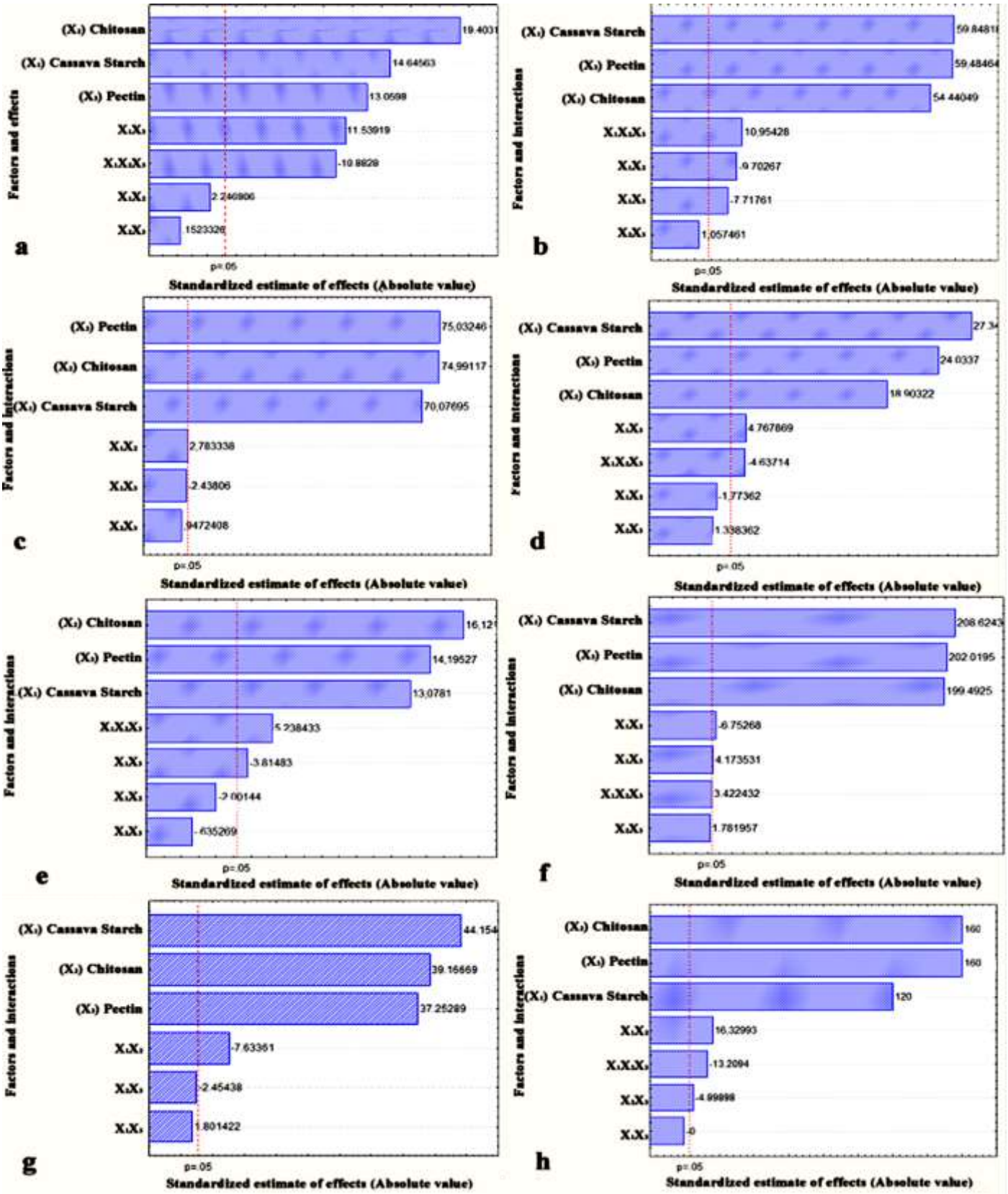

Figure 1. Pareto graph of standardized effects for factors and their interactions on fruit firmness (N) (a), soluble solids content (%) (b), pH (c), titratable acidity (% of citric acid) (d), SS/TA ratio (e), lightness of pulp color (L) (f), physiological loss (%) (g), and appearance (9-1 scale) (h) in 'Tommy Atkins' mangoes coated with chitosan, cassava starch and pectin coatings.

Table 3. Variance analysis for adjustment of models to physiological loss (%), appearance, lightness of pulp color (L), chroma (C), and hue angle (H) in 'Tommy Atkins' mangoes coated with chitosan, cassava starch and pectin coatings.

Source	SS	DF	MS	F	S
Physiological loss					
Quadratic model	0.706787	5	0.141357	19.7621	0.006367*
Residual	0.028612	4	0.007153		
Lack of fit	0.000132	1	0.000132	0.0139	0.913645
Pure error	0.028480	3	0.009493		
Adjusted total	0.735399	9	0.081711		
R-squared = 0.9611			Adjusted R-squared = 0.9125		
External appearance					
Cubic model	3.189286	4	0.797321	5.57011	0.043745*
Residual	0.715714	5	0.143143		
Lack of fit	0.685714	2	0.342857	34.28571	0.008582*
Pure error	0.030000	3	0.010000		
Adjusted total	3.905000	9	0.433889		
R-squared = 0.9980			Adjusted R-squared = 0.9939		
Lightness					
Cubic model	20.93224	6	3.488707	28.76888	0.009569*
Residual	0.36380	3	0.121267		
Lack of fit	0.00000	0	0.000000		
Pure error	0.36380	3	0.121267		
Adjusted total	21.29604	9	2.366227		
R-squared = 0.9829			Adjusted R-squared = 0.9488		
FF					
Cubic model	1209.916	6	201.6527	35.47737	0.007049
Residual	17.052	3	5.6840		
Lack of fit	0.000	0	0.0000		
Pure error	17.052	3	5.6840		
Adjusted total	1226.968	9	136.3298		
R-squared = 0.9861			Adjusted R-squared = 0.9583		
SS					
Cubic model	10.86817	6	1.811361	37.40549	0.006524*
Residual	0.14527	3	0.048425		
Lack of fit	0.00000	0	0.000000		
Pure error	0.14527	3	0.048425		
Adjusted total	11.01344	9	1.223716		
R-squared= 0.9868			Adjusted R-squared = 0.9604		
pH					
Quadratic model	0.093748	5	0.018750	7.881487	0.033787*
Residual	0.009516	4	0.002379		
Lack of fit	0.002656	1	0.002656	1.161424	0.360107
Pure error	0.006860	3	0.002287		
Adjusted total	0.103264	9	0.011474		
R-squared= 0.9078			Adjusted R-squared = 0.7927		

Table 3. Contd.

TA					
Cubic model	0.101793	6	0.016966	20.52528	0.015590*
Residual	0.002480	3	0.000827		
Lack of fit	0.000000	0	0.000000		
Pure error	0.002480	3	0.000827		
Adjusted total	0.104273	9	0.011586		
R-squared= 0.9762			Adjusted R-squared = 0.9287		
SS/TA					
Cubic model	90.277420	6	15.046240	7.94971	0.058598*
Residual	5.678030	3	1.892680		
Lack of fit	0.000000	0	0.000000		
Pure error	5.678030	3	1.892680		
Adjusted total	95.955450	9	10.661720		
R-squared= 0.9408			Adjustest R-squared = 0.8225		

*Effects considered statistically significant ($p < 0.05$) at 95% of confidence.

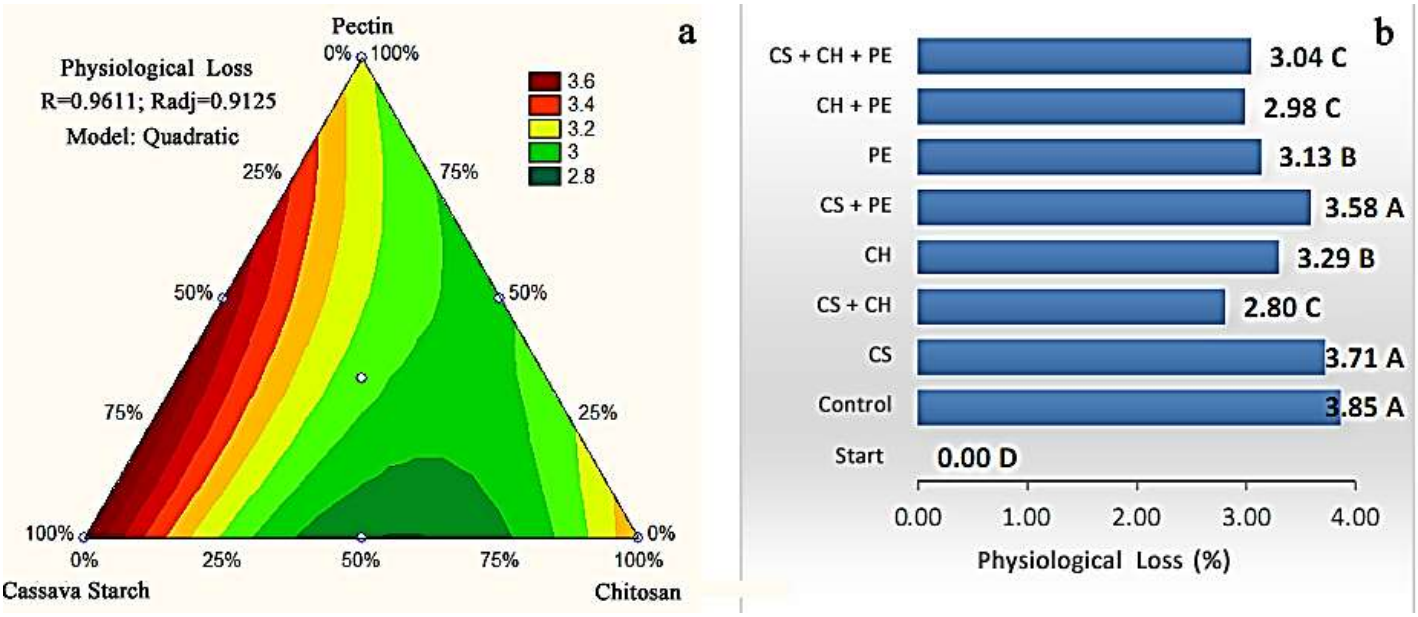

Figure 2. Response surface contour curves (a) and Scott-Knott test (b) for physiological loss at the twenty-eighth day of storage (13°C and 90% of RH).

Lima et al. (2012) reported divergent results using cassava starch in 'Tommy Atkins' mangoes, obtaining scores lower than the control treatment. Fruit appearance evaluation is extremely useful to estimate the time of commercialization, because fruit must get to large consumer markets with acceptable visual quality for consumption and marketing. Figure 4 presents response surface contour curves (a) obtained from mathematical model and Scott-Knott test (b) at 5% of probability for pulp color: lightness (L). Mango fruits pulp color suffered a decrease in lightness and at day 28 of storage, demonstrating that ripening process occurred and was represented by the changes in pulp variables during storage period.

Study of mango conservation using cassava starch performed by Serpa et al. (2014) verified that there was no effect of coatings on L variables, causing reduction of pulp lightness in 'Palmer' mangoes from 80.58 to 70.07 during a 10-days storage at room temperature.

When the cubic model for L, from response surface of

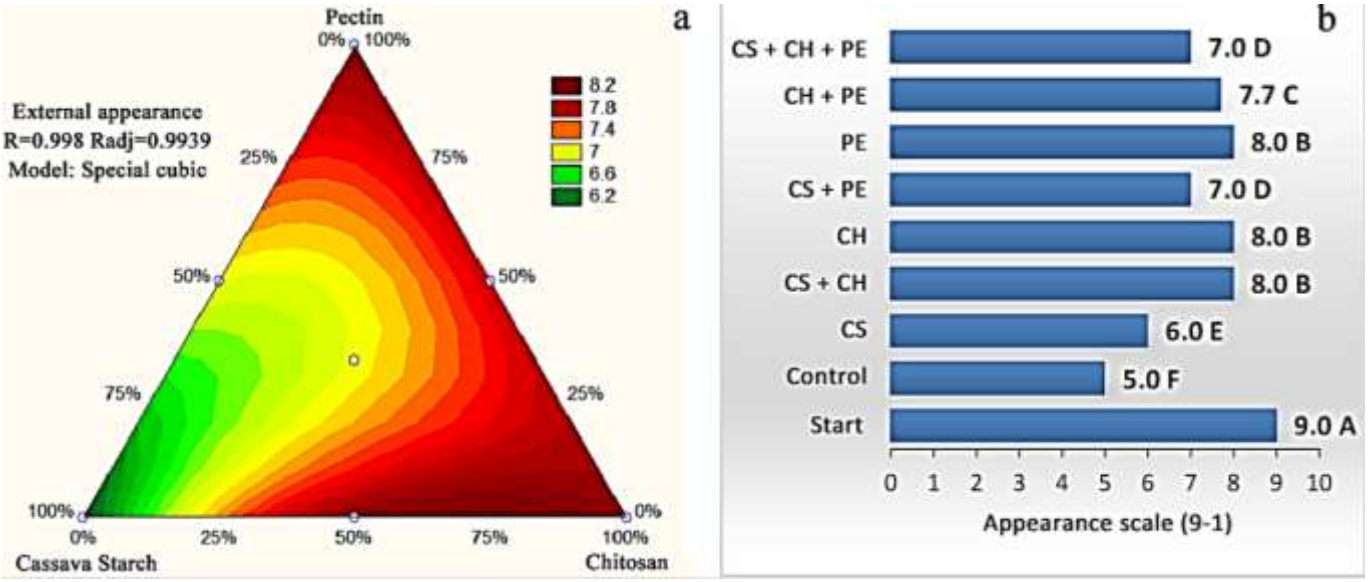

Figure 3. Response surface contour curves (a) and Scott-Knott test (b) for external appearance scale (9-1 scale) at twenty-eighth storage day (13°C and 90% of RH).

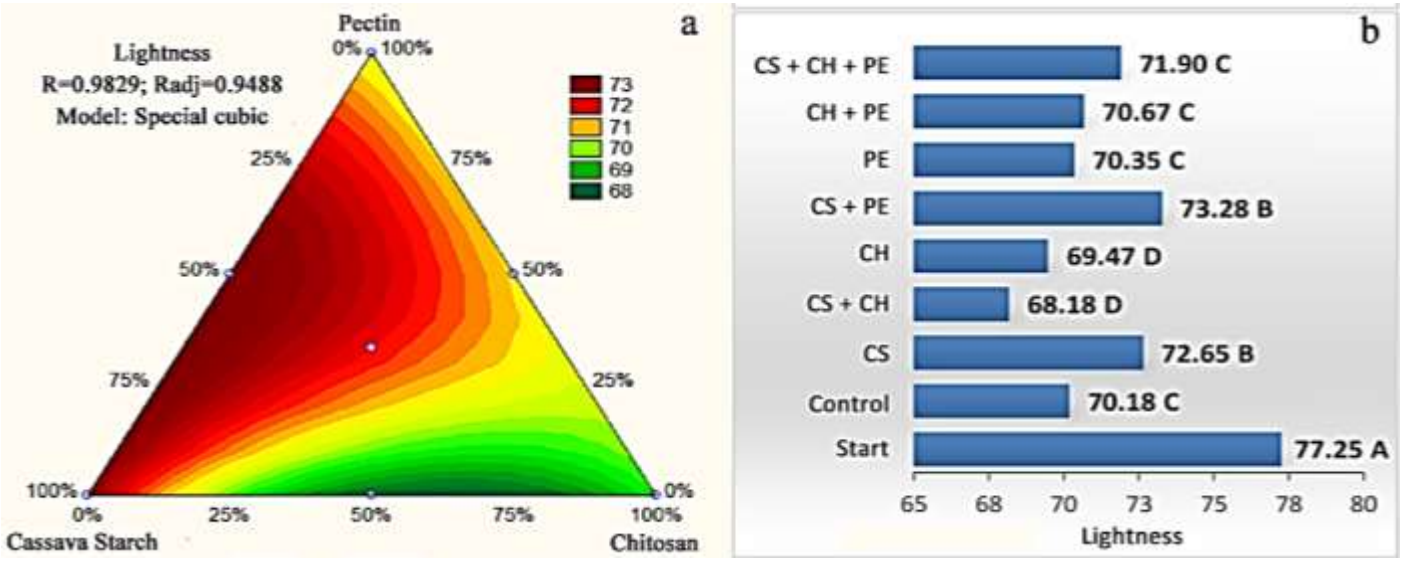

Figure 4. Response surface contour curve (a) and Scott-Knott test (b) to lightness of pulp at 28 day of storage (13°C and 90% of RH).

pulp color of mangos during 28 days of storage was analyzed, it was shown that the models are adequate to describe the mixture of the three components, because in the presented graphs for ternary mixtures with cubic model, there is a higher interaction of mixtures with the three polymers while in the graph with quadratic model, it is noted, a higher synergic interaction in binary mixtures that involves cassava starch and pectin.

Lightness of mango's pulp was influenced by coatings at day 28. There was significant difference with Scott-Knott test within treatments. Fruits coated with CS and CS+PE presented values of pulp lightness higher than the other treatments. Lightness is related to brightness, which varies from 0 (black) to 100% (white) (Ferreira, 2003). Different results were found by Azerêdo (2013) using different coatings of cassava starch and chitosan in 'Tommy Atkins' mangoes where coated fruits presented higher values of pulp lightness at the end of storage period. Other studies carried with Tommy Atkins (Amariz et al., 2010) and Palmer (Braz et al., 2007) cultivars

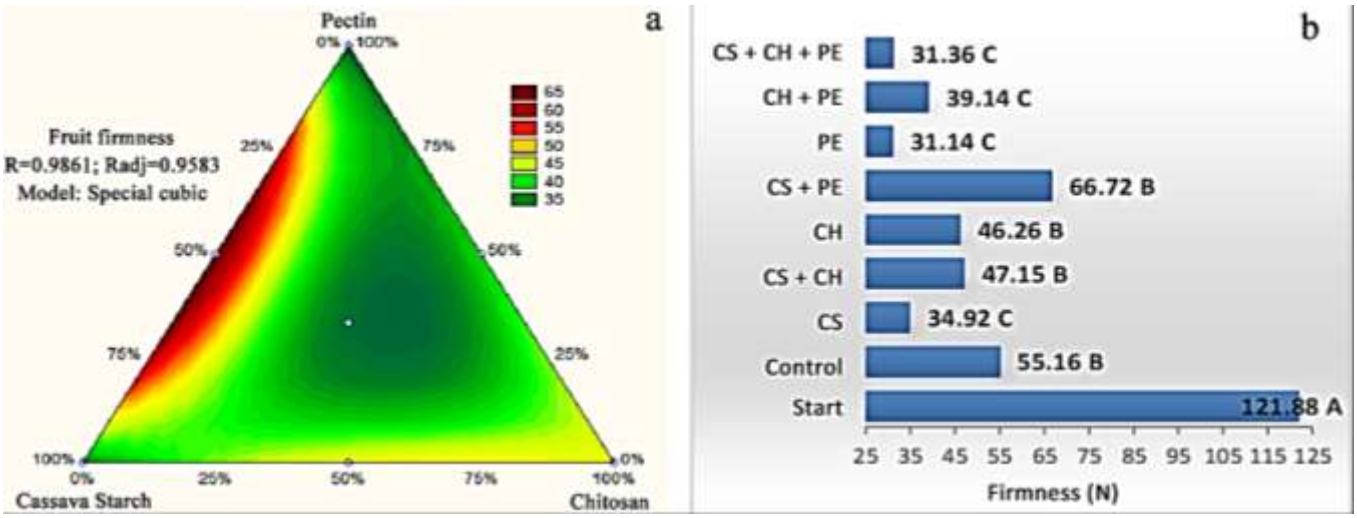

Figure 5. Response surface contour curves (a) and Scott-Knott test (b) for fruit firmness (Newton) at twenty-eighth day of storage (13°C and 90% of RH).

showed decrease in pulp brightness during storage period as well, indicating quality conservation (Chien et al., 2007).

Pectin and cassava starch created a semipermeable coat around the fruit that reduces gases exchange with the environment, modifying the atmosphere inside the fruit (Wills and Goulding, 2015), interrupting carotenoids synthesis process. Similar results were reported in 'Tommy Atkins' mangoes treated with 1.0% CMC + 0. 2% dextrin, 0.8% CMC + 0.5% dextrin and dextrin (Amariz et al., 2010).

Main effects are statistically significant at 95% confidence. For fruit firmness, soluble solids content, titratable acidity and SS/TA ratio variables, the x_1*x_3 double interaction and $x_1*x_2*x_3$ cubic interaction may be considered significant at 95% of confidence, as well as x_1*x_2 double interaction for pH and soluble solids. Other interactions between factors are not so significant.

Figure 5 shows response surface contour curves (a) obtained from mathematical model application and Scott-Knott test (b) at 5% probability for fruit firmness (Newton). Fruits coating did not avoid decrease in fruit firmness of mango fruits during storage period (Figure 5b). A decrease of 55.16% was observed in fruit firmness, from zero (121.88 N) to day 28 of storage (55.16 N) to control fruits; even though, fruits were still marketable. Among coated fruits, it was verified that the proportion of cassava starch and pectin showed higher values than other coatings (Figure 5a), not differing from control fruits.

$x_1*x_2*x_3$ cubic interaction, x_2*x_3 double interaction and isolated cassava starch and pectin coatings presented the lowest values of fruit firmness at day 28 of storage, which has no effect on holding reactions that reduce fruit firmness of fruits.

Firmness is considered one of the most important attributes of fruits quality, since it affects transport resistance, postharvest conservation techniques, and microorganism's attacks (Wills and Goulding, 2015). It is also one of the characteristics of texture and corresponds to the level of plant tissue compressive strength. It is related to the composition and pectin solubilization from cellular wall as well as middle lamella (Chitarra and Chitarra, 2005).

Firmness is also related to the amount of water in the cells, or cell turgor (Chitarra and Chitarra, 2005), which decreases with storage. Firmness decrease is also associated with the insoluble pectin fractions conversion to soluble forms during maturation. Through maturation, proto pectin enzymes and pectin methylesterase are responsible for hydrolysis and solubilization of pectic substances, contributing to firmness reduction (Wills and Goulding, 2015).

Lima et al. (2012) verified in postharvest conservation of 'Tommy Atkins' mangoes, after 15 days of storage at 10°C and 88% of RH, higher firmness of control fruits as compared to fruits coated with 3% cassava starch + Anise (*Pimpinella anisum*) extract. Cissé et al. (2015) also verified that coating systems with chitosan and lactoperoxidase in all coating treatments with concentrations of 1 to 1.5%, isolated or in mixtures, ensured higher firmness in control fruits of 'Kent' mango.

Results reported by Azerêdo et al. (2016) differ, where fruits coated with cassava starch and chitosan in similar concentrations to this study showed, at 29 days of storage, firmness values higher than uncoated fruits. An increase in soluble solids content of fruits was observed in all treatments at day 28 (Figure 6a and b).

$x_1*x_2*x_3$ cubic interaction with mixtures of the three polymers in equal proportions showed the highest values of soluble solids content at day 28 of storage, not holding the reactions that breaks down carbohydrates and stored reserve of the fruit, differently from x_1*x_2 and x_2*x_3

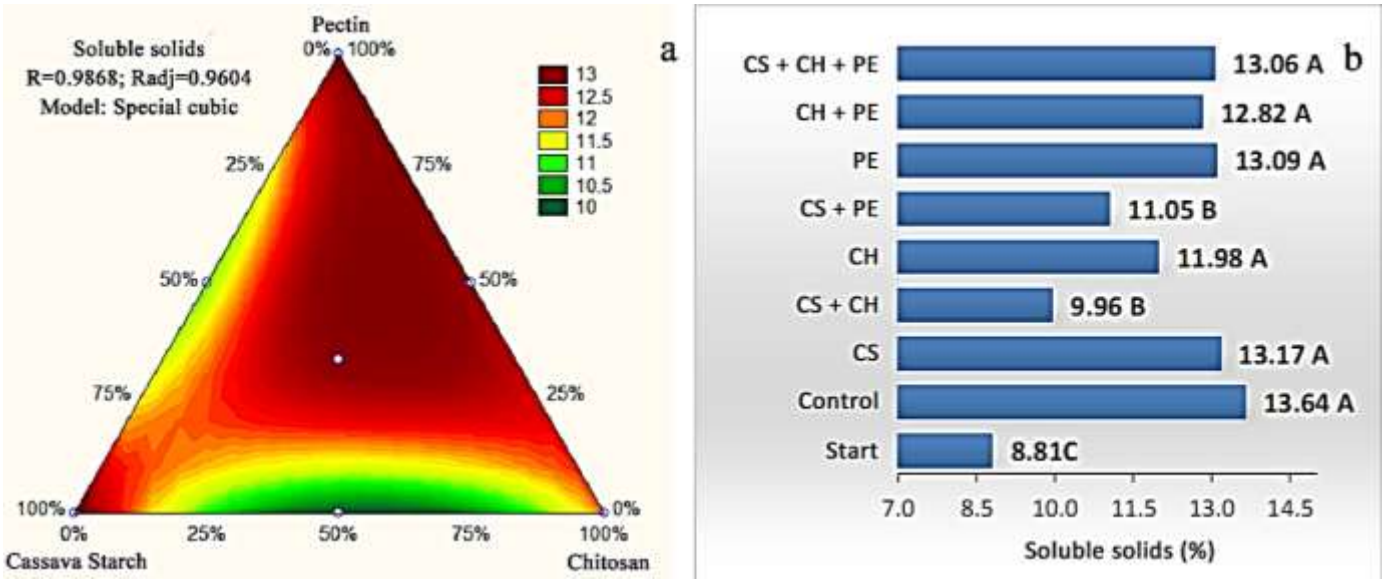

Figure 6. Response surface contour curves (a) and Scott-Knott test (b) for soluble solids content (%) at the twenty-eighth day of storage (13°C and 90% of RH).

double interaction which had lower values of soluble solids content than the other treatments analyzed at day 28 of storage.

Significant difference was reported for soluble solids content between analyzed treatments. Mango fruits coated with 1:1 mixture of chitosan and cassava starch and 1:1 of pectin and cassava starch had lower values of soluble solids content within treatments, under the experimental conditions. Mangoes coated with mixtures of equal parts (1:1) of chitosan and cassava starch had a 27% reduction of soluble solids content related to control group. This occur due to an specific characteristic of combination coating formulation which avoids physiological loss and gas exchanges with the increase of CO_2 levels and O_2 reduction, what prevents polysaccharides and stored reserves of mango fruits from breaking down.

According to Medeiros et al. (2012), soluble solids content is used as maturity indicators and determines fruit quality, having a great role in its taste. Results showed, in general, that fruits coated with a homogeneous coating of ½ chitosan and cassava starch sustained their soluble solids content, as compared to control fruits group. Fruits from control treatment showed soluble solids content variation from 8.81 to 13.64%, while coated fruits held values between 8.81 and 9.96%, confirming that coatings reduced metabolic activity of the fruit, slowing down its ripening.

Normally, soluble solids content increases during maturation due to fruit's polysaccharides degradation. There is also an increase in soluble solids content when water loss is increased, causing sugar concentration to increase in fruits tissues. Reduced concentration of O_2 influences respiration processes causing decline in ethylene production and, consequently, expanding maturation period (Kader, 2002).

This sugar buildup is due to the starch degradation (Cissé et al., 2015). The increase in soluble solids content indicates maturation evolution (Silva, 2015). This way, retarding the increase in soluble solids content implies extending the conservation period (Medeiros et al., 2012).

Through the mango's maturation, starch degradation occurs due, mainly, to the α-amylase and β-amylase enzymes actions. Sugars found in mango are glucose, fructose and sucrose, with the last one being found in larger quantity than the others, and having a major contribution to soluble solids content (Cissé et al., 2015).

When the buildup of soluble solids is retarded, there is a delay in ripening process. This is probably due to metabolic activity reduction (Silva et al., 2001). This attribute delay may be achieved with biodegradable coatings such as chitosan (Cissé et al., 2015), cassava starch (Serpa el al., 2014), and mixture of cassava starch and chitosan (Azerêdo et al., 2016).

Medeiros et al. (2012) reported in 'Tommy Atkins' mangoes coated with chitosan and pectin, a significant effect during the experimental period until day 28, an increase from 11 to 17%, with buildup of soluble solids in control fruits, while coated mangoes maintained themselves relatively stable during this period, from 11 to 13%. Figure 7 shows response surface contour curves (a) obtained from mathematical model application and Scott-Knott test (b) at 5% of probability for pH.

A decrease in pH from 3.55 at timing of zero to 3.40 at day 28 of storage from control fruits was observed

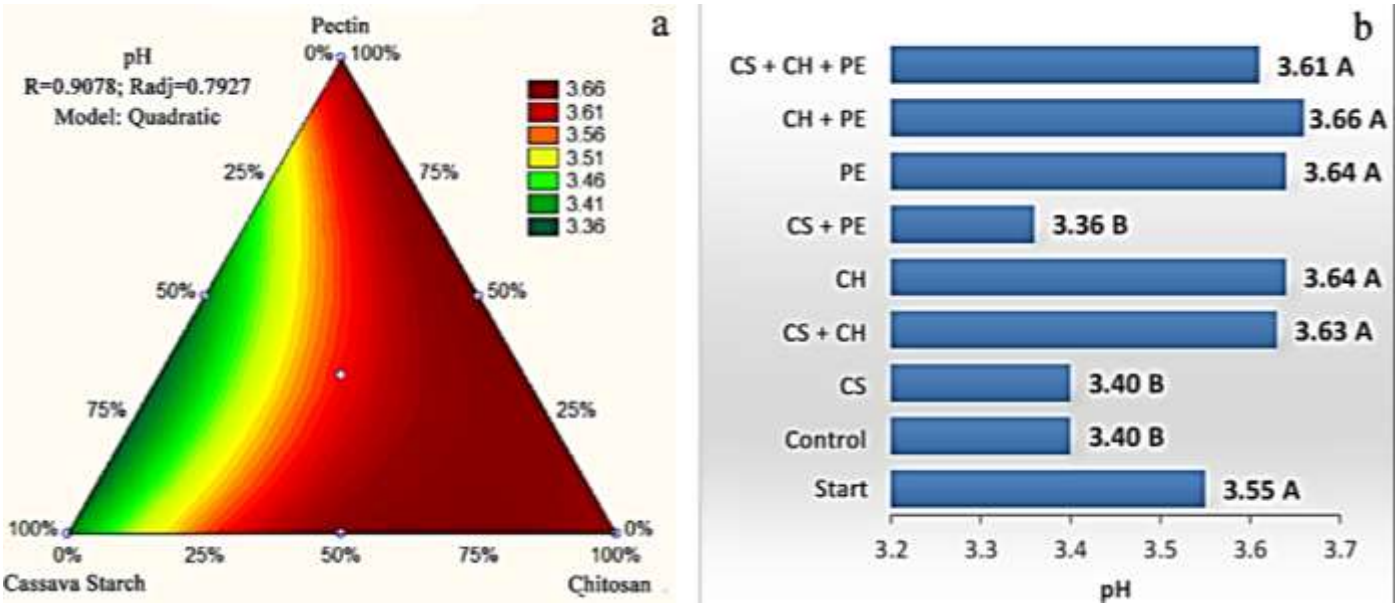

Figure 7. Response surface contour curves (a) and Scott-Knott test (b) for pH at twenty-eighth day of storage (13°C and 90% of RH).

(Figure 5b). Within the coated fruits, it was verified that 1:1 proportion of cassava starch and pectin and isolated cassava starch showed lower values then other coatings (Figure 5a), not differing from control fruits. All these treatments reported high physiological loss at the end of storage period and higher soluble solids content values due to breaking down of stored reserves, that causes increase in acidity with the solutes concentration and breaking down of stored reserves (amid and pectic substances) which reduced pH values.

x_1*x_2 double interaction and chitosan and pectin isolated coatings reported the highest values of pH at day 28, higher than the control fruits. This result shows that a change occurred in the atmosphere around the fruit, caused by the use of coatings that promoted a semipermeable coating on fruits surface, modifying endogenous concentration of CO_2 and O_2 and slowing down the maturation process.

The change in pH is due to sugar formation, acids, and concentration of solutes due to physiological loss throughout maturation. pH increases with the period of storage of fruits (Moraes et al., 2012). Results clearly shows the relationship between pH and acidity, with increase in pH (Figure 7) as the acidity decreases (Figure 8), making the fruit more palatable (Serpa et al., 2014) and more mature.

The use of some coatings decelerates pH changes, affecting the acidity (Moraes et al., 2012). Possibly, these events occur due to reduction in respiration process (Trigo et al., 2012). Azerêdo (2013) verified pH reduction with ripening of fruits up to the twentieth day of storage, followed by a significant increase at day 29, existing differences between coated fruits and control treatment. Figure 8 presents response surface contour curves (a) obtained from mathematical model application and Scott-Knott test (b) at 5% of probability for titratable acidity.

Increase in titratable acidity was observed from 0.73 at timing zero to 0.85% of citric acids at day 28 of storage for control fruits (Figure 5b). Some authors reported that during the storage period, mangoes from 'Tommy Atkins' cultivar had increase in values of acidity followed by a significant reduction with the use of coatings or modified atmosphere packaging (Yamashita et al., 2001; Jeronimo et al., 2007; Ribeiro et al., 2009; Amariz et al., 2010). The high physiological loss and soluble solids content of control fruits at the end of storage period cause, firstly, solute concentration and rapid increase in acidity due to breakage of stored reserves (amid and structural substances) from control fruits.

Titratable acidity of fruits was verified to be altered with coating type. Fruits that had high values of soluble solids content and physiological loss showed higher acidity, caused by the loss of solutes concentration and increase in organic acids from the breakdown of substances such as amid and stored reserves of cell walls.

x_1*x_2*x_3 cubic interaction and x_1*x_3 double interaction showed lower values of acidity caused by a lower loss of weight and soluble solids content, and higher values of acidity due to greater weight and soluble solids content values with a statistical difference for each coating in isolated forms at day 28 of storage.

Cissé et al. (2015) considered that the ripening reduced acidity in coated and uncoated mango fruits, which was not seen in this study. According to Chitarra and Chitarra (2005), normally, content of organic acids decreases with fruits maturation due to its use as respiratory substrate or

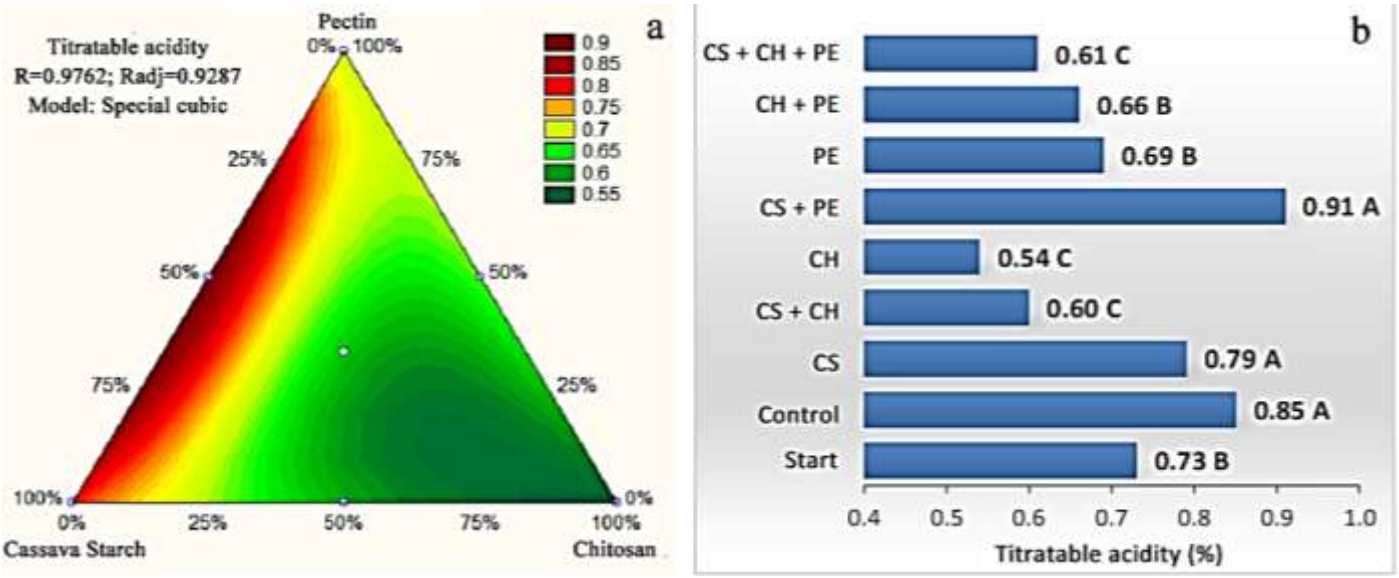

Figure 8. Response surface contour curves (a) and Scott-Knott test (b) for titratable acidity (% of citric acid) at 28 day of storage (13°C and 90% of RH).

in sugar conversion, however phenolic compounds have acidy nature as well, maybe contributing to acidity in some way. According to Alves et al. (2000), an increase in acidity is caused by release of galacturonic acids that increases with fruit ripening by the action of pectin methylesterase and polygalacturonase enzymes.

Fruits that reported decrease in titratable acidity at the end of storage period are probably related to the use of acids as carbon skeleton structure in respiratory process as explained by Kays (1991). According to Chitarra and Chitarra (2005), the content of acids in plants may decrease with maturation, because of transformation of substrates to synthesize phenolic compounds, lipids, and natural aromas.

Similar behavior for titratable acidity reported in treatments with higher values were also detected by Amariz et al. (2010) with coatings based in carboxymethyl cellulose and dextrin in 'Tommy Atkins' mangoes under refrigeration due to increase in values of acidity to coated and control fruits at the twentieth day, with reduction of values throughout the storage period. Figure 9 shows response surface contour curves (a) obtained from mathematical model application and Scott-Knott test (b) at 5% of probability for SS/TA ratio.

Significant difference was observed for SS/TA ratio within analyzed treatments, graph of contour curves and Scott-Knott test (b) at 5% reported in Figure 9a and b. Increase was reported in SS/TA ratio from 12.17 at zero time to 15.95 at day 28 of storage in control fruits (Figure 5b). It is verified that fruits SS/TA ratio changed with the type of coating. Fruits that had reduced values showed low soluble solids content and/or titratable acidity at the end of storage. There was significant effect for $x_1*x_2*x_3$ cubic interaction. x_1*x_2 double interaction presented higher and lower values of SS/TA ratio, not having statistical difference for each coating in isolated form at day 28 of storage.

The response surface contour curve shows that the use of coatings in mixtures with cassava starch and chitosan and pectin at 1:1 proportion had beneficial effects in SS/TA ratio. The beneficial effects of these coatings on reducing maturation velocity of mangoes may be verified when SS/TA ratio values are analyzed.

Azerêdo (2016) verified, in 'Tommy Atkins' mango fruits storage for 32 days, fruits coated with cassava starch had lower value as compared to the control fruits and other treatments. SS/TA ratio is one of the most used indexes to determine maturation, being taste indicator (Chitarra and Chitarra, 2005).

Conclusion

Mangoes ripening, fruit firmness, external appearance, lightness of pulp color and pH of all treatments reduced. Soluble solids content and SS/TA ratio increased during the 28 days of storage. The coating based in cassava starch and chitosan at 50% proportion was more efficient in containing physiological loss, slowing down the soluble solids buildup and sustaining the pH and SS/TA ratio of 'Tommy Atkins' mango fruits. Coated fruits had sustained external appearance and quality to commercialize product at the end of storage period.

Cassava starch and chitosan coating provided better conservation of 'Tommy Atkins' mango fruits for a 28 days period of storage at 13°C and 90% of RH.

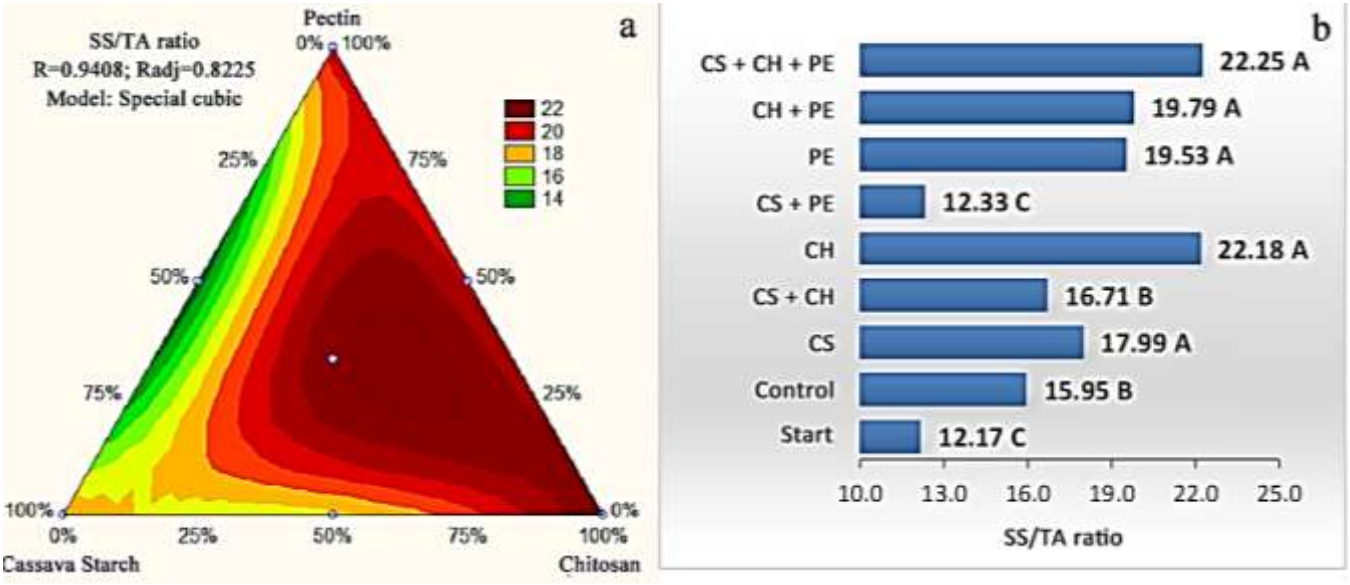

Figure 9. Response surface contour curves (a) and Scott-Knott test (b) for SS/TA ratio at 28 day of storage (13°C and 90% of RH).

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Influence of seasonality on the yield and composition of the essential oil of *Siparuna guianensis* Aublet

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The plant *Siparuna guianensis* is used in traditional medicine and has been the target of studies on the development of new drugs for the control of pests and vector insects. The present study was aimed to evaluate the seasonal influence on the content and composition of the essential oil of *S. guianensis*. The experiment was conducted for 12 months evaluating the yield of the essential oil of leaves throughout the seasons (autumn, winter, spring and summer). The chemical composition of the essential oil was obtained by gas chromatography-mass spectrometry (GC-MS). The highest oil yield was in autumn and winter, comprising 0.33 and 0.29% (w/w), respectively. The major compound identified was β-myrcene (48.59-24.2%), followed by epicurzerenone (27.24-13.7%) being the most abundant; germacrene D showed lower values of 9.93% in autumn and 13.5-14.34% in the other seasons, besides curzerene that had no production in autumn. The ɣ-elemene component had a higher production of 7.29% in autumn. Compound 2-undecanone did not show significant seasonal changes, with percentages being between 7.26 and 5.43%. The monoterpenes and sesquiterpenes showed similar levels in autumn; however, in the other seasons (winter, spring, and summer), the sesquiterpenes presented higher concentration, reaching 68.54% in summer. The components identified in the essential oil of *S. guianensis* exhibit interesting biological activities, making this essential oil a promising compound for the development of new biodegradable drugs, repellents, and insecticides. The knowledge about the yield and seasonal composition is fundamental to optimize and maximize the obtainment of compounds of interest for the production of new drugs.

Key words: Medicinal plant, negramina, seasonal production, essential oil, gas chromatography-mass spectrometry (GC-MS).

INTRODUCTION

Medicinal plants have been used since ancient times owing to their biological activities and aromatic properties. Among the products obtained, the essential oils are remarkable and have attracted the attention of researchers. Essential oils are complex mixtures of volatile and lipophilic substances produced by the secondary metabolism of plants, obtained primarily by distillation, where leaves are normally used, although different parts of the plant can also be used in this process. These compounds are primarily used in perfumes, cosmetics, food and pharmaceutical industries (Teixeira et al., 2013; Okereke et al., 2017).

Siparuna guianensis Aublet is a neotropical species, and in Brazil, it is primarily found in the Cerrado biome, where it is both native and abundant and primarily known by the popular name of “negramina.” The use of this plant was identified in Central and South America in the traditional medicine through remedy baths, and juice and tea were also prepared using its leaves to combat fevers, rheumatic diseases, cramps, and high blood pressures. It was also used as an antibiotic, aromatic, diuretic, and stimulant (Approbato and Godoy, 2006; Furtado, 2006; Souza and Felfile, 2006; Valentini et al., 2010a; Pereira and Silva, 2017).

Other ethnobotanical studies have revealed the traditional use of this plant for combating sinuses, headaches, migraines, and body aches, acting as an analgesic and anti-inflammatory agent (Valentini et al., 2010a; Andrade et al., 2015). It has also been reported to be used for fluctuation of consciousness between hypoactive and hyperactive states, neck pains, muscle strains, fears, vomiting, and seizures (Pagania et al., 2017). The essential oil of this plant has also been studied for the biological control of pests, wherein it has been recently used for the control of *Aedes aegypti* Linnaeus, 1762 (Diptera: Culicidae) and *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae) with interesting results (Aguiar et al., 2015).

The plant has also been evaluated for its activity against pathological bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis* and *Staphylococcus aureus*; against the filamentous fungi such as *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus carbonarius*, and *Penicillium commune*; and against the epimastigotes of *Trypanosoma cruzi* (Andrade et al., 2015). It has also been evaluated for its activity against the promastigotes of *Leishmania amazonensis* (Andrade et al., 2016) and as a repellent (Aguiar et al., 2015).

Several promising results can be observed in the literature confirming the biological activity of *S. guianensis* for various applications. However, less information is available on the variation of the chemical composition of the oil of this plant in relation to the period of harvest of the plant. Therefore, the objective of this study was to evaluate the influence of seasonality on the content and yield of the essential oil of *S. guianensis*, with the intention of subsidizing the production of essential oil for commercial purposes.

MATERIALS AND METHODS

Study area

Leaves were collected from *S. guianensis* in Gurupi-TO, Brazil (11°43'45" S, 49°04'07" W), for the extraction of the essential oil. Taxonomic identification was confirmed by experts at the herbarium of the Federal University of Tocantins (Campus-Porto Nacional), where the samples were deposited with reference number 10.496. The research was registered and approved by Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, no. 010580/2013-1.

Obtaining the essential oil

The leaves of *S. guianensis* were removed from the plant between 7:00 and 8:00 AM. They were then crushed and submitted to hydrodistillation extraction in a Clevenger apparatus (Aguiar et al., 2015). A mixture of 300 g of fresh crushed leaves and 1000 mL of distilled water was heated in a 2-L flask, and the vapors generated were directed to a condensation column coupled to a cooling system. At the end of the distillation process, an aromatic watery phase and another less dense organic phase containing the wet essential oil were obtained, and the organic phase was dried using sodium sulfate. The yield was calculated in relation to the weight of the dried essential oil with the weight of the leaves *in natura* (Silva et al., 2016). The collection period of *S. guianensis* included the four seasons of the year, with six extractions in each season.

Gas chromatography and mass spectrophometry (GC-MS)

The chemical composition of the essential oil was assessed in triplicate and identified by gas chromatography (GC-FID) using a Chemito 8510 GC instrument (Chemito Technologies Ltd, Mumbai, India Pvt.). The separation of the constituents of the sample was performed by a large caliber capillary column BP-5 (30 × 0.53mm i.d., 1.0 mm film thickness). Then, 0.03 mL of the essential oil was injected through a Hamilton syringe into the GC with a cover of 1.0 mL. Hydrogen was used as the drag gas at a flow rate of 5 mL/min and a pressure of 20 psi. The temperature of the GC oven was programmed at 70 to 210°C using a heating ramp at a rate of 2.5°C/min, and the injector and detector temperatures (FID) were maintained at 230°C. The GC-MS analysis with a DSQ MS screen was performed on a Thermo Electron Corporation equipment, Waltham, MA, USA, using a BP-5 (30 × 0.25 × 0.25 mm) capillary column. Helium was used as the drag gas at a flow rate of 1 mL/min and 1:20 split. The temperature of the column was programmed to range from 65 to 210°C using a heating ramp at a rate of 3°C/min. Mass spectra were obtained in the range of 40 to 650 amu, operating at 70 V, and the source was maintained at 200°C (Aguiar et al., 2015).

Identification of compounds

The components present in the essential oil were identified through the computerized comparison of the mass spectrum obtained with those contained in the NIST library database of the mass spectrum of GC-MS, according to the methodology adopted by Moronkola et al. (2017).

Statistical analyses

Analysis of variance was performed by the Tukey’s test ($P < 0.05$).

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Statistical analyses were performed using SISVAR 4.6 (Ferreira, 2001), and the graphs were produced using SIGMA PLOT 11.0 (Systat Software, Inc. San Jose, USA).

RESULTS AND DISCUSSION

Phytochemical studies have shown that essential oils can be obtained from dried and fresh leaves, as well as from stems and fruits; however, the leaves comprise the part of the plant with the highest yield in extraction according to the literature on *S*. *guianensis* (Aguiar et al., 2015); therefore, the leaves were chosen as the object of this study. The extraction of the essential oil from the plant was performed by the hydrodistillation process using the Clevenger apparatus. The leaves were collected during the first hours of the day to avoid loss of volatile material and were immediately sent to the laboratory for extraction.

The percentage values found in this study were calculated from the weight of the oil obtained by the weight of the leaves *in natura* (w/w). The seasons of autumn (April-June) and winter (July-September) are characterized by a drought period in the Cerrado biome with greater temperature variation between the day and night period, whereas the spring and summer seasons present higher values of precipitation and smaller temperature variations along the day, as observed in Figure 2.

The extraction yields in autumn and winter were 0.33 and 0.29% (w/w), respectively. In the spring and summer seasons, the amount of oil obtained from the leaves of *S. guianensis* was lower, with 0.18 and 0.22% (w/w), respectively (Figure 1). These results are consistent with the data found in the literature on the yield of oil extraction from *S. guianensis* leaves, with values ranging from 0.10 to 0.61% (w/w), in terms of quantity and seasonal variation, as shown in Figure 1 (Castellani et al. 2006; Valentini et al., 2010b). Castellani et al. (2006) also obtained higher levels of essential oil in autumn and lower in the spring for leaves of *S. guianensis*.

Analyses of the chemical composition were carried out using samples collected during the four seasons. Figure 3 presents the chromatograms referring to the composition using GC/MS, which enabled the determination of the chemical constituents present in the essential oil of *S. guianensis.* Table 1 shows the composition values of the essential oil of *S. guianensis* in the four seasons of the year, with 97.23% of the sample collected in autumn, 97.82% in winter, 97.06% in spring, and 100.00% in summer. The data obtained from the qualitative and quantitative determinations are presented in Table 1.

Autumn was the period with the highest yield of the essential oil, a period characterized by the end of the rains and the beginning of the dry season. Water stress during this period favors the production of some metabolites, such as the terpenoids that are the primary components of the essential oils (Castellani et al., 2006; Yang et al., 2014). Such metabolites are contained in secretory cells, epidermal cells, cavities, channels, or glandular trichomes (Yang et al., 2014).

Liu et al. (2011) clarified that the greatest accumulation of metabolites is attributed to the closure of plant stomata in the prolonged drought period. The effect of water stress can be observed in other studies, such as that of Jaleel (2008), where *Catharanthus roseus L.* produced more ajmalicine under stress conditions. Valentini et al. (2010b) studied *S. guianensis* in Minas Gerais, Brazil and found that in the dry season, the production of the essential oil reached 0.61%, whereas in the rainy season, it was about 0.10% (w/w).

The oil obtained in the autumn season had the following major compounds: β-myrcene (48.59%), epicurzerenone (19.31%), germacrene D (9.93%), ɣ-elemene (7.39%), and 2-undecanone (5.43%). β-Myrcene was the component that mostly contributed to the increase in oil production. In the other seasons, it was observed that the concentration of oil obtained was directly proportional to the concentration of this component (Table 1), evidencing that the period of water stress favors its production.

The availability of water changes the production of metabolites, and stress positively stimulates the production of some metabolites such as cyanogenic glycosides, glucosinolates, anthocyanins, alkaloids, and some terpenoids (Liu et al., 2011). Samples collected during winter showed the following chemical composition: β-myrcene (40.20%), germacrene D (14.09%), epicurzerenone (13.70%), 2-undecanone (6.05%), curzerene (5.47%), and ɣ-elemene (5.01%), as described in Table 1. This season is also characterized by drought, promoting water stress.

Samples collected during the seasons of spring and summer showed inferior yields compared to those collected during the other two seasons (Figure 1), periods characterized by high precipitation, as shown in Figure 2. Spring was the season with a lower yield, a period characterized by fruiting and sprouting of the plant. This result was consistent with the results for other plants (Ganzera et al., 2008). Valentini et al. (2010a) concluded that the amount of oil of *S. guianensis* remains constant throughout the year, and during the fruiting period, the oil content reduces in the leaves and branches; however, it is compensated by the essential oil present in the fruits.

Samples collected during the spring season showed the following major composition: β-myrcene (34.67%), germacrene D (14.34%), epicurzerenone (18.16%), 2-undecanone (6.09%), curzerene (5.91%), and ɣ-elemene (5.05%), as shown in Table 1. Samples collected during summer showed the same major compounds, that is, β-myrcene (24.20%), germacrene D (13.50%), epicurzerenone (27.24%), 2-undecanone (7.26%), curzerene (7.17%), and ɣ-elemene (5.94%), as shown in Table 1. Although the spring and summer seasons had

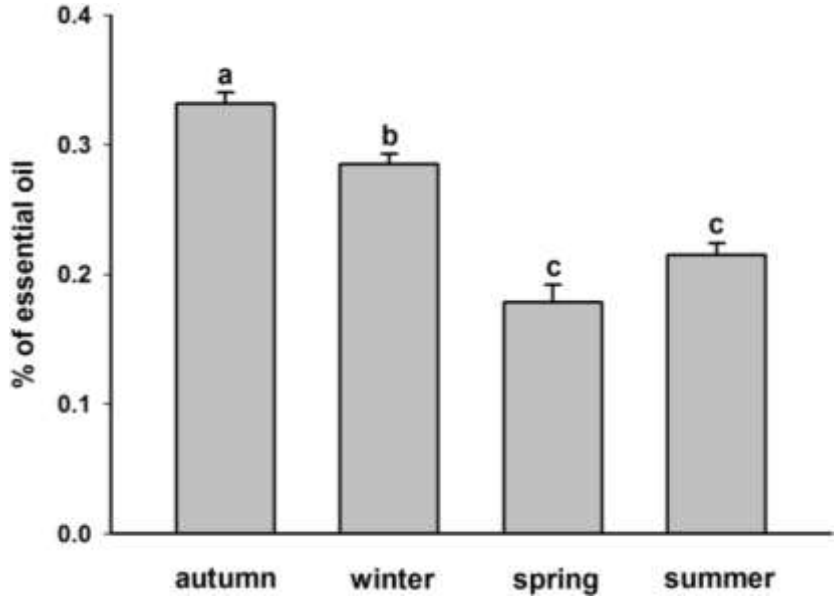

Figure 1. Production of essential oil of *S. guianensis* in relation to the seasons of the year. Different letters up the columns indicate significant differences ($P < 0.05$). Analysis of variance was performed by Tukey's test.

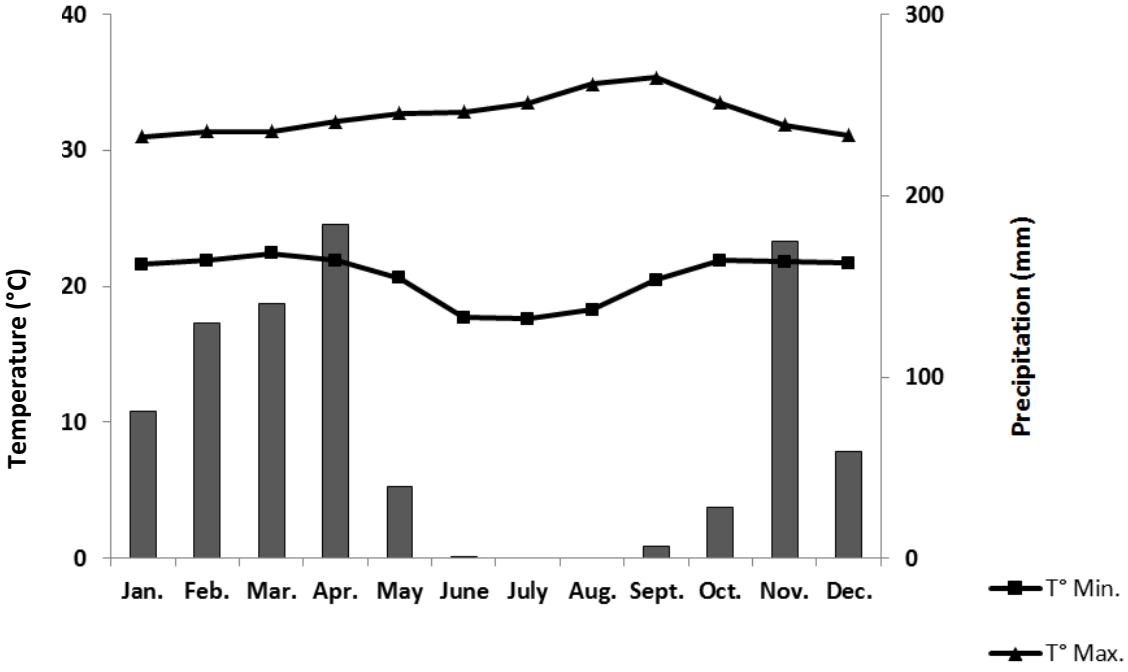

Figure 2. Maximum (T° Max) and minimum (T° Min) temperatures (°C) and rainfall (mm) during the period from January to December 2016 at the Research Station, Federal University of Tocantins/University Campus of Gurupi, Tocantins, automatic station INMET, 2016.

higher radiation rates, which is one of the factors that increase the production of essential oils, it is noted that water stress plays a major role in the production of essential oils for this species.

The fact that these plants are shrubs and groves that develop in the interior of the forest in an environment with low radiation is less relevant, differing from other medicinal plants that present higher production of

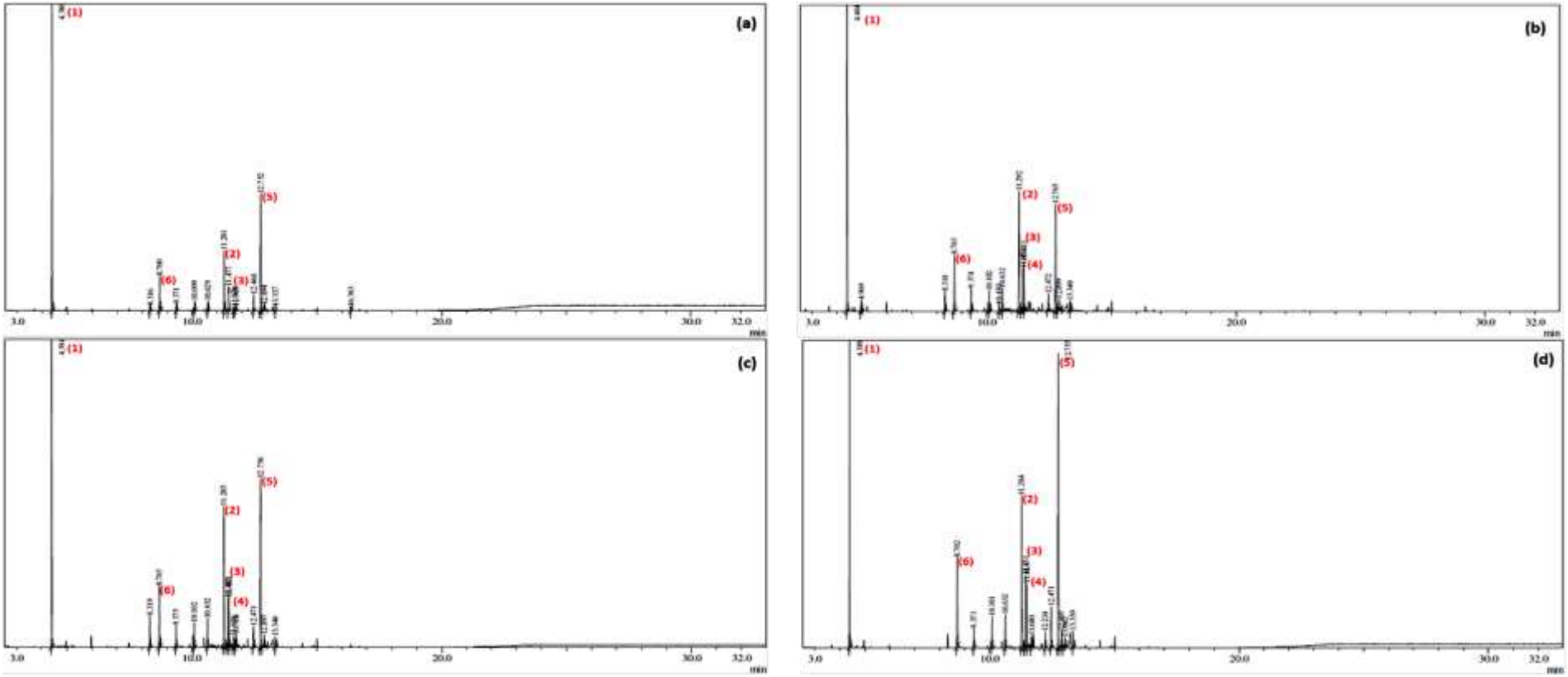

Figure 3. Mass chromatograms of the essential oil collected in autumn (a), winter (b), spring (c), and summer (d) seasons, evidencing the major components, β-myrcene (1), germacrene D (2), ɣ-elemene (3), curzerene (4), epicurzerenone (5), and 2-undecanone (6).

essential oils in periods with higher radiation (Liu et al., 2011). The production of essential oils in the spring season did not show significant changes compared to the production in the summer season; however, it was observed that the content of β-myrcene in the summer (24.20%) was inferior when compared to that in the spring (34.67%), as shown in Table 1. The difference in the concentration of β-myrcene is compensated by the content of epicurzerenone that had yields of 27.24 and 18.16% in the summer and spring seasons, respectively (Table 1).

The concentration of epicurzerenone in the summer (27.24%) is superior when compared to that in the other seasons, with 19.31% in autumn and 13.70% in winter, as depicted in Table 1. These data show that the relevant factor for the production of this secondary metabolite is not water stress but solar radiation. Therefore, a higher composition was found in the summer, even after taking into account the different quantities extracted throughout the year. Liu et al. (2011) concluded that the amount of available radiation and the wavelength type are the factors that alter the composition of the essential oil; the incidence of UV radiation is a factor that contributes to the increased production of certain secondary metabolites.

The composition of the metabolite 2-undecanone presented lower yield in the autumn

Table 1. Chemical composition, concentrations (%), and Kovats index for the essential oil of *S. guianensis.*

Compound	Concentrations (%)				Ric*
	Autumn	Winter	Spring	Summer	
Isoterpilonene	0.94	-	-	-	925
β-Myrcene	48.59	40.2	34.67	24.2	986
Pseudolimonen	-	1.57	-	-	1019
2-Undecanone	5.43	6.05	6.09	7.26	1271
α-Cubene	-	-	-	1.02	1352
ɣ-Elemene	7.39	5.01	4.5	5.94	1439
Elixene	-	4.72	4.5	4.04	1435
ɣ-Muurolene	0.67	1.27	1.36	1.34	1441
δ-Cadinene	0.57	-	1.25	-	1478
Germacrene D	9.93	14.09	14.34	13.5	1528
Curzerene	-	5.47	5.91	7.17	1542
Spathulenol	2.19	1.69	2.03	3.55	1547
α-Cadinol	0.62	1.39	1.32	1.87	1592
Germacrene B	1.59	2.66	2.93	2.87	1613
Epicurzerenone	19.31	13.7	18.16	27.24	1611
Non identified (%)	2.77	2.18	2.94	-	-
Total identified (%)	97.23	97.82	97.06	100.0	-

Ric* = Calculated retention index.

season (5.43%) and this is due to the amount of oil extracted, which was higher than that in the other seasons. The concentration in the other seasons ranged from 6.05 and 7.26%, as shown in Table 1. In autumn, germacrene D production (9.93%) was lower than that in the other seasons (13.50-14.34%), as shown in Table 1, a period characterized by the appearance of flower buds, evidencing that flowering reduces the production of this metabolite or that this metabolite migrates to the flowers.

For the metabolite curzerene, as similar phenomenon was observed but with greater intensity, this metabolite was not identified in the composition analysis done in autumn. However, the metabolite ɣ-elemene (7.39%) presented a higher composition in autumn, indicating that the flowering period stimulates its production and storage in the leaves, consequently increasing the composition of this metabolite in the essential oil when compared to the composition observed during the other seasons (4.50-5.9%), as depicted in Table 1. Flowering and fruiting periods can also quantitatively and qualitatively alter the extracted oils (Ganzera et al., 2008).

Variations in the production of secondary metabolites, especially in medicinal plants, have been studied for their relevance. The genetic factor is preponderant regarding the chemical composition. However, in the same plant species, the essential oil can vary quantitatively and qualitatively depending on the climate, soil composition, organ from which it was extracted, plant age, vegetative cycle stage, and the technique used for extraction (Simões et al., 2010). These conditions promote changes in the composition and, consequently, in the activity of the final product.

The essential oils extracted from the leaves of *S. guianensis* in three Brazilian cities, Belém common Mojú (both in the state of Pará) and Rio Branco, in the state of Acre, have been investigated. Analysis of the chemical composition revealed that in the first municipality, the primary constituents were germaerona (23.2%), germacrene B (8.0%), and atractilona (31.4%); whereas in Mojú, epi-α-bisabolol (25.1%) and espatulenol (15.7%) were identified. The samples from Rio Branco presented the following composition: Espatulenol (22.0%), selin-11-en-4-α-ol (19.4%), β-eudesmol (10.0%), and elemol (10.0%) (Zoghbi et al., 1998). Studies performed in Gurupi-Tocantins, Brazil, found another composition; β-myrcene (79.71%) and 2-undecanone (14.58%) (Aguiar et al., 2015).

The primary constituent, comprising around 90%, of most essential oils are monoterpenes (C10), followed by sesquiterpenes (C15). Several monoterpenes are linked to pollination and sesquiterpenes are most often related to the protection against fungi and bacteria (Simões et al., 2010; Silvia et al., 2011). The essential oils have specific functions in development and cellular respiration and have an important role in primary metabolism. A majority of terpenoids have the function of mediating the relationships between the environment and the plant.

Its production follows two pathways, the cytosolic pathway or the acetate-mevalonate pathway and the plastid pathway or the methylerythritol phosphate pathway, both of which produce isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP).

Table 2. Chemical group and biological activity of the major components of the essential oil of *S. guianensis*.

Compound	Chemical group	Autumn (%)	Winter (%)	Spring (%)	Summer (%)	Biological activity	Author
β-Myrcene	Hydrocarbons monoterpenes	48.59	40.20	34.67	24.20	Antifungal, antioxidant, and anti-inflammatory activities	Bonamin et al., 2014; Ciftci et al., 2011; Lorenzetti et al., 1991
Germacrene D	Hydrocarbons sesquiterpenes	9.93	14.09	14.34	13.50	Antibacterial activity and a precursor in the biosynthesis of sesquiterpenes	Murari et al., 2008; Bulow et al., 2000
ɣ-Elemene	Hydrocarbons sesquiterpenes	7.39	5.01	4.50	5.94	Antitumor activity notable for being efficient in several types of cancer	(Costa et al., 2010)
Curzerene	Oxygenated sesquiterpenes	-	5.47	5.91	7.17	Antiproliferative effect on tumor cells	(Costa et al., 2010; Zhong et al., 2011)
Epicurzerenone	Oxygenated sesquiterpenes	19.31	13.70	18.16	27.24	Antitumor, antibacterial, and antioxidant activities	(Ammon et al., 1991; Collection of China Traditional and Herbal Medicine, 1996)
2-Undecanone	Cetone	5.43	6.05	6.09	7.26	Insect repellent activity and is nontoxic	(Whang et al., 2008; Aguiar et al., 2015)

Monoterpenes such as menthol, geraniol, linalos, and citral are relevant in the perfumes and flavoring industries. Sesquiterpenes such as farnesol, zingiberane, and cariofileno that act in the defense can be used as chemotherapeutic agents and in the treatment of glaucoma (Coteau et al., 2000; Yang et al., 2014).

The chemical structures of the major components of *S. guianensis* essential oil are presented in Figure 4. The biological activities found in the literature for these components are described in Table 2, including the chemical group to which they belong.

β-Myrcene is a hydrocarbon monoterpene that has antifungal, antioxidant, and anti-inflammatory activities (Lorenzetti et al., 1991; Ciftci et al., 2011; Bonamin et al., 2014). Germacrene D is a hydrocarbon sesquiterpene that has antibacterial activity (Murari et al., 2008) and is studied as a precursor in the biosynthesis of sesquiterpenes (Bulow et al., 2000). The metabolite ɣ-elemene is a sesquiterpene hydrocarbon that has antitumor activity notable for being efficient against several types of cancer (Costa et al., 2010). It has been reported that curzerene (an oxygenated sesquiterpene) has an antiproliferative effect on tumor cells (Costa et al., 2010; Zhong et al., 2011).

Epicurzerenone, another oxygenated sesquiterpene, is a major compound in *Curcuma sichuanensis* (Zhou et al., 2007), and it has been used in traditional Chinese medicine owing to its antitumor, antibacterial, and antioxidant activities (Ammon et al., 1991; Collection of China Traditional and Herbal Medicine, 1996). 2-Undecanone, a methyl nonyl ketone, is a naturally occurring nontoxic compound with insect repellent activity (Whang et al., 2008; Aguiar et al., 2015).

Conclusion

In the autumn and winter seasons, the yields were higher due to water stress and flowering period. The major component was β-myrcene, with the highest yield in autumn, evidencing that these phenomena positively affect the production of this metabolite. In the spring and summer seasons, the yields were lower; however, the second major component showed higher yield in the summer, indicating that the solar radiation index is more relevant for the production of this metabolite. The chemical composition of *S. guianenis* presented variations when compared with the literature data, in addition to variations throughout the successive seasons. The components identified in the essential oil of *S. guianensis* exhibit interesting biological activities, making this essential oil a promising compound for the development of new biodegradable drugs and insecticides.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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