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Full Length Research Paper

Comparison of various commercial products for phenol-guanidine-based classical swine fever virus RNA extraction

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TRIzol®, TRI Reagent®, and RNAzol® are widely used commercial reagents for the extraction of cellular or viral RNA. Several other brand name products, some of which are advertised for the processing of specific sample types such as blood, are also available. Here, we compare the efficiency of these products for classical swine fever virus RNA extraction from cell culture supernatant, serum, and tonsil tissue, assessed by quantitative RT-PCR. Furthermore, the detection of a synthetic RNA transcript used as an internal positive control for extraction and RT-qCR was compared as well. Most tested products showed a similar extraction efficiency, and none of the products recommended for specific sample types performed better than the all-purpose reagents. We also show that the homogenization method for tissue samples has a significant impact on the detection efficiency of the RNA after extraction from the homogenized tissue. Homogenization of 100 mg tissue in 5 ml cell culture medium and using an UltraTurrax® tissue grinder yielded the best results, whereas TissueLyser®-mediated homogenization in 1 ml cell culture medium or direct homogenization in RNA extraction medium proved to be less efficient.

Key words: RNA extraction, homogenization, comparison, TRIzol, TRI reagent, reverse transcription-quantitative PCR (RT-qPCR).

INTRODUCTION

Nucleic acids (NA) can be extracted by various methods. For diagnostic purposes, today most methods basically follow the same principle: samples are being lysed in a protein-denaturing agent such as guanidine and/or phenol, followed by purification of the NA from the precipitated proteins, either by centrifugation resulting in organic/-inorganic phase separation followed by NA precipitation, or by NA adsorption to silica membranes or coated magnetic beads followed by washing and subsequent elution.

One of the most widely used methods is the isolation of total cellular or viral RNA by TRIzol® which is a mono-phasic solution of phenol and guanidine isothiocyanate. The extraction protocol was initially developed by Chomczynski and Sacchi (Chomczynski, 1993; Chomczynski and Sacchi, 1987). Today, several additional

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commercial products are available, many of them being advertised to be superior for specific sample types, for example, TRI Reagent® BD for processing blood derivatives. We have previously shown, that full-length viral RNA is still detectable after long-term storage of clinical samples in TRIzol® (Hofmann et al., 2000). Since the advent of reverse transcription-quantitative PCR (RT-qPCR) for the detection of cellular or viral RNA, the consistent isolation and purification of RNA has become a crucial step in any laboratory employing RT-qPCR.

Classical swine fever (CSF) is one of the most devastating pig diseases worldwide (Penrith et al., 2011). It is caused by CSF virus (CSFV) which belongs to the genus pestivirus within the family Flaviviridae. CSFV has an unsegmented, plus-oriented, single-stranded RNA genome. Whereas the virus was traditionally detected by virus isolation on susceptible cell lines, RT-qPCR has become the method of choice for CSFV detection, based on its superior sensitivity and specificity (Hoffmann et al., 2009). Furthermore RT-qPCR still allows detecting CSFV-specific RNA in samples that do not contain any infectious virus anymore. CSFV can be readily detected in serum and specific organs, in particular the tonsils of infected pigs. Since CSFV replicates in commonly used cell cultures without producing any cytopathic effect, RT-qPCR is often also used to identify CSFV upon cell culture infection.

Whereas numerous studies have been published which compare different RNA extraction procedures, both for cellular (Ruettger et al., 2010; Kong et al., 2006) and for viral (Deng et al., 2005; Guarino et al., 1997; Scheibner et al., 2000) RNA extraction, no published data are available on the comparison of various commercial products that are all based on the phenol-guanidine principle. In the present study we compare the performance of TRIzol® with several other brand name products based on the same principle for the extraction of CSFV RNA from cell culture supernatant, serum and tonsils.

MATERIALS AND METHODS

CSFV-positive samples

Clarified supernatant from SK-6 swine kidney cell cultures infected with the moderately virulent CSFV strain Alfort/187 (Greiser-Wilke et al., 1990), and serum and tonsils collected 7 days post infection from a pig infected with the highly virulent CSFV strain Koslov (Kaden et al., 2001) were used for the comparative RNA extraction. All extractions were done with the same sample materials.

Extraction media and RNA extraction

TRIzol® (Invitrogen, Carlsbad, CA, USA) was compared to the following alternative brand name products (all from Molecular Research Center, Inc., Cincinnati, OH, USA): TRI Reagent®, TRI Reagent® BD, TRI Reagent® LS, TRI Reagent® RT, TRI Reagent® RT-Blood, TRI Reagent® RT-Liquid Samples, RNAzol®.

All RNA extractions were run in triplicates and were performed according to the respective manufacturer's protocol, except that 10 µl of a 1 mg/ml solution of glycogen (type III, from rabbit liver; Sigma, Buchs, Switzerland) and 10 µl of an *in vitro*-transcribed EGFP RNA (Hoffmann et al., 2006) corresponding to 10^4 RNA copies were added immediately before sample extraction. Precipitated RNA was dissolved in 20 µl RNase-free H_2O.

Tissue homogenization

Aliquots of 100 mg of tonsil epithelium were homogenized in isolation medium [Eagles Minimal Essential Medium supplemented with 2 % horse serum and antibiotics (EMEM)], either in 5 ml in an UltraTurrax® tissue grinder, or in 1 ml in the TissueLyser® homogenizer (Qiagen, Hilden, Germany). To assess the suitability of RNA extraction media (REM) for direct one-step homogenization and RNA extraction, 100 mg slices of tonsil tissue were also homogenized in the TissueLyser® in 1 ml of TRIzol®, TRI Reagent®, TRI Reagent® RT-Blood, respectively.

RT-qPCR

All samples were tested in triplicates in a CSFV-specific RT-qPCR (Hoffmann et al., 2005) for the presence of viral RNA. The added EGFP RNA was used as an internal positive control (IPC) to monitor both extraction and RT-qPCR. Mean values and standard deviations for the cycle of threshold (Ct) value of the 9 replicates for all REM (that is, 3 independent RNA extractions that were each tested in triplicates in the RT-qPCR) were calculated and used to compare the efficiency and robustness of RNA extraction.

RESULTS AND DISCUSSION

Quantitative detection of CSFV RNA by RT-qPCR following extraction in various guanidine/phenol-based extraction media was compared by analyzing two different liquid sample types, that is, cell culture supernatant or serum. Furthermore, tissue specimens that had been homogenized either in 1 or 5 ml EMEM, or directly in 1 ml of selected REM before RNA extraction, respectively, were also included in the study. Raw Ct values were normalized in order to refer to the same original volume/weight of sample before extraction. For example when results of UltraTurrax®-and TissueLyser®-homogenized tissue were compared, 2.3 Ct were subtracted from the mean of the UltraTurrax®-derived Ct values to take in account the 5 times lower amount of homogenized tissue used for RNA extraction, due to the different volumes used for homogenization (that is, 5 ml for UltraTurrax® versus 1 ml EMEM for TissueLyser®). Differences in original sample amount due to the varying volumes recommended by the manufacturers of the REM to be used for extraction were taken into account as well.

The synthetic EGFP RNA added as IPC prior to extraction of cell culture supernatant was detected with nearly the same efficiency with all REM (Figure 1A), whereas the IPC added to serum samples was detected less consistently, illustrated as greater Ct fluctuations between the REMs. Tri Reagent® showed the lowest Ct values for IPC detection and was the only product that yielded sample type-independent, similar results. In most

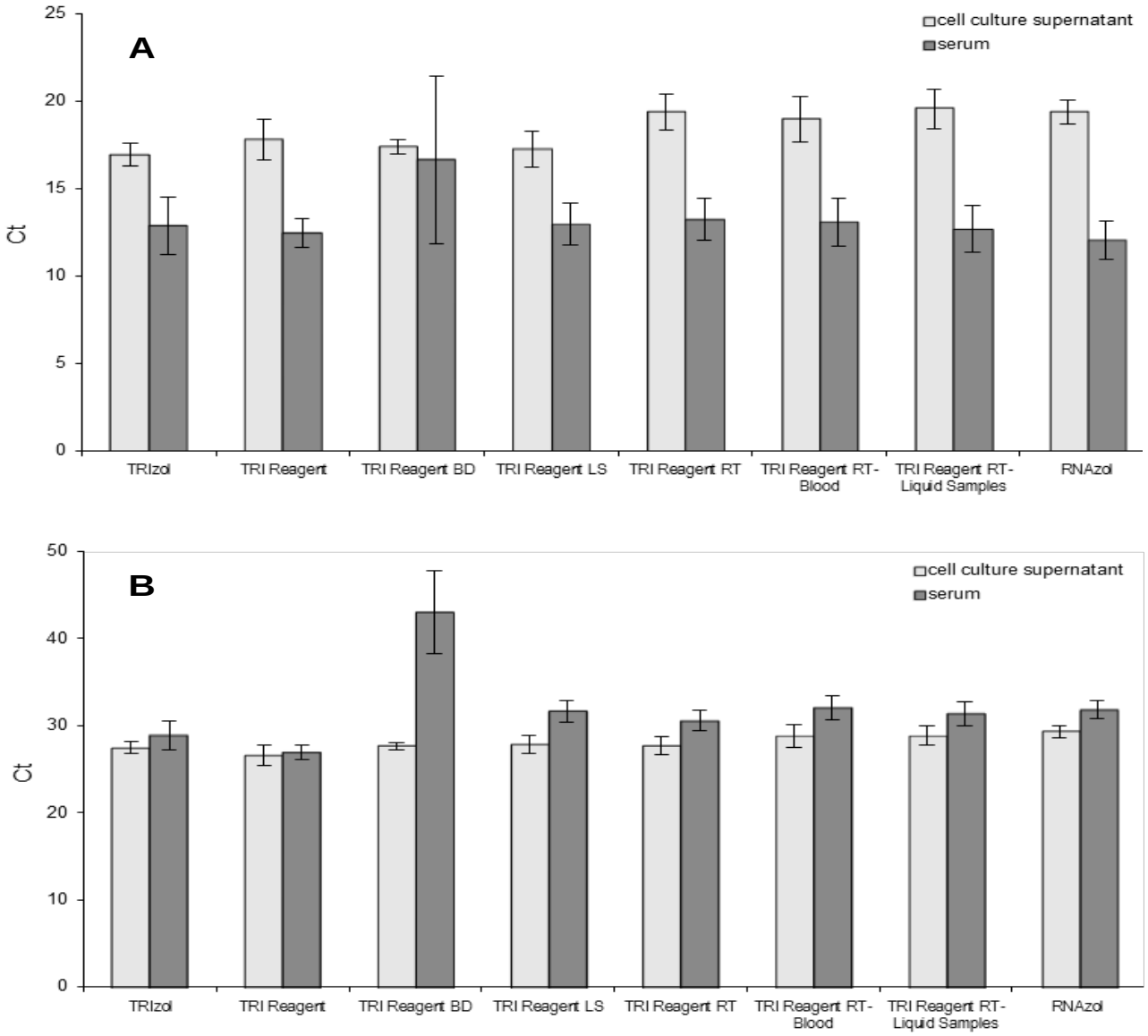

Figure 1. Comparison of RNA extraction media for EGFP IPC (A) and CSFV (B) RNA from cell culture supernatant and serum. Sample volume-normalized Ct values are shown. Error bars indicate standard deviation of the 9 individual RT-qPCR reactions (triplicates from 3 individual extractions).

extractions, IPC detection in serum was less efficient than in cell culture supernatant. In particular, a 100-fold inhibition of the IPC detection was observed after Tri Reagent® BD extraction which is advertised as advantageous for blood derivates (BD). These results indicates that none of the RNA extraction protocols led to a quantitative recovery of the spiked IPC RNA or was able to completely remove RT-qPCR inhibitors present in serum but not in cell culture supernatant.

When the efficiency of CSFV RNA extraction was compared, again only minor differences but the same tendency as for IPC detection was observed (Figure 1B). For the detection of viral RNA from CSFV-positive cell culture supernatant, TRIzol® and TRI Reagent® BD performed slightly better than the other products. However, when viral RNA was extracted from serum, all extractions showed a very similar efficiency, except TRI Reagent® BD which as in IPC detection again showed an inferior efficacy and a larger fluctuation. These results suggest that none of the REM from the TRI Reagent product line nor RNAzol® performed better than TRIzol®. In particular the TRI Reagent® products advertised for specific sample types did not lead to more efficient RNA extraction than the all-purpose products.

RNA extraction from tonsil tissue samples from a CSFV-infected pig was also compared between TRIzol®, TRI Reagent® and TRI Reagent® RT. As shown in Figure 2, the efficiency of CSFV (A) and IPC (B) RNA extraction

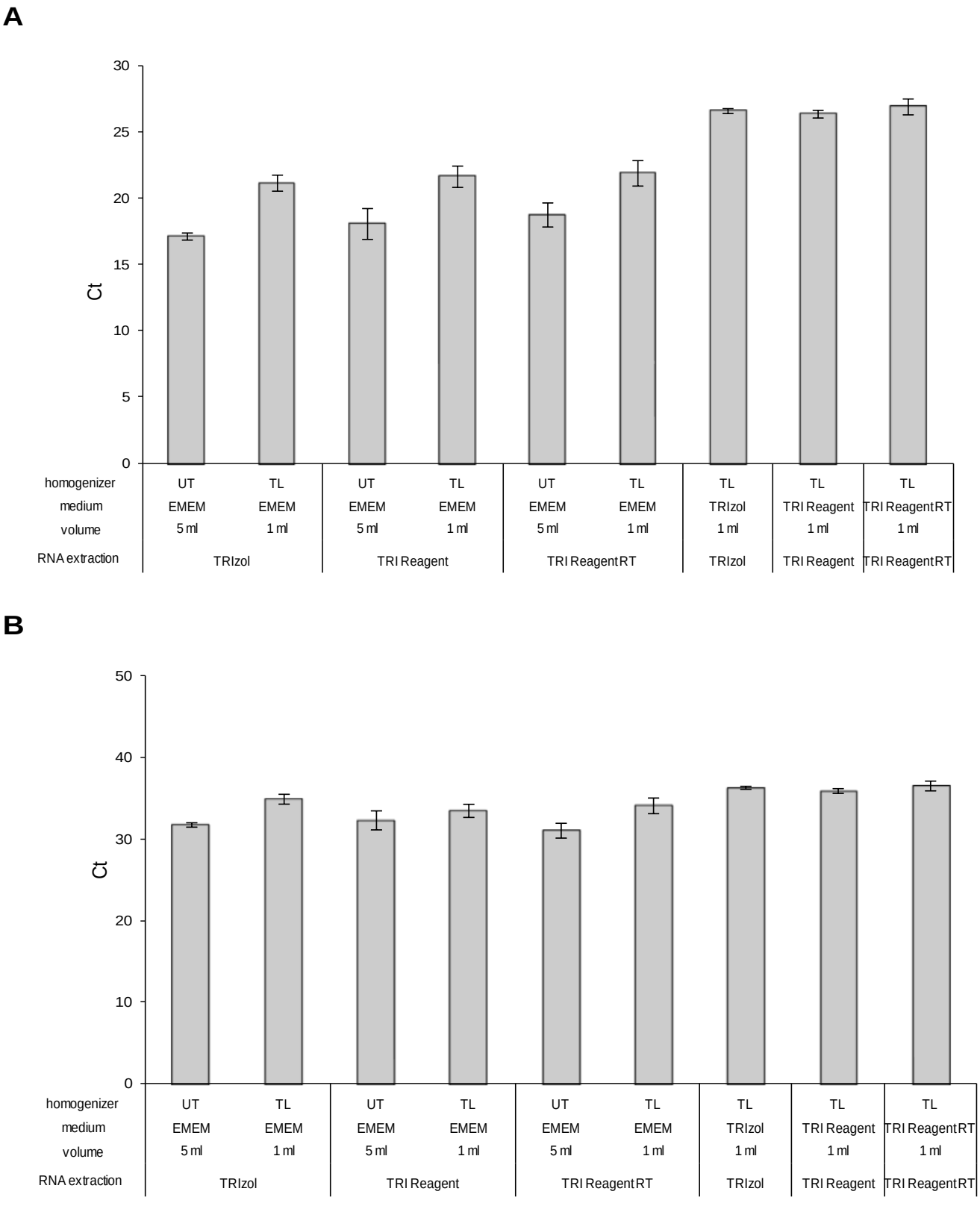

Figure 2. Comparison of homogenization and RNA extraction media for CSFV (A) and EGFP IPC (B) RNA from tonsil tissue. 100 mg tissue was homogenized with an UltraTurrax® grinder (UT) in 5 ml EMEM, or with a TissueLyser® homogenizer (TL), either in 1 ml of EMEM or directly in 1 ml of TRIzol®, TRI Reagent®, or TRI Reagent® RT, respectively. Tissue weight-normalized Ct values are shown. Error bars indicate the standard deviation of the 9 individual RT-qPCR reactions (triplicates from 3 individual homogenizations/extractions).

was mainly dependent on the method used for homogenization of the tissue. The best results were obtained when the 100 mg tissue sample was homogenized in 5 ml EMEM. All 3 REM yielded comparable Ct values that were distinctly lower than if the tissue had been extracted in a smaller volume (1 ml) and using a different homogenization device. On the other hand, direct homogenization in the REM before RNA extraction was significantly less efficient. Results for IPC detection were similar to the CSFV data. Again RNA detection was most efficient in those samples that had been extracted in EMEM in a relatively large volume. Spiked IPC RNA added to the samples homogenized directly in the REM again yielded higher Ct values for all 3 REM. This indicates that the most important factor for efficient RNA extraction is the medium and the volume used for homogenizing the tissue. Differences between the 5 ml and the 1 ml homogenization in EMEM could be due to a different efficiency of the UltraTurrax® compared with the TissueLyser®. However, since the 5 ml homogenization also showed lower Ct values for IPC detection, it is more likely that the volume itself is a critical factor, for example, by leading to a higher dilution of tissue fragments that could interfere with RNA extraction or inhibit the RT-qPCR. Direct homogenization in REM was clearly inferior, most likely due to an inefficient homogenization due to the "tanning" effect of phenol that rendered the tissue more solid. Furthermore, tissue homogenization in EMEM allows - in contrast to protein denaturing agents - infectious virus detection by inoculating susceptible cell cultures.

In conclusion, our data demonstrate that no major differences in RNA extraction/detection efficiency exist between the REM compared in this study. None of the TRI Reagent® products recommended either for blood derivates or for liquid samples performed better than the all-purpose TRIzol® or TRI Reagent®, suggesting that these 2 REM can be used for virtually any sample type. Virus-containing samples should be homogenized in a large volume (for example, 5 ml) of EMEM for efficient RNA extraction and detection by RT-qPCR, respectively, and to allow isolation and subsequent characterization of infectious virus present in the sample.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Genetic diversity in African nutmeg (*Monodora myristica*) accessions from South Eastern Nigeria

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Twenty-one accessions of African nutmeg (*Monodora myristica* Gaertn. Dunal), an endangered spice plant, were collected from the South-East and South-South regions of Nigeria and analyzed for genetic diversity using random amplified polymorphic DNA (RAPD) markers. Twenty-one (21) decamer primers were tested out of which 10 that gave reproducible band patterns were selected for the study. A total of 77 bands were generated, ranging from 3 for OPB17 to 13 for OPT07, and were all polymorphic. The mean polymorphic information content (*PIC*) and genetic diversity (H_e) were 0.673 and 0.697, respectively, indicating high genetic variation among the accessions. Cluster analysis delineated the accessions into four major groups. The maximum similarity index (0.88) based on Dice coefficient was recorded between AGL-01 and CRS-01 while the least (0.13) was between UGA-02 and EKW 01. The derived data was thus able to determine the extent of molecular variation underlying RAPD size polymorphism. Results obtained from this study proved that RAPD could be successfully used as a molecular tool for diversity study in *M. myristica*. The distributive pattern of genetic variation of *M. myristica* accessions provides important baseline data for conservation and improvement strategies for this species.

Key words: African nutmeg, random amplified polymorphic DNA (RAPD), genetic variation, polymorphic information content (PIC), similarity index, *Monodora myristica.*

INTRODUCTION

Monodora myristica (Gaertn.) Dunal also known as Calabash nutmeg, Jamaican nutmeg or African nutmeg, is a useful but neglected tropical tree of the family Annonaceae. It is perennial and found mainly in the evergreen and deciduous forests of tropical African countries, (GRIN, 1985; Iwu, 1993). The plant can grow up to 35 m in height and 2 m in girth with conspicuous, attractive and scented flowers. The large subspherical fruits contain brown, oval, aromatic seeds (Figure 1) which when ground are used as a spice or condiment in African cuisines, providing a flavour resembling that of nutmeg, *Myristica fragrans* (Celtnet Recipes, 2011). In addition, the bark, seed and leaves are used in treating various ailments in African traditional medicine, (Erukainure et al., 2012).

Genetic diversity plays an important role in the survival and adaptability of a species (Frankham, 2005) and is

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Figure 1. Parts of *Monodora myristica* plant. A = Trunk, B = Leaf, C = Flower, D = Broken pod with seeds, E = Intact pods.

essential for its conservation and management (Razvi et al., 2013). When genetic diversity is low, the species is susceptible to diseases or natural hazards and the entire crop species could easily be wiped out.

Molecular tools have been found to be more useful and accurate in the study of inter-species and intra-species genetic diversity in several plants. Randomly amplified polymorphic DNA (RAPD) markers being technically easy to use and fast, have been more commonly used and successfully too, for determination of inter- and intra-species genetic diversity in plants. Some of these include *Zingiber officinale (*Ashrat et al., 2014), *Andrographis paniculata* (Gosh et al., 2014), Phaseolus *vulgaris (*Razvi et al., 2013), *Ocimum* spp. (Sairkar et al., 2012) *Chrysanthemum* (Martin et al., 2002), *Annona crassiflora* (Cota et al., 2011), *Prosopis* (Goswami and Ranade, 1999), date palm (Corniquel and Mercier, 1994), *Papaya* (Stiles et al., 1993), poplars (Bradshaw et al., 1994) and amaranths (Ranade et al., 1997). However, to date, no such attempt has been reported for *M. myristica.*

M. myristica is listed presently under Kew's difficult seeds due to its inability to grow easily outside its natural habitat (Burkill, 1985; Baskin and Baskin, 1998). The plant is largely harvested from the wild and greatly affected by wild fires, urbanization, reckless and uncontrolled felling of trees for timber and firewood without replanting. As part of our conservation efforts to rescue this important species from extinction, there is need, therefore, to determine the extent of genetic diversity to guide the selection of a core collection in a local gene bank. This information could also be used as a guide for further documentation of available genetic and phenotypic variations towards initiating a breeding program for this underutilized crop species. The present study was carried out with this in mind, and should provide the much needed baseline data or information for further studies.

Table 1. Accession codes and location information for 21 accessions of *M. myristica.*

Name	State	LGA	Latitude	longitude
AKS 02	Akwa Ibom	Ikono	05°09.119’	007°48.483’
CRS 01	Cross River	Calabar municipal	04°57.440’	008°19.775
AGL02	Anambra	Anaocha	6°5’33”	7°2’59”
AGL08	Anambra	Anaocha	6°5’30”	7°2’54”
AGL03	Anambra	Anaocha	6°5’14”	7°2’44”
EKW01	Anambra	Aguata	6°0’56”	7°5’10”
AGL09	Anambra	Anaocha	6°5’21”	7°2’45”
UMN04	Anambra	Aguata	6°2’4”	7°3’1”
AGL07	Anambra	Anaocha	6°5’28”	7°2’54”
UMN01	Anambra	Aguata	6°2’8”	7°3’3”
AGL01	Anambra	Anaocha	n/a	n/a
UGA02	Anambra	Aguata	5°56’37”	7°4’53”
AGL10	Anambra	Anaocha	6°5’28”	7°2’54”
UGA01	Anambra	Aguata	5°56’34”	7°4’50”
UMN02	Anambra	Aguata	6°2’6”	7°2’59”
UMN03	Anambra	Aguata	6°2’2”	7°2’53”
AGL06	Anambra	Anaocha	6°5’28”	7°2’54”
AGL05	Anambra	Anaocha	6°5’28”	7°2’50”
AKS01	Akwa ibom	Ikono	05°09.115’	007°48.478’
AGL04	Anambra	Anaocha	6°5’22”	7°2’55”
AGL11	Anambra	Anaocha	6°5’28”	7°2’53

n/a: not obtained

Table 2. RAPD Primer sequences used for the study.

Primer	Sequence (5’ to 3’)
OPT14	TCACCTCCTG
OPT07	GGCAGGCTGT
OPH06	ACGCATCGCA
OPT16	GGTGAACGCT
OPT20	GACCAATGCC
OPT13	GAGGAGCATC
OPB17	AGGGAACGAG
OPT04	CACAGAGGGA
OPB12	CCTTGACGCA
OPB11	GTAGACCCGT

MATERIALS AND METHODS

Sample collection

Young, healthy, leaf samples were collected from 21 accessions of *M. myristica* from different locations in South East and South South Nigeria. The accession codes and collection sites are given in Table 1. The collections were preserved in silica gel during transportation to the laboratory.

DNA extraction, PCR amplification and RAPD analysis

All laboratory experiments were carried out at the International Institute of Tropical Agriculture, Ibadan, Nigeria. Total genomic DNA was extracted from 6 g of young leaves per accession using the modified cetyl trimethyl ammonium bromide (CTAB) method, as adopted by Razvi et al. (2013). After extraction, the yield of DNA was measured using a Nanodrop (ND-1000) UV Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8% agarose gel. Polymerase chain reactions (PCR) were carried out in a Peltier thermal cycler (BioRAD DNA engine) using the primers listed in Table 2. The PCR amplifications were performed as follows: 49 cycles of 94°C for 20 s (denaturation), 38°C for 40 s (annealing) and 72°C for 1 min (elongation) followed by a final extension step of 7 min at 72°C. Amplicons were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide and bands were visualised and photographed under a UV trans-illuminator (GDS-8000, Bio-imaging system).

For RAPD analysis, 21 10-mer oligonucleotide primers (Operon Technologies Inc., CA. USA) were tested and ten were selected and used for characterization of genotypes based on their ability to produce reproducible bands. The selected RAPD primers along with their base sequences are presented in Table 2. The amplified bands as seen in the gel pictures were scored. For each primer, the amplified bands were scored as present (1) or absent (0). Summary statistics for the genetic data based on the following parameters: genetic diversity as described by Weir (1996) and polymorphic information content as described by Botstein et al. (1980), were calculated using the Powermarker software version 3.25 (Liu and Muse, 2005).

The similarity indices were determined using the Dice’s method (dij= b+c/2a+(b+c) where dij is dissimilarity between units i and j, xi, xj: variable values for units i and j a: number of variables where xi = presence and xj = presence, b: number of variables where xi = presence and xj = absence, c: number of variables where xi = absence

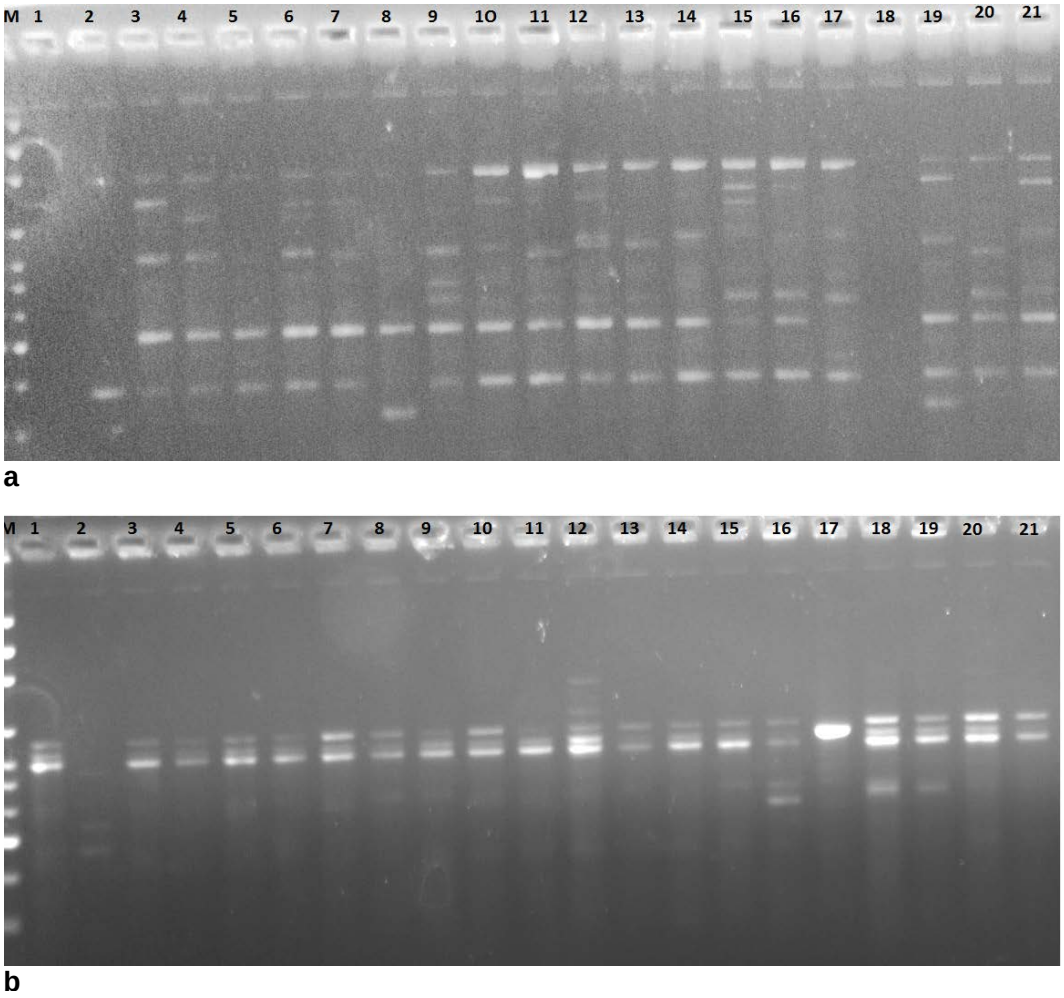

Figure 2. Typical RAPD profiles of 21 accessions of *M. myristica* amplified with a) OPT16 and b)OPH06. M, marker DNA; 1, AKS02; 2, CRS01; 3, AGL02; 4, AGL08; 5, AGL03; 6, EKW01; 7, AGL09; 8, UMN04; 9,AG07; 10, UMN01; 11, AG01; 12, UGA02; 13, AGL10; 14, UGA01; 15, UMN02; 16, UMN03; 17, AGL06; 18, AGL05; 19, AKS01; 20, AGL04; 21, AGL11.

Table 3. Summary statistics for genetic information based on 10 RAPD markers on 21 accessions of *M. myristica*.

Marker	Number of Bands	Gene Diversity	PIC
OPT14	7	0.698	0.683
OPT07	13	0.880	0.869
OPT16	10	0.939	0.935
OPH06	11	0.813	0.791
OPT20	3	0.608	0.562
OPT13	10	0.540	0.525
OPB17	3	0.558	0.481
OPT04	7	0.766	0.746
OPB12	7	0.640	0.617
OPB11	6	0.535	0.517
Mean	7.7	0.698	0.673

PIC, Polymorphic information content.

and xj = presence with the software Darwin 5.0 (Perrier and Jacquemoud-Collet, 2006). Hierarchical cluster analysis using the unweighted pair group method with arithmetical means (UPGMA) was carried out using the Darwin 5.0 software.

RESULTS

Different bands were observed for most accessions, indicating a high level of polymorphism among the species. Typical RAPD profiles for selected primers are shown in Figure 2. The primer OPT07 had the highest number of bands (13) while OPB17 and OPT20 had the lowest with 3 bands each (Table 3). The average similarity index based on Dice's similarity matrix ranged from 0.13 to 0.88 (Table 4) and the genetic differences obtained based on the 10 RAPD markers ranged from 0.540 to 0.938 with an average index of 0.698 (Table 3). The dendogram further delineated the accessions into four clusters with several

Table 4. Similarity indices based on the Dice's method among 21 accessions of *Monodora myristica* in South-eastern Nigeria.

	AKS-2	CRS1	AGL2	AGL8	AGL3	EKW1	AGL9	UMN4	AGL7	UMN1	AGL1	UGA2	AGL10	UGA1	UMN2	UMN3	AGL6	AGL5	AKS1	AGL4
CRS1	0.75																			
AGL2	0.39	0.81																		
AGL8	0.26	0.75	0.31																	
AGL3	0.28	0.73	0.45	0.17																
EKW1	0.35	0.78	0.19	0.21	0.32															
AGL9	0.39	0.80	0.36	0.28	0.29	0.22														
UMN4	0.25	0.76	0.37	0.25	0.26	0.33	0.37													
AGL7	0.41	0.73	0.39	0.37	0.39	0.32	0.39	0.43												
UMN1	0.39	0.80	0.31	0.43	0.44	0.34	0.44	0.45	0.28											
AGL1	0.60	0.88	0.40	0.62	0.62	0.38	0.56	0.64	0.44	0.32										
UGA2	0.42	0.76	0.25	0.33	0.43	0.13	0.30	0.40	0.38	0.39	0.40									
AGL10	0.46	0.68	0.33	0.35	0.37	0.24	0.26	0.49	0.28	0.38	0.46	0.28								
UGA1	0.44	0.64	0.44	0.38	0.35	0.27	0.30	0.46	0.35	0.47	0.54	0.31	0.21							
UMN2	0.44	0.53	0.62	0.44	0.35	0.51	0.41	0.47	0.43	0.48	0.57	0.52	0.43	0.46						
UMN3	0.52	0.61	0.58	0.57	0.50	0.53	0.50	0.55	0.45	0.47	0.40	0.54	0.41	0.53	0.30					
AGL6	0.54	0.86	0.74	0.60	0.64	0.65	0.64	0.68	0.63	0.62	0.72	0.64	0.59	0.67	0.64	0.74				
AGL5	0.41	0.74	0.49	0.45	0.38	0.47	0.38	0.39	0.47	0.35	0.38	0.48	0.49	0.47	0.48	0.38	0.66			
AKS1	0.42	0.81	0.39	0.37	0.39	0.40	0.28	0.35	0.53	0.46	0.55	0.46	0.46	0.49	0.44	0.52	0.60	0.36		
AGL4	0.41	0.68	0.50	0.35	0.31	0.38	0.37	0.44	0.36	0.42	0.42	0.36	0.28	0.37	0.37	0.46	0.59	0.40	0.46	
AGL11	0.33	0.70	0.41	0.27	0.29	0.32	0.23	0.43	0.43	0.45	0.59	0.35	0.19	0.35	0.35	0.41	0.53	0.38	0.33	0.25

sub-clusters, indicating genetic variations among the accessions. Fourteen accessions, namely, AGL11, AGL10, AGL09, UGA01, AGL04, UGA-02, EKW-01, AGL-02, UMN04, AKS02, AGL03, AGL08, AKS01 and AGL05 made up cluster 1 with many sub clusters, while 5 accessions, namely, UMN01, AGL07, AGL01, UMN-03 and UMN-02 fell into cluster 2; cluster 3 had accession AGL-06 only while cluster 4 had CRS 01 which was the most distant accession (Figure 3).

DISCUSSION

The present study was an effort to document available genetic variation in African nutmeg populapopulations in the South East and South South zones of Nigeria. The study is particularly critical as the spice is endangered and difficult to propagate.

The number of fragments observed in this study using RAPD markers was satisfactory for interpretation and conversion into molecular data for the populations studied. According to Ferreira and Grattapaglia (1995), RAPD markers are sensitive and may generate different quantities of amplified fragments depending on the quality and quantity of the DNA used, as well as the amplification conditions.

The mean genetic diversity of 0.698 obtained in the present study was quite high compared to values from other species using similar techniques. An average of 0.31 was reported for *Anonna crassiflora* (Cota et al., 2011). The high values obtained in our study are quite encouraging and conform to that expected for tree species which generally present greater genetic variation within populations (Porth and El-Kassaby, 2014). The genetic similarity indices usually range from zero to one, with values closer to one indicating greater genetic diversity. In the present study it ranged from 0.13 (between UGA-02 and EKW-01) to 0.88 (between AGL-01 and CRS-01), indicating high intra-specific variation in this species. The closest similarity was obtained between accessions UGA-02 and EKW-01 from Aguata Local Government area of Anambra state. However, the both correlation

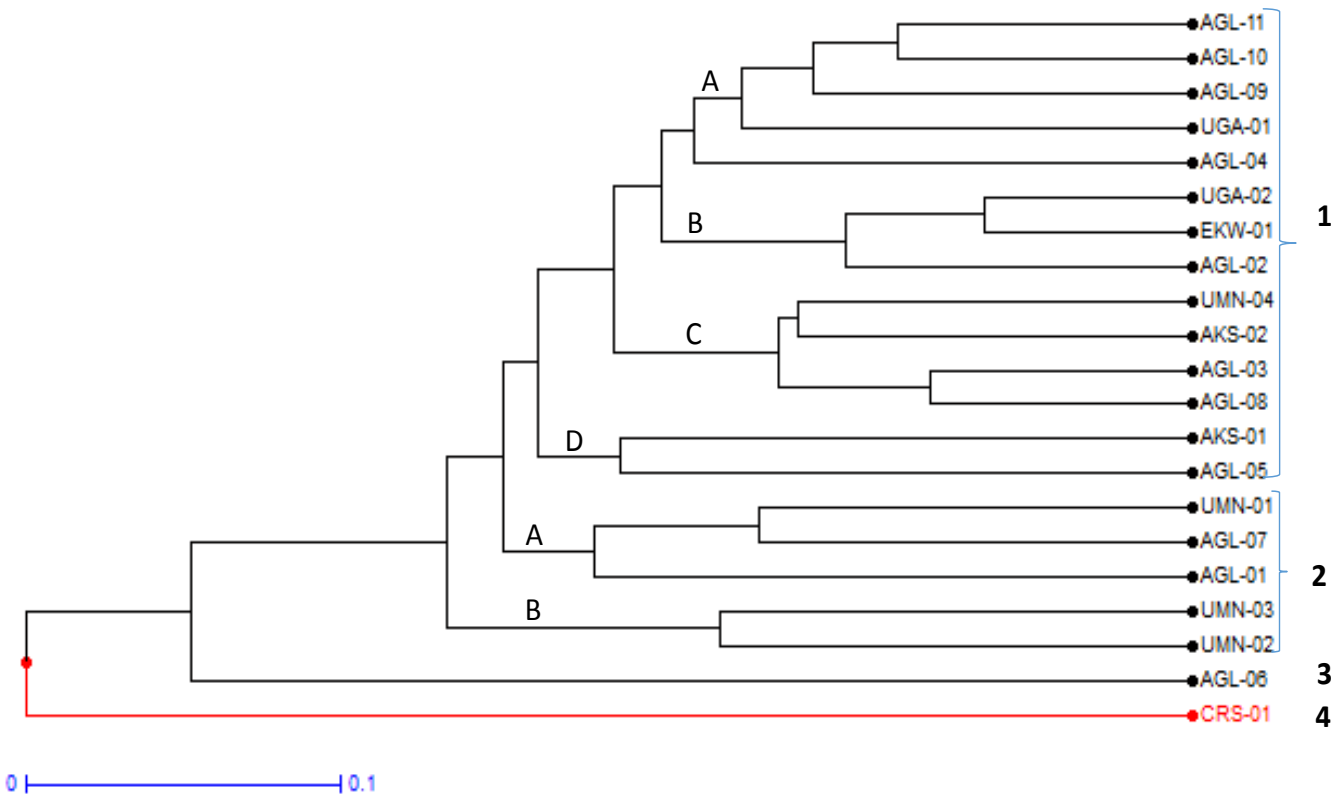

Figure 3. Dendogram based on UPGMA for genetic data in the 21 accessions of *M. myristica.*

between genetic and geographical distances of the accessions did not generally indicate much tendency towards isolation by distance as further illustrated in the clustering pattern, except for CRS-01 (the only accession from Cross River State) which was quite distinct from all the rest. CRS-01 accession was obtained from a coastal region which has an average monthly rainfall of 305.708 mm and total annual rainfall volume of 3668.5mm, which is quite high when compared with the volume of rainfall in other locations. This accession appears to have evolved special adaptations over the years to cope with the higher annual rainfall experienced in this zone, even though such features could not be clearly ascertained from this study. The distribution of the 20 accessions in four different clusters indicates that even though samples were selected from different geographical areas, evolutionary forces such as genetic drift and natural selection pressure remain the major causes of diversity among the accessions. They produce divergent phylogenetic branching which can be recognized because the molecular sequences on which they are based share a common ancestor.

The RAPD-PCR technique has not been used previously in diversity studies on *M. myristica* but has been successfully used for diversity studies in various other species including *Changium symrymioedes* (Fu et al., 2003), *Eremanthus erythropappus* (Freitas et al., 2008), *Ocimum* spp. (Saikar et al., 2012), *Hybiscus* spp. (Prasad, 2014) and *Annona crassiflora* (Cota et al., 2011) among others.

The RAPD pattern observed in this study was able to distinguish the minute differences among the accessions. Molecular markers, unlike morpho-agronomic traits, are not influenced by environmental conditions and, therefore, are more reliable tools not only to characterize genotypes, but also to measure genetic relationship more precisely (Razvi et al., 2013).

The present investigation has shown clearly that *M. myristica* is rich in diversity despite reported losses due to urbanization, unsustainable harvests and indiscriminate felling of trees for firewood and timber. Apart from maintaining food and health security, a rich genetic diversity is a basic resource for improvement programs. It also helps the species to withstand different biotic and abiotic stresses under changing environmental conditions (Porth and El-Kassaby, 2014).

The identification and development of primers that can generate reproducible bands in this species is useful for further studies on improvement, taxonomy and conservation. Information provided in this study with the use of RAPD-PCR has shown it clearly to be quite efficient for genetic variability studies in *M. myristica* and probably other related species and biological specimens. The scope can, however, be increased by using more informative and co-dominant markers in further genetic con-

servation efforts to safeguard this highly valued butundomesticated spice tree species from extinction.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Assessment of over time changes of moisture, cyanide and selected nutrients of stored dry leaves from cassava *(Manihot esculenta* Crantz*)*

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Most fresh agricultural products are quickly perishable and various methods of preservation are necessary. Cassava leaves from different types of cassava (bitter, sweet and wild) were (1) dried un-pounded and (2) dried pounded in a tunnel solar dryer, filled in high density polyethylene material, sealed and placed into opaque cartons. The packing materials were purposively chosen to limit water, oxygen and light access. The complete drying was when samples were completely brittle. The storage was done at room temperature at Sokoine University of Agriculture, Morogoro, Tanzania. The main purpose of the study was to estimate shelf life by evaluating satisfactory quality in terms of nutritional values, dryness and organoleptic parameters. Water, cyanide, ascorbic acid, β-carotene, protein, iron, phosphorus, potassium and zinc were chemically analyzed at zero, three, six, nine and twelve months of storage. Dryness and organoleptic parameters were also evaluated at these different storage lengths. Processing procedure had significant effect only on water (p=0.0358), cyanide (p=0.0189) and β-carotene (p=0.0214) contents. Storage time affected water, cyanide, ascorbic acid, β-carotene, protein, iron, phosphorus, potassium and zinc significantly (p<.0001). Water content increased by 6.8% and ascorbic acid decreased to zero while β-carotene, protein, minerals and cyanide showed slight decline during the storage period. The optimum storage time under the conditions was judged to be six months for nutrients and organoleptic parameters stability.

Key words: Cassava leaves, solar drying, storage time, Rwanda.

INTRODUCTION

Agriculture is a substantial food source for rural and urban populations, and also a reliable source of income through selling fresh or processed products (Legg and Tresh, 2000). However, most fresh agricultural products are usually seasonal and quickly perishable. Hunger and malnutrition can exist in spite of adequate food production because of uneven distribution, deterioration and losses of available resources. To make foods available throughout the year,

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humans have developed various methods of preservation to keep food produced in one harvest for gradual consumption until next harvest. Microorganisms and enzymes that promote spoilage in foods thrive well in foods with high moisture contents and thus drying works as a preservation method simply by reducing the water content of the products and making it unavailable for chemical reactions and growth of microorganisms (Emebu and Anyika, 2011). Dry food products can be distributed and stored at ambient temperatures and this is affordable and common system in rural world, where cooling facilities are not available. As examples, maize, rice and bean are usually dried for extending storage period. According to Mills (1989), their respective equilibrium moisture contents for a safe storage are 13.5, 13.0 and 15.0%, in controlled storage conditions of temperature (27°C) and relative humidity (70%). In rural world, storage of the dry products at uncontrolled ambient temperature and relative humidity is common, especially in developing countries, but their storing lives are not stable. Among food preservation methods by reducing water to equilibrium levels, sun drying is the simplest, inexpensive and commonly adequate for rural and poor communities. However, solar drying offers the following over sun drying: faster drying rate, greater retention of nutrients and organoleptic qualities (Eze, 2010). In addition, minimizing exposure to rain, dust and insects by solar dryers reduces contamination and biological hazards. A sensory evaluation of solar dried cassava leaves in Rwanda showed a greater retention of color, taste, aroma and texture (Umuhozariho et al., 2013). Solar drying has also some advantages over the conventional drying with respect to cost and adaptability to small scale farmers. In reality, solar dryers are promising means for tropical countries to meet their requirements as the available amount of solar energy in most cases are sufficient to cover the required heat for small dryers.

Cassava (*Manihot esculenta* Cruntz) is a staple root crop in many countries of the tropics and particularly in sub-Saharan Africa (Huzsvai and Rajkai, 2009; Legg et al., 2006). According to FAO (2013), cassava can be produced efficiently without the need for mechanization or purchased inputs, and in marginal areas with poor soils and unpredictable rainfall. In fact, ccassava is known to tolerate prolonged drought conditions and low nutrient soils (Leihner, 2002). In Rwanda, cassava is described as “classic food security crop” because it offers the advantage of a harvest even in situations of erratic rainfall and infertile soils (Mushiyimana et al., 2011). In low altitude regions of Rwanda, cassava is among main crop plants and one of the priority crops that are being promoted for economic development and poverty reduction in the agricultural sector (MINECOFIN, 2007). Achidi et al. (2005) indicate that millions of tonnes of cassava leaves are harvested and used as vegetables by many families, especially in Africa, and provide protein, vitamins and minerals (Akinwale et al., 2010; Priadi et al., 2009). They are usually utilized freshly harvested. As it applies to other vegetable products, cassava leaves price varies much according to season and market location. Leaves are available as seasonal surpluses during certain parts of the year (rainy season) and go to waste due to improper processing, pre-packaging, handling, distribution and marketing. During the peak season, vegetables in general are sold at very low prices and some are simply wasted (TCARC, 2007). This reduces income for farmers, adding to the people’s poverty.

For preservation issues in rural communities, cassava leaves are sun dried and consumed at family level during the off-season. For example in low land areas of Rwanda, where cassava is the principal crop, cassava leaves are sun dried to brittle, stored in different types of containers, without any concern about water vapor, air and light access for gradual consumption in long dry season.

However, shelf life of dry food products is for finite period, depending on the type of the product, final moisture content, packaging material and storage conditions (Boyer and Mckinney, 2009; Fellows, 2009). James and Kuipers (2003) and Thomas (2008) mentioned that optimization of storage conditions, specifically by controlling moisture, temperature, oxygen and light, is very important to postpone rotting and spoilage of food products. Therefore, containers would be not only for containing, but also for protecting the food products from outside influences, precisely from water, gases and light entry (Marsh and Bugusu, 2007).

In the present study, leaves from different varieties of *M. esculenta* (bitter, sweet and wild) were been processed by drying, using a tunnel solar dryer, and packed in opaque, water and air proof material for storage at ambient temperature. The main purpose of the study was to esti-mate shelf life by periodically evaluating satisfactory quality in terms of nutritional values, dryness, smell and appearance (color) of the improved solar dried cassava leaves product called *isombe* and *kisanvu*, respectively in Rwanda and Tanzania.

MATERIALS AND METHODS

Collection of cassava leaves

Tender cassava leaves, the first matured up to leaf position five were harvested from three different cassava varieties, named “Seruruseke” (5280), ISAR 1961 and “Igicucu” were chosen for sweet, bitter and wild, respectively. In order to minimize the effects of age, environment and soil type on chemical composition, leaves of same age were selected from similar plot at Rwanda Agricultural Board (RAB)’s field at the Karama Research Station, in Bugesera District of Eastern Province of Rwanda.

Sample preparation

Samples were collected in the field and transported in closed polyethylene bags, which were stored in a cool box containing ice.

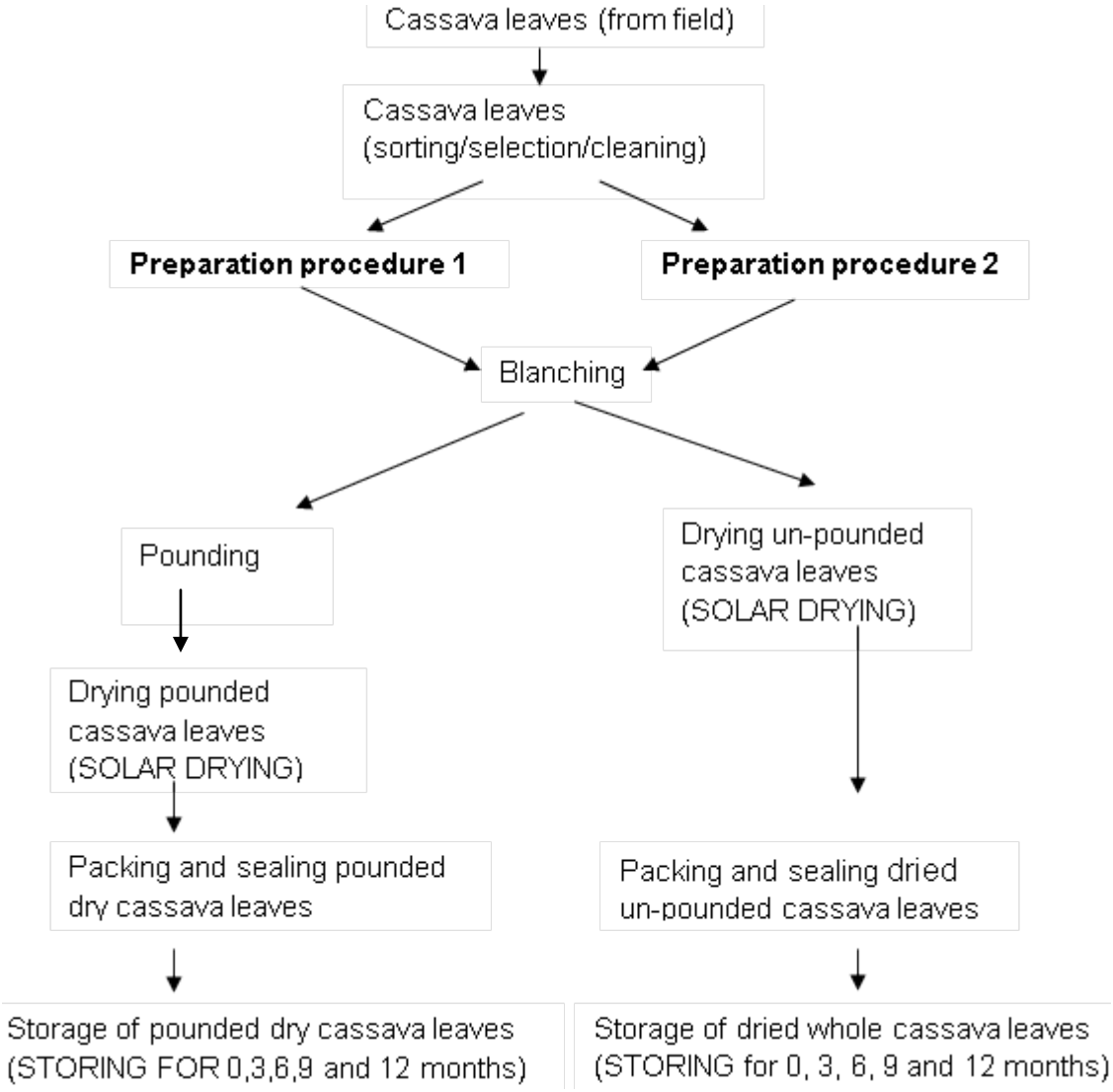

Figure 1. Flow chart of preparation procedures of cassava leaves.

Each sample was divided into two portions after blanching, first portion was dried un-pounded and for the second portion drying was done after pounding. Blanching was done by submersion in boiling water for 4-5 min, and then immediately cooled in tap water at ambient temperature as described by Kendal et al. (2010).

Two different preparation procedures were conducted, namely: (1) drying un-pounded and (2) drying pounded leaves. Pounding was done using wooden mortar and pestle, while drying was done using a tunnel solar dryer at Sokoine University of Agriculture. The products obtained by the two different preparation procedures (Figure 1) were assessed for dryness, color and smell/odor, and chemically analyzed for moisture, cyanide, protein, vitamins and minerals (Ca, Fe, K, P and Zn) at 0, 3, 6, 9, and 12 months of storage. The first four chemical analyses were conducted at Sokoine University of Agriculture laboratories, while vitamins (ascorbic acid and β-carotene) analyses were done at the Tanzania Food and Drug Authority (TFDA), in Dar-Es-Salaam. All chemical analyses were carried out in quadruple.

Drying, packing and storing

Temperatures inside the dryer were recorded at 8 a.m., noon and 8 p.m. each day, averaging 38°C. The complete drying was reached when the samples became entirely brittle. The dried samples were immediately filled in plastic bags. To avoid absorption of water, each bag was sealed in high density polyethylene (HDPT) material and placed into opaque cartons. HDPT and opaque carton were purposively chosen to limit oxygen, water and light access. Storage was done at room temperature, at Sokoine University of Agriculture (SUA), Morogoro, Tanzania. Each of the three test cassava species/varieties (bitter, sweet and wild) was treated separately.

Moisture, cyanide (HCN) and nutrients determination

Moisture content of samples was determined as outlined in AOAC (1995), official method 934.01. Cyanide (HCN) levels in the samples were determined by alkaline titrating method as described

Table 1. Effect of cassava species, processing procedures and storage time on water, cyanide and nutrients of stored dry cassava leaves.

Chemical content	Cassava type	P-value	
		Processing procedure	Storage time
Water	0.9086	0.0358*	<.0001**
HCN	0.2420	0.0189*	<.0001**
β-carotene	0.1504	0.0214*	<.0001**
Protein	0.0662	0.1704	<.0001**
Fe	0.0604	0.0639	<.0001**
Ca	0.1484	0.0797	<.0001**
P	0.1645	0.4232	<.0001**
K	0.3440	0.5625	<.0001**
Zn	0.1505	0.7478	<.0001**

*Significant effect (p<0.05), ** highly significant effect (p<0.01).

by AOAC (1995), official method 915.03B. Minerals, sample ashes and solutions were obtained respectively by official methods 965.09 and 982.23 described by AOAC (1995). Total phosphorus (P) was obtained using ascorbic acid blue color procedure and by reading the absorbance at a wavelength of 884 nm on a UNICAM 5625 UV/visible spectrometer (Okalebo et al., 1993). Calcium (Ca) and potassium (K) were measured by flame photometry, reading their absorbance at 422.7 and 766.5 nm, respectively on a Cole-Parmer instrument, Model 2655-00 Digital flame Analyzer. Iron (Fe) and zinc (Zn) were determined by reading their absorbance at 248.3 and 213.9 nm, respectively on a UNICAM 919 Atomic Absorption Spectrometer (AAS) using Hollow Cathode lamps (Okalebo et al., 1993). Crude protein content was determined by using the micro-Kjeldahl method (AOAC, 1995), official method 920.87. Vitamin C (ascorbic acid) content was determined as outlined by ISO (1984), method 6557/2. B-carotene was measured using a high performance liquid chromatography (HPLC), equipped with a photodiode array (PDA) detector fitted with a 436 nm wavelength. For sample preparation, aliquots were extracted by solvent n-hexane (Priadi et al, 2009; Tee Siong and Lam, 1992). Further extraction and clean-up was done using a dispersive Solid Phase Extraction (dSPE) technique as described in AOAC (2007), official method 2007 0.1.

Statistical analysis

Data from the chemical analysis of the samples were subjected to statistical analysis, using SAS 9.2 (SAS Institute, 2008). Longitudinal analysis techniques which account for correlation among observations over time and equally spaced measurements were used as suggested by Agresti (2007) and, Tiwari and Shukla (2011). The effect was judged significant at p<0.05.

RESULTS AND DISCUSSION

As revealed by Mills (1989), agricultural products change physically and chemically and need to be managed. For “dried pounded” and “dried un-pounded” cassava leaves from bitter, sweet and wild, the final moisture content was on average 4.6% and were completely brittle at packing time. As it is mentioned by James and Kuipers (2003), green vegetables contains less sugar, and thus, dryness to brittle can be considered as safe moisture content levels. In general, at brittle, water contents of green vegetables are between 4-8%, depending on the type of vegetable. Thus, dried at 4.6%, cassava leaves were at safe moisture content level or in equilibrium with a present temperature and relative humidity of the air; but, in ambient conditions, storage temperature and relative humidity were uncontrolled and could not be kept for keeping the leaves quality.

In the conditions, over time physical and chemical changes were to be evaluated because stored agricultural products are influenced by many factors that determine their keeping quality, including product condition, storage container, length of storage and type of handling (Mills, 1989). At nine and twelve months of storage, the dryness changed from brittle to pliable. The appearance did not noticeably change during the storage time of one year while the odor characteristic became more pronounced at pliable than at brittle dryness.

Results of chemical analyses of the samples (just after drying the un-pounded and pounded leaves) were statistically analyzed and effects of cassava type, processing procedure and storage period are shown in Table 1. From the table, cassava types did not have significant influence on the overtime changes of moisture, cyanide, ascorbic acid, β-carotene, protein, Iron, calcium, phosphorus, potassium and zinc contents (p>0.05). Processing procedure had significant effect only on moisture (p=0.0358), cyanide (p=0.0189) and β-carotene (p=0.0214) while storage time affected all the chemical contents significantly (p<0.0001). Mean concentrations of moisture, cyanide, ascorbic acid, β-carotene, protein, iron, calcium, phosphorous, potassium and zinc contents of stored un-pounded and pounded dry cassava leaves, at different period of storage (0, 3, 6, 9 and 12 months) are given in Table 2. From the table, for each cassava type an

Table 2. Mean levels of cyanide, moisture and selected nutrients of cassava leaves according to cassava type, drying procedure and storage time.

Time (month)	Dried and stored un-pounded cassava leaves										Dried and stored pounded cassava leaves									
	%	mg/kg	mg/100 g		%	mg/kg					mg/kg	%	mg/100 g		%	mg/kg				
	MC	HCN	AA	**B**-C	CP	Fe	Ca	P	K	Zn	HCN	MC	AA	**B**-C	CP	Fe	Ca	P	K	Zn
					Bitter											Bitter				
0	4.7	562.8	75 x 10^{-5}	43.7	36.8	222.5	6956.1	4590.4	15520.6	61.9	413.6	4.6	70 x 10^{-5}	39.1	36.6	215.2	6360.2	4454.6	14693.3	59.7
3	5.4	562.5	-	43.7	36.7	221.5	6956.1	4588.2	15518.8	60.8	413.1	5.6	-	38.7	36.2	213.9	6359.4	4453.3	14691.1	59.1
6	6.3	562.1	-	43.1	36.5	220.7	6955.3	4586.6	15515.6	58.9	412.5	6.8	-	38.2	35.9	212.2	6358.1	4451.8	14689.9	58.4
9	8.1	561.8	-	42.5	35.7	219.9	6955.1	4584.4	15513.8	56.6	412.1	8.4	-	37.8	35.5	211.3	6356.6	4450.4	14688.9	56.1
12	10.8	560.9	-	41.6	35.2	218.6	6954.5	4580.4	15511.7	55.7	411.7	11.1	-	37.1	34.9	210.4	6354.7	4448.7	14687.7	55.2
					Sweet											Sweet				
0	4.7	467.7	75 x 10^{-5}	39.8	35.2	221.8	6587.0	4212.0	14358.1	65.5	352.4	4.6	70 x 10^{-5}	29.1	35.1	215.3	6124.7	3957.2	12922.2	62.1
3	5.5	467.7	-	39.7	35.2	220.7	6587.0	4211.8	14356.9	64.3	352.1	5.7	-	28.4	34.7	214.2	6123.5	3955.6	12920.8	61.5
6	6.2	467. 4	-	39.1	34.8	219.6	6587.0	4209.2	14353.4	62.5	352.0	6.7	-	27.5	34.4	213.7	6122.2	3954.1	12919.4	60.9
9	7.8	467. 1	-	38.4	34.4	218.1	6586.1	4207.7	14350.1	60.7	351.8	8.4	-	26.5	33.8	212.5	6121.1	3952.7	12917. 6	59.6
12	10.5	466. 3	-	37.5	33.8	217.2	6585.2	4205.8	14348.3	59.8	351. 4	11.1	-	25.3	33.1	211.3	6119.5	3950.8	12915.7	58.6
					Wild											Wild				
0	4.8	873.3	90 x 10^{-5}	65.9	36.3	243.0	8070.2	4877.4	16157.4	73.2	684.9	4.6	80 x 10^{-5}	63.5	36.1	228.9	7894.8	4607.2	15765.7	69.5
3	5.8	873.1	-	65.7	36.2	242.6	8070.1	4875.7	16155.8	72.3	684.4	5.9	-	62.8	35.7	227.7	7893.9	4605.4	15763.8	68.4
6	6.5	871.8	-	65.1	36.0	241.5	8068.2	4873.5	16153.2	70.5	684.1	6.7	-	62.2	35.3	226.8	7892.5	4603.1	15761.9	67.8
9	7.8	871.4	-	64.3	35.6	240.8	8067.6	4870.7	16151.6	68.7	683.7	8.2	-	61.6	34.9	224.2	7890.4	4601.3	15759.8	67.4
12	10.5	870.7	-	63.5	36.1	238.9	8067.4	4868.8	16149.8	65.7	683.2	11.1	-	60.8	34.3	223.8	7888.9	4598.9	15757.9	65.2

Values are means of ten independent determinations in quadruple. MC= Moisture content, HCN= hydrogen cyanide, AA=ascorbic acid, β-C=β-carotene, CP=crude protein, Fe=iron, Ca=calcium, P=phosphorus, K=potassium, Zn=zinc.

processing procedure, only moisture content increased over time, while cyanide, protein, β-carotene and minerals slightly decreased as time increased. The influence of processing procedure on sample contents during storage was not surprising as some samples were pounded while others were un-pounded. Their pieces had different sizes and it is known that pounding and slicing in small pieces increase the surface available for water evaporation or absorption depending on relative humidity of the storage room (FAO, 1995). In this study, water increased with time. The increase of water during dry food storage has also been reported by Gupta et al. (2012). The increase may be attributed to water vapor absorption through packaging material. In fact, all packaging materials may be permeable to water vapor and air at a certain extent (Marsh and Bugusu, 2007; Brody et al., 2002). According to the same authors, the best barriers may have low permeability which is said to be increased by elevated and variable temperatures of storage room and a recommended storage temperature for dry foods is 21°C/70°F. However, under ambient conditions, temperatures are not controlled. In Morogoro, where the dry products were stored, the average annual temperature varies between 25 and 30°C according to altitude and season (Tanzania Minister of State, Planning and Parastatal sector Reform 1997). The temperatures are high

and not stable during the year. Higher ambient temperature also accelerates oxidative degradation of foods, and the oxidative rate is promoted when the cellular integrity is destroyed (Boon et al., 2010). Besides, considerable temperature differences within container are a major driving force for moisture translocation and condensation and microorganisms and enzymes that promote spoilage in foods thrive well in foods with high moisture contents (Mills, 1989). Taga et al. (2008) noted cyanide liberation from residual linamarin after foods are processed and therefore, the noticeable increase of cyanide characteristic smell during storage period may be caused by the liberated free cyanide.

Nutrient contents deterioration of stored foods is inevitable to some extent. Among deteriorative reactions that cause food components decomposition are enzymatic and non-enzymatic oxidations. All these reactions are known to take place in the presence of oxygen, favored by water, and promoted by light (Bonilla et al., 2010; Kim et al., 2005; Gibis et al., 2011). Enzymatic reaction was excluded by blanching before drying, light was limited by opaque carton, but as reported by Brody et al. (2002), increase of water in stored dry foods through the package is inevitable. Also, despite the hermetic sealing before storing, residual oxygen is unavoidably present in the package headspace and product interstices (Kim et al., 2005), and as mentioned by Brody et al. (2002), all packaging material may be permeable, not only to water vapor, but also to air that contain oxygen. Ascorbic acid, β-carotene, protein, iron, calcium, phosphorous, potassium and zinc decreases may be attributed to the deteriorative reactions. The continuous decline of nutrient contents, especially vitamin C of dried green leaves during storage has been also reported by Negi and Roy (2001). For physical and nutritional quality, dry green vegetables may be consumed before losing its dryness and for the dried cassava leaves in this study, at six months of storage, the products were still brittle, but at nine months, the structure changed to pliable and moisture content to 8.3 and 11 percent at nine and twelve months of storage respectively. The observation was in agreement with what was reported by Boyer and Mckinney (2009) that, in general, vegetables dried until they are brittle, packed in airtight, light and moisture-proof packaging material can be stored for six months at room temperature and dry place.

Conclusion and recommendations

Storage time affected water, cyanide, ascorbic acid, β-carotene, protein, iron, phosphorus, potassium and zinc significantly ($p<0.0001$). Water content increased by 6.8% and ascorbic acid decreased to zero while β-carotene, protein, minerals and cyanide showed slight decline during the storage period. The optimum storage time under the conditions was judged to be six months for nutrients and organoleptic parameters stability. Storage at uncontrollable high and variable ambient temperatures such as these of Morogoro (25-30°C), promotes deteriorative reactions which are the main causes of nutritive and sensory quality losses of the dry foods, even when stored in opaque, water and air proof, and hermetically sealed containers.

When the adequate drying, storage and packing conditions are not combined, storage life can be very limited, less than six months. Therefore, food quality control of dry foods in rural areas, where appropriate packaging materials are not available, is recommended to ensure healthier and safe foods. At industrial level, promoting dry food staffs such as dry cassava leaf products could include water absorber use for safe and stable storage because water inevitably increase within containers.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Volatile compounds produced in two traditional fermented foods of the Congo: Nsamba (palm wine) and bikedi (retted cassava dough)

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The "nsamba" (palm wine) and "bikedi" (retted cassava dough) are respectively produced by fermentation from the sap of the oil palm (*Elaeis guineensis* Jack) and lactic acid fermentation from cassava root (*Manihot cassava* Crantz). The study is focused on the determination of volatile compounds present in these two traditional fermented foods of Congo at the end of fermentation. The characterization of these volatile compounds at the end of fermentation for "nsamba" and "bikedi" revealed as the main compounds esters, terpenes, fatty acids and long chain alcohols. Indeed, it was noted: for "nsamba" 86% esters (ethyl caprylate, ethyl decenoate, N-ethyl decanoic, ethyl laurate) and decanoic acid; for "bikedi" 43% terpenes and 37% alcohols: estragol, limonene, linalol, myrcene and menthol.

Key words: Palm wine, dough, cassava, aroma, fermentation.

INTRODUCTION

In Congo, people have developed, since immemorial times, at the level of family or operative units, traditional processes of fermentation to produce fermented foods and beverages. Within the identification and characterization of constituents responsible for flavor, two traditional fermented foods have been the subject of our study because of their economic, cultural and sociological impact to the population and the type of plant raw material, the root and the sap. It is about the retted cassava roots "bikedi" and palm wine "nsamba".

Food aromas are the main compounds responsible for the taste of foods, more particularly fermented foods. Several bacteria and yeasts synthesize aromas molecules during their metabolism (Belitz and Grosch, 1999; Monroy-Rivera et al., 1990; Bourgeois and Larpent-Gourgaud, 1990; Malonga et al., 1993).

Some specific aromas synthetised by microorganisms can be used as additives. It is the case for aromatic compounds produced by lactic acid bacteria, which can enhance the savour of many foods (Spinnler and Desmazeaud, 1996; Takeoka, 1998; Campbell-Platt, 1987). The cassava (*Manihot esculenta Crantz*) belongs to the botanical family of Euphorbiaceae. It is a perennial plant that can reach about 2-4 m height according to the variety. Leaves and tuberous roots are the main food products from this plant (Gomez and Valdivieso, 1985; Louembe et al., 2001; Louembe and Kobawila, 2003).

The cassava (*M. esculenta Crantz*) is an important

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source of calories because it covers 60% of daily caloric needs of populations in tropical Africa and Central America (Nartley, 1968). Cassava roots are a few inches of the soil surface and are 5 to 10 number. In Central Africa, particularly in Congo, the cassava root is consumed mainly as fufu (flour from fermented root) or chikwangue (cassava bread from fermented dough) (Louembe et al., 2001; Kobawila, 2003; Achi and Akomas, 2006). The fermentation of cassava roots is a lactic fermentation involving the lactic acid bacteria, mainly *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Lactobacillus plantarum*; other bacteria, the *Bacillus* (*Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus polymyxa*), the Enterobacteria (*Actinomycetes*, *Streptococcus* spp.); the yeasts, the *Saccharomyces cerevisiae* species and the genus *Candida*; and the molds.

The main metabolites products are lactic acid (0.45%), butyric acid (0.40%), acetic acid (0.145%), propionic acid and ethanol (0.305%).

Among the local beverages consumed in Congo is palm wine. The sweet sap is obtained from the male inflorescences of oil palm-tree *Elaeis guineensis Jack*, which is left to rest for a few hours or days for fermentation to obtain the fermented palm wine (the type of fermentation is alcoholic) (Brassir, 1962; Faparusi and Brassir, 1972; Malonga et al., 1995; Lasekan and Abbas, 2010; Ho et al., 2007). Among the identified micro-organisms, we noted mainly *S. cerevisiae* and *L. lactis* (Amoa-Awua et al., 2007); others also present were *Bacillus*, *Corynebacterium*, *Pseudomonas* and *Hanseniaspora*. The main metabolites produced are ethanol (3.27%), acetic acid (3.95%), isobutyric acid (0.91%) and isovaleric acid (0.42%) (Malonga et al., 1995; Nur Aimi et al., 2013).

MATERIALS AND METHODS

Cassava roots aged 18 months were gotten from plantations around Brazzaville. The "bikedi" from a production workshop located in the south of Brazzaville. The fermented sap of the oil palm (*Elaeis guineensis Jack*) was collected in the vicinity of Brazzaville.

Preparation of retted cassava roots

The internal production process was made with cassava roots whole or peeled. Fresh roots were immersed in water (or peeled and immersed) for fermentation two to five days. The retted tubers were removed from the water, peeled, washed and drained (or washed and drained in the case of pre-peeled tubers). The fermented tubers were then defibrated by extracting the central fiber; they fall in clumps to give retted cassava dough.

Collection of the oil palm-tree sap

Oil palm-tree male inflorescence was cut and the sap which escapes was collected in the sterile bottles during several hours.

Preparation of samples

Case of "bikedi"

Samples of tubers were placed in tubes noted (T_0, T_1, T_2, T_3 and T_m) corresponding to tubers before soaking in water (T_0), after one, two and three days of soaking (T_1, T_2, T_3), 48 and 72 h after soaking and fermented cassava roots (T_f tube of accomplished product). For each sample of tubers (T_0, T_1, T_2, T_3 and T_f), grinding was done with Waring Blendor. 15 g obtained pulp were weighed and placed in a test tube. The tubes were then noted TT_0, TT_1, TT_2, TT_3 and TT_f. For each sample, three trials were prepared.

Case of "nsamba"

For each sample of "nsamba", V_0, V_1, V_2, V_3 and S correspond respectively to the beginning (V_0) after one, two and three days of fermentation (V_1, V_2 and V_3) and palm wine (S). The sample was stirred to mix well, then 15 g of the obtained liquid was placed in test tubes, respectively noted as TV_0, TV_1, TV_2, TV_3 and TS. For each sample three trials were prepared.

The analysis was realized with the fermented products accomplished (T_f and TS) which were appreciated by tasters panel.

Preparation and analysis of extracts

20 ml of ethyl ether (solvent 1) were added to the first tube and 20 ml of cyclohexane (solvent 2) was added in the last two tubes. Then all the tubes were placed for 3 min in vortex for sedimentation and finally, the supernatants were collected in screws tubes which were placed in the refrigerator. Analysis of samples was carried out by gas chromatography (GC) coupled with mass spectrometry (GC / MS). The apparatus used was a Hewlett Packard 5973/6890, equipped with an injector (280°C) and a HP-5 column (25 m x 2.25 mm, 0. 25 µm film thickness). The temperature programme was 50°C (5 min) the temperature was increased to 300°C at the rate of 5°C / min. The carrier gas was helium at a flow rate of 1.1 ml / min. The injected volume was 1 µl of the sample diluted at 10% (v/v) with acetone sample. Retention indices of all aroma compounds were determined according to Van Den Dool approach (Van Den Dool and Kratz, 1963). The identification of compounds was performed by comparing their mass spectra with those presented by Mc Lafferty (Mc Lafferty and Stauffer, 1989), Adams (Adams, 2001) and Joulain (Joulain and Konig, 1998), and their retention indices with those given in literature.

RESULTS AND DISCUSSION

The results of the identification and quantification indicate: a) for "nsamba" (palm wine) 15 compounds (Table 1), including: 86.05% esters; 6.48% of fatty acids, octanoic, decanoic, dodecanoic and hexadecanoic acids; 0.67% of terpene represented by (E)-beta-farnesene and dillapiole; 0.59% phenyl ethylic alcohol; 0.18% of aldehyde, tridecanal. b) For the "bikedi" (fermented cassava paste) 51 compounds (Table 2) including: 41.19% terpenes; 37.11% alcohols such as linalool, estragol, anethole and cravacol. The estragole is the most represented (33.82%); 6.18% methyl esters with angelate, citronnellyl formate, ethyl decanoate, bornyl acetate, geranyl acetate, and thymol methyl ester; 4.57% alkanes $nC_{12}H_{26}$, $nC_{15}H_{32}$, $nC_{16}H_{34}$; 1.51% fatty acids with nonanoic,

Table 1. Compounds identified in the extract of “nsamba"

Chemical constituent	Percentage (%)
Ethyl hexanoate	1.7135
Phenyl-ethylic alcohol	0.5946
Octanoic acid	1.8381
Ethyl caprylate	28.2519
Phenyl ethyl acetate	0.3402
Ethyl 3-phenyl propionate	0.2417
Decanoic acid	3.6444
Ethyl 9-decenoate	23.7702
N–Ethyl decanoate	22.5624
(E)-Beta-farnesene	0.3903
Tridecanal	0.1781
Dodecanoic acid	0.8117
Ethyl laurate	3.7615
Dill apiole	0.2777
Ethyl palmitate	0.3072
Hexadecanoic acid	0.1922
Ethyl 9-hexadecanoate	1.4718
Isomeric ethyl 9- hexadecanoate	0.2517
Ethyl palmitate	1.8893
Ethyl oleate	0.3251
Ethyl oleate	0.9766
Ethyl stearate	0.1981

Table 2. Compounds identified in the extract of "bikedi"

Chemical constituent	Percentage (%)
Myrcene	2.4749
Limonene + eucalyptol + para-cymene	23.9185
(Z) beta ocimene	0.7087
(E) beta ocimene	0.5456
Gamma terpinene	1.7114
2-Nonanone	1.0451
Linalol GIVAUDAN	2.4808
CIS-ROSE OXIDE	0.5274
Ocimene « Neo-allo »	0.7592
Camphor	0.5182
2-Methyl butyl angelate	0.2858
Menthone	2.4612
Iso-menthone	1.1662
Terpinene-4-ol	1.1721
Methyl salicylate	0.7165
Estragol	33.8179
Methyl thymol ether	0.7945
Methyl carvacrol ether	0.4405
L-Carvone	0.3358
Piperitone	0.4528
Citronnellyl formiate	1.8292
Nonanoic acid	0.6219
Bornyl acetate	1.6852
Anethol	0.6951
2-Undecanone	0.2734
Methyl nonylcetone	0.7623
Carvacrol	0.1182
Citronnellyl acetate	0.4974
Neryl acetate	0.1557
Dehydro acetic acid	0.5773

decanoic and dehydro acetic acids.

The identification and quantification of responsible compounds for aromas in the two fermented traditional foods "bikedi" and "nsamba" reveal a clear difference of compounds between the two extracts. They showed the presence of terpene hydrocarbons in larger amounts (41.19%) than alcohol (37.11%) in the aromatic extract of "bikedi." As for the aroma of "nsamba", they are mostly composed of fatty acid esters (86.05%) with mainly ethyl caprylate (28.25%) (Anli et al., 2007). This difference is due, in part, to the constituents of the sap of oil palm and of cassava root and, secondly, the microorganisms involved in the fermentation and the ability of these microorganisms to synthesize aroma compounds from the present products. The microorganisms produce intra and extra cellular enzymes which contribute to generative reactions for flavor and aromas. The aldehydes, ketones and carboxylic acids may result from a degradation reaction of oxidation catalysed by lipoxigenase and hyperoxidase enzymes (Leejeerajumnean et al., 2001; Zhao et al., 2006; Nzigamasabo, 2012).

In the "bikedi" limonene representing 23.92% of the aromas could come from the breakdown of sugars. It allows the production of mevalonic acid and leads to the formation of geranyl pyrophosphate (GPP), a precursor of limonene (Nyako, 1977; Mann and Davidson, 1994).

Limonene is used in food and pharmaceutical industry to flavor bitter alkaloids. It possesses also phytotherapeutic virtues because it is recognized as an anticancer agent (Daeschel et al., 1988; Tsuda et al., 2004). Estragole (33.82%) was also identified in these extracts. It is used as a food aroma in some liqueurs and in perfumery (Scheier, 1979). The estragole is also suspected to be carcinogenic and genotoxic (Annan et al., 2003).

Among the phenols, we noted the presence of carvacol and thymol. Phenols have the antioxidant power and the most interesting bactericidal or bacteriostatic activity (Richard, 1992).

Myrcene, monoterpene hydrocarbon, identified in "bikedi" and "nsamba" is a key molecule for the synthesis of various compounds, including vitamins A and E but also the geraniol and its derivatives. It allows the reduction of the sensitivity to pain by increasing endogenous morphinopeptides (Andah and Muller, 1973).

In "nsamba", the following compounds were identified: tridecanal, dillapiole, dodecanoic acid and ethyl stearate. This qualitative difference may be support of the difference in aromatic typical of these foods (Umoh et al.,

1985; Onyango et al., 2004; Lasekan and Abbas, 2010).

Conclusion

The characteristic aromatic notes of "nsamba" could be due to the presence of: a) constituents compounds of the oil palm-tree sap, rich in carbohydrates, fats and proteins, while the cassava root, raw material of "bikedi", is essentially rich in carbohydrates; b) mixed populations of yeasts and bacteria whose the action lead to alcoholic and lactic acid fermentation, respectively for the oil palm-tree sap and the cassava root as well as the particular synthesis of aromas molecules according to the micro-organisms species. The qualitative difference in the composition of these two fermented foods is at the basis of the difference in the aromatic notes between "bikedi" and "nsamba". The characterization of these volatile compounds is an important piece of information for understanding the perception of odours, flavor and aromas (Shittu and Adedokun, 2010; Biasioli et al., 2011; Fiches et al., 2013).

In perspective, to improve the taste and aromas of the products, it is possible to use the technique of starters which is more suitable to the use of pure cultures in traditional methods as it is indicated by the studies of Ko (1985), Nout et al. (1987, 1992, 1993), and Tuncel et al. (1989). The knowledge of the biochemical phenomenon occurring in these food fermentations could allow to improve the traditional processes of production for obtaining products which have better quality.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Moringa extracts used in sugarcane juice treatment and effects on ethanolic fermentation

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The objective of this study was to evaluate the effects of sugarcane juice treatment using *Moringa oleifera* leaf and seeds extracts on ethanolic fermentation. The experiment was arranged in a split plot statistical design, with four replications. Main treatments were three sedimentation agents (synthetic polyelectrolyte, moringa leaf and seed extracts) and control while the secondary treatments were two sugarcane varieties (RB867515 and CTC4). Extracted sugarcane juice was clarified by simple defecation with pH adjusted to 6.0. The flocculating agents were added in a decanter before the limed juice. After then, the juice was standardized to 16° Brix at pH 4.5, and musts were inoculated with yeast *Saccharomyces cerevisiae* strain, FT858. At the end of the fermentation process, wines were recovered by centrifugation. In all experimental stages, extracted juice, clarified juice and wine were chemically and technologically characterized. The use of moringa leaf and seed extracts as sedimentation adjuvants did not increase the sedimentation speed of impurities. However, there was a high sludge compaction, which was essential for maintenance of yeast and bud population at the beginning of fermentation, and yeast budding rate in the end. The use of different sedimentation agents as adjuvants in juice treatment did not affect wine quality and ethanol yield.

Key words: Juice clarification, *Moringa oleifera* Lamarck, simple defecation, polyelectrolyte, flocculating agents, *saccharomyces cerevisiae.*

INTRODUCTION

Ethanol is one of the mostly produced biofuels in the world, with the United States and Brazil being the major producers. The estimated production for the season, 2014/2015, is approximately 28.37 billion liters only in Brazil (CONAB, 2014). In this country, ethanol is produced from sugarcane. After extraction, cane juice is treated to remove soluble and insoluble impurities, and submitted to a fermentation process by yeast inoculation, which metabolizes sugars and produces the ethanol that is recovered via distillation process.

Juice treatment is essential to remove some materials and yeast inhibitory compounds, such as dirt, bagasse, acids and phenolic compounds. This process begins with sieving extracted juice to remove some bagasse, followed

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by a dirt removal through hydrocyclone equipment. After physical treatment, calcium hydroxide is added to the juice until pH 6.0 and juice is heated to 100-105°C. A chemical reaction between added calcium and the phosphate present in the juice occurs, and insoluble compounds such as calcium phosphate precipitate and adsorbed biomolecules like acids and phenolic compounds are formed. Juice is placed in a decanter and without stirring for 1 to 3 h to remove these precipitates (Steindl, 2010).

In order to accelerate particle sedimentation, synthetic acrylamide-based polyelectrolytes are used in the clarification process, because they react with the calcium phosphate precipitates to form flakes with higher mass and density which accelerate precipitation (Doherty, 2009).

However, acrylamide has carcinogenic and neurotoxic action in animals and humans (WHO, 2002). Thus, some countries as the United States restrict the use of this product to the maximum of 5 mg/L of juice in sugar production (FDA, 2014). It is also important to know that the clarified juice by this treatment could contain significant amounts of acrylamide. In the fermentation process, the yeasts may absorb this molecule and, when destined to animal food after ethanol production process, may be toxic. This product is sold in Brazil at an average price of $6.00/Kg, and the amount used is approximately 1 kg per 150-250 tons of processed cane (Hassuani, 2012). Considering these problems, the search for molecules to replace synthetic polyelectrolytes in juice treatment has intensified. Among the alternative products, leaf and seed extracts of moringa (*M. oleifera* Lamarck) are considered as they are widely used as flocculating agents in water treatment (Sarpong and Richardson, 2010).

The seed contain a protein (MO2.1) that has a coagulant action. This protein presents molecular weight about 6-30 kDa, and isoeletric point between 10 and 11. It is formed by glutamate, arginine, proline, glycine, valine, serine, among others amino acids (Gassenschmidt et al., 1995). It should be noted that the coagulation/flocculation mechanism of moringa protein is similar to the polyelectrolyte, once positive charges are presents in molecule that adsorb particles of the medium (Borba, 2001). Costa et al. (2014) observed that moringa leaf and seed extracts also had flocculation effects when used to treat sugarcane juice for sugar production. The objective of this study was to evaluate the flocculation effects of moringa leaf and seed extracts treatment on ethanol production during sugarcane juice fermentation.

MATERIALS AND METHODS

The experiment was conducted at the Sugar and Ethanol Technology Laboratory of the Department of Technology, UNESP, Jaboticabal-SP, Brazil, in 2013/2014 season. The juice of two sugarcane varieties; RB867515 (fourth ratoon harvested in July, 2013) and CTC4 (second ratoon harvested in August, 2013), both ripening between July and September (mid-season), was extracted. The sugarcane plants were collected from commercial sugarcane fields in Jaboticabal-SP, Brazil during the practical industrialization period. The experiment was arranged in a split-plot statistical design, with four replications. Main treatments comprised different sedimentation adjuvants; and secondary treatments were represented by different varieties.

Sugarcane stalks were manually harvested without trash burn and processed using a laboratory scale cane crusher. The raw material was analyzed for total soluble solids (Brix), sucrose content (Pol), purity, reducing sugars (RS), total reducing sugars (TRS), cane fiber, pH, total acidity, soluble ashes (CTC, 2005) and total phenolic compounds (TPC) (Folin and Ciocalteu, 1927).

The extracted juice was filtered in a 60 mm-mesh filter and submitted to a clarification unit by simple defecation process, through a pH adjustment to 6.0 with $Ca(OH)_2$ 6°Bé, and heating until ebullition. Then 1 L of heated-juice was kept for 20 min in a laboratory decanting system that contained the flocculating adjuvants. Sedimentation speed and sludge volume were evaluated (CTC, 2005). The clarified juice was removed using a siphon.

The sedimentation adjuvants used were: conventional synthetic polymer (Flomex 9034), moringa leaf extract (Ghasi et al., 2000) and moringa seed extract (Bhatia et al., 2007) at the doses of 1.5, 5 and 100 mg/L, respectively. Untreated clarified juice was used as control. These concentrations were established in previous assays, where the concentrations from 1 - 500 mg/L of extracts and 1 - 5 mg/L of polymer were tested. Analysis of Brix, pH, soluble ashes and total acidity were performed in clarified juice to characterize the treatments.

For musts preparation, the clarified juice was standardized to 16° Brix at pH 4.5 with H_2SO_4 5 mol/L. Total reducing sugars (TRS), total phenolic compounds (TPC) and total acidity were analyzed.

The yeast strain, FT858, was then inoculated at 10% of cell concentration (m/v), in 250 ml of must. First, 100 ml of must was added and kept for 1 h, and then another 150 ml was added. Yeast cell, bud viability and yeast budding rates were analyzed (Lee et al., 1981) after 1 h of the second must feeding when Brix was ≤ 1 (end of the fermentation process).

Wines were centrifuged at 2500 x *g*, at 30°C and total residual reducing sugars (TRRS), total acidity (CTC, 2005), glycerol (Copersucar, 2001) and alcohol content (Ebuliometter) were analyzed. The results were submitted to analysis of variance (ANOVA) using F test and the means were compared by Tukey test at 5% of probability.

RESULTS AND DISCUSSION

Characterization of flocculating agents

Table 1 shows the results of Brix, pH, soluble ashes and total acidity of the three flocculating agents studied. The total soluble solids values obtained from moringa leaf and seed extracts treatments differed from water (0°). These results were similar to that of Costa (2013), who observed Brix values of 1.3 and 1.5 of moringa seeds and leaf, respectively.

The pH values of the flocculating agents were 6.0 for the extracts and 6.5 for synthetic polymer. These results do not corroborate with those of Costa (2013), who obtained values of 3.3 and 7.2 for moringa seed and leaf extracts, respectively. This difference could indicate that time of season influence the acid quantity in the moringa seed. Considering the polymer, the difference could be the water quality used.

Extracted juice characterization

Table 2 shows the results of Brix, Pol, Purity, RS and

Table 1. Results for Brix, pH, Soluble Ashes and Total Acidity of sugarcane juice treated with moringa leaf, seed extracts and synthetic polymer.

Extract	Brix	pH	Soluble ashes (%)	Total acidity (g/L H_2SO_4)
Seed	1.5	5.80	0.09	0.17
Leaf	1.9	5.15	0.43	0.89
Polymer	0.2	6.50	0.01	-

Table 2. ANOVA results for Brix, sucrose content (Pol), purity, reducing sugars (RS) and total reducing sugars (TRS) of sugarcane varieties RB867515 and CTC4, in 2013/2014 Season.

Varieties (V)	Brix	Pol	Purity (%)	RS (%)	TRS (%)
RB867515	18.23B	15.22B	83.5A	0.84A	14.87B
CTC4	21.33A	18.57A	87.0B	0.29B	18.73A
LSD	0.52	0.02	0.02	0.03	0.21
F test	270.28**	168337.50**	480533.99**	1743.06**	2496.6**
CV (%)	1.17	0.06	0.01	2.87	0.56

Means followed by the same letter are not significantly different according to Tukey test at 0.05 of probability. ns, not significant; *significant at 0.05 **; significant at 0.01.

Table 3. ANOVA results for fiber, total acidity, soluble ashes, turbidity and total phenolic Compounds (TPC) of sugarcane varieties RB867515 and CTC4, in 2013/2014 season.

Varieties (V)	Fiber (%)	Acidity (g/L)	Soluble ashes (%)	Turbidity (NTU)	TPC (mg/L)
RB867515	11.54A	0.98A	0.60A	661A	553B
CTC4	11.15B	0.77B	0.42B	658A	658A
LSD	0.02	0.06	0.09	158.42	2.01
F test	2281.50**	68.74**	28.43**	0.00ns	20823.21**
CV (%)	0.09	3.48	7.98	10.59	0.15

Means followed by the same letter are not significantly different according Tukey test at 0.05 of probability. ns , not significant; *significant at 0.05 **; significant at 0.01**.

TRS obtained in extracted juice from sugarcane varieties, RB867515 and CTC4. Both sugarcane varieties were in their practical industrialization period, as Pol was higher than 14%, RS lower than 1% and TRS higher than 14%, according to the earlier reports of Ripoli and Ripoli (2009). The variety, CTC4, contained higher sugar levels than RB867515.

The fiber content was 11.3%, pH 5.0, total acidity of 0.7 g/L of H_2SO_4, 0.5% of soluble ashes and TPC about 600 mg/L (Table 3). These data are in accordance with the sugar content with regards to the ripening stage of both varieties.

Juice clarification process

Figure 1 shows the values obtained for sedimentation speed (Figure 1A) and sludge volume (Figure 1B) when three sedimentation adjuvants were added in the juice from two sugarcane varieties. The use of synthetic polyelectrolyte promoted higher flake sedimentation speed for both varieties. These results are similar to that of Costa et al. (2014), who evaluated moringa leaf extract and synthetic polymer for cane juice clarification with pH 7.0 in sugar production, and found values of 0.5 and 3.5 cm/min, respectively.

Despite the fact that the sedimentation speed was higher when the synthetic polymer was used, the sludge volume after 20 min was similar to treatments in which moringa extracts were used (Figure 1B). These values were lower than those obtained by Thai et al. (2012), who used synthetic polymer as flocculating agent in sugar production and observed sludge formation of about 200 ml. Nevertheless, when these adjuvants were not used in the juice of the variety RB867515, there was low impurities precipitation, which resulted in high sludge volume (Figure 1B), as also observed by Costa et al. (2014).

Must preparation

After treatment, clarified juices were subjected to an

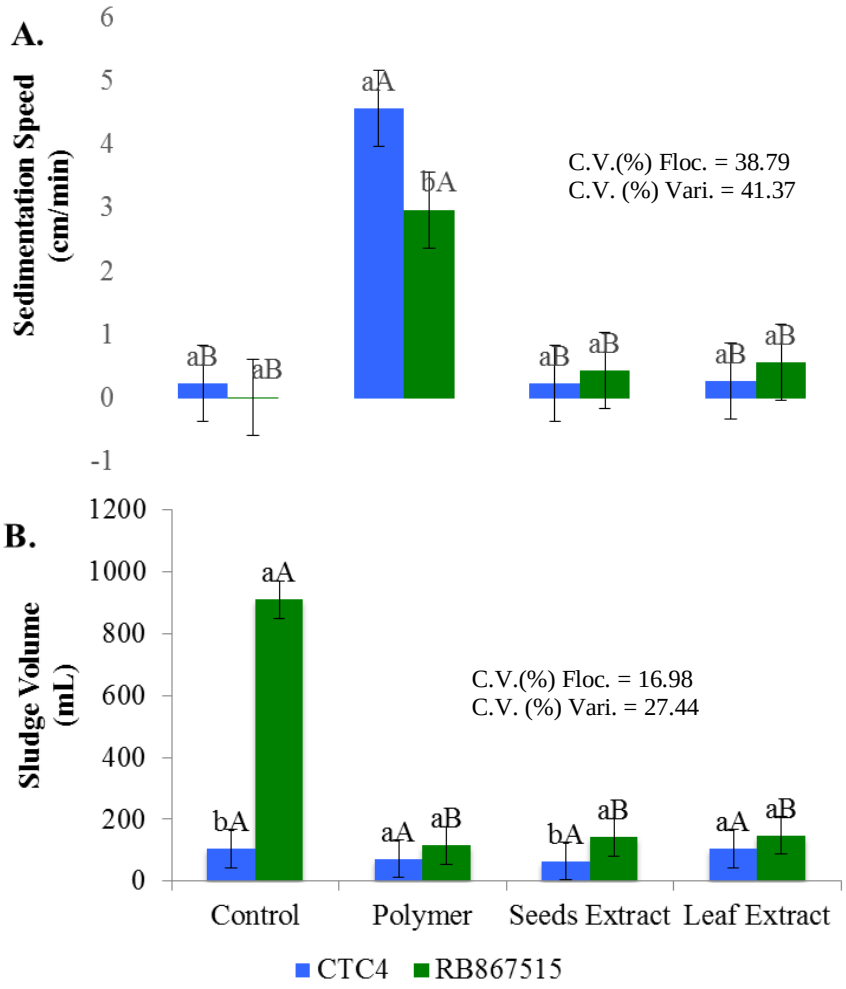

Figure 1. Interaction between flocculating agents and sugarcane varieties for: **A)** sedimentation speed; and **B)** sludge volume, during juice clarification. Jaboticabal-SP, Brazil; Season 2013/2014. Lower case letters compare varieties and upper case letters compare flocculating agents.

adjustment soluble solid; pH, TRS, TPC and total acidity were analyzed in musts. The TRS values were between 12.8 and 13.7%. No significant reductions in must TRS was found when the different flocculating agents were used in the clarification process. The same behavior also was verified when musts from the two sugarcane varieties were compared. This was expected since the juice clarification process does not remove sugars (Rein, 2012). The use of sedimentation adjuvants in juice clarification did not reduce the TPC values which were between 368 and 401 mg/L. Nevertheless, there was a reduction in the concentration of these biomolecules in relation to extracted juice. This result was similar to the observations of Costa et al. (2014). The TPC in clarification process are important in the ethanol industry, since these compounds act as yeast inhibitors during fermentation reducing yeast cell viability and conse-quently ethanol yield (Ravaneli et al., 2011).

Musts prepared from the variety RB867515 had higher amount of TPC (504 mg/L) than that of CTC4 (262 mg/L). These values were different from those obtained in the extracted juice (Table 3). Considering that the sugarcane ripening directly impacts in juice clarification process (Ripoli and Ripoli, 2009), especially in TPC removal (Mutton et al., 2010), probably the CTC4 cane quality allowed higher removal of these molecules during juice treatment.

Synthetic polymer caused significant reductions ($p < 0.05$) in must acidity in relation to control treatment. However, all the treatments had total acidity between 1.0 and 1.2 g/L of H_2SO_4 (Table 4). These results are important, since acids can act as yeast inhibitors during fermentation as earlier found by Camolez and Mutton (2005). The cane juice obtained from CTC4 variety had lower acidity values. The increase in must acidity in relation to extracted juice occurs probably because the

Table 4. Total acidity in musts and total acidity, total residual reducing sugars (TRRS), glycerol and alcohol content in wines obtained during fermentation of the sugarcane varieties of sugarcane varieties RB867515 and CTC4, in 2013/2014 Season.

Flocculating agent (F)	Total acidity (g/L H_2SO_4)	Total acidity (g/L H_2SO_4)	TRRS (%)	Glycerol (%)	Alcohol content (%)
Control	1.16A	2.37A	0.12A	1.95A	8.6A
Polymer	1.08B	2.20A	0.10A	1.98A	8.7A
Seed	1.10AB	2.31A	0.16A	2.03A	8.8A
Leaf	1.15AB	2.26A	0.14A	1.94A	8.9A
LSD	0.07	0.24	0.08	0.26	0.64
F test	4.28*	1.29ns	1.32ns	0.40ns	0.75ns
CV (%)	4.45	7.71	42.87	9.32	5.23
Varieties (V)					
RB867515	1.35A	2.46A	0.22A	1.81B	8.5B
CTC4	0.89B	2.11B	0.04B	2.14A	9.0A
LSD	0.11	0.23	0.07	0.21	0.28
F test	101.12**	13.02*	36.18**	13.70*	13.80**
CV (%)	11.48	12.11	64.20	12.79	3.74
F test F x V	4.77ns	1.74ns	1.98ns	0.82ns	1.24ns

Means followed by the same letter are not significantly different according to Tukey test at 0.05 of probability. Ns, not significant; *significant at 0.05 **significant at 0.01**. FxV, interaction between flocculating agentes (main treatments) and sugarcane varieties (secondary treatments).

pH was adjusted to 4.5 with sulphuric acid.

Fermentation process

The yeast cell, bud viability and budding rate were 85, 98 and 15%, respectively, which were considered adequate for fermentation according to Amorim (2005). There was a reduction of 15% in yeasts cell viability in the beginning of the fermentation in RB867515 and untreated control. In other treatments, yeast viability remained between 85 and 90% (Figure 2A). Yeast bud viability was also reduced in this same treatment (Figure 2B). When no sedimentation adjuvant is used in cane juice clarification process, probably a higher amount of impurities remain in the juice as calcium phosphate compounds, which are inhibitory to yeasts during fermentation. As a consequence, cell viability and stored trehalose (reserve carbohydrate used by yeast in stress condition) are negatively affected (Walker, 1998; Steindl, 2010). However, these treatments had no negative impact on yeast budding rate, which remained between 10.3 and 13.4% during fermentation.

There were no significant differences ($p > 0.05$) amongst treatments in terms of yeast cell viability. The means ranged from 84 to 88%. The same behavior was observed in yeast bud viability, which was higher than 96% (Figure 2C). These results indicate that the yeast FT858 was adapted to the substrate, and was not affected by treatments. Yeast budding rate in the RB867515 untreated control was significantly (50%) lower than treated musts, which had 30% budding rate. This rate was the triple of that found in the beginning of the fermentation process. However, this behavior was already expected, because in the end of fermentation, there is low sugar concentration, which favors yeast glycolytic pathway, resulting in high energy production (ATP) and biomass (Venturini Filho et al., 2013).

Wine characterization

Table 4 shows the results obtained for Brix, total acidity, glycerol and alcohol content in wines. The use of different sedimentation adjuvants did not directly affect wine characteristics. However, when wines from two sugarcane varieties were compared, musts from the variety CTC4 showed lower means of total acidity and total residual reducing sugars, which were about 2.11 g/L and 0.04%, respectively. These results are similar to Moreira et al. (2013), who studied the ethanolic fermentation using the yeast CAT-1, and found 2.02 g/L of total acidity and 0.04% of TRRS. Acid production by yeasts during fermentation process is undesired, because sugar is spent to produce this metabolite, at the cost of lower ethanol yield (Camolez and Mutton, 2005). The low TRRS observed in this study indicate high sugar assimilation by yeasts during fermentation. Low ethanol and glycerol yield were observed in wines obtained from sugarcane CTC4 fermentation, with means of 9 and 2.14%, respectively. These results were higher than those obtained by Ferrari (2013), who observed values between 6.8 and 7.8 for ethanol and 0.23 to 0.4% for

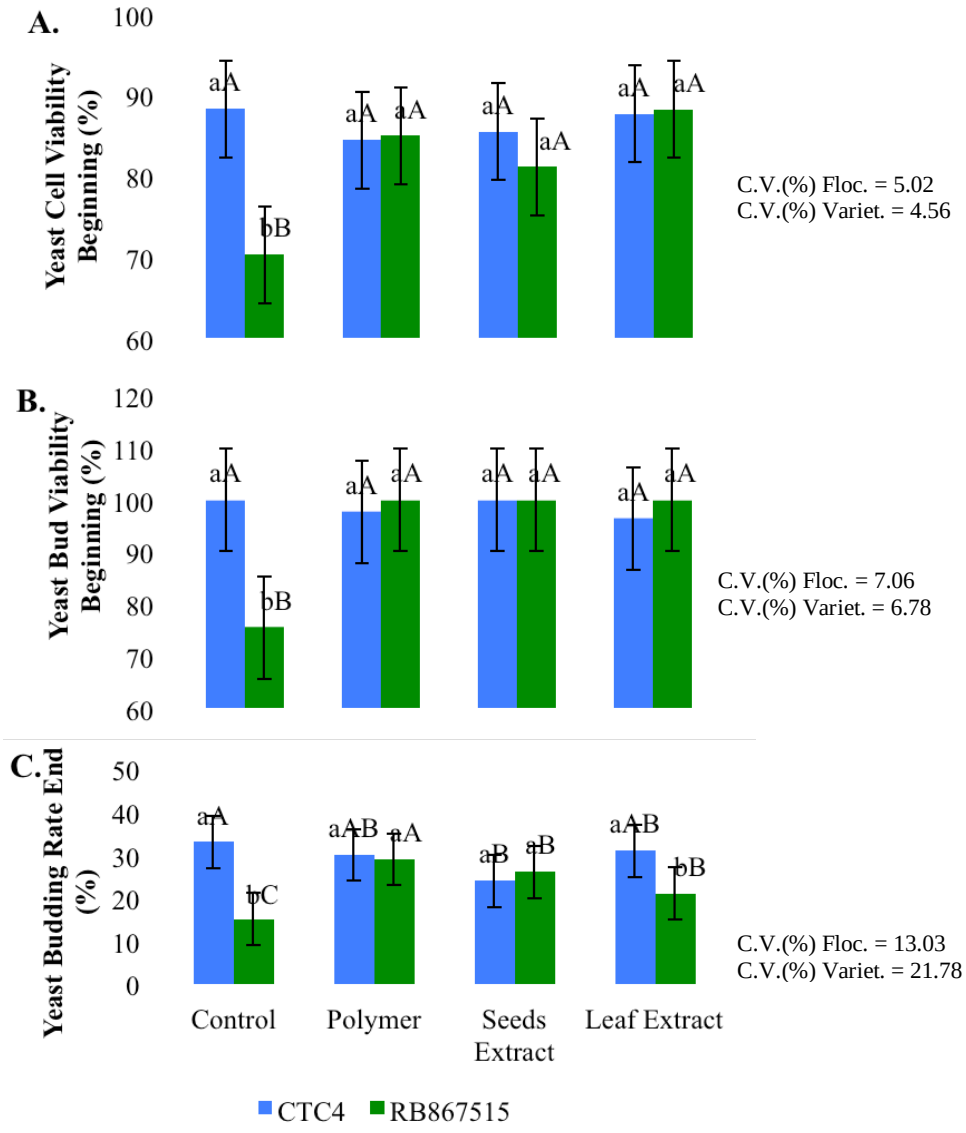

Figure 2. Interaction between flocculating agents and sugarcane varieties for: **A,** yeast FT858 cell viability in beggining of fermentation; **B,** yeast FT858 budding rate in beggining of fermentation; **C,** yeast FT858 budding rate in end of fermentation in Jaboticabal-SP, Brazil (Season 2013/2014). Lower case letters compare varieties and upper case letters compare flocculating agents.

glycerol production in industrial scale. These compounds are obtained from the same yeast metabolic pathway, and their production is inversely proportional (Nevoigt and Stahl, 1997; Wang et al., 2001; Ferrari, 2013). In this study, wines from the variety CTC4 presented higher ethanol and glycerol levels than those from RB867515, probably as a result of higher TRRS in wines. Also, it is important to emphasize that yeasts always produce glycerol during ethanolic fermentation, because it is essential for NAD regeneration and to maintain the metabolic balance. Under stress conditions such as bacterial contamination or osmotic stress, an increase in the production of this sugar-alcohol is also observed (Ren et al., 2012).

Conclusion

The use of moringa leaf and seed extracts as sedimentation adjuvants in cane juice treatment for ethanol production does not increase flake sedimentation speed. However the sludge compaction is higher when compared with the untreated control. The use of flocculating agents in cane juice treatment is essential to maintain

yeast cell and bud viability in the beginning of the fermentation, and budding rate in the end of the process, when low quality sugarcane is used (RB867515).

The use of different flocculating adjuvants in cane juice treatment does not affect wine quality and ethanol yield.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Evaluation of the antioxidant effects of different forms of *Schisandra chinesis* in emulsion-type sausages during chilled storage

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The effects of different forms of *Schisandra chinesis* extract (20% *S. chinensis* juice, 20% *S. chinensis* ethanol extract, and 20% *S. chinensis* water extract) on physico-chemical characteristics and color in emulsion-type sausage were evaluated. Physico-chemical characteristics and color were determined during storage. Thiobarbituric acid reactive substance (TBARS) and residual nitrite (RN) values decreased significantly in treatments with 20% *S. chinensis* juice and 20% *S. chinensis* ethanol extract due to a lower pH. However, control treatments and treatment with 20% *S. chinensis* water extract did not have a significant effect on physico-chemical characteristics during storage. In addition, no remarkable differences were observed in total plate counts (TPCs, antimicrobial effect) or color in any treatment as the storage time increased. Our results suggest that 20% *S. chinensis* ethanol extract was the best antioxidant agent to reduce lipid oxidation and RN during storage.

Key words: *Schisandra chinensis* juice, *Schisandra chinensis* ethanol extract, *Schisandra chinensis* boiling water extract, physico-chemical characteristics, color, emulsion-type sausage.

INTRODUCTION

The appearance of meat, especially its color, is an indicator of freshness (Ismail et al., 2008). Therefore, discoloration and lipid oxidation in meat products are important parameters that determine quality and acceptability by consumers. Consumers may be concerned about the application of antioxidants that prevent color change and lipid oxidation in meat and meat products during storage. In recent years, natural antioxidants have been accepted as alternatives to widely used synthetic antioxidants. For example, in some regions, the use of nitrite, butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) have been restricted and the use of synthetic antioxidants has been reduced, primarily due to their toxic properties (Hettiararchchy et al., 1996; European

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Abbreviations: TBARS, Thiobarbituric acid reactive substance; **RN**, residual nitrite; **TPCs**, total plate counts.

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Union, 2006). Thus, using natural agents as *Schisandra chinensis* (*S. chinensis*) has very good antioxidant potential, as they are considered natural.

S. chinensis is a perennial climbing plant that grows abundantly in the northern regions of China, Korea, Japan and Russia. The berries of *S. chinensis* are called 'wu wei zi' in China or 'omija' in Korea because it possesses all five basic flavors: sour, sweet, salty, sharp, and bitter (Panossian and Wikman, 2008; Hwang, 2012). *S. chinensis* is extensively used as a therapeutic drug, tea, wine and functional food (Hwang, 2012), as well as an alternative medicine to treat liver diseases (Sheng et al., 2011). Bio-active compounds from *S. chinensis* include lignans (types A-E), volatile oil (includes ingredients such as cadinene, ylangene and chamigrenol) and polysaccharides (Hwang, 2012; Yang et al., 2011). The lignans isolated from *S. chinensis* might be effective in the treatment or prevention of a variety of human diseases, including cardiac diseases and immune diseases (Hwang, 2012). *S. chinensis* polysaccharides have antioxidant activities that protect cells from the damaging effects of reactive species and pharmacological properties that prevent chronic diseases (Song et al., 2010). Furthermore, Sheng et al. (2011) reported that the use of *S. chinensis* as a traditional Chinese medicine (TCM) as anti-lipid peroxidative properties could prevent free radical induced problems. However, knowledge about *S. chinensis* on antimicrobial effect in meat industry has not been reported.

Although the effects of *S. chinensis* in animals have been studied, there are few studies on the use of *S. chinensis* extracts on emulsion-type sausages during chilled storage. The objective of the present work was to determine the effectiveness of different forms of *S. chinesis* as antioxidant agent in emulsion-type sausage during chilled storage.

MATERIALS AND METHODS

Preparation of *S. chinensis* extracts

The *S. chinensis* fruits used in this study were purchased from a local market (Munkyong, South Korea). To obtain *S. chinensis* juice, approximately 500 g of *S. chinensis* fruit was processed in a juicer (KJ-303, Kwang Jin Co., South Korea). To obtain *S. chinensis* ethanol extract and *S. chinensis* water extract, 500 g of *S. chinensis* fruit was suspended in 1,000 mL 70% ethanol and distilled water for 30 min at room temperature and extracted by steam distillation at 60°C, respectively.

Sausage preparation

Sausages were made following standard methods used in emulsion-type sausages. Fresh boneless pork was obtained from a local meat market and excess fat and connective tissue were removed. Pork meat was ground using a 5-mm grinder plate. The ground pork was divided into four groups: control (no additives), T1 (20% *S. chinensis* juice), T2 (20% *S. chinensis* ethanol extract), and T3 (20% *S. chinensis* water extract). The ingredients used in the sausage formulations were: 55% ground pork meat, 15% fat, 5.3% cornstarch, 3% sausage seasoning (containing 0.4% nitrite), 1.5% salt, 0.2% polyphosphate, and 20% iced water. The control was the basal sausage containing 2000 mL ice water (20% ice water) as 20% for a 10 kg sausage. To make 20% *S. chinensis* juice, 20% *S. chinensis* ethanol extract, or 20% *S. chinensis* water extract, the extract was added to ice water, adjusted to 2000 ml and then mixed together. All other ingredients were mixed with ground pork using a cutting chopper. When the emulsion was processed, heat was generated. The generated heat was absorbed by the iced water. During the chopping process, the meat was cut into finer particle sizes which encourage protein extraction. Fat was added to solubilized meat proteins when emulsions were thoroughly formed. The batter was then mixed for 5 min in an emulsifier (Kenmix Electronic, model FP800, Kenwood Ltd., New Hampshire, UK). The emulsified meat batter was stuffed into polyvinylidene chloride casings (50 mm in diameter, Viskase Corporation, Chicago, IL) and cooked at 75°C for 70 min in a cooking chamber (NU-VUES-3, Food Service System, USA). All samples were cooled in ice water for 2 h and stored at 4°C. The samples were analyzed at 0, 10, 20, and 30 days of storage at 4°C. All experiments were performed in triplicate.

pH measurement

pH was determined following the AOAC method (1990). To measure pH, 10 g of sausage was homogenized in a blender with 90 mL of distilled water. A digital pH meter (Model 520A, Orion, USA) was used to record pH.

Thiobarbituric acid reactive substance (TBARS)

Lipid oxidation was determined by the TBARS assay following the method described Witte et al. (1970). A 20 g sample was homogenized with 50 mL of 20% trichloroacetic acid solution (in 2 M phosphate solution) in a blender and mixed with 50 mL of distilled water. After samples were filtered through No. 1 filter paper; 5 mL of TBA solution (0.005 M in water) was mixed with 5 mL of the filtered solution in a test tube. The test tubes were placed in darkness at room temperature. After 15 h, the absorbance of the supernatant was recorded at 532 nm using an ultra-violet/visible (UV/VIS) spectrophotometer (UV-24D, Shimadzu, Tokyo, Japan). TBARS values were expressed as mg malondialdehyde (MDA) per kg sausage.

Residual nitrite (RN) content

The RN measurement was estimated using the colorimetric procedure described in AOAC (1990). A 5 g sausage sample was homogenized with 50 mL of distilled water for 2 min. The mixture was heated for 10 min at 40°C in a boiling water bath; 5 mL of saturated HgCl solution was added to the mixture and the resulting mixture was heated for 2 h at 80°C in a boiling water bath, and then cooled to room temperature. A 10-mL supernatant solution sample was mixed with 1 mL of sulfanilamide and kept at room temperature for 15 min. Absorbance was measured at 540 nm using a UV-VIS spectrophotometer (UV-24D). The RN content was expressed as mg per kg of sausage.

Total plate counts (TPCs)

A 10 g sausage sample was homogenized with 90 mL sterile peptone water using a stomacher (Laboratory Equipment, London,

Table 1. Effect of different forms of *S. chinensis* on physico-chemical characteristics of emulsion- type sausages during storage at 4°C.

Item	Treatments[1)]	Storage time (days)			
		0	10	20	30
pH	Control	6.65±0.03^{aA}	6.46±0.03^{bA}	6.30±0.05^{cAB}	6.28±0.10^{cA}
	T1	6.47±0.04^{aB}	6.34±0.04^{bB}	6.23±0.08^{cB}	6.16±0.04^{cB}
	T2	6.41±0.04^{aB}	6.28±0.04^{bB}	6.21±0.06^{bB}	6.12±0.04^{cB}
	T3	6.59±0.04^{aA}	6.46±0.04^{bA}	6.38±0.026^{cA}	6.40±0.04^{bcA}
TBARS (mg MDA/kg)	Control	0.29±0.02^{dA}	0.39±0.06^{cA}	0.43±0.02^{bA}	0.50±0.02^{aA}
	T1	0.23±0.02^{dB}	0.29±0.03^{cB}	0.37±0.01^{bB}	0.42±0.02^{aB}
	T2	0.23±0.02^{dB}	0.29±0.03^{cB}	0.35±0.01^{bB}	0.39±0.02^{aC}
	T3	0.28±0.03^{cA}	0.38±0.02^{bA}	0.41±0.02^{bA}	0.48±0.01^{aAB}
Residual nitrite (mg/kg)	Control	7.36±0.11^{aA}	7.12±0.09^{bA}	5.76±0.16^{cA}	5.16±0.12^{dA}
	T1	6.00±0.39^{aB}	5.61±0.19^{bB}	4.73±0.11^{cB}	4.18±0.07^{dC}
	T2	5.76±0.13^{aB}	5.13±0.07^{bC}	4.11±0.10^{cC}	3.76±0.14^{dD}
	T3	7.29±0.25^{aA}	7.03±0.14^{bA}	5.74±0.06^{cA}	4.81±0.08^{dB}
TPCs (log CFU/g)	Control	1.36±0.01^{dA}	3.59±0.29^{cA}	5.45±0.04^{bA}	7.02±0.04^{aA}
	T1	1.37±0.01^{dA}	3.73±0.07^{cA}	5.39±0.07^{bA}	6.53±0.09^{aB}
	T2	1.37±0.01^{dA}	3.65±0.10^{cA}	5.18±0.07^{bA}	6.47±0.06^{aB}
	T3	1.36±0.02^{dA}	3.76±0.14^{cA}	5.41±0.05^{bA}	6.80±0.04^{aA}

$^{a-d}$Means within row with different superscripts are significantly different (p<0.05). $^{A-D}$Means within columns with different superscripts are significantly different (p<0.05). [1]Control: no *S. chinensis*, T1: 20% *S. chinensis* juice. T2: 20% *S. chinensis* extract, T3: 20% *S. chinensis* water extract.

UK). The samples were serially diluted (10^{-1} to 10^{-8}) and 100 µL of dilutions (10^{-4} to 10^{-8}) were spread-plated on plate count agar (Difco Laboratory, Detroit, MI). The agar plates were incubated at 35°C for 48 h. The colonies were counted and expressed as logarithmic colony forming units (CFU) per g sausage.

Color evaluation

Color was evaluated using a Minolta Chromameter (Minolta CR-300, Tokyo, Japan) that had been standardized with a white plate (lightness, L* = 96.16; redness, a* = 0.10; yellowness, b* = 1.90). Five random readings were taken for each sausage point.

Statistical analysis

All data were analyzed by analysis of variance (ANOVA) using the SAS General Linear Model (GLM) procedure (SAS, 2002). Duncan's multiple range test was used to determine significant differences among treatment means at the 5% level (Duncan, 1955).

RESULTS AND DISCUSSION

Physico-chemical characteristics of emulsion-type sausage

Table 1 shows the effects of different forms of *S. chinensis* on physico-chemical characteristics of emulsion-type sausage during chilled storage. Statistical differences (P < 0.05) were observed in pH, TBARS, RN, and TPCs between treatments and storage times. However, TPCs did not differ in any treatment at 0, 10 or 20 days of storage.

For pH, the lowest pH value was observed in T2 (20% *S. chinensis* ethanol extract), followed by T1 (20% *S. chinensis* juice). Treatments with 20% *S. chinensis* water extract (T3) and the control had similar pH values (6.65 through 6.28) during storage. These differences have been attributed to the spectrum of compounds extracted with the different materials used. Overall, pH values decreased as storage time increased in all treatments. Antioxidant effectiveness relies on pH (Xiong et al., 1993). Therefore, the low pH values may be due to the anti-oxidants present in *S. chinensis*. An increase in meat pH could be due to the accumulation of metabolites by bacteria and deamination of proteins (Jay, 1996). Ibrahim et al. (2011) reported that lamb patties containing ginseng extract as a source of antioxidants had the lowest pH values after nine days of storage.

TBARS values, which are indicators of the degree of lipid oxidation, increased significantly over time in all treatments. The order of effectiveness of different forms of *S. chinensis* as antioxidants in decreasing TBARS values is T2 >T1 > T3 = control. Thus, the 20% *S. chinensis* ethanol extract (T2) had the highest antioxidant properties. The antioxidant activity of *S. chinensis* has been attributed to a group of lignans or phenolic compounds

Table 2. Effect of different forms of *S. chinensis* on color of emulsion-type sausages during storage at 4°C.

Item	Treatment[1]	Storage time (days)			
		0	10	20	30
L* (lightness)	Control	67.75±0.54^{aA}	67.13±0.23^{aA}	65.57±0.31^{bA}	64.40±0.36^{cA}
	T1	68.09±0.27^{aA}	65.63±0.31^{bB}	63.99±0.38^{cB}	63.84±0.18^{cAB}
	T2	67.81±0.56^{aA}	65.21±0.09^{bB}	64.07±0.81^{cB}	63.61±0.37^{cB}
	T3	67.93±0.11^{aA}	65.61±0.15^{bB}	65.70±0.28^{bA}	64.35±0.26^{cA}
a* (redness)	Control	8.13±0.08^{cA}	8.26±0.05^{bAB}	8.37±0.13^{abB}	8.55±0.14^{aB}
	T1	8.12±0.05^{dA}	8.36±0.10^{cA}	8.61±0.10^{bA}	8.82±0.08^{aA}
	T2	8.04±0.12^{dA}	8.38±0.06^{cA}	8.61±0.06^{bA}	8.79±0.12^{aA}
	T3	8.09±0.06^{cA}	8.21±0.09^{bcB}	8.37±0.06^{bB}	8.55±0.13^{aB}
b* (yellowness)	Control	8.02±0.10^{aA}	7.73±0.21^{abA}	7.47±0.29^{bA}	7.56±0.09^{bA}
	T1	8.05±0.04^{aA}	7.98±0.06^{aA}	7.45±0.21^{bA}	7.72±0.07^{cA}
	T2	8.11±0.06^{aA}	7.91±0.15^{abA}	7.71±0.28^{abA}	7.76±0.16^{bA}
	T3	8.05±0.04^{aA}	7.79±0.17^{abA}	7.53±0.41^{abA}	7.76±0.20^{bA}

$^{a-c}$Means within row with different superscripts are significantly different (p<0.05). $^{A-B}$Means within columns with different superscripts are significantly different (p<0.05). [1]Control: no *S. chinensis*, T1: 20% *S. chinensis* juice. T2: 20% *S. chinensis* extract, T3: 20% *S. chinensis* water extract.

(Song et al., 2010; Toda et al., 1988). According to Osada et al. (2000), the antioxidant action of *S. chinensis* powder (SCP) might inhibit cholesterol oxidation in meat and meat products during storage or processing because of their phenolic compounds. Our results agrees with the results of Jin and Park (2013), who reported that TBARS values decreased with increasing levels of *S. chinensis* powder in cooked pork sausages.

As the storage time increased, the RN content declined in all treatments. The RN content was affected (P < 0.05) by both storage days and treatments. The efficacy of the different forms of *S. chinensis* extracts in decreasing the RN content was greatest in T2 (20% *S. chinensis* ethanol extract), followed by T1, T3 and the control. Adding *S. chinensis* extract to emulsion-type sausages increased the antioxidant activity, which was similar to the results of Hah et al. (2006). The antioxidant effects of *S. chinensis* in meat products have been reported by several researchers (Kim et al., 2000; Kim et al., 2008). For example, the phenolic compounds in *S. chinensis* increase the nitrite scavenging activity. Furthermore, nitrites could react with certain amines in some foods to form carcinogenic nitrosamines, leading to various cancers (Van Maanen et al., 1998; Mirvish et al., 2000). RN reduction in emulsion type sausages may be related to a lower pH and the presence of *S. chinensis*, both of which are important factors controlling nitrite reactions.

In the current study, all treatments showed an increase in TPCs values over storage. Our observation is that after 30 days of storage, the TPCs in all treatments exceeded the maximum permissible levels (6 $\log_{10}$ cfu/g) recommended by ICMSF (1986) for human consumption. Overall, these results indicate that the use of *S. chinensis* extract in emulsion-type sausages did not bring antimicrobial activity.

The color of emulsion-type sausages

The effects of different forms of *S. chinensis* on the color of emulsion-type sausages during storage are presented in Table 2. At 10, 20 and 30 days of storage, all *S. chinensis* treatments had some effects (P < 0.05) on L* and a* values in emulsion type sausages. However, no differences were observed for L* and a* values at 0 d or for b* values at 0 through 30 days. There were statistically differences (P < 0.05) in L*, a*, and b* values in all treatments as the number of storage days increased. In general, meat discoloration is closely related to myoglobin oxidation caused by lipid oxidation (Yin and Faustman, 1993). In the current study, using different forms of *S. chinensis* extracts did not improve the color of emulsion-type sausages during storage.

Conclusions

During storage, the addition of 20% *S. chinensis* juice and 20% *S. chinensis* ethanol extracts as antioxidant agents decreased TBRAS (lipid oxidation) and residual nitrite (RN) in emulsion-type sausages, due to a decrease in pH. However, our findings do not confirm a benefit of different forms of *S. chinensis* extracts on total plate counts (TPCs) or color stability of emulsion-type sausages during storage. *S. chinensis* may be a promising source of antioxidant agents that extend the shelf life of emulsion-type sausages

and prevent lipid oxidation during storage.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Evaluation of yacon (*Smallanthus sonchifolius*) extracts as a potential antioxidant source in emulsion-type sausage during refrigerated storage

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Yacon (*Smallanthus sonchifolius*) is a plant that grows in the Andean highlands of South America. Recent studies have shown that yacon is effective in improving antioxidant activity. In this study, the effects of different forms of yacon extracts (20% yacon water extract, 20% yacon juice, and 20% yacon extract) on the lipid oxidation and color of emulsion-type sausage were evaluated. Significant decrease in pH and residual nitrite (RN) values were observed as well as increased thiobarbituric acid reactive substances (TBARS) in all treatments over time. However, during the 30 days of storage, no differences were seen in pH values in any treatment. For color, differences in L*, a*, and b* due to each yacon extract were small, except at 0 days of storage. In conclusion, the addition of 20% yacon extract to emulsion-type sausages was most effective in improving lipid oxidative stability, which could lower pH.

Key words: Yacon water extract, yacon juice, yacon extract, lipid oxidation, color, emulsion-type sausage.

INTRODUCTION

Yacon (*Smallanthus sonchifolius*) is an herbaceous plant that grows in the Andean highlands of South America (Grau and Rea, 1997; Castro et al., 2012; Saldaña et al., 2014). It has been used as food and medicine because of its juicy tuberous root and medicinal properties (Park and Han, 2013). The tuberous roots produced by this plant are similar to sweet potatoes in appearance, but they have a relatively low energy value despite the sweet taste and crunchy flesh (Aybar et al., 2001; Lachman et al., 2003). In terms of nutritional and medicinal properties, yacon has abundant fructans with low glucose, which is potentially beneficial in the control of diabetes (Yan et al., 1999). Studies have shown that yacon tuber extracts have a hypoglycemic effect in individuals with diabetes and in weight reduction (Aybar et al., 2001; Lachman et al., 2003). Tubers are generally used as a source of natural sweetener

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Abbreviation: TBARS, Thiobarbituric acid reactive substances; **RN**, residual nitrite.

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for individuals with renal disorders and digestive problems (Park and Han, 2013). Yacon leaves are used either fresh or dried as a tea, which has anti-diabetic properties (Simonovska et al., 2003). For example, in Brazil, the dried leaves were used as the medicinal ingredient to make yacon tea and said to be antidiabetics (Aybar et al., 2001). The study conducted by Volpato et al. (1997) using yacon leaves showed lowering blood glucose. The tuberous roots, leaves, and stems of yacon contain up to 80% water, 0.3-21.18% proteins, 0.28-26.85% fiber, minerals such as Ca, K, and Mg (Lachman et al., 2003), as well as polyphenol compounds (up to 3.8% dry weight), which exhibit antioxidant activity (Castro et al., 2012).

In general, natural antioxidants, which occur innately in plants, may help to counter the detrimental effects of reactive oxygen species (ROS) and free radicals that cause lipid oxidation via phenolic compounds. The main characteristic of antioxidants such as phenolic acids and polyphenols is their ability to trap free radicals and inhibit oxidative mechanisms (Borkataky et al., 2013). It was previously demonstrated that the major antioxidant compounds in yacon roots are chlorogenic acid and tryptophan (Castro et al., 2012). Takenaka et al. (2003) also reported that caffeic acid derivatives in yacon root belonged to five groups: chlorogenic acid, 3,5-dicaffeoylquinic acid, 2,4- or 3,5-dicaffeoylatraric acid, 2,5-dicaffeoylatraric acid, and 2,3,5- or 2,4,5-tricaffeo- ylatraric acid.

At present, the antioxidant properties in yacon have been studied in detail, but there are no studies on the effect of different forms of yacon extracts on antioxidants and color of meat and meat products. Hence, the objective of this study was to evaluate the effectiveness of different forms of yacon extracts on thiobarbituric acid reactive substances (TBARS), residual nitrite (RN), and color changes in emulsion-type sausage during refrigerated storage.

MATERIALS AND METHODS

Preparation of yacon extract

Yacon roots used in this study were purchased from a local market (Munkyong, South Korea). To prepare yacon water extracts, 500 g of yacon roots was weighed and extracted with 1,000 ml distilled water using steam distillation at 70°C for 5 h. For yacon juices, approximately 500 g of yacon roots was washed and squeezed using a juicer (KJ-303; Kwang Jin Co.; South Korea). Yacon extracts were prepared using the following procedure: 500 g of yacon roots was mixed with 1, 000 ml of 70% ethanol for 30 min, extracted by steam distillation at 70°C for 5 h, and then cooled to room temperature.

Sausage preparations

Emulsion-type sausages were prepared using fresh boneless pork trimmed of excessive fat and connective tissue, and ground through a 5 mm plate. The emulsion type sausages formulation consisted of four treatments [a no-additive control, 20% yacon water extract (T1), 20% yacon juice (T2), or 20% yacon extract (T3)]. The amount of the ingredients, expressed per 10 kg of sausage, are as follows: 55% ground pork meat, 15% fat, 5.3% cornstarch, 3% sausage seasoning (containing 0.4% nitrite), 1.5% salt, 0.2% polyphosphate, and 20% iced water. The control was 2, 000 ml ice water (20% ice water) as 20% for a 10 kg sausage. For preparation of 20% yacon water extract 20% yacon juice, and 20% yacon extract, the extract was added to ice water until the volume was adjusted to 2, 000 ml and mixed together. All treatments were then added to the ground meat, mixed, and homogenized in a chopper to obtain finer meat particles for greater protein extraction. Fat was also added to the formulation after proteins were solubilized. After mixing in an emulsifier (Kenmix Electronic, model FP800; Kenwood Ltd.; New Hampshire, UK) for 5 min, the batter was stuffed into polyvinylidene chloride casings (50 mm diameter; Viskase Corporation; Chicago, IL). During the emulsification process, ice water was added to absorb the generated heat. Samples were cooked for 70 min in a cooking chamber (NU-VUES-3; Food Service System; USA) until the temperature reached 75°C. All samples were cooled in ice water for 2 h, stored at 4°C, and analyzed after 0, 10, 20, and 30 days of storage. All parameters were determined in triplicate.

pH

The pH was determined according to the method of AOAC (1990). Approximately 10 g of sausage was added to 90 ml of distilled water and homogenized using a blender. pH was measured using a digital pH meter (Model 520A; Orion, USA).

Thiobarbituric acid reactive substance (TBARS)

The TBARS assay was determined according to a previous study (Witte et al., 1970). Approximately 20 g of sample was weighed and homogenized in 50 ml of 20% trichloroacetic acid solution (in 2 M phosphate solution) using a blender. The sausage homogenate was then blended with 50 ml of distilled water. Samples were filtered through No. 1 filter paper, and 5 ml of the filtered solution in a test tube was blended with 5 ml TBA solution (0.005 M in water). Tubes were kept in the dark at room temperature for 15 min before use. Absorbance of the supernatant was determined at 532 nm using an ultraviolet/visible (UV/VIS) spectrophotometer (UV-24D; Shimadzu; Tokyo, Japan). The amount of TBARS was expressed as mg malondialdehyde (MDA) per kg sausage.

Residual nitrite (RN)

RN was determined according to AOAC methods (1990). Approximately 5 g of sausage was mixed with 50 ml of distilled water for 2 min, heated for 10 min at 40°C in a boiling water bath, and then mixed with 5 ml of saturated HgCl solution. Mixtures were then heated again in a boiling water bath at 80°C for 2 h. After cooling to room temperature, the supernatant was added to 1 ml of sulfamilamide and stored at room temperature for 15 min. The absorbance of this solution was measured in an UV-VIS spectrophotometer (UV-24D) at 540 nm and RN values were expressed as mg per kg of sausage.

Color analysis

Determination of sausage sample color was performed with a Minolta Chromameter (Minolta CR-300; Tokyo, Japan) calibrated using a white standard plate. Values for the white standard tile were as follows: lightness, L* = 96.16; redness, a* = 0.10; and yellowness, b* = 1.90. Measurements were taken at five randomly selected locations on each sample.

Table 1. Effect of different forms of yacon (*Smallanthus sonchifolius*) extracts on pH, TBARS, and RN in emulsified sausage during storage at 4°C.

Parameter	Treatments	Storage time (days)			
		0	10	20	30
pH	Control	6.60±0.05^{aA}	6.45±0.03^{bA}	6.28±0.08^{cAB}	6.29±0.11^{cA}
	T1	6.64±0.02^{aA}	6.44±0.04^{bA}	6.37±0.02^{bA}	6.28±0.09^{cA}
	T2	6.44±0.04^{aB}	6.34±0.06^{bB}	6.23±0.05^{cB}	6.15±0.05^{cA}
	T3	6.43±0.06^{aB}	6.26±0.06^{bB}	6.22±0.05^{bcB}	6.13±0.07^{cA}
TBAR (mg MDA/kg)	Control	0.30±0.02^{dA}	0.39±0.01^{cA}	0.45±0.03^{bA}	0.50±0.01^{aA}
	T1	0.27±0.02^{dAB}	0.32±0.03^{cB}	0.37±0.03^{bBC}	0.46±0.02^{aB}
	T2	0.25±0.02^{bB}	0.29±0.05^{bB}	0.40±0.01^{aB}	0.44±0.02^{aB}
	T3	0.23±0.02^{cC}	0.29±0.03^{bB}	0.34±0.03^{aC}	0.38±0.03^{aC}
RN (mg/kg)	Control	7.41±0.13^{aA}	7.19±0.07^{bA}	5.77±0.10^{cA}	5.24±0.12^{dA}
	T1	7.03±0.09^{aB}	6.83±0.11^{bB}	4.92±0.06^{cB}	4.26±0.11^{dB}
	T2	6.58±0.22^{aC}	5.97±0.10^{bC}	4.79±0.17^{cB}	4.16±0.08^{dB}
	T3	5.94±0.07^{aD}	5.57±0.10^{bD}	4.29±0.08^{cC}	3.91±0.19^{dC}

$^{a-d}$Means within row with different superscripts are significantly different (p<0.05); $^{A-D}$Means within columns with different superscripts are significantly different (p<0.05); [1]Control, no yacon; T1: 20% yacon water extract; T2: 20% yacon juice; T3: 20% yacon extract.

Statistical analysis

All data were subjected to an analysis of variance (ANOVA) using the General Linear Model (GLM) of the SAS procedure (SAS, 2002). Differences among treatment means were determined using Duncan's multiple range tests, and significance was defined at the 5% level (Duncan, 1955).

RESULTS AND DISCUSSION

The effects of addition of different forms of yacon extracts on pH, TBARS, and RN contents during storage are presented in Table 1. Overall, there were significant differences (p<0.05) in pH, TBARS, and RN values among emulsion-type sausages with different forms of yacon extracts over time. However, during 30 days of storage, no differences (p>0.05) were observed in pH values in any treatment. As storage days increased, all treatments showed reduced pH levels, indicating that the number of storage days significantly affected pH levels. Sausages with addition of different forms of yacon extracts showed lower pH values compared to controls, due to the presence of antioxidant. Treatment with 20% yacon juice (T2) and 20% yacon extract (T3) resulted in lower pH values than other treatments over time. Similar findings were also observed by Kim et al. (2010), who found that different forms of garlic decreased pH in emulsion-type sausages during storage, with pH values ranging from 5.19 to 6.38. According to Aksu (2007), pH values play an important role in antioxidant effectiveness when antioxidants are added to meat and meat products. In the current study, a reduction in TBARS values was observed after treatment with different forms of yacon extracts in emulsion-type sausages over time compared to the control. In addition, all treatments tended to increase TBARS values over increasing storage days.

Among the antioxidants tested, 20% yacon extract (T3) resulted in the most significant reduction in TBARS, while 20% yacon water extract (T1) was the least effective in reducing lipid oxidation in emulsion-type sausage. This finding agreed with Rababah et al. (2011), who reported that addition of plant extracts to goat meat decreased TBARS values concomitant with increased storage days. In general, antioxidants are compounds that retard autoxidation by interrupting free radical chain reactions such as lipid peroxidation (Nawar, 1996). Thus, the mechanism by which different forms of yacon extracts reduce lipid oxidation could be related to either inhibition of free radical formation or interruption of the chelation of free ions released from hemoproteins (Shahidi, 2000). Overall RN values in all sausages decreased as storage days increased, and RN values in sausages with three different forms of yacon extracts (T1, T2, and T3) were lower compared to controls. The inhibiting effect on RN values in emulsion-type sausage was highest with 20% yacon extract (T3), followed by 20% yacon juice (T2), and then 20% yacon water extract (T1) during 30 days of storage. The reduced RN values may be due to decreased pH and antioxidant effects. These results are in agreement with results reported by Kim et al. (2002), who demonstrated that the nitrite-scavenging effect (NSE) was dependent on the pH and increased with the concentration of garlic extract. Nitrite has been extensively used for color and flavor development; however, the use of nitrates has been reduced

Table 2. Effect of different forms of yacon (*Smallanthus sonchifolius*) extracts on color in emulsified sausage during storage at 4°C.

Parameter	Treatments	Storage time (days)			
		0	10	20	30
L* (lightness)	Control	67.96±0.14^{aA}	67.10±0.30^{bA}	65.66±0.33^{cA}	64.38±0.14^{dA}
	T1	68.19±0.18^{aA}	65.73±0.44^{bB}	64.70±0.48^{cB}	63.81±0.20^{dBC}
	T2	68.09±0.27^{aA}	65.56±0.17^{bB}	64.03±0.42^{cB}	63.94±0.07^{cB}
	T3	67.81±0.56^{aA}	65.27±0.07^{bB}	63.99±0.22^{cB}	63.51±0.22^{dC}
a* (redness)	Control	8.16±0.11^{bA}	8.30±0.04^{abB}	8.46±0.10^{aB}	8.48±0.17^{aB}
	T1	8.09±0.06^{cA}	8.32±0.06^{bAB}	8.50±0.09^{aB}	8.57±0.13^{aAB}
	T2	8.16±0.09^{cA}	8.41±0.09^{bAB}	8.79±0.09^{aA}	8.81±0.14^{aA}
	T3	8.18±0.03^{cA}	8.46±0.11^{bA}	8.78±0.11^{aA}	8.79±0.12^{aA}
b* (yellowness)	Control	7.99±0.09^{aA}	7.70±0.18^{abB}	7.46±0.26^{bB}	7.56±0.09^{bB}
	T1	8.07±0.02^{aA}	7.73±0.13^{bB}	7.78±0.16^{bAB}	7.73±0.14^{bAB}
	T2	7.99±0.11^{aA}	7.98±0.06^{aAB}	7.57±0.24^{bB}	7.63±0.11^{bB}
	T3	8.11±0.07^{aA}	8.00±0.30^{abA}	8.01±0.03^{abA}	7.91±0.07^{bA}

$^{a-d}$Means within row with different superscripts are significantly different (p<0.05); $^{A-C}$Means within columns with different superscripts are significantly different (p<0.05); Control, no yacon; T1, 20% yacon water extract; T2, 20% yacon juice; T3, 20% yacon extract.

because of their toxic properties (European Union, 2006). In view of the growing consumer demand for food with natural additives (for example, yacon extracts), RN reduction associated with decreased pH is desirable.

Analysis of the color of emulsion-type sausages with three forms of yacon extracts at different storage days is shown in Table 2. At 0 days of storage, treatments were not significantly different (p> 0.05) based on L*, a*, and b* values. Overall changes in color of emulsion-type sausages showed increased a* values and decreased L* and b* values in all treatments as the storage length increased, but differences in L*, a*, and b* values of emulsion-type sausages induced by the three different forms of yacon extracts were small, indicating that yacon extracts were less effective in improving color stability of emulsion-type sausages. However, Rohlík et al. (2010) observed a positive effect of antioxidants (rosemary extract) on color stability and elimination of color varieties in different parts of dried sausages.

Conclusions

The findings of this study suggested that adding different forms of yacon extracts to emulsion-type sausages decreased pH, TBARS (lipid oxidation) and RN values when compared with the controls over time. Among antioxidant used in this study, 20% yacon extract caused the most significant reduction in lipid oxidation and RN of emulsion-type sausages. However, inclusion of different forms of yacon extracts was less effective in improving color stability of emulsion-type sausages. In order to fully understand the effectiveness of yacon extracts, further research on organoleptic properties, color stability, and sensory evaluation during refrigerated storage should be done.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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