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COMBINED APPROACH FOR THE INVESTIGATION OF DOMINANT FERMENTING MICROBIOTA IN TWO TRADITIONAL SOURDOUGHS PRODUCED IN SICILY

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ABSTRACT

In order to explore the community of lactic acid bacteria (LAB) and yeasts present in two major typical Sicilian sourdoughs, seven mature sourdoughs for "Pane Nero di Castelvetrano" (CV1 - CV3 samples) and "Pane di Monreale" (MR1 - MR4 samples) were analysed through a culture-dependent and culture-independent approach. The highest values of microbial counts were revealed in MR1 sourdough. In particular, LAB counts were at about 10^9 CFU/g in media specific for typical sourdough LAB, such as SDB and SFM, while levels of 10^6 CFU/g were registered for yeasts. The total DNA form each sourdough sample was extracted and subjected to a multiplex-PCR in order to recognize the major groups of LAB. Seventy-six LAB with a rod shape, presumptively *Lactobacillus*, were phenotypically grouped and subjected to a genotypic identification by sequencing of the 16S rRNA gene and further confirmed by species-specific PCRs. Yeasts were isolated and identified by a combined genotypic approach consisting of restriction fragment length polymorphism (RFLP) of 5.8S rRNA gene and sequencing of D1/D2 domain of the 26S rRNA gene. The LAB species identified were *Lactobacillus sanfranciscensis*, *Lactobacillus paralimentarius*, *Lactobacillus brevis* and *Lactobacillus coryniformis*. Among yeasts, *Saccharomyces cerevisiae*, *Pichia guiliermondii*, *Pichia segobiensis*, *Rhodotorula acuta* and *Rhodotorula mucilaginosa* were the species hosted in Sicilian sourdough. The multiplex PCR carried out on total DNA of sourdoughs allowed the rapid identification of the majority of sourdough lactobacilli but the culture-dependent methodology was confirmed to be necessary for the detection of the species, such as *Lactobacillus coryniformis* not included in the system.

1. Introduction

Cereals constitute a major source of dietary nutrients throughout the world (Blandino et al., 2003). They contain the macronutrients (proteins, lipids and carbohydrates) and supply important minerals, vitamins and other micronutrients required by humans for growth and maintenance (Topping, 2007). Dry cereals can only be eaten after grinding and mixing with water (Salovaara, 1998). After a while, thanks to the action of several biological agents including bacteria, yeasts and filamentous fungi, which determine the saccharification of starch in the raw materials and affect microbial protein supply (Herrera-Saldana et al., 1990), the mixture results in the formation of a product characterized by sour aroma.

Cereal-based fermented foods are produced all over the world. Some products, such as sourdoughs employed to produce bread and sweet baked goods, are common to many societies and, for this reason, they have been well characterized for their microbial diversity which play a major role in the determination of the final products. However, the majority of cereal fermented products are local, often obtained with raw materials produced within

The ecological composition of traditional and typical food products, although characterised by a limited diffusion, deserve to be investigated. As a matter of fact, unlike industrial foods processed with a few microbial strains able to dominate the fermentations, artisanal niche products with no commercial starters added are source of high biodiversity. Thus, several strains with different aptitudes, including health promoting features, may be found and used to maintain some characteristics over time (Settanni et al., 2013; Alfonzo et al., *in press*). Moreover, a survey of the dominant populations and strains of a given product is of paramount importance for the valorisation of the food itself, since some strains may be used as marker microorganisms to be searched for the authenticity of raw materials, technology of transformation, as well as production area. With this regard, Pane Nero di Castelvetrano and Pane di Monreale restricted areas, and processed following traditional recipes (Ventimiglia et al., 2015). Thus, the resulting products are characterised by a strong typicality, reflecting the usages of the different cities, even though they are located at a distance of a few kilometres from one another.

Sicily is a region of the South Italy were several niche food products link their history to the production area. Among typical products, those manufactured with sourdough technology are gaining more and more interest for their organoleptic characteristics and also for their prolonged shelf-life, compared to baked goods processed with baker's yeast addition. Different sourdough breads are produced throughout this region, the most known are Pagnotta del Dittaino, Pane nero di Castelvetrano, Pane di Lentini and Pane di Piana degli Albanesi (Minervini et al., 2010; Corona et al., 2016).

Furthermore, recent studies of the bacterial community that characterizes Sicilian sourdoughs highlighted the differences between the populations of lactic acid bacteria in sourdough produced in different areas (Ventimiglia et al., 2015).

represent two typical breads of western Sicily, well appreciated throughout the island and also in the continental Italy. Both breads are obtained with sourdough technology employing *Triticum durum* flour. However, the production of Pane Nero di Castelvetrano involves the use of sourdough as a leavening agent and the mixture of two semolina: whole wheat blond sicilian and ancient varieties of local durum wheat (Tumminia) in the ratio of 30% (w/w). Tummina (*Triticum durum* Desf. var. *reichenbachii*) cultivated in a few towns within Valle del Belìce area (western Sicily), which provides the final bread with the typical dark brown appearance (Giancaspro et al., 2016). The microorganisms hosted in raw materials employed in sourdough might be defining for production (Alfonzo et al., 2013). Sourdough is a complex ecosystem characterized mainly by the presence of lactic acid bacteria (LAB) and yeasts (De Vuyst et al., 2014).

For the above reasons, the aims of the present work were to detect, isolate and identify the dominant LAB and yeast population of the sourdoughs used to produce Pane Nero di Castelvetrano and Pane di Monreale by means of a combined approach including classical and culture-independent microbiological techniques.

2. Materials and methods

2.1.Sourdough sample collection and pH measurement

Sourdoughs used in Pane Nero di Castelvetrano and Pane di Monreale production (Table 1) were collected from bakeries located in Castelvetrano (Trapani province) and Monreale (Palermo province). Samples were taken from five bakeries (CV1 and MR1-MR4), one farm house (CV2) and one restaurant (CV3) prior refreshment with new semolina.

The values of pH were determined electrometrically by means of pH-Meter BASIC 20+ (Crison Instrument S.A., Barcelona, Spain).

Table 1. Characteristics of sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Sample	Production level	Dough Yield	Baker's yeast (%)	Fermentation time (h)	Fermentation temperature (°C)	pH
CV1	Bakery-artisanal	180	0	5	18-20	3.48 ± 0.03
CV2	Home-made	170	0	8	Ambient	3.79 ± 0.02
CV3	Home-made	175	0	8	Ambient	4.02 ± 0.02
MR1	Bakery-artisanal	230	0.3	6	30	3.37 ± 0.01
MR2	Bakery-artisanal	160	0.5	5	18	4.34 ± 0.02
MR3	Bakery-artisanal	180	0.5	6	18	4.30 ± 0.01
MR4	Bakery-artisanal	180	0	8	16	4.38 ± 0.01

[dough yield (DY) = weight of dough/weight of flour × 100]

2.2.Microbiological analysis

Each sample (25 g) was suspended in Ringer's solution (225 mL) (SigmaeAldrich, Milan, Italy), homogenised in a stomacher (BagMixer 400; Interscience, Saint Nom, France) for 2 min at maximum speed, and then serially diluted.

LAB were counted on two generic agar media commonly used for this bacterial group: de Man-Rogosa-Sharpe (MRS) (Oxoid, Milan, Italy) and M17 (Oxoid) for generic food rod and coccus LAB, respectively; Sour Dough Bacteria (SDB) (Kline and Sugihara, 1971) as specific medium for LAB associated with the sourdough matrices. All media were added with cycloeximide (10 mg/ml), to avoid yeast growth. Petri dishes were anaerobically incubated at 30°C for 48 h. Tota l yeasts were estimated on Yeast Glucose Chloramphenicol (YGC) agar (Liofilchem, Roseto degli Abruzzi, Italy), incubated aerobically at 25°C for 48 h and on Wallerstein laboratory (WL) nutrient agar incubated aerobically at 28°C for 72 h. All microbiological counts were carried out in duplicate.

2.3.Genotypic investigation of sourdough *Lactobacillus*

The composition of lactobacilli in the sourdoughs was first investigated by the approach consisting of the combined multiplex PCR technique described by Settanni et al. (2005). Total DNA of sourdoughs were extracted from 500 mg of pellet (by centrifugation at 10.000 × g for 5 min) of the first dilutions used for the microbial analysis. Extraction was performed with the FastDNA® Pro Soil-Direct Kit (MP Biomedicals, CA, USA) following manufacturer's instructions.

To ascertain the presence of lactobacilli in all sourdoughs, they were first typed by a multiplex PCR assay named Grouping-multiplex PCR. Subsequently, four multiplex PCR assays, named Group1-, Group2-, Group3-, and Group4-multiplex PCRs, were performed in order to discriminate

Lactobacillus representatives of each group at the species level (Settanni et al., 2005).

2.4.Isolation,phenotypic characterization and identification of rod LAB

After growth, colonies of various shape (at least five with identical morphology) Gram-positive [Gregersen KOH method (Gregersen, 1978)] and catalase negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding 5% H_2O_2) bacteria (presumptive LAB) were randomly picked up from agar plates and transferred to the corresponding broth media. The isolates were purified by successive sub-culturing and stored in glycerol at −80°C until further experimentations.

The rod cell morphology of the isolates was confirmed by observation with an optical microscope (Olympus, BX60). Subsequently, the presumptive rod LAB isolates were subjected to further phenotypic assays and were grouped on the basis of their growth characteristics: growth at 15 and 45°C; acid production from arabinose, ribose, xylose and sucrose; and CO_2 production from glucose. The test for CO_2 production was carried out in Durham's tubes with MRS broth. Positive results for this test indicated a hetero-fermentative metabolism.

A representative percentage of isolates from each phenotypic group was identified genotypically by 16S rRNA sequencing. Genomic DNA for PCR assays was prepared from sourdoughs isolates after their overnight growth in MRS broth at 30°C. Cells were harvested, and DNA was extracted using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Crude cell extracts were used as templates for PCR.

PCR reactions were performed as described by Weisburg et al. (1991). PCR products were visualized by UV transillumination. The amplicons corresponding in size to the molecular weight of the 16S rRNA genes were excised and purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy). The resulting DNA was sequenced using the same primers employed for the PCR amplifications. The sequences were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997) and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) (Chun et al., 2007) databases.

Lactobacillus brevis, Lactobacillus paralimentarius and *Lactobacillus sanfranciscensis* were verified by the multiplex PCR strategy reported by Settanni et al. (2005).

2.5.Isolation and identification of yeasts

Yeasts were collected from YGC and WL media. At least five colonies per morphology were randomly collected from agar plates, purified to homogeneity after several sub-culturing steps onto WL medium and subjected to genetic characterization. Genomic DNA for PCR assays was extracted as reported for LAB.

In order to perform the identification of yeasts, all isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1/ITS4 (Esteve-Zarzoso et al., 1999) by means of T1 Thermocycler (Biometra, Göttingen, Germany) and subsequently the amplicons were digested with the with the restriction endonucleases *Cfo*I, *Hae*III, and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37°C for 8 h. ITS amplicons as well as their restriction fragments were analyzed twice on agarose gel using at first 1.5% (w/v) agarose and then 3 % (w/v) agarose in 1 × TBE buffer. Standard DNA ladders were 1kb Plus DNA Ladder (Invitrogen) and GeneRuler 50 pb DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany).

Sequencing of the D1/D2 domain of the large subunit 26S rRNA gene was performed for the representative isolates of each ITS group. Gene amplification was carried out using the primers NL1 and NL4 (Invitrogen, Milan, Italy) as described by Kurtzman and

Robnett 1998. PCR products (600-bp) were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions and then subjected to sequencing at MWG Biotech AG (Ebersberg, Germany). The sequences obtained were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul et al. 1997).

2.6.Statistical analysis

Statistical analysis of microbiological counts were performed using Statistica software (StatSoft Inc., Tulsa, OK). Data from microbiological data were analysed using a generalised linear model (GLM) that included the effects of samples and media. The means and pairwise comparisons were evaluated with a post-hoc Tukey's test. A P value <0.05 was deemed significant.

3. Results and discussions

Microbiological analysis and pH measurement

The main microbiological characteristics of the sourdoughs collected in western Sicily are reported in Table 2. pH ranged between 3.37 and 4.38 that is reported as the typical range of values for several Italian sourdoughs (Valmorri et al., 2006; Minervini et al., 2015). The highest counts were registered on SDB for all samples and on MRS for five samples, while those on M17 showed the lowest values. The levels of sourdough LAB estimated on SDB were above 10^7 CFU/g for almost all samples except MR1 that showed concentrations of about 10^9 CFU/g. The levels of yeasts registered on WL were above 10^5CFU/g, while those displayed by YGC results were lower of approximately one order of magnitude except for the sample MR1. Yeast/LAB ratio was 1:100 or 1:10, reflecting the typical proportion among lactic and acetic acid characterizing the mature sourdoughs (Corsetti and Settanni 2007). The fermentation temperature, has affected the chemical and microbiological characteristics of sourdoughs. MR1 was fermented at temperature of 30 °C, that is optimal for the growth of several LAB and this explains the very high levels of LAB registered for this sample. On the contrary, the other sourdoughs characterized by lower counts were refreshed for shorter times (almost 6 – 8 h) and fermented at lower temperatures (ambient or 16 – 20°C). This was reflected also in the final pH which was lowest for MR1 samples. For this samples, the acidification velocity was also influenced by the DY = 230 (Banu et al., 2011; Vogelmann and Hertel, 2011) which was higher compared to that of the other samples (DY = 160-180).

3.1.Genotypic investigation of sourdough *Lactobacillus* composition

The multiplex-PCR showed in **Fig. 1** allowed to distinguish the lactobacilli composition of the sourdoughs as a function of the amplicons generated by each group (Settanni et al. 2005, 2006). In particular, all sourdoughs except MR1 showed a band of 280 bp typical for the lattobacilli belonging to group III: *L. brevis*, *Lactobacillus fructivorans*, *Lactobacillus hilgardii* and *Lactobacillus sanfranciscensis*. The samples CV1, MR2, MR3 and MR4 showed comparable results for the presence of 3 amplicons of ca. 180, 280 and 1100 bp. In this samples, the group II (*Lactobacillus alimentarius*, *Lactobacillus paralimentarius*, *Lactobacillus farciminis*, and *Lactobacillus mindensis*) and IV (*Lactobacillus fermentum*, *Lactobacillus frumenti*, *Lactobacillus panis* and *Lactobacillus pontis*) were also revealed. CV2 and CV3 samples showed merely the amplicon at 280 bp. The first step of Grouping-multiplex PCR enabled the detection of *Lactobacillus* species *in situ* without the need of colony isolation and cultivation. The presence of species of Groups II, III and IV in sourdough samples under study, confirmed the constant presence of LAB commonly found in Italian sourdoughs (Settanni et al., 2005; Minervini et al., 2012; Reale et al., 2011).

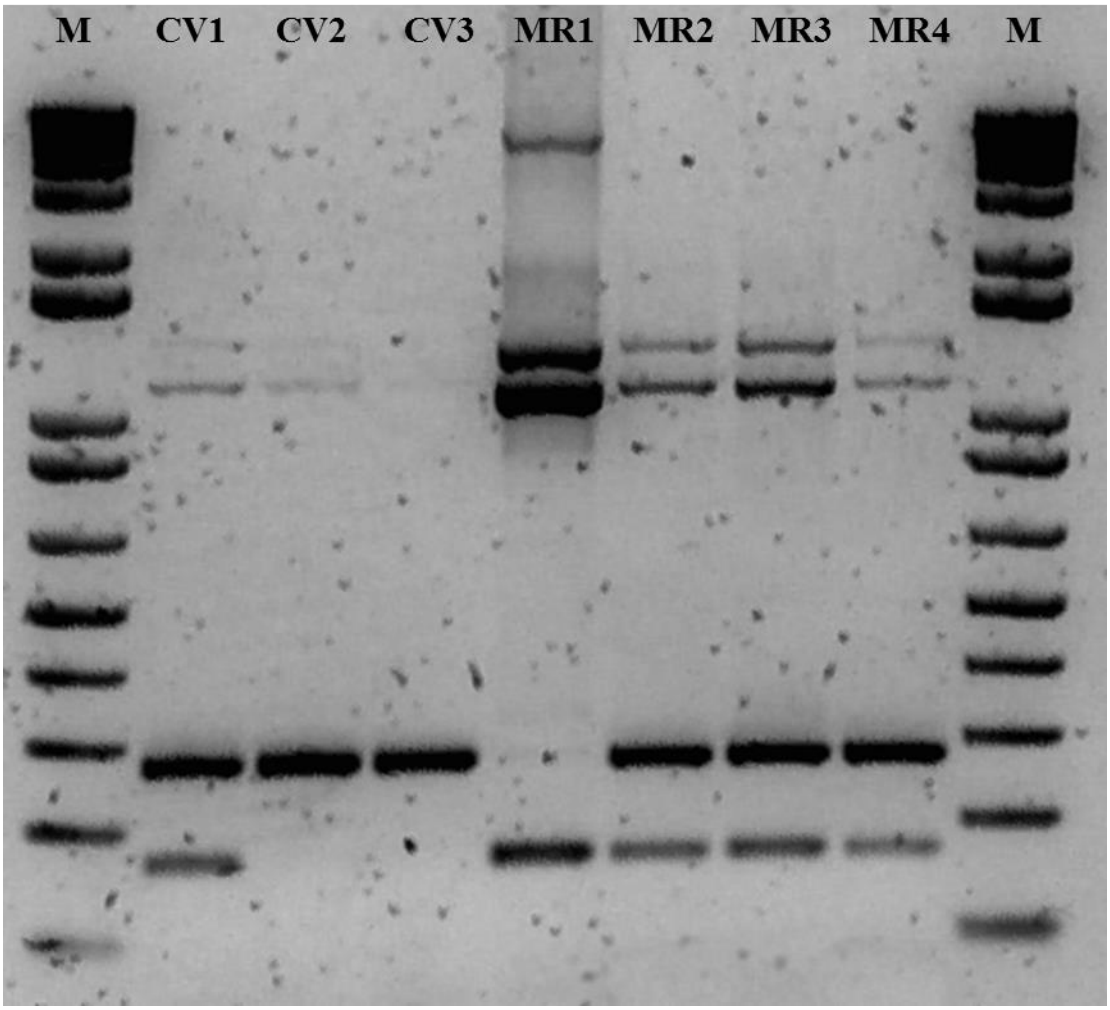

Figure 1. Grouping-multiplex PCR (first step) assay. Abbreviations: M, 1-kb DNA molecular size markers (Invitrogen).

Table 2. Microbiological counts (log CFU/g) of sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Sample	LAB				Yeasts	
	MRS	M17	SDB		YGC	WL
CV1	6.90 ± 0.05^{b}	5.21 ± 0.22^{a}	7.61 ± 0.35^{b}		4.42 ± 0.08^{a}	5.58 ± 0.11^{bc}
CV2	5.80 ± 0.14^{a}	4.99 ± 0.13^{a}	7.65 ± 0.17^{b}		4.45 ± 0.08^{a}	5.08 ± 0.25^{c}
CV3	5.63 ± 0.13^{a}	5.00 ± 0.22^{a}	7.69 ± 0.12^{b}		4.18 ± 0.21^{a}	5.19 ± 0.15^{c}
MR1	7.90 ± 0.05^{c}	4.95 ± 0.16^{a}	9.30 ± 0.09^{a}		6.36 ± 0.13^{c}	6.85 ± 0.18^{a}
MR2	6.88 ± 0.05^{b}	6.27 ± 0.20^{b}	7.30 ± 0.21^{b}		5.18 ± 0.32^{b}	5.37 ± 0.20^{c}
MR3	6.84 ± 0.12^{b}	6.01 ± 0.24^{b}	7.57 ± 0.16^{b}		5.45 ± 0.09^{b}	5.88 ± 0.10^{b}
MR4	6.83 ± 0.21^{b}	6.23 ± 0.16^{b}	7.54 ± 0.28^{b}		4.31 ± 0.21^{a}	5.18 ± 0.02^{c}
Statistical significance	***	***	**		**	***

Data within a column followed by the same letter are not significantly different according to Tukey's test.
Abbreviations: MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; M17, medium 17 agar for mesophilic coccus LAB; SDB, sourdough bacteria agar for typical sourdough LAB; YGC, Yeast Extract Glucose Chloramphenicol Agar for yeast; WL, Wallerstein Laboratory Nutrient agar for yeasts.

Table 3. Phenotypic grouping of the rod LAB isolated from sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Characters	Clusters			
	A (n=32)	B (n=26)	C (n=10)	D (n=8)
Growth:				
15°C	+	+	+	+
45°C	-	-	-	-
Acid production from:				
arabinose	-	+	-	-
ribose	-	+	+	-
xylose	-	+	-	-
sucrose	-	+	+	+
CO_2 from glucose	+	+	+	-

3.2.Isolation, phenotypic characterization and identification of rod LAB

A total of 209 colonies were collected from the seven sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production. All cultures were microscopically inspected and, after Gram determination and catalase test, 76 isolates were classified as presumptive lactobacilli. Based on the combination of the phenotypic features evaluated, the 76 LAB cultures were separated into 4 groups (Table 3). The largest groups were groups A and B (32 and 26 isolates, respectively). All groups included mesophilic lactobacilli growing at 15°C but not at 45°C. Groups A and D were characterized by an obligately hetero-fermentative metabolism showing the ability to produce CO_2 from glucose.The isolates representative of each phenotypic group, for all sourdoughs, were also identified by sequencing of the 16S rRNA gene. The sequences were compared with those available in two distinct databases; all isolates processed were clearly identified as rod LAB, since sequence similarity was higher than 97% in both databases (Table 4) and all species belonged to the genus *Lactobacillus*. The species detected were *L. sanfranciscensis* (n=10), *L. brevis* (n=6), *L. paralimentarius* (n=3) and *L. coryniformis* (n=3). All the species identified are commonly associated with wheat, wheat flour and bakery products (Corsetti et al., 2007; Alfonzo et al., 2013; Ventimiglia et al., 2015). The strains of *L. brevis*, *L. sanfranciscensis* and *L. paralimentarius* were also analyzed with species-specific PCR strategies (Settanni et al., 2005). This technique confirmed their identity. For *L. coryniformis* the unequivocal identification was obtained by applying the sequencing of the 16S rRNA gene because the multiplex PCR assay does not include this species. *L. coryniformis* is a species that is commonly dominant in oat and sorghum sourdoughs and exceptionally can be found in sourdoughs made with durum wheat flour (De Vuyst et al., 2005; Hüttner et al., 2010; Sekwati-Monang et al., 2011), but it is commonly found in raw materials (Alfonzo et al., 2013; Alfonzo et al., in press).

Table 4. Identification of rod LAB strains isolated from sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Strain	Amplicon Multiplex PCR (bp)	Species	Sourdough Sample	% similarity (accession no. of closest relative) by:		Sequence length (bp)
				GenBank	EzTaxon	
LABCM02	134	*Lactobacillus sanfranciscensis*	CV1	99 (AJ422037.1)	99.51 (X76327)	1,421

LABCM08	502	*Lactobacillus brevis*	CV1	99 (KU315055.1)	99.55 (KI271266)	1,348
LABCM34	521	*Lactobacillus paralimentarius*	CV1	97 (AJ422034.1)	97.25 (BAMH01000179)	1,447
LABCM59	502	*L. brevis*	CV2	99 (KU555383.1)	99.03 (KI271266)	1,343
LABCM71	134	*L. sanfranciscensis*	CV2	99 (KM822616.1)	99.58 (X76327)	1,419
LABCM78	n.d.	*Lactobacillus coryniformis*	CV2	99 (KX430830.1)	99.79 (GL544638)	1,432
LABCM84	502	*L. brevis*	CV3	100 (KF975703.1)	99.66 (KI271266)	1,171
LABCM85	134	*L. sanfranciscensis*	CV3	99 (AJ422037.1)	99.37 (X76327)	1,423
LABCM86	502	*L. brevis*	CV3	99 (KX010095.1)	98.64 (KI271266)	1,253
LABCM99	n.d.	*L. coryniformis*	MR1	97 (KX430830.1)	97.68 (GL544638)	1,340
LABCM109	521	*L. paralimentarius*	MR1	98 (KC755102.1)	97.43 (BAMH01000179)	1,404
LABCM111	134	*L. sanfranciscensis*	MR2	98 (CP002461.1)	98.26 (X76327)	1,329
LABCM113	502	*L. brevis*	MR2	99 (KU315055.1)	99.85 (KI271266)	1,341
LABCM121	134	*L. sanfranciscensis*	MR2	99 (KM822617.1)	98.82 (X76327)	1,440
LABCM130	134	*L. sanfranciscensis*	MR2	99 (CP002461.1)	99.72 (X76327)	1,419
LABCM138	134	*L. sanfranciscensis*	MR3	99 (KM822615.1)	99.55 (X76327)	1,354
LABCM140	134	*L. sanfranciscensis*	MR3	99 (AJ422037.1)	99.64 (X76327)	1,404
LABCM164	502	*L. brevis*	MR3	99 (EU177644.1)	99.86 (KI271266)	1,428
LABCM174	134	*L. sanfranciscensis*	MR4	100 (CP002461.1)	99.78 (X76327)	1,344
LABCM181	521	*L. paralimentarius*	MR4	100 (KC755102.1)	99.93 (BAMH01000179)	1,353
LABCM186	134	*L. sanfranciscensis*	MR4	99 (AJ422037.1)	99.08 (X76327)	1,401
LABCM188	n.d.	*L. coryniformis*	MR4	97 (KX430830.1)	97.05 (GL544638)	1,357

Table 5. Molecular identification and distribution of yeasts isolated in sourdough used for Pane Nero di Castelvetrano and Pane di Monreale production.

Strain	Species	R. P.	5.8S-ITS PCR	Size of restriction fragments			% similarity[a] (accession no. of closest relative) by:	Isolation source
				*Cfo*I	*Hae*III	*Hinf*I		
YCM125	*P. guilliermondii*	I	650	300+265+60	400+115+90	320+300	99 (EU807915)	MR3
YCM140	*P. segobiensis*	II	640	300+285	490+140	310+310	99 (DQ409151.1)	CV1, CV2, CV3
YCM120	*R. acuta*	III	670	355+315	650	250+190+185	99 (KP216512.1)	MR3
YCM156	*R.mucilaginosa*	IV	630	320+240+80	425+215	340+225+75	99 (AF335986.1)	CV2
YCM02	*S. cerevisiae*	V	850	360+340+140 +50	320+225+165+135	385+125+50	99 (GU138462.1)	MR1, MR2, MR3, MR4

Abbreviation: R.P., restriction profile.
All values for the 5.8S-ITS PCR, 26S PCR and restriction fragments are given in bp.
[a] According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database.

3.3.Isolation and identification of yeasts

A total of 101 colonies of yeasts were isolated from count plates. They were purified to homogeneity and grouped on the basis of colony appearance on WL medium. The yeasts representative of each morphology were subjected to the molecular identification. The results of the restriction analysis of 5.8S-ITS region were used to cluster the isolates into five groups (Table 5). The sequencing of D1/D2 domain of the 26S rRNA gene identified five species: *Pichia guilliermondii* (group I), *Pichia segobiensis* (group II), *Rhodotorula acuta* (group III), *Rhodotorula mucilaginosa* (group IV), *Saccharomyces cerevisiae* (group V).

The majority of yeasts belonged to the species *S. cerevisiae* and *Pichia segobiensis.* In particular, *S. cerevisiae* were found in all sourdoughs used for Pane di Monreale production while, *P. segobiensis* in all sourdoughs used for Pane Nero di Castelvetrano production. The presence of *S.*

cerevisiae might be due to cross-contamination by conventional bread production (Vrancken et al., 2010). *Rhodotorula acuta* and *P. guilliermondii* were both found only in the sample MR3 while, *R. mucilaginosa* in the sample CV2. Among these yeasts only *S. cerevisiae* was previously isolated from Sicilian sourdoughs (Pulvirenti et al., 2001; Giannone et al., 2010) while, *R. mucilaginosa* from traditional sourdoughs collected from western region in Inner Mongolia of China (Zhang et al., 2011) and *P. guilliermondii* from French and Turkish sourdoughs (Lhomme et al., 2016; Yağmur et al., 2016). The other two yeast species isolated in the present work are not commonly associated with sourdoughs.

4. Conclusions

Sourdough is a specific and stressful ecosystem inhabited by LAB, mainly hetero-fermentative lactobacilli, and yeasts. The results of this study confirmed the codominance of hetero-fermentative lactobacilli in typical Sicilian sourdoughs and the high levels of *L. sanfranciscensis* that is considered the key sourdough LAB. *S. cerevisiae* dominated the yeast community on certain sourdoughs, probably due to the cross-contamination. Works are being prepared to investigate the technological properties of the dominant strains in order to develop *ad hoc* starter cultures constituting stable elements for the typicality of the Sicilian sourdough productions.

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STUDIES REGARDING QUALITY PARAMETERS OF TRADITIONAL RAW-DRIED SALAMI FROM PRIVATE PRODUCER FROM THE WEST PART OF ROMANIA

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ABSTRACT

The main objectives of this paper are the studies of physic-chemical characteristics of traditional raw-dried salami, produced in the West part of Romania.

During three experimental years (2013 – 2015) it have been analyzed several samples of raw-dried salami from the same producer from the West part of Romania, as follow: water content, nitrites, sodium chloride and oxidation stage of fats through Kreiss reaction. Measurements were carried out according to the standards SR ISO 17025/2005, ISO 1442/1997, STAS 9065/5-1973, STAS 9065/9-74, quarterly in the Laboratory of physical-chemical and bacteriological determinations at the same private producer.

The results of the physical-chemical analysis of the studied raw-dried salami samples were in accordance with the legal requirements established by Romanian law. All samples of raw-dried salami have excellent nutritional characteristics being proper for consumption.

1. Introduction

The raw-dried (fermented) meat products represent a group of food products with a wide variety of flavours and textures, very appreciated by consumers looking for new taste experiences. This type of meat products has been prepared since ancient times, it is well-known that drying is one of the first methods used to preserve meat. At present, even if through particularities of manufacturing recipes and production methods it exists a wide range of products, they have common specific characteristics: microbial stability, special flavour and intense colour, long preservation period (Meat Milk, 2013).

Raw-dried salami are a class of meat products obtained by mixing meat and chopped fat with salt, carbohydrates, curing agents and adjuvants, spices and other food additives. The mixture is stuffed into membranes/forms and undergoes a maturation process. In most fermented salamis, maturation comprises a fermentation phase generated by the own meat enzymes and by synthesized through bacteria, moulds and yeasts, followed by a curing - drying phase. During the ripening – drying period and fermentation, a specific complex processes occurs, controlled physical, biochemical and microbiological, with an important role in training, improving and balancing of physic-chemical and sensory and preservation of those products (Meat Milk, 2013).

Knowledge of physic-chemical and sensory characteristics of meat is necessary in solving problems of storage, determination of equipment capacity, heating and freezing

treatments, appreciation of sanitation. The physical characteristics of meat and meat products are important in determining both storage conditions and energy needed in different stages of technological processing (Cocan *et al.*, 2015).

With the purpose to dissolve other chemicals in its mass, water is a necessary nutrient for the activity of bacterial enzyme. The water content of food has to be a good indicator of the preponderance of one or another of the degradation reactions that might occur during storage of food. The water content is specified in standards as an essential element according to the established conditions and shelf life prediction of food, also the water content influences consumer perception to some product features (freshness) (Mathlouthi, 2001).

2. Materials and methods

2.1. Materials:

There where obtained and analyzed two types of raw-dried salami, during three experimental years, 2013-2015.

2.2. Methods:

Physic-chemical analysis of water content, nitrites, sodium chloride and oxidation stage of fats were carried out quarterly in the Laboratory of physical-chemical and bacteriological determinations at the same private producer from the West part of Romania, according to the following standards:

ISO 1442:1997. Meat and meat products. Determination of water content.

SR ISO 17025/2005. General requirements for the competence of laborators for testing and calibration. Kreiss reaction.

STAS 9065/5-1973. Determination of sodium chloride from meat and meat products.

STAS 9065/9-74. Determination of nitrite content from meat and meat products.

3. Results and discussions

3.1. Determination of water content in food products of animal origin (oven drying method)

Determination of water content from samples of raw-dried salami, studied between 2013 and 2015 were carried out quarterly in the Laboratory of physico-chemical and bacteriological determinations at the same private producers from the West part of Romania.

The values of the water content of the studied raw-dried salami samples are given as average of three determinations in Figure 1. In 2013 we recorded values of the water content above the maximum admitted limit of 35%, legislated by Order 210/2006, in the I. (35.15%), third (35.8%) and fourth (37.8%) Quarters, only in the II. Quarter the value of water content (34.7%) was below the admitted limit (Figure 1).

However the results are in accordance with literature studies that record values of the water content between 24.3% and 53% (Zanardi *et al.*, 2010).

During 2014 and 2015 the studied sample raw-dried salami registered values of the water content between 32.36% (II. Quarter) and 33.55% (IV. Quarter) in 2014 and much lower in 2015, between 27.18% (II. Quarter) and 30.20% (IV. Quarter) (Figure 1), all values under the admissible legal limit of 35%.

3.2. Determination of sodium chloride of meat and meat products

The sodium chloride (NaCl) content in meat products influences the formation of taste and also on the decrease of water activity. Removing of water during drying influence the development and decay of pathogenic microorganisms positive involved in the fermentation. Another benefit of using sodium chloride refers to the myofibrillar proteins that are important for binding and strengthening of the salami mass, thus causing a certain resistance of the product (Gunter and Hautzinger, 2007).

Sodium chloride in raw salami and sausages, is added in percentage of 2% to 3.2%. The studied samples of raw-dried salami recorded higher values of sodium chloride in 2013, between 5.21% (IV. Quarter) and 5.67% (I. Quarter) (Figure 2).

During the experimental years 2014 and 2015, the analysis showed a decrease in the sodium chloride content, once with implementation of a rigorous control through the HACCP system. Thus, in 2014 the values range between 4.8% and 5.15%, and in 2015 between 4.15% and 4.65% (Figure 2), all values framing below the maximum legal limit of 6%. The results are in accordance with other literature studies which show NaCl content of 3.51-5.20% in raw-dried Italian salami. (Zanardi *et al.*, 2010).

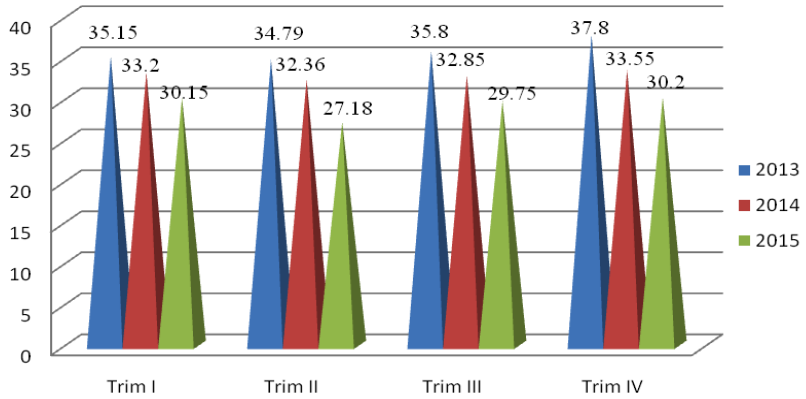

Figure 1. Water content (%) of raw-dried salami samples studied between 2013 and 2015

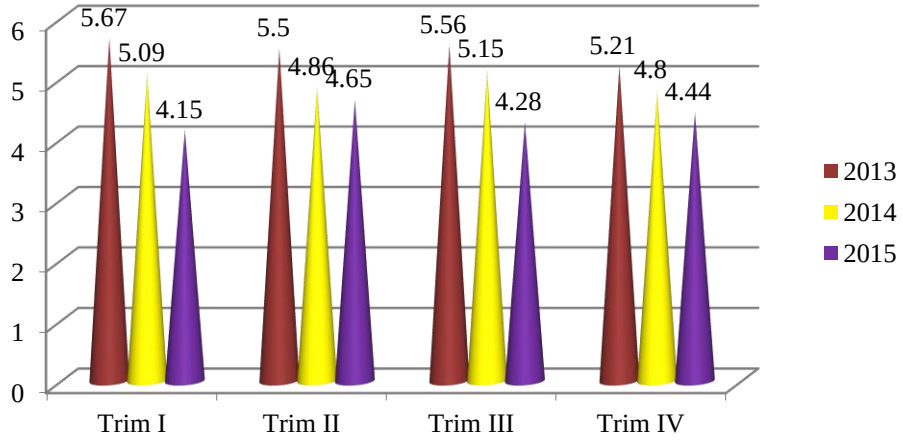

Figure 2. NaCl content (%) of raw-dried salami samples studied between 2013 and 2015

3.3. Determination of nitrites in meat and meat products

Nitrite can be used in low concentrations without endangering human health, with purpose to preserve and colour meat products. According to literature studies traces of nitrite are not poisonous, besides the effect of reddening; it has a number of additional benefits, so that the meat industry depends very much by this substance. In meat products, it is recommended a level of 150 mg/kg (0.015%) nitrite, but to reduce the risk of nitrite overdose, a safe dosage is achieved in a homogeneous mixture, formed by 99.5% sodium chloride and 0.5% nitrite (Gunter and Hautzinger, 2007).

Order 210/2006 concerning the admissibility conditions for the physic-chemical properties for meat products set a maximum admitted limit for nitrite of 7 mg/100 g (0.007%). The nitrate content in the studied samples of raw-dried salami, during three years (2013-2015), records values of 4.7-5.9 mg/100 g (2013), 3.8-4.6 mg/100 g (2014) and 2.7-3.4 mg/100g (2015) (Figure 3). All registered values are below the legal limit of 7 mg/100 g , but higher values were registered in 2013 (4.9-5.9 mg/100g), before the implementation of the HACCP system.

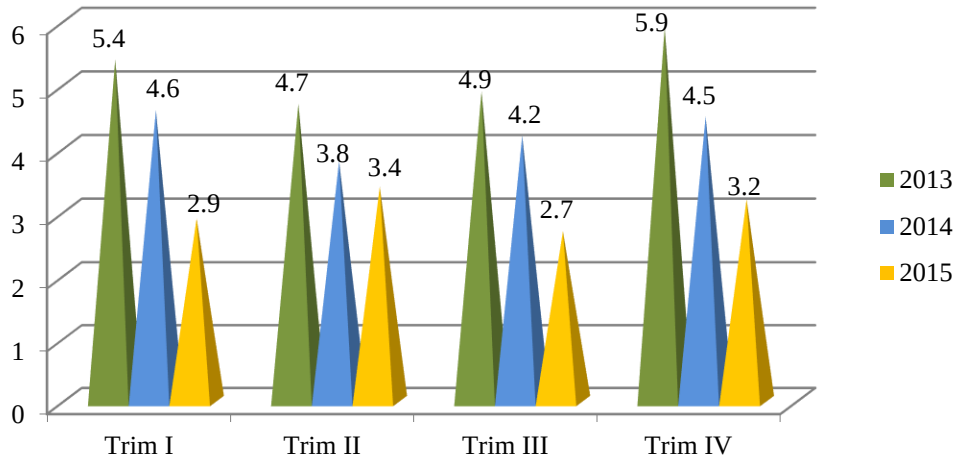

Figure 3. Nitrite content (mg/100g) of raw-dried salami samples studied between 2013 and 2015

3.4. Determination of the oxidation stage of fats through Kreiss reaction

Kreiss reaction was negative in all studied samples of raw-dried salami, during all the three experimental years (2013-2015), the results being in accordance with the values according to SR ISO 17025 (General requirements for the competence of testing and calibration laboratories).

4. Conclusions

The researches of this paper are interdisciplinary, including technological, economic and nutritional sciences, to respond to new challenges of the society. Important points that should be taken into account refer to the amount of food for daily intake, to different types of consumers and dietary diversity associated with lifestyles, which forms the base of health.

The results of the physical-chemical analysis performed for the studied samples of raw-dried salami were within the allowed legal requirements; all samples of raw-dried salami have excellent nutritional characteristics being suitable for consumption.

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COMPARATIVE STUDY REGARDING PHYSICAL-CHEMICAL CHARACTERISTICS OF CHICKEN POLISH SAUSAGES WITH NATURAL EXTRACTS AND CHEMICAL ADDITIVES

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ABSTRACT
The purpose of the paper is to present the study regarding the influence of natural extracts (*Kombucha* extracts) on the nutritional quality of polish sausages comparative with polish sausages with chemical additives. The products (classic polish sausages with chicken meat and polish sausages with chicken meat and kombucha extracts) were obtained at a private company from Timisoara and the quality control of the studied products were performed at the laboratory for physic-chemical and bacteriological analyzes at the same company. The physic chemical analyzes were done according to the following standars: ISO 1442/1997 (water content), SR ISO 1444/2008 (fat content), STAS 9065/5-1973 (sodium chloride content), SR ISO 937:2007 (nitrogen content), SR ISO 17025 (Kreiss reaction), STAS 9065/9-74 (nitrites content).
The obtained results show that the natural extracts (*Kombucha* extract) used in polish sausages with chicken meat have a high influence on the physic-chemical parameters, the obtained product has high nutritional value, without chemical additives.

1. Introduction

As the food consumption increased worldwide, food safety has become a concern of great interest around the world. Increased international trade of food products has led to new opportunities for food pathogens to reach the farest regions in the world. Increasing public concerns regarding food contamination with microbiological, chemical and radioactive pollutants determined official public health organizations and the World Health Organization to step up prevention and control effects. Thus any food product intended to be marketed is analyzed in terms of physical-chemical, microbiological and food innocuousness (Mencinicopschi *et al*., 2006).

Meat products are products that spoil easily due to high protein content that is why meat and meat products must be kept and preserved at low temperatures, to increase the elapsed time until alteration. Unlike pork, chicken and chicken products are low-fat, they are more easily digested and assimilated (Olaru *et al*., 2001).

The protein content of chicken meat range between 19.8% and 22.8%, the water content between 67.5% and 72.1% and the fat content between 4% and 11.5% (Olaru *et al*., 2001).

Current trends in the food industry are directed to product quality, aiming to produce assortments with high nutritional value, without additives, preservatives, heavy metals or other compounds with negative impacts for human health. The possibility to improve traditional meat products quality through nutritional

intervention brings public awareness of foods with added biologically active compounds, which contribute to their superior capitalization, good organism functioning and health (Gunter and Hautzinger, 2007).

Kombucha represents microorganisms that coexist in symbiosis, representing a very complex biological system which is formed of bacteria and fungi (yeast), involved in sugar conversion in substances with remarkable healing effects. Kombucha contains high quantities of vitamin C and B complex (B1, B2, B3, B6, B12), with rejuvenating properties and stimulating natural resistance of the human organism (Dudea, 2016).

Chicken polish sausages belong to the category of smoked/pasteurized meat products prepared without structure. Polish sausages are fresh products which have homogeneous composition, seasoned and treated by smoking and boiling (Banu, 1997).

Poultry products and polish sausages in particular are preferentially consumed by children. In this regard, this study analyses two types of chicken polish sausages: classics polish sausages with chicken meat, with synthesis conservation additives and polish sausages with chicken meat and natural extracts (*Kombucha*) to preserve the product.

2. Materials and methods

The main raw and auxiliary materials that enter the manufacturing recipe of classic polish sausages, are: chicken breasts, bacon, ice flakes as raw materials and polyphosphates, vitamin C, mix salt, nutmeg, white pepper, garlic granules, paprika, powdered milk 20%, sugar as auxiliary materials. Natural membrane used is edible. We obtained smoked and cooked meat products in cylindrical form pieces with 10-15 cm lenght and 20-22 mm diameter.

The recipe for obtaining premium polish sausages with chicken breast and Kombucha extract, include the following raw and auxiliary materials: chicken breast, chicken meat, ice, Herba pure, spices for sausages (knacker frankfurter efr), salt.

The technological process for manufacturing premium polish sausages with *Kombucha* extract is identical to the process for obtaining classic polish sausages with chicken meat, with following technological operations: preparation of raw materials, preparation of spices, cutting, mixing, shredding, filling in membranes, binding, putting on sticks, showering, cooling, packaging, labeling, storage and transportation.

The products (chiken polish sausages classic and premium with *Kombucha* extract) were obtained at a private company from Timisoara and the physic-chemical and microbiological analysis for quality control were performed in the Laboratory for physical-chemical and becteriological analysis at the same company, according to the following standards: ISO 1442/1997 for water content, SR ISO 1444/2008 for free fat content, STAS 9065/5-1973 for sodium chloride, SR ISO 937: 2007 for nitrogen content, SR ISO 17025 for the oxidation stage of fats through Kreiss reaction, STAS 9065/9-74 for nitrites.

The physic-chemical properties of meat products (boiled and double smoked) have been established according to the Order 210/2006 regarding the admissibility conditions of the physic-chemical properties of cold cuts meat products type.

3. Results and discussions

Results are given as mean of quarterly values (January, April, July, October) ± SD (standard deviation), in three years of study, 2013-2015.

Physical-chemical analysis results for the studied polish sausages are given in Tables 1-5.

The water content in meat products is a factor for assessing the nutritional value (the higher the water content is, the lower the nutritional value), but also for assessing the power of conservation (the lower the water content is, the strength of the preservation is better) (Mencinicopschi *et al*., 2006).

The mean annual value of the water content in the studied samples of classic polish

sausages with chicken meat, is *63.50%±1.4389* in 2015 and *64.50%±1.923, in 2014*, lower than the mean annual value for premium polish sausages with chicken breast and *Kombucha* extract, ranges between *65.45%±1.1524* in 2014 and *67.12%±0.2231* in 2015 (Table 2) and below the legal maximum limit of 70%.

Also, the obtained values are in accordance with data obtained in other literature, between 53.67% and 59.22% (Cocan *et al.*, 2015).

The mean annual value of protein content in the studied sausages polish samples registered differences, ranges between *12.98%±0.9416* in 2014 and *14.63%±2.7801* in 2013 and in the case of premium polish sausages with chicken meat and *Kombucha* extract, between *16.77%±0.5104* in 2015 and *17.90 %±0.6739* (Table 2), in accordance with legal minimum protein level of 10% (Order 210/2006).

The results are in accordance with literature studies showing that polish sausages with chicken meat and with various natural extracts have a protein content of 14.0% and a fat content of 19.8% (Gunter and Hautzinger, 2007).

Animal nutrition influences the sensory characteristics of meat, tissue proportion and content in nutritive substances (proteins, fats, vitamins, and minerals). Administration of growth regulators in animal nutrition (tissue preparations, antibiotics, micronutrients, amino acids, hormones, anti-hormones) increases yields, but causes a decrease of the meat quality (Pop, 2012).

The legal maximum allowed value for fat in polish sausages is 26 %. The mean annual value of the fat content in the studied samples of classic polish sausages with chicken meat, ranges between *16.60% ±2.1620* in 2014 and *18.22%±3.0362* in 2015, higher then the values registered for premium polish sausages with chicken meat and *Kombucha* extract, between *12.01%±1.6017* in 2013 and *12.66%±1.3215* in 2014 (Table 3).

The studied samples of classic polish sausages with chicken meat (Table 4), registered mean annual values for sodium chloride between *2.10%±0.1255* in 2015 and *2.29%±02609* in 2014, lower than the mean annual values for premium polish sausages with chicken meat and *Kombucha* extract, between *2.31%±0.0775* in 2013 and *2.55%±0.0714* in 2014.

As for the mean annual values of the nitrite content in samples of classic polish sausages with chicken meat, they vary between 5.03mg/100 g *±0.2217* in 2014 and *5.53mg/100 g ±0.6702*, below the legal maximum limit of 7 mg/100 g (Table 5).

Consumption of nitrite is often considered a health risk because is contained in cured meat products. Studies showed that 5% of the dietary nitrate (ingested mainly with vegetables) and of the nitrate endogenously synthesized is reduced to nitrite by bacteria in the oral cavity, being the main source of nitrite for humans. Also these studies showed that cured meat products contribute only 9 to 16 % of the total nitrite supply and consumers concern against cured meat products because of their nitrite content is not justified (Schmid, 2006).

Table 1. Annual mean values of water content (%) in studied samples of polish sausages

Sample	Experimental years		
	2013	**2015**	**2015**
Polish sausages with chicken breast	64.28 ±1.3203	64.50±1.9238	63.50±1.4389
Premium polish sausages with chicken breasts and *Kombucha* extract	67.00±0.4779	65.45 ±1.1524	67.12±0.2231

Table 2. Annual mean values of protein content (%) in studied samples of polish sausages

Sample	Experimental years		
	2013	**2015**	**2015**
Polish sausages with chicken breast	14.63±2.7801	12.98±0.9416	13.09±1.9024
Premium polish sausages with chicken breasts and *Kombucha* extract	17.11±1.8318	17.90±0.6739	16.77±0.5104

Table 3. Annual mean values of fat content (%) in studied samples of polish sausages

Sample	Experimental years		
	2013	**2015**	**2015**
Polish sausages with chicken breast	16.83±1.3100	16.60±2.1620	18.22±3.0362
Premium polish sausages with chicken breasts and *Kombucha* extract	12.01±1.6017	12.66±1.3215	12.38±1.3215

Table 4. Annual mean values of NaCl content (%) in studied samples of polish sausages

Sample	Experimental years		
	2013	**2015**	**2015**
Polish sausages with chicken breast	2.28±0.1457	2.29±0.2609	2.10±0.1255
Premium polish sausages with chicken breasts and *Kombucha* extract	2.31±0.0775	2.55 ±2.5575	2.37±0.1169

Table 5. Annual mean values of nitrite content (mg/100g) in studied samples of polish sausages

Sample	Experimental years		
	2013	**2015**	**2015**
Polish sausages with chicken breast	5.20±0.2944	5.03±0.2217	5.53±0.6702

4. Conclusions

Following the results obtained after production and the physical-chemical analysis of 2 types of polish sausages, with chicken meat and chemical additives and natural extracts (*Kombucha* extract), we can conclude that the nutritional characteristics can be improved with obtaining safe products.

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CHEMICAL COMPOSITIONS AND CHARACTERISTIC OF SAWAI (*PANGASIANODON HYPOPHTHALMUS*) MEAT

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ABSTRACT

Chemical compositions and thermal property of Sawai meat were studied. High protein (55.36% dry basis) and lipid (40.96% dry basis) contents were found in Sawai meat. Fractionation of nitrogenous constituents revealed that myofibrillar protein (71.81%) was the major component in the muscles; myosin heavy chain (MHC) and actin were the predominant proteins. Triglyceride was the main lipid (80.79%) in Sawai meat, followed by phospholipid. Sawai meat had monounsaturated fatty acids as the major component and was rich in oleic acid and palmitic acid. Docosahexaenoic acid (DHA; 22:6), eicosapentaenoic acid (EPA; 20:5) and arachidonic acid (ARA; 20:4) were also found in Sawai lipid. Magnesium (Mg) was the dominant mineral in Sawai meat, followed by calcium (Ca). Zinc (Zn) and iron (Fe) were also found at high concentrations. Sawai meat exhibited thermal transition temperatures (T_{max}) of 54.33 and 78.00°C. for myosin and actin, respectively. These data could be useful for consumers and food industry.

1. Introduction

Catfish such as Sawai (*Pangasianodon hypophthalmus*) has been increasingly produced and consumed due to its taste and useful nutrients. Viet Nam is the largest producer followed by Thailand, Cambodia, Lao People's Democratic Republic, Myanmar, Bangladesh and China (Orban et al., 2008). Sawai is a fast growing species which reached market sizes within 8 months. So, it has become an economically important cultured freshwater fish in Thailand. This fish is mainly exported as frozen fillet. Recently, frozen catfish fillet market has been increasing due to its meat qualities (Wang & Hsieh, 2016). Catfish meat has been also reported as a good source of lipid and unsaturated fatty acids (Wang et al., 2012). However, the chemical composition of fish

meat is influenced by various factors such species, size, feeding fish habits, environmental conditions and season (Balçik Misir et al., 2014; Suárez et al., 2015). Variation of chemical compositions and characteristics of fish muscle would affect the nutritional value and the organoleptic properties of fish. For example, marine fish contains high amount of n-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) but monounsaturated fatty acid (MUFA) was dominant in freshwater fish (Di Lena et al., 2016; Trbović et al., 2013).

Generally, fish meat quality can be varied with species, more likely governed by compositions and thermal property of tissue. There are many reports on the chemical compositions and muscle characteristics of marine fish species (Balçik Misir et al., 2014; Suárez et al., 2015). However, information on that content of freshwater fish species such as catfish is limited. The understanding of the chemical and thermal properties including lipid class, fatty acid profile and mineral content of Sawai meat could provide the nutritive values of this species. Nevertheless, information regarding chemical compositions and thermal property of Sawai meat, especially cultured in Thailand, is scarce. Therefore, the objective of this research was to study the chemical composition and thermal property of meat from Sawai cultured in Thailand.

2. Materials and methods

2.1. Chemicals

Chloroform, methanol and sulfuric acid were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), acrylamide,*N,N,N,N*-tetramethyl ethylene diamine (TEMED) and bis-acrylamide were obtained from Fluka (Buchs, Switzerland). ß-mercaptoethanol (BME) was procured from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), coomassie Blue R-250 and protein molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals used were of analytical grade.

2.2. Sample preparation

Sawai (*P. hypophthalmus*) with an average weight of 2 kg were obtained from fish farm in Ubon Ratchathani province, Thailand. Three different lots (10 fish each) were used for the study. The live samples were placed in plastic box added oxygen and transported to the Food Science and Technology Program, Ubon Ratchathani Rajabhat University, Ubon Ratchathani province, Thailand. Upon the arrival, fish were immediately washed and filleted. The fillets were placed in polyethylene bags and kept in ice at a meat/ice ratio of 1:3 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla province, Thailand within 9 h. The fillets were ground to obtain uniformity using a blender (Type AY46, Moulinex, Group SEB Thailand Ltd., Bangkok, Thailand). The minced samples were placed in polyethylene bags and kept in ice (0-2°C) until the analyses. The storage time was not longer than 5 h.

2.3. Analyses

2.3.1. Proximate Analysis

Fish meat was analyzed for moisture, protein, fat, and ash contents according to AOAC methods (AOAC, 1999) described in the analytical numbers of 950.46, 920.153, 960.39 and 928.08, respectively. The values were expressed as % of wet weight basis.

Determination of hydroxyproline content

The hydroxyproline content of fish muscle was analyzed according to the method of Bergman and Loxley (1963) with a slight modification. The sample was hydrolyzed with 6 M HCl in a screw cap tube at 110°C for 24 h in an oil bath (model B-490, BUCHI, Flawil, Switzerland). The hydrolyzed sample was clarified with activated carbon and filtered through Whatman No. 4 filter paper. The filtrate was then neutralized with 10 M NaOH to obtain a pH of 6.0–6.5. The neutralized sample (0.1 mL) was transferred into a test tube and isopropanol (0.2 mL) was added and mixed well; 0.1 mL of oxidant solution (mixture of 7% chloramine T and 0.1 M acetate/citrate buffer, pH 6, at a ratio of 1:4 (v/v)) was added and mixed thoroughly. A 1.3 mL of Ehrlich's reagent solution (mixture of solution A; 2 g of *p*-dimethylamino-benzaldehyde in 3 mL of 60% perchloric acid) and isopropanol at a ratio of 3:13 (v/v) were added and mixed. The mixture was heated at 60°C for 25 min in a water bath (Memmert, Schwabach, Germany) and then cooled for 2-3 min using running water. The solution was diluted to 5 mL with

isopropanol. Absorbance of the mixture was measured at the wavelength of 558 nm. Hydroxyproline standard solution, with concentration ranging from 0 to 60 ppm, was also included. Hydroxyproline content was calculated and expressed as mg/g of sample.

2.3.2. Determination of protein and non-protein nitrogenous compounds

Non-protein nitrogenous constituents, sarcoplasmic protein, myofibrillar protein, alkali-soluble protein, and stromal protein in fish meat were fractionated according to the method of Hashimoto et al. (1979). Nitrogen content in each fraction was measured by the Kjeldahl method (AOAC, 1999). Protein patterns of different fractions were determined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) with 10% running gel and 4% stacking gel, as described by Laemmli (1970). Samples (10 μg protein) determined by the Biuret test (Robinson & Hogden, 1940) were loaded. After separation, proteins were stained and destained.

2.3.3. Determination of lipid composition and fatty acid profile

Lipid in the fish was extracted by the Bligh and Dyer method (Bligh & Dyer, 1959). The lipid compositions were determined by thin-layer chromatography/flame ionization detector (TLC-FID). Scanned quartz rods (silica gel powder-coated Chromarod S III) were dipped in 3% boric acid solution for 5 min, dried and rescanned with the TLC-FID analyzer. The sample solution (1 μL) was spotted on the rod and the separation was carried out with the mixture of benzene: chloroform: acetic acid (52:20:0.7) for approximately 35 min. Then, the rods were dried in an oven (105°C) for 5 min before analyzing with the flame ionization detector. The analytical conditions were H_2 flow rate of 160 mL/min, air flow rate of 2000 mL/min and scanning speed of 30 s/scan. Retention time of lipid composition standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as % of total lipid.

The fatty acid compositions were determined as fatty acid methyl esters (FAME) using a gas chromatography, GC-14A (Shimadzu, Kyoto, Japan) equipped with fused silica capillary column Carbowax-30 M (30 m, 0.25 mm ID) and flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 170°C and was increased to 225°C with a rate of 1°C/min and then held at 220°C for an additional 20 min. The detector temperature was set at 270°C, while the temperature at the injection port was maintained at 250°C. Retention time of FAME standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as % of total lipid (AOAC, 1999).

2.3.4. Determination of mineral content

Copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni), iron (Fe), calcium (Ca), magnesium (Mg) and lead (Pb) contents were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Perkin–Elmer, Model 4300 DV, Norwalk, CT, USA) according to the method of AOAC (AOAC, 1999). Ground fish meat (4 g) was mixed well with 4 mL of nitric acid. The mixture was heated on the hot plate until digestion was completed. The digested samples were transferred to a volumetric flask and the volume was made up to 10 mL with deionised water. The solution was subjected to (ICP-OES) analysis. Flow rates of argon to plasma, auxiliary and nebulizer were kept at 15, 0.2, and 0.8 L/min, respectively. Sample flow rate was set at 1.5 mL/min. The wavelengths for analysis of Fe, Cu, Mn, Ni, Zn, Ca, Mg and Pb were 238.2, 327.4, 257.6, 231.6, 206.2, 317.9, 285.2 and 220.4 nm, respectively. The concentration of mineral was calculated and expressed as mg/kg sample.

2.3.5. Differential scanning calorimetry (DSC)

Thermal transitions of Sawai meat were measured using a differential scanning calorimeter (DSC; Perkin–Elmer, Model DSCM, Norwalk, CT, USA). The samples (15-20 mg) were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. An empty hermetic pan was used as a reference. Calibration was made using Indium thermogram. The samples were scanned at 1°C/min over the range of 20-100°C. Thermal transition temperature (T_{max}) was measured, and the denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve.

2.4. Statistical analysis

For each experiment, fish minces from 10 randomly selected fish were used as the composite sample. The composite sample was divided into 3 replications, each of which were considered experimental units (n = 3). Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel & Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussions

3.1. Proximate composition and hydroxyproline content in Sawai meat

Sawai meat contained 70.34% moisture, 16.42% protein, 12.15% fat, 0.86% ash and 0.20 mg/g sample hydroxyproline. High protein (55.36%, dry weight basis) and lipid (40.96%, dry weight basis) contents were found in Sawai meat. These result indicated that Sawai meat could be served as a good source of proteins and lipid. The slight difference in chemical composition was observed between Sawai and that reported for other fish species. Edible parts (dorsal, ventral and lateral line muscle) of giant catfish (*Pangasianodon gigas*) contained 75.51-81.67% moisture, 14.36-19.00% protein, 0.54-8.60% fat, 1.11-1.47% ash and 0.73-0.83 mg/g hydroxyproline (Chaijan et al., 2010). Trbović et. al. (2013) also reported that proximate composition of common carp (*Cyprinus carpio*) consisted of 71.44-77.47% moisture, 15.14-18.28% protein, 3.02-11.57% fat, and 1.05-1.06% ash. The different chemical content between Sawai and other fish species might be due to the different fish species and feeding conditions. Haard (1992) reported that fish are classified into 4 groups according to their lipid content including lean fish (<2%); low-fat fish (2-4%); medium-fat fish (4-8%) and high-fat fish (>8%). Base on lipid content, Sawai in the present study is classified to high-fat fish. Differences in chemical compositions might be associated with the differences in sensory properties, nutritional value, and shelf-life of different fish.

3.2. Proteins and non-protein nitrogenous compounds

The contents of different proteins and non-protein nitrogenous components in Sawai meat are shown in Table 1.

Table 1. Nitrogenous constituents of Sawai meat

Components	Nitrogen content (mg N/g meat)
Non-protein nitrogen	2.36±0.05*
Sarcoplasmic protein	6.05±0.25 (23.36) **
Myofibrillar protein	18.60±0.10 (71.81)
Alkali-soluble protein	0.35±0.07 (1.36)
Stromal protein	0.88±0.02 (3.41)

*Means ± SD (n=3).

**Numbers in parenthesis represent percentage distribution.

Myofibrillar protein (71.81% of total protein) was the major protein in Sawai meat, followed by sarcoplasmic protein (23.36% of total protein). Fish sarcoplasmic proteins mainly consist of myoglobin and enzymes (Hui Y. H., 2012). This finding was in agreement with Chaijan et al. (2010) and Sriket et al. (2013) who reported that myofibrillar and sarcoplasmic proteins were the major proteins in fish and shellfish muscle. Alkali-soluble

protein and stromal protein were found as the minor components in Sawai meat. Alkali-soluble protein was reported as a cross-linked myofibrillar protein (Kristinsson et al., 2005), and collagen was presented as the major protein in stroma (Gökoğlu & Yerlikaya, 2015). Sawai meat contained 2.36 mg N/g meat of non-protein nitrogenous compound. Non-protein nitrogenous constituents including free amino acids, nucleotide, peptides and betaine play a crucial role in fish and shellfish flavor (Sikorski et al., 1990; Sriket et al., 2013). Additionally, the changes in non-protein nitrogenous compounds could be used as a quality index for monitoring the shelf-life of fish.Protein patterns of whole fish meat and different. Protein fractions from Sawai meat are shown in Fig. 1.

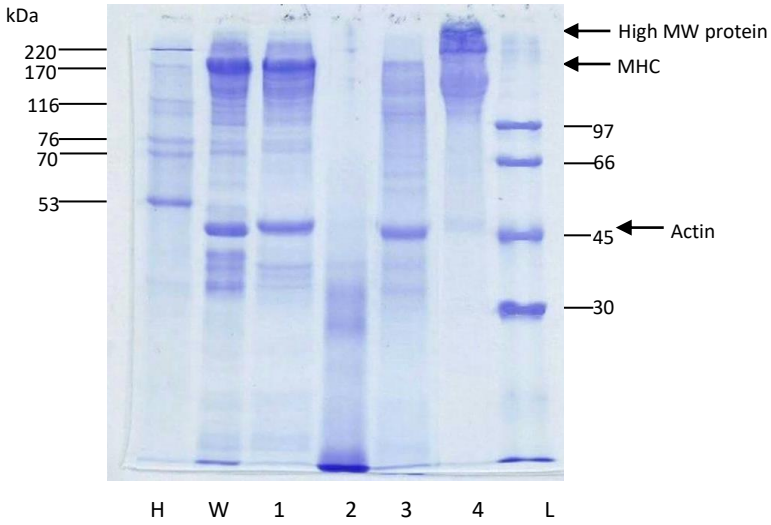

Figure 1. Protein patterns of Sawai meat and various protein fractions (H: high molecular weight protein markers; W: whole meat; 1, 2, 3, 4: myofibrillar, sarcoplasmic, alkali-soluble and stromal protein fractions, respectively; L: low molecular weight protein markers; MHC: myosin heavy chain).

Myosin heavy chain (MHC) was the dominant protein component in Sawai meat, followed by actin. This result was in agreement with previously reported in other species such as Asian hard clam, *Meretrix lusoria*, (Karnjanapratum et al., 2013) and freshwater prawn, *Macrobrachium rosenbergii* (Sriket et al., 2013). MHC and actin are the major proteins in myofibrillar protein and constitute around 40-60% of the total protein in fish and shellfish (Hui, 2012). Two major protein bands, corresponding to MHC and actin, were observed in myofibrillar protein fraction (Fig. 1). Generally, sarcoplasmic protein fraction expressed mainly low-molecular-weight proteins (Fig. 1). For the alkali soluble protein fraction, the protein with MW of 45 kDa was present as the major proteins. Stromal protein fraction contained proteins with MW of 116 and 220 kDa. It was noted that there were some cross-links with high MW protein in this fraction as evidenced by the presence of the protein band close to the stacking gel.

3.3. Lipid composition and fatty acid profile of Sawai meat

Table 2. Lipid compositions of Sawai meat

Composition	Content (% of total lipid)
Phospholipid	17.07 ± 0.55^{b}
Triglyceride	80.79 ± 0.28^{a}
Diglyceride	1.58 ± 0.06^{c}
Monoglyceride	0.45 ± 0.03^{d}
Free fatty acid	0.11 ± 0.01^{e}

*Means ± SD (n=3). Different superscripts indicate the significant differences ($p < 0.05$).

In general, triglyceride, phospholipids, diglyceride, monoglyceride and free fatty acid were observed in Sawai lipid (Table 2). Triglyceride was the major component (80.79%) of lipid in Sawai meat, followed by phospholipid (17.07%) and traces amounts of diglyceride, monoglyceride and free fatty acid (Table 2). The result was similar to Senphan and Benjakul (2015) who reported that triglyceride was the main component in striped catfish (*P. hypophthalmus*) lipid. It was noted that free fatty acid content was very low in Sawai lipid. This might be due to very low lipase activity in Sawai meat during postmortem storage. However, it was reported that free fatty acid tends to be high in low lipid tissue (Oliveira & Bechtel, 2006). The fatty acid profile of lipid extracted from Sawai meat is shown in Table 3. Oleic acid (18:1) is the most abundant fatty acid (35 g/100 g) in Sawai meat, followed by palmitic acid (16:0). Fish meat also contains many n-3 polyunsaturated fatty acids (n-3 PUFA) and n-6 polyunsaturated fatty acids (n-6 PUFA) such as eicosapentaenoic acid (EPA: C20:5n-3), docosahexaenoic acid (DHA: C22:6n-3) linoleic acid (C18:2n-6) and arachidonic acid (ARA: 20:4n-6). The consumptions of fish oil rich in DHA and EPA have been reported to reduce risk of cardiovascular and neurological diseases (Khawaja, et al., 2014) EPA, DHA and ARA were also reported to maintain cell membrane structure integrity and proved as the precursors of eicosanoids hormones in the human body (Ateş et al., 2013; Inhamuns & Franco, 2008).

Table 3. Fatty acid compositions (g/100g) of lipid from Sawai meat

Fatty acids	**Content**
Caproic acid C6:0	0.31
Caprylic acid C8:0	0.06
Capric acid C10:0	0.07
Lauric acid C12:0	1.01
Myristic acid C14:0	1.48
Myristoleic acid C14:1	0.03
Pentadecanoic acid C15:0	0.17
Palmitic acid C16:0	23.87
Palmitoleic acid C16:1 n-7	3.85
Heptadcanoic acid C17:0	0.25
Stearic acid C18:0	6.72
Cis-9-Octadecenoic acid C18:1 n-9	35.67
Cis-Vaccenic acid C18:1 n-7	2.32
Cis-9,12-Octadecadienoic acid C18:2 n-6	12.37
Cis-9,12,15-Octadecatrienoic acid C18:3 n-3	0.65
Cis-6,9,12-Octadecadienoic acid C18:3 n-6	0.17
Moroctic acid C18:4 n-3	0.08
Arachidic acid C20:0	0.11
Cis-11-Eicosenoic acid C20:1 n-9	0.67

Cis-11,14-Eicosadienoic acid C20:2 n-6	0.64
Cis-8,11,14-Eicosatrienoic acid C20:3 n-6	0.79
Cis-11,14,17-Eicosatrienoic acid C20:3 n-3	0.10
Cis-5,8,11,14-Eicosatetraenoic acid C20:4 n-6 (ARA)	1.63
Eicosatetraenoic acid C20:4 n-3	0.10
Cis-5,8,11,14,17-Eicosapentaenoic acid C20:5 n-3 (EPA)	0.37
Behenic acid C22:0	0.05
Cis-13-Docosenoic acid C22:1 n-9	0.05
Cis-13,16-Docosenoic acid C22:2	0.03
Cis-4,7,10,13,16,19-docosahexaenoic acid C22-6 n-3 (DHA)	2.51
Tricosanoic acid C23:0	0.04
Lignoceric acid C24:0	0.51
Nervonic acid C24:1	0.04
MUFA	42.58
PUFA	19.44
UFA	62.02
SFA	34.60

MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UFA: unsaturated fatty acids; SFA: saturated fatty acids.

In addition, as shown in Table 3, MUFAs (42.58 g/100g) were found as the major fatty acids in Sawai meat followed by SFAs (34.60 g/100g) and PUFAs (19.44 g/100g). Caldironi and Manes (2006) revealed that to reduce the risk of cardiovascular disease, a diet should provide a 1.5:1:1 relation among, MUFA, PUFA and SFA. Generally, the fatty acid composition of fish muscle is influenced by fish species, season, diet and wild or cultured types. However, high unsaturated fatty acids (62.02 g/100g, Table 3) found in lipid extracted from Sawai meat might be implied that Sawai meat is susceptible to lipid oxidation during processing and storage. Sawai fillet exported to European countries is not successful because the yellow discoloration of the fillets due to lipid oxidation has been reported (Khawaja et al., 2014).

3.4. Mineral content in Sawai meat

The content of different minerals in the Sawai meat is shown in Table 4.

Table 4. Mineral contents in Sawai meat

Minerals	Content (mg/kg)
Cu	$4.36 \pm 0.06^{*e}$
Mn	ND
Zn	50.35 ± 0.03^{c}
Ni	ND
Fe	27.41 ± 0.05^{d}
Pb	ND
Ca	439.21 ± 6.55^{b}
Mg	$1{,}662 \pm 11.84^{a}$

*Means ± SD (n=3).

ND: Non-detectable. Different superscripts indicate the significant differences ($p < 0.05$).

From the results, it was found that Mg (1,662 mg/kg) was the dominant mineral in Sawai meats, followed by Ca (439.21 mg/kg). Thanonkaew et al. (2006) reported that Ca is an essential for muscle contraction, hard tissue structure and as a cofactor for enzymatic activity. Zn (50.35 mg/kg) and Fe (27.41 mg/kg) were also found at high amounts (Table 4). Zn is a metal required by a wide variety of metal-dependent enzymes. Iron is recommended for pregnant woman (Bogard et al., 2015). High content of Ca (1023.71-1225.55 mg/kg), followed by Zn (10.79-17.29 mg/kg) and Fe (6.81-9.34 mg/kg) in plaice (*Pleuronectes platessa*) and garfish *(Belone belone)* was also reported (Staszowska et al., 2013). Low levels of Ca (40.1 mg/kg), Zn (3.48 mg/kg) and Fe (12.0 mg/kg) in African catfish (*Clarias gariepinus*) were found. Calcium ranging from 8.6 to 1,900 mg/100 g, zinc from 0.6 to 4.7 mg/100 g and iron from 0.34 to 19 mg/100 g were reported for fish and shellfish (Bogard et al., 2015). Mineral contents in marine and freshwater fish can vary due to the difference in species, sex, feeding and living evironment. Major sources of minerals for fish are water and feed.

From the result, Sawai meat would be recommended as good source of minerals due to high content of calcium, zinc and iron (Table 4). However, on the other hand, Fe and Cu have been known as the major catalysts for lipid oxidation (Chaiyasit et al., 2007; Thanonkaew et al., 2006). Those minerals might contribute to the oxidation of fish meat during processing and storage. High content of Fe found in Sawai meat might be implied that the fish meat is susceptible to lipid oxidation. Additionally, high contents of unsaturated fatty acids in Sawai meat (Table 3) might cause the meat to be more prone to oxidation. No toxic mineral such as lead (Pb) was detectable in Sawai meat.

3.5. Thermal transition of Sawai meat

T_{max} and ΔH of Sawai meat are shown in Table 5.

Table 5. T_{max} and enthalpy of whole meat from Sawai

Sample	T_{max} I (ºC)	ΔH (J/g)	T_{max} II (ºC)	ΔH (J/g)
Sawai meat	54.33± 1.02*	1.17± 0.01	78.00± 1.40	0.32± 0.01

*Means ± SD (n=3).

DSC analysis was used to determine the thermal transition or unfolding temperature of protein and also to quantify the enthalpy of conformational transition (John & Shastri, 1998). Sawai meat revealed two major transition peaks, corresponding to myosin and actin peaks. T_{max} of the first (54.33°C) and second (78.00°C) peaks, with ΔH of 1.17 and 0.32 J/g, respectively, were observed. The result was slightly difference from Techarang and Apichartsrangkoon (2015) who reported two major endothermic transitions, with T_{max} of 56.09 (myosin) and 68.09°C (actin) in *P. hypophthalmus*. Aussanasuwannakul et al. (2012) reported that T_{max} of the first and second peaks of rainbow trout (*Oncorhynchus mykiss*) fillets were 38.89-44.27 and 77.27-78.19°C, respectively. The differences in thermal properties between Sawai meat and other species may be due to the differences in species, season, habitat, temperature, feed, etc. (Aussanasuwannakul et al., 2012; Sriket et al., 2013).

4. Conclusion

Sawai meat can serve as a good source of nutrients, especially protein, lipid and minerals for human consumption. Triglyceride was the main component in Sawai lipid. MUFA and oleic acid were the major fatty acids in Sawai lipid. Sawai meat was also rich in unsaturated fatty acids and essential elements. These data would be benefit for consumers and food industry.

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THE EFFECT OF DIFFERENT FRYING CONDITIONS ON THE COLOR PARAMETERS OF PURPLE SWEET POTATO *(Ipomoea batatas Poiret)* SLICES

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ABSTRACT
The aim of the research was to analyze of the surface color measurement to the pre-frying and post-frying purple sweet potato (Ipomea batatas Poiret) slices by using a combining device i.e. the digital camera and the graphic software. It is believe that such analysis method able to observe colors and an average in a value of L*a*b* units of food ingredient surfaces. The digital images of purple sweet potato slices could be displayed on the computer screen or printed on a certain piece of paper their colors and structures can be analyzed. To do so, the method of digital image application is used, namely a digital camera Canon Ixus 145 without using the camera light and with the distance focus of 10 cm from the object. Following that, the resulted images, were plotted against colors on the Adobe Photoshop CS5. The results show that the use of the Adobe Photoshop CS5 software is extremely helpful in measuring colors of purple sweet potato chips due to the fact that it could bring a consistency when defining colors of pre-frying and post-frying slices with the system model of L*a*b* units. Overall, this study found that there is no difference in colors between pre-frying purple sweet potatoes and the post-frying ones in various treatments by the variance analysis and continued to the Duncan Multiple Range Test (DMRT) at a confidence level of 95 percent ($p<0.05$), except those in a fractional number of treatments.

1. Introduction

A food visual appearance is still a primary consideration for consumers to assess and decide to buy a food product. The food color is relatively one important parameter for consumers to select foods that would be consumed. This, at once, becomes a decision in accepting those food ingredients, whether it is proper to be accepted by consumers. The visual on colors sometimes is more preferred by consumers rather than observation on other quality parameters and it provides a clue regarding chemical changes in food ingredients (Winarno, 2002). The digital image is a process aiming to manipulate and analyze color images in which involves many visual perceptions and supported by a computer. The image processing aims to see the detail of image qualities in order to be interpreted easily by humans or machines (Wijaya et al., 2007).

Overall, there are three measurement models of a color space, those are the RGB (red, green, blue) model (for televisions, computer screens, scanners, and digital cameras), the CYMK (cyan, magenta, yellow, black) model (for printing industries) and the CIE Lab model or also called as the L*a*b* color model (used in laboratories of

color measurement) (Fernandez et al., 2005). The color model most commonly used is the L*a*b* color model since it has a homogenous color distribution (Leon et al., 2005, Zhang et al., 2010). The homogeneity of color perceptions carries an appropriate guarantee of color differences and is essential for the process of segmentation (Dong & Xie, 2005). This color space is able to depict all colors that could be seen by human eyes and is often used as a reference of color spaces (Yam & Papadakis, 2004).

The color measurement of food ingredients in L*a*b units is an international standard of a color measurement developed by the Commission Internationale d'Eclairage (CIE) in 1976. The L*a*b color model comprises 3 components, those are the L* dimension as luminance (color brightness) whose the value ranges from 0 to 100, in which 0 is for black and 100 is for white; the a* dimension describing the color types of green-red, in which a negative indicates a green color and the reverse indicates a red color; and the b* dimension for the color types of blue-yellow, in which a negative indicates a green color and the reverse indicates a yellow color. The a* and b* dimensions are color dimensions opposite to each other ranging from -120 to +120. The L*a*b is an independent device that provides consistent colors regardless of the inputs or outputs. It is specifically devices of digital cameras, scanners, monitors, and printers (Widiasri, 2013; Lukinac et al., 2009; Yam and Papadakis, 2004).

Soaking in the sodium bicarbonate ($NaHCO_3$) solution aims for crisping and would produce the CO_2 gas. As the concentration of $NaHCO_3$ improves, more gas will be generated in food ingredients when the frying process is conducted. This gas builds some pores in the food ingredients. Due to a large number of pores in the ingredients, their mass becomes lower and will be friable to loads or outer forces exposed to them. As more pores are built, the texture of chips produced will be much crispier (Shinta et al., 1995; Putranto et al., 2013). The $NaHCO_3$ is one of cake improvers and a firming agent of fried foods in the form of white powder. As the concentration of $NaHCO_3$ and the frying temperature increase, the hardness value of chips will decline (Winarno, 2002).

2.Materials and methods

2.1. Tools and material

The tools used in this study were knifes, peel removers, stoves, pans, plastic washbasins, food jars, plastic bags, manual chip slicers, digital scales, thermometers, a digital camera Canon Ixus 145 (DIGIC 4, 16.0 MP, 28-224 mm, 8x optical, 16x zoom plus, 1600 max. ISO value, ½.3 type CCD, 2.7-inch LCD), and the Adobe Photoshop CS5 program (Adobe System, 2010).

The main material used in this research was the purple sweet potato obtained from famers in the Sare area, Aceh Besar Regency, Aceh Province, Indonesia. Purple sweet potatoes used in this study were cropped in approximately 4-5 months of age after planting. In addition, supporting materials included in this research were sodium bicarbonate ($NaHCO_3$), salt (NaCl), water, and Bimoli brand cooking oil produced by PT. Salim Ivomas Pratama Tbk.

2.2. Research procedures

The fresh purple sweet potatoes were washed to get rid of any dirt, then they were weighed in 500 grams and sliced in various thicknesses of 1 mm, 2 mm, and 3 mm. After slicing, each of them were weighed before soaking in a solution of 2% salt and 1 L of water within 10 minutes. Following that, the next soaking used a solution of $NaHCO_3$ at concentrations of 1 gram/liter water, 3 grams/liter water, and 5 grams/liter water within 30 minutes respectively in order to enable chips to be crispy. Then, the analysis of pre-frying purple sweet potatoes was conducted.

The purple sweet potato slices were fried on the stove at the temperatures ranging from 145^{o}C to 150^{o}C for 2-3 minutes. The volume of cooking oil was 2 L for 500 grams of sweet

potato slices from various thicknesses and $NaHCO_3$ concentrations. The cooking oil was replaced in each completed frying so as each treatment was conducted with the new cooking oil. A thermometer was inserted directly into the oil in order to keep the temperature range desired. Since the temperature stability of frying purple sweet potato chips would determine colors and crispness of chips produced, the chips from each treatment, after frying, were weighed and putted into jars. Finally, the color measurement of post-frying sweet potato slices was carried out.

2.3. The color measurement

The method of digital image taking was conducted with a Canon Ixus 145 digital camera having a resolution of 16 megapixels without the camera light and with the focus distance of 10 cm from objects in light rooms. The digital image taking was performed in two times of repetitions in each treatment of ingredients. The digital images obtained were saved in the SanDisk Ultra 30 MB/s memory card at a capacity of 8 GB in the PNG (Portable Network Graphics) format. Those images, then, were transferred to a PC to be plotted against colors on Adobe Photoshop CS5, thereby obtaining the RGB (Red, Green, Blue) values at a color intensity ranging from 0 to 225 (Magdić and Dobričević, 2007; Lukinac et al., 2009). Hue angles were obtained using the method described by Precil (1953), that is:

$$\Delta E_{RGB} = \sqrt{\left[(R-R_0)^2 + (G-G_0)^2 + (B-B_0)^2\right]}$$

Where R_0, G_0 and B_0 indicate the color parameters of sweet potato slices. The calculation of R, G, and B values were shown by the 0^o (red), 60^o (yellow), 120^o (green), 180^o (cyan), 240^o (blue), 300^o (magenta) degrees. The average values of each color of purple sweet potato slices were presented as the final result of the color determination plotted along R, G, B axises (Figure 1).

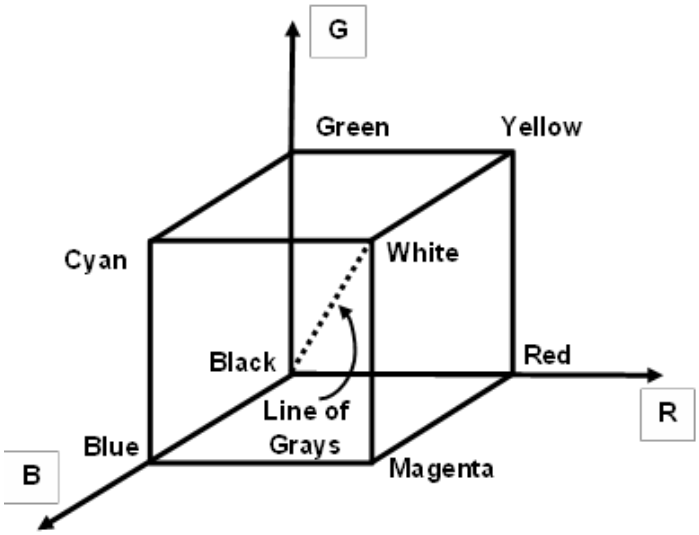

Figure 1. The graphic representation of color spaces along R, G and B axis (White, 2003).

Accordingly, the values of X, Y, Z could be calculated using an equation from the Commission on Illumination (CIE) (White, 2003) as follows:

X = 0,607R+0,174G+0,201B
Y = 0,299R+0,587G+0,114B
Z = 0.066G+1.117B

Subsequently, by using the system of Hunter-Lab, the values of a* and b* that would be plotted into the L*a*b* (Hunter-Lab, 2008) units were gained; therefore digital images of purple sweet potato chips were obtained.

L* = 25(100Y/100)1/3-16

a* = 500[(X/98.071)1/3-(Y/100)]1/3

b* = 200[(Y/100)1/3-

(Z/118.225)]1/3

2.4. The experiment design

This research used the Randomized Completely Design with 3 treatments and 2 repetitions. Factors that were tested included the purple sweet potato thicknesses of 1 mm, 2 mm, and 3 mm as well as the sodium bicarbonate ($NaHCO_3$) concentrations of 1 gram/liter, 3 grams/liter and 5 grams/liter. Hence, there were 9 combinations of the treatment and 2 repetitions so as the total was 18 experimental units. Data obtained, then, was subjected to Analysis of Variance (ANOVA) using a SAS software, version 9.1.3 (SAS Institute Inc., 2006) and was tested further by the Duncan Multiple Range Test (DMRT) at the uncertainty of 5% (Gomez and Gomez, 1984; Sastrosupadi, 2000; Steel and Torie, 1980).

3.Results and discussions

There are several reasons to select the Adobe Photoshop as a color measurement method. Firstly, this software has numerous features of picture editing and an analysis capability comparable to other more expensive softwares. Secondly, this software also provides a more advance capability to manage colors and create consistent colors rather than other graphic softwares. Furthermore, it is available in many computer laboratories and also supported by the manufacturer and users (Yam et al., 2004).

The average values of pre-frying and post-frying color measurements from various treatments and thicknesses of purples sweet potato slices were obtained using the Adobe Photoshop CS5. The L*, a*, and b* values from purple sweet potato slices could be seen in Table 1. The L* average values of purple sweet potato slices gained range from 48.01 (the lowest) to 74.10 (the highest). The former was obtained from a treatment of 3 grams/1 L water $NaHCO_3$ concentration and a slice thickness of 2 mm (K2S2) and the latter was obtained from a treatment of 5 grams/1 L water $NaHCO_3$ concentration and a slice thickness of 3 mm (K3S3). Whereas, the a* average values of purple sweet potato slices gained range from -8.28 (the lowest) to 27.04 (the highest). The former was obtained from a treatment of 3 grams/1 L water $NaHCO_3$ concentration and a slice thickness of 3 mm (K2S3) and the latter was obtained from a treatment of 1 gram/1 L water $NaHCO_3$ concentration (K1S1) and a slice thickness of 1 mm (K1S1). Whilst, the b* average values of purple sweet potato slices gained range from 3.59 (the lowest) to 64.49 (the highest). The former was obtained from a treatment of 3 gram/1 L water $NaHCO_3$ concentration and a slice thickness of 1 mm (K2S1) and the latter was obtained from a treatment of 5 grams/1 L water $NaHCO_3$ concentration and a slice thickness of 1 mm (K3S1).

This color measurement is extremely important in food industries since a large amount of information, nowadays, could be received from measurements at the level of pixels that would enable better characterizations of food color images. The measurement in identifying food colors commonly used is the one with the model of L*a*b* units, due to the homogenous distributions so as the results resemble colors perceived by humans (Leon et al., 2006; Papadakis et al., 2000; Segnini et al., 1999).

In brief, the results show that there is no significant difference among colors created from the treatment of various $NaHCO_3$ concentrations and the different thicknesses of purple sweet potato slices (L*: K1S1, K2S1, K3S1; a*: K1S1, K2S1, K1S2, K2S2, K3S2, K1S3, K2S3, K3S3; b*: K1S1, K2S1, K2S2, K3S2, K1S3, K2S3, K3S3).

Table 1. The L*a*b* unit values of pre-frying and post-frying purple sweet potato slices

Initial		K1S1	K2S1	K3S1	K1S2	K2S2	K3S2	K1S3	K2S3	3S3
L*	Pre	62.73 ± 3.48 a	66.66 ± 5.15 a	63,39 ± 2.48 a	65,50 ± 7.19 a	67,87 ± 2.50 a	70,54 ± 1.30 a	70,33 ± 11.73 a	68,69 ± 7.39	74,10 ± 3.59 a
	Post	62,43 ± 5.03 a	57.25 ± 14.08 a	55,27 ± 1.81 a	51,43 ± 0.00 b	48,01 ± 0.00 b	57,08 ± 8.02 b	60,25 ± 0.79 a	65,55 ± 4.99	60,47 ± 9.72 a
a*	Pre	27,04 ± 16.14 a	10,59 ± 13.37 a	13,45 ± 1.97 a	8,73 ± 6.18 a	3,00 ± 4.16 a	6,76 ± 12.77 a	3,51 ± 6.21 a	4,04 ± 14.58 a	-2,42 ± 0.13 a
	Post	7,41 ± 4.76 a	6,31 ± 10.87 a	6,26 ± 1.44 b	2,84 ± 11.15 a	6,25 ± 2.25 a	3,09 ± 2.97 a	5,97 ± 4.37 a	-8,28 ± 8.45 a	-3,54 ± 7.96 a
b*	Pre	12,44 ± 1.86 a	3,59 ± 1.05 a	8,56 ± 5.55 a	11,64 ± 0.13 a	5,84 ± 8.46 a	13,16 ± 4.68 a	12,50 ± 4.37 a	11,22 ± 1.35 a	10,87 ± 1.08 a
	Post	6,55 ± 1.35 a	6,85 ± 4.51 a	64,49 ± 17.90 b	4,82 ± 0.91 b	14,22 ± 9.59 a	11,45 ± 6.48 a	4,07 ± 9.48 a	12,94 ± 12.55 a	13,41 ± 1.89 a

The notification of same words in each column shows that there is no statistically significant difference (P>0.05)

Descriptions:

K1S1 = the $NaHCO_3$ concentration of 1 gram and the thickness of 1 mm
K2S1 = the $NaHCO_3$ concentration of 3 grams and the thickness of 1 mm K3S1 = the $NaHCO_3$ concentration of 5 grams and the thickness of 1 mm K1S2 = the $NaHCO_3$ concentration of 1 gram and the thickness of 2 mm K2S2 = the $NaHCO_3$ concentration of 3 grams and the thickness of 2 mm K3S2 = the $NaHCO_3$ concentration of 5 grams and the thickness of 2 mm K1S3 = the $NaHCO_3$ concentration of 1 gram and the thickness of 3 mm K2S3 = the $NaHCO_3$ concentration of 3 grams and the thickness of 3 mm K3S3 = the $NaHCO_3$ concentration of 5 grams and the thickness of 3 mm

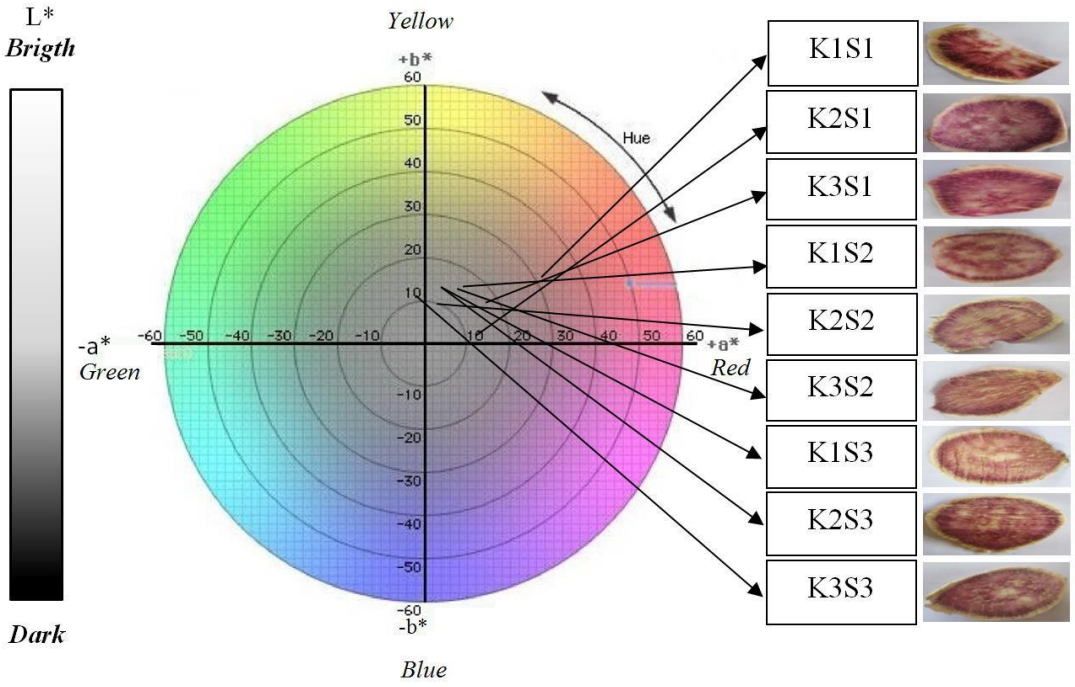

Figure 2. The graph of Pre-frying L*a*b values (Adopted from Konicaminolta, 2003)

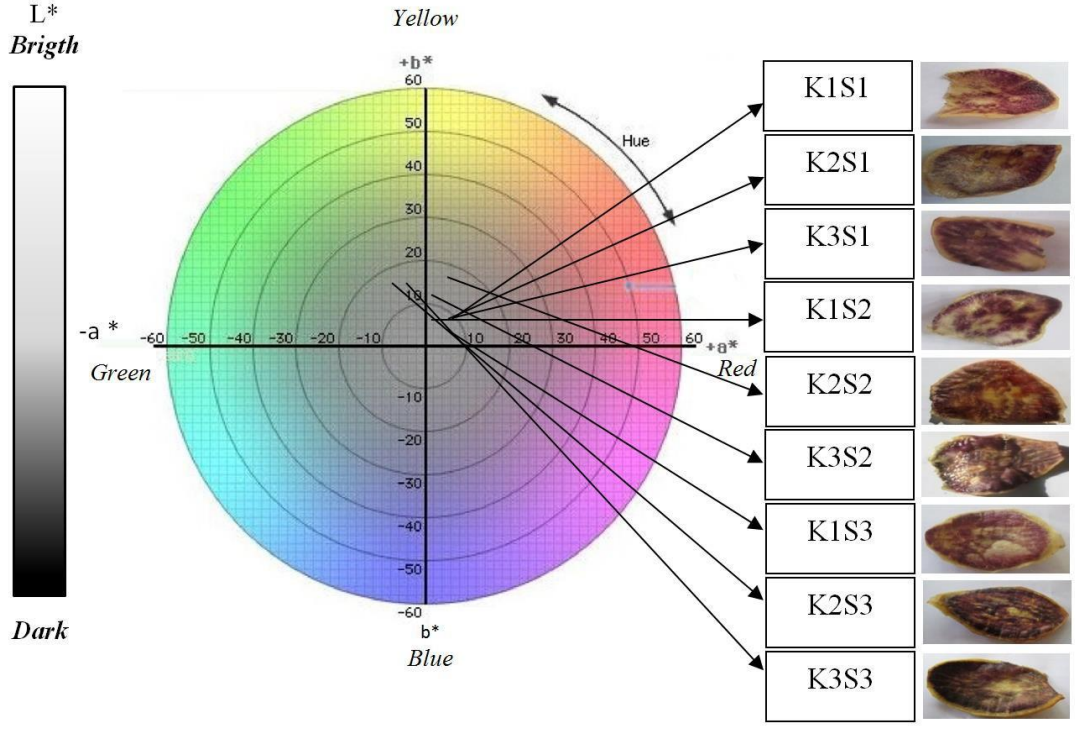

Figure 3. The graph of Post-frying L*a*b values (Adopted from Konicaminolta, 2003)

Hoever, several other treatments show a significant difference between those of pre-frying and post-frying (L*: K1S2, K2S2, K3S2; a*: K3S1; b*: K3S1,

K1S2) (Table 1). Figure 2 and Figure 3 also show the different L*a*b* values gained. Apparently, it is due to the inhomogeneous color of those purple sweet potato slices resulting in the different L*a*b* values of each thickness and $NaHCO_3$ concentration.

4.Conclusions

The use of the Adobe Photoshop CS5 software is very helpful in analyzing colors of purple sweet potatoes as it could provide a consistency in defining colors of pre-frying and post-frying slices with the system model of L*a*b* units. Overall, there is no significant difference among several treatments of pre-frying and post-frying purple sweet potatoes by the Duncan Multiple Range Test (DMRT) at the confidence level of 95 percent ($P<0.05$), except in a fractional number of treatments. Consumer decision on food products, particularly is based on the visual perception and this becomes commonly the only one immediate decision of the food product acceptance. This coverage eventually suggests that an analysis model of this system could be used in analyzing colors of a food product, particularly in observing the color distribution of food ingredient surfaces having the average values within L*a*b* units. Later on, it is necessary to conduct a further research, in particular for the application on different food ingredients and products.

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KINETIC STUDY OF SOLID FERMENTATION OF AMORPHOPHALLUS ONCOPHYLLUS FLOUR USING ASPERGILLUS NIGER AND ITS PREBIOTIC POTENTIAL

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ABSTRACT
Amorphophallus oncophyllus tuber is rich of glucomannan, a promising prebiotic compound. Prebiotic potential of glucomannan improves significantly after hydrolizing. Cellulase and mannanase are commonly applied to hydrolyze the mannan in liquid enzymatic reaction. However, exceptional swelling characteristic of glucomannan limits the hydrolysis of high concentration of mannan in the liquid reaction. The objective of this work was to study the effect of fermentation time and temperature on chemical composition and prebiotic potential of fermented A.oncophyllus flour using Aspergillus niger. Kinetic model of the glucomannan hydrolysis was also determined. In general, the fermentation decreased pH, glucomannan, fat, and calcium oxalate but increased spore number, protein and reducing sugars. This study showed the □-mannanase activity tripled after the fermentation, which in turn improved ability to hydrolyze glucomannan and led to produce more oligosaccharides. Prebiotic potential increased almost five times after fermentation. The highest prebiotic potential of fermented A. oncophyllus was achieved at 5-day fermentation at room temperature. The fermentation reduced absorbance intensity of some functional groups in the wavelength range of 2400-400 cm-1, but not 4000-2400 cm-1. Moreover, the fermentation also effectively reduced 65.7% of calcium oxalate which caused people's objection in consuming the tuber. The first-order sequential kinetic model was in good agreement with experimental data to predict glucomannan hydrolysis at various temperatures (R2> 0.939) thus can be used to control the reaction. The model confirmed production of reducing sugars was maximum at 4-day fermentation at room temperature. The kinetic constants decreased with increasing temperature due to enzyme denaturation.

1. Introduction

Prebiotic is non-digestible food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract and encourage the growth of the good bacteria. All prebiotics are described as short-chain carbohydrates with a degree of polymerization between two and about sixty. Since these oligosaccharides are unhydrolyzed in the upper small intestine hence they reach the large intestine and serve as substrates for beneficial

bacterial metabolisms such as the change in the composition of short chain fatty acids, increase fecal weight, and immune system modulation. These confer benefits upon the host well-being and health (Saad et al., 2013; Valcheva and Dieleman, 2016). After the concept of prebiotic was introduced, many studies have been conducting to identify food components which have prebiotic activities. It is broadly known that fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and inulin have prebiotic activity. In search of other prebiotic ingredients, glucomannan showed a promising prebiotic activity (Tester and Al-Ghazzewi., 2013).

Manno-oligosaccharides (MOS) are oligosaccharides derivative from hydrolyzed glucomannan which are indigestible in the human gut (Tester and Al-Ghazzewi, 2016). The hydrolyzed glucomannan has been reported to demonstrate higher prebiotic activities than unhydrolyzed one (Al-Ghazzewi et al., 2007, Chen et al., 2005). This property of the hydrolyzed glucomannan makes it as a potential prebiotic ingredient with wide possible applications in foods, feeds, and healthcare/pharmaceutical products. The other works commonly produce MOS by applied only β-mannanase or cellulase to hydrolyze the glucomannan in liquid enzymatic phase. However, Mikkelson et al. (2013) highlighted it is not only work of one enzyme which required to hydrolyze the linked of glucomannan but a simultaneous work of enzymes mainly endo-1,4-β-mannanases (mannanase, EC 7.2.1.78) and endo-β-glucanases (EG, endoglucanase, EC 3.2.1.4). Moreover, glucomannan is soluble neutral heteropolysaccharide which shows exceptionally high swelling characteristics when hydrated. This characteristic reduces the possibility to use high glucomannan concentration thus limits the production of hydrolyzed glucomannan through liquid enzymatic reaction. Hence, other methods to hydrolyze glucomannan were in search to overcome the swelling characteristic of glucomannan in liquid enzymatic reaction.

Solid state fermentation was a promising method to produce a high capacity of hydrolyzed glucomannan due to low used in water. This fermentation has been successfully applied to produce pectin oligosaccharides from orange peel by fungal multi-enzymes of Aspergillus japonicus (Li et al., 2016). Aspergillus niger is commonly known as an excellent enzyme producer, including β-mannanase (Huang et al., 2014) dan β-glucanase (Hong et al., 2001). Glucomannan content in Amorphophallus oncophyllus tuber is up to 55 g/g. The glucomannan composes of 1 part of glucose and 2 parts of mannose joined with ß-1,4 glycosidic bond linkages, in which 0.233 g glucose corresponds to 0.35 g mannose (Ohtsuki, 1967). Despite in vivo prebiotic activity of glucomannan A. oncophyllus (Harmayani et al., 2014), the activity of the polysaccharides after hydrolyzing through solid fermentation has not been studied. Hence, the objective of this research was to study the chemical composition and prebiotic potential of fermented A. oncophyllus by using A. niger. Moreover, the kinetic model of glucomannan hydrolysis was also investigated.

2. Materials and methods

2.1.Materials

A. oncophyllus flour (80-100 mesh) contained 0.68 g glucomannan /g was obtained from local market. A. niger FNCC 6018 obtained from Center of Food and Nutrition Studies, University of Gadjah Mada (Yogyakarta, Indonesia). L-Bio® powder contained a mixture of freeze dried probiotic bacteria including Lactobacillus acidophilus, Bifidobacterium longum, and Lactobacillus rhamnosus (Lapi Laboratories). deMan-Rogosa-Sharpe Broth (MRS Broth), deMan-Rogosa-Sharpe Agar (MRS Agar), H2SO4 p.a (Merck, 96%), NaOH p.a (Merck, 99%), KNaTartrat p.a (Merck), phenol p.a (99%) were obtained from Merck. 3,5 dinitrosalicylic acid (DNS), Ringer's solution ¼ strength tablet and soluble starch were purchased from Sigma-Aldrich.

2.2.Fermentation

Sterilized A. oncophyllus flour (270 g) was sprayed with 230 ml A. niger suspension (2.07x106 spore/ml). After mixed homogenously, the mixture was poured to 18 Petri dishes and incubated at three different incubation temperatures e.g. room temperature (28-30^{o}), 37° and 45°C, six petri dishes for each temperature incubation. A petri dish was removed from the incubator everyday and subsequently preheated at 80°C for 15 min to stop the fermentation. Keep the fermentation sample at -20°C until ready to be analyzed. All the samples were prepared in duplicate.

2.3.Determination of the Fermentation Samples

The samples (1 g) were extracted with 9 ml of distilled water and stirred for 1h at room temperature. The suspensions were centrifuged (Hettich EBA 20, UK) at 2000 x g for 10 min. The supernatants were kept for pH determination (Handylab pH 11/14, Schott, UK) and any other analysis.

2.3.1.Spore Number

The supernatant obtained from section 2.3 was vortexed for 10 s and subsequently dropped on the hemocytometer (Improved Neubauer, Weber, UK) for spore counting. The supernatants were diluted necessary to obtain convenience concentration for spore counting.

2.3.2.Determination of β-mannanase Activity and Reducing Sugars

The mannanase activity of fermented A. oncophyllus was determined based on the method of Sugiyama et al. (1973). One ml of the diluted supernatant, 1 ml of dissolved starch (1%) and 1 ml of acetate buffer (0.1 M, pH 5) were placed in a test tube. After incubated at 40°C for 1 h, the mixture was boiled for 5 min to stop the reaction. Total reducing sugars of the mixture were determined by the dinitrosalicylic acid method (Miller, 1959). One unit of β-mannanase activity was expressed as the amount of β-mannanase that produces reducing sugars equivalent to 1 μg of glucose per minute under the assay conditions.

2.3.3.Determination of Glucomannan

The amount of glucomannan was determined followed the method of He et al. (2001) which was measured by the phenol-sulfuric acid method. Two ml of the diluted supernatant, 500 μl of 4% phenol and 2.5 ml of 96% sulfuric acid were placed in a capped test tube. For the blank, the supernatant was replaced with distilled water. The amount of glucomannan was determined by using a spectrophotometer at 490 nm and use glucose as a standard.

2.3.4.Determination of Prebiotic Potential

The prebiotic potential of fermented A. oncophyllus was determined by utilized the fermented flour as a substrate for probiotic bacteria. Viable bacteria colonies were enumerated based on the method of Miles and Misra (Collins, 1984). A mixture of fermented A. oncophyllus flour (4%), MRS Broth media and probiotic bacteria were placed in the test tubes and incubated at 37°C for 1 day. Decimal dilutions of the broth were prepared using sterile Ringer's solution. Five μL were dropped onto 3–4 days old MRS agar plates and then incubated at 37° C for 2–3 days. Viable cell counts were expressed as colony forming units per ml (cfu/ml). Dilutions with less than 10 or more than 150 colonies were discarded.

2.3.5.The Kinetics Modeling

Kinetic model of hydrolysis of glucomannan from A. oncophyllus flour using solid state fermentation was adapted from Zhuang et al. (2009) who proposed the first-order sequential kinetic model for hydrolysis of xylan. In this study, glucomannan was decomposed into oligosaccharides and monosaccharides which were reducing sugars. These reducing sugars thus degraded into decomposition product.

$$(\mathrm{G}) \xrightarrow{k1} (\mathrm{R}) \xrightarrow{k2} (\mathrm{D})$$

where G, R, D represent concentration of glucomannan, reducing sugar, and decomposition product, respectively.

Following the model of Zhuang et al. (2009), the relationship between the yield of reducing sugars (Y_R) and reaction time on glucomannan hydrolysis was therefore:

$$Y_R = \frac{C_R}{C_{G0}} = \frac{k_1}{k_2 - k_1}\left(e^{-k_1 t}\right) + \left(\frac{C_{R0}}{C_{G0}} - \frac{k_1}{k_2 - k_1}\right)\left(e^{-k_2 t}\right) \quad [1]$$

where t, C_R, C_{R0}, and C_{G0} represent fermentation time, a concentration of reducing sugars, an initial reducing sugars and an initial of glucomannan, respectively, while k_1 and k_2 were the kinetic constants. The kinetic constants were determined from the yield of reducing sugars at various reaction times by optimization of their values by fitting the model to the experimental data using solver facilities of Ms-Excel. The model was statistically validated through the R^2 value.

3. Results and discussions

3.1.Spore Enumeration

Figure 1 (top) shows the effect of temperature and fermentation time on A. niger spore production. All the graphs of Figure 1 (top) show a similar pattern of spore production consisted of lag, log, and stationary phase. At a room temperature and 37°C, A. niger showed 2-day lag phase period, followed with another 2-day of the log phase and 1-day of the stationary phase period. Meanwhile, A. niger that incubated at 45°C showed another longer day of the lag phase compared than those of other temperatures. The log phase just took 1 day before started the stationary phase for another day.

In all temperatures, the amount of spore number increased significantly by the end of fermentation. The highest spore production was observed at room temperature incubation which increased from 3.57x 10^6 ml^{-1} to 7.19 x 10^7 ml^{-1} over 5-day fermentation. This trend was in agreement with Shehu and Bello (2011) who reported that five species of Aspergillus including A. niger have an optimum growth temperature between 30°C and 35°C. Ibrahim et al. (2012) specifically reported the growth of A. niger at 28°C and 30°C was similar but the growth reduced at higher temperatures than 30ºC.

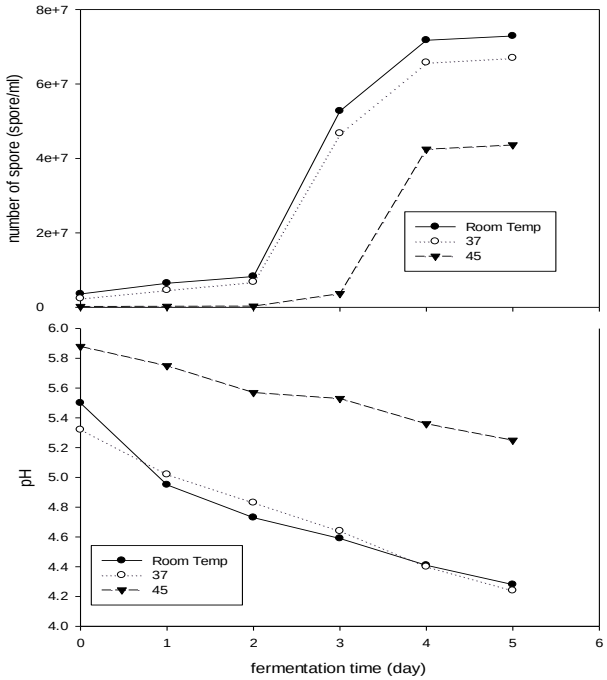

Figure 1. Effect of fermentation temperature and incubation time of solid state fermentation of A. oncophyllus by using A. niger on spore number (top) and pH (bottom)

3.2.pH

Profile of pH development of A. oncophyllus fermentation incubated at different temperatures is presented in Figure 1 (bottom). All the graphs of Figure 1 (bottom) showed a similar trend in which the pH decreased over fermentation period. HPLC graphs (data not shown) demonstrated the area of citric acid increased almost double over 5-day fermentation. This indicated a higher concentration of citric acid was found in longer fermentation incubation samples. Rasoulnia and Mousavi (2016) reported the pH drops from 6 to 3 over 7-day fermentation of power plant residual ash using A. niger in a bubble column bioreactor. Apart of citric acid, A. niger was also reported to have the ability to produce other organic acids such as gluconic acid, oxalic acid and malic acid (Rasoulnia and Mousavi, 2016; Mai et al., 2016). Hence, these kinds of acids might be produced in this fermentation too and contributed to the pH decrease.

The maximum decrease of pH was observed at room temperature in which the pH decreased from 5.50 to 4.28 over 5-day fermentation. Meanwhile, the pH slightly decreased from 5.88 to 5.25 at 45°C. These results were suggested that the decrease of pH was in correlation with the spore production as discussed in section 3.1

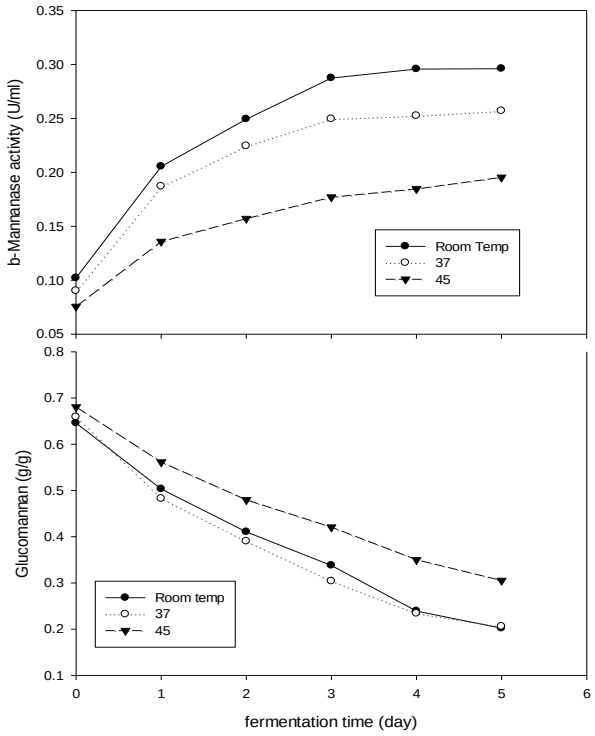

Figure 2. Effect of fermentation temperature and incubation time of solid state fermentation of A. oncophyllus by using A. niger on β-mannanase activity (top) and glucomannan content (bottom).

3.3.β-mannanase Activity

Glucomannan hydrolysis involves two major enzymes: β-mannanases and β-glucanases (Mikkelson et al., 2013). The β-mannanases attack the internal glycosidic bonds of glucomannan, releasing shorter β-1,4-manno-oligosaccharides (van Zyl et al., 2010). A. niger is one of microorganisms that produce a high activity of mananase enzyme on various substrates, such as rice husks and Gmelina arborea (Ibrahim et al, 2012 and Adesina et al., 2013).

Figure 2 (top) shows a development of β-mannanase activity during incubation of A. niger at different temperatures. The enzyme activity was detected since day 0 and steadily

increased over fermentation period. This result was contradictive with Ibrahim et al. (2012) who reported none of β-mannanase activity was observed on day 0 in the fermentation of rice husk by using A. niger. However, our found was supported by Shimahara et al. (1975) who reported that Amorphophallus konjac tuber itself demonstrates to have β-mannanase activity. No wonder that in this research the enzyme activity was observed since the fermentation began. Adesina et al. (2013) found that the β-mananase activity was affected by fermentation period. They showed that the enzyme activity of A. niger was steadily rose from 0 to 12-day of solid fermentation of Gmelina arborea.

The highest enzyme activity was achieved at room temperature when the highest growth of A. niger was observed, while the lowest activity was found at 45^{o}C. It was suggested that high temperature causes denaturation or inactivation of enzymes which result in the decreases of enzyme activity. It was reported that the enzyme activity of A. niger was higher at room temperature (Thomas et al., 2016). A similar result was reported by Adesina et al. (2013) and Ibrahim et al. (2012) who found the highest mannanase activity occurred at 30^{o}C. Moreover, Ibrahim et al. (2012) reported enzyme activity of A. niger at 28^{o} and 30^{o}C was similar while no enzyme activity was observed at 45^{o}C.

3.4.Glucomannan Content

Effect of temperature and time of A. onchophyllus fermentation on glucomannan content is presented in Figure 2 (bottom). At all temperatures, glucomannan content reduced gradually over fermentation time. Glucomannan was the main carbon source in A. oncophyllus flour which was used for energy source during A. niger metabolism and activities, hence the content decreased in line with the period of incubation. This decrease was a negatively correlation with the β-mannanase activity which reported in section 3.3. The higher the mannanase activity, the more the decrease of glucomannan concentration was observed. Increasing fermentation temperature resulted in higher glucomannan concentration still remained in the substrate due to denaturation of mannanase enzyme in higher temperature. About one-third of the unhydrolyzed glucomannan was still detected in the end of fermentations of room temperature and 37^{o}C, while higher glucomannan content was found at 45^{o}C.

3.5.Total Reducing Sugars

Hydrolysis of glucomannan releases oligosaccharides and monosaccharaides which are reducing sugars. Figure 3 (top) shows reducing sugars gradually increased over 5-day for all temperatures. At the beginning of fermentation, the flour contained 0.24 g reducing sugar/g dried samples. The fastest rate of hydrolysis was found in the early of an enzymatic reaction. A similar result was reported by Mikkelson et al. (2013).

At the end of fermentation time, reducing sugars of room temperature fermentation was almost double than the native one. Meanwhile, fermentation at 45^{o}C showed the lowest rise of reducing sugars from 0.22 to 0.30 g/g dried samples. This result suggested that the increase of reducing sugars was in line with the development of β-mannanase activity as discussed in section 3.3. The activity of the enzyme tended to decrease in higher temperature due to denaturation of the protein. As a consequent, the enzyme could not work optimum to hydrolyze glucomannan at a higher temperature and resulted in releasing a lower concentration of reducing sugars. A similar result was reported by Chen et al. (2013) and Mikkelson et al. (2013). Chen et al. (2013) found reducing sugar increases during 7 h of hydrolyzing glucomannan with β-mannanase. Mikkelson et al. (2013) observed a significant increase of reducing sugars over 48 h of hydrolysis glucomannan by using Trichorderma reesei mannanase to produce glucomannooligosaccharides.

3.6.Prebiotic Potential

In this study, the enzymatic reaction through fermentation was conducted to degrade

glucomannan of A. oncophyllus and produced reducing sugars including MOS which was reported to show higher prebiotic potential. The prebiotic ingredients stimulate the growth and activity of one or a number of probiotic bacteria in the colon. The probiotic bacteria consume the prebiotic ingredients for their metabolism and releases short-chain fatty acids such as acetic acid, butyric acid, propionic acid, and lactic acid (Zhao and Geng, 2016). In this study, the prebiotic potential of the fermented A. oncophyllus flour was determined by using the fermented flour as a substrate to support the growth of a group of mixture probiotic bacteria consist of Lactobacillus acidophilus, L. casei, L. salivarius, Bifidobacterium infatis, B. labtis, B. longum dan Lactococcus lactis.

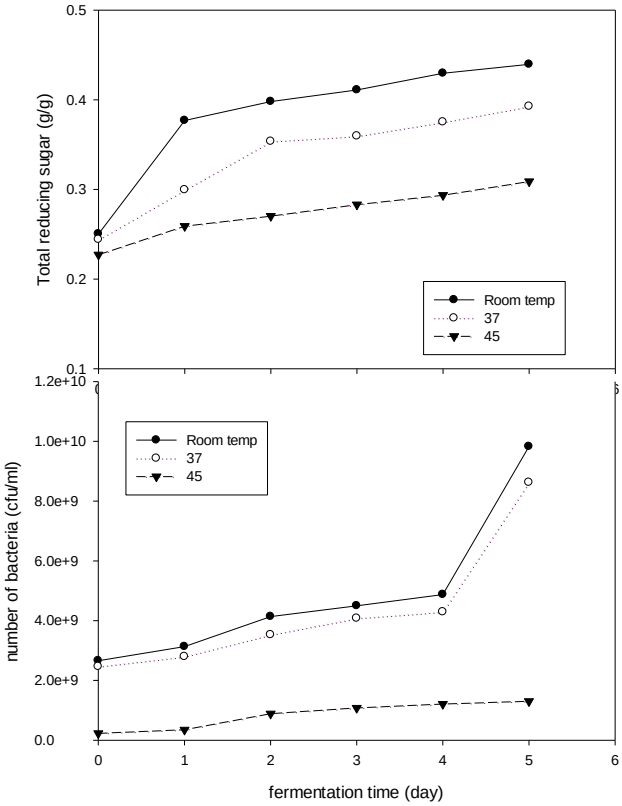

Figure 3. Effect of fermentation temperature and incubation time of solid state fermentation of A. oncophyllus by using A. niger on total reducing sugar (top) and prebiotic potential (bottom).

Figure 3 (bottom) shows the influence of temperature and time incubation on a prebiotic potential of fermented A. oncophyllus. At the beginning of the reaction, the tuber was already able to support the growth of probiotic bacteria. This suggested that even the native tuber contain compounds with prebiotic activity and could be used as a medium for the growth of probiotic bacteria. In this study, the A. oncophyllus flour contained glucomannan up to 0.68 g/g as a major compound. This result was in accordance with Harmayani et al. (2014) who reported in vivo study of prebiotic potential of glucomannan of A. oncophyllus. Their in vivo study showed that the major compound of A. oncophyllus was able to support the growth of probiotic bacteria and reduce the amount of E. coli bacteria in the digestive tract of mice.

Our study showed that hydrolysis of glucomannan of the tuber through solid fermentation permitted an improvement of the prebiotic potential of the tuber. More colony

number of probiotic bacteria was found at longer fermentation period. The prebiotic potential was also affected by temperature. A less growth of the probiotic bacteria was demonstrated on the samples incubated at 45°C. This could be due to less content of oligosaccharides during fermentation at that temperature than that of other temperatures. This result was in line with the mannanase activity of Figure 2 (top) which shows that the highest enzyme activity was found at room temperature but then decreased with increasing fermentation temperature. Less enzyme activity resulted in lower oligosaccharide production which in turn performed less support on the probiotic bacteria growth. Some authors reported the improvement of prebiotic activities of hydrolyzed glucomannan through enzymatic reaction by using β-mannanase or cellulase. Al Ghazzewi et al. (2007) reported that the hydrolyzed konjac glucomannan stimulate the growth of lactobacilli and bifidobacteria as well as inhibit pathogens when it uses as sole carbon source. Chen et al. (2005) reported that hydrolyzed glucomannan exerts a greater prebiotic effect than does unhydrolyzed glucomannan in Balb/c mice. Yeh et al. (2010) highlighted that the prebiotic effect of partially-hydrolysed konjac glucomannan is responsible for a greater protection of fecal water-induced DNA damage than that of unhydrolyzed konjac glucomannan.

3.7.Kinetic Model

Fitting the yield of reducing sugars at various fermentation time base on equation [1] is presented in Figure 4. Predicted yield data demonstrated a parabolic pattern, particularly at room temperature reaction. It was predicted that the yield of room temperature incubation began to decrease at 5-day when the yield of other temperatures was still increasing. Figure 4 shows good agreements between experimental data and predicted yield for all temperature reactions, as presented with R^2 values of the fitting which were close to unity (Table 1).

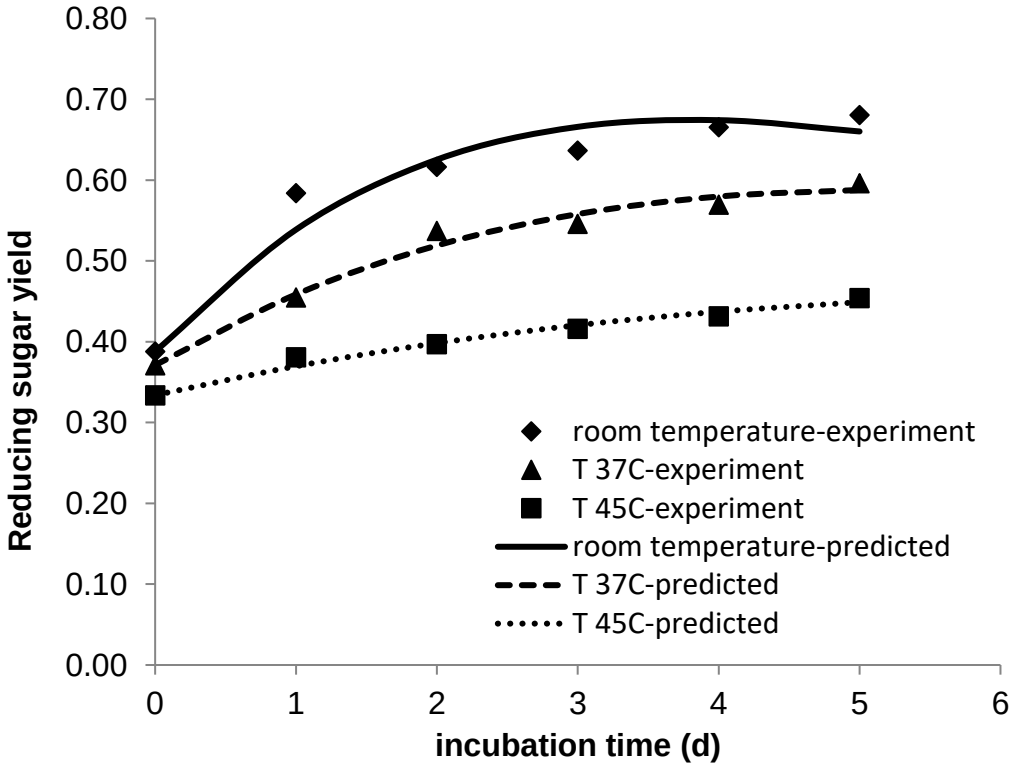

Figure 4. Plotting experimental data and predicted data of yield from glucomannan hydrolysis at various temperatures.

The kinetic constants of k_1 and k_2 of equation [1] were determined by nonlinear regression (Table 1). Both kinetic constants decreased with increasing temperature. This result was in line with the enzyme activity which decreased in higher temperature. This trend was opposed to Zhuang et al. (2009) who conducted hydrolysis of xylan with hot liquid water without involving any enzymes. Hence, it was suggested that this reverse effect of temperature on the kinetic rate was due to enzyme denaturation (section 3.4). At room temperature, the k_1 value was almost double over k_2 value. This suggested that at this temperature production of reducing sugars was faster than the decomposition ones. It implied that room temperature was a suitable condition for producing reducing sugars including the oligosaccharides and monosaccharides. At 45°C, however, k_2 was slightly higher than k_1 which indicated decomposition of reducing sugars was faster at the higher temperature. Arrhenius equation was applied to represent the relation between kinetic constants and temperature (Figure 5). This plotting resulted in higher value of pre-exponential factor (A) and activation energy (E) of k_1 than those of k_2 (Table 1).

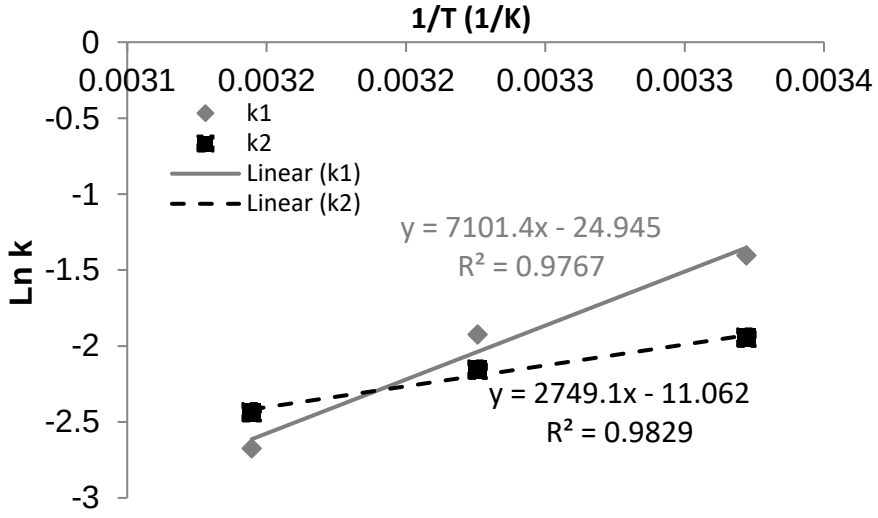

Figure 5. Arrhenius plot of ln k and 1/T from glucomannan hydrolysis at various temperatures.

Table 1. Values of k1, k2 and R2 of the kinetic model (equation [1]) and values of pre-exponential factors and activation energies of Arrhenius equation.

Temp (°C)	**k_1 (d^{-1})**	**k_2 (d^{-1})**	**R^2**
room temperature	0.2457	0.1428	0.9394
37	0.1458	0.1156	0.9814
45	0.0689	0.0873	0.9773
E (kJ/mol)	59.03	6.78×10^{10}	
A (d^{-1})	22.86	6.36×10^{4}	

3.8.FTIR Determination

FTIR analysis of the sample of 5-day fermentation at room temperature was compared to that of A. oncophyllus flour as the control. The FTIR spectra of the samples in the wavelength range of 4000-400 cm^{-1} are shown in Figure 6. In general, the spectra of both samples demonstrated some peaks at similar wavelength range but the difference in intensity of the absorbance was observed. Basically, the peak spectra were in agreement with data of Chua et al. (2012).

The samples had similar absorbance intensity at a wide band of 3700-3000 cm^{-1} which attributed to O-H stretching vibration of the glucomannan and ~2900 cm^{-1} which assigned to alkyl (C-H) groups. Fermentation by using A. niger did not affect on these peaks. Both samples demonstrated comparable peaks in the range of 2400-400 cm^{-1}, however, the absorbance intensities of the fermented sample were lower than those of the control. This indicated that the fermentation affected and cleaved functional groups in this wavelength.

Some peaks that observed in 2000-1000 cm^{-1} were carbonyl of acetyl groups (~1720 cm^{-1}), absorbed water (~1650 cm^{-1}), angular deformation of C-H (~1410 and 1370 cm^{-1}), C-O ether bond (1150 cm^{-1}) and C-O alcohol bond (~1070 and ~1020 cm^{-1}). At 1000-400 cm^{-1} the characteristic peaks were assigned to β-glucosidic and β-mannosidic linkages which observed at 900-800 cm^{-1} (Chua et al., 2012).

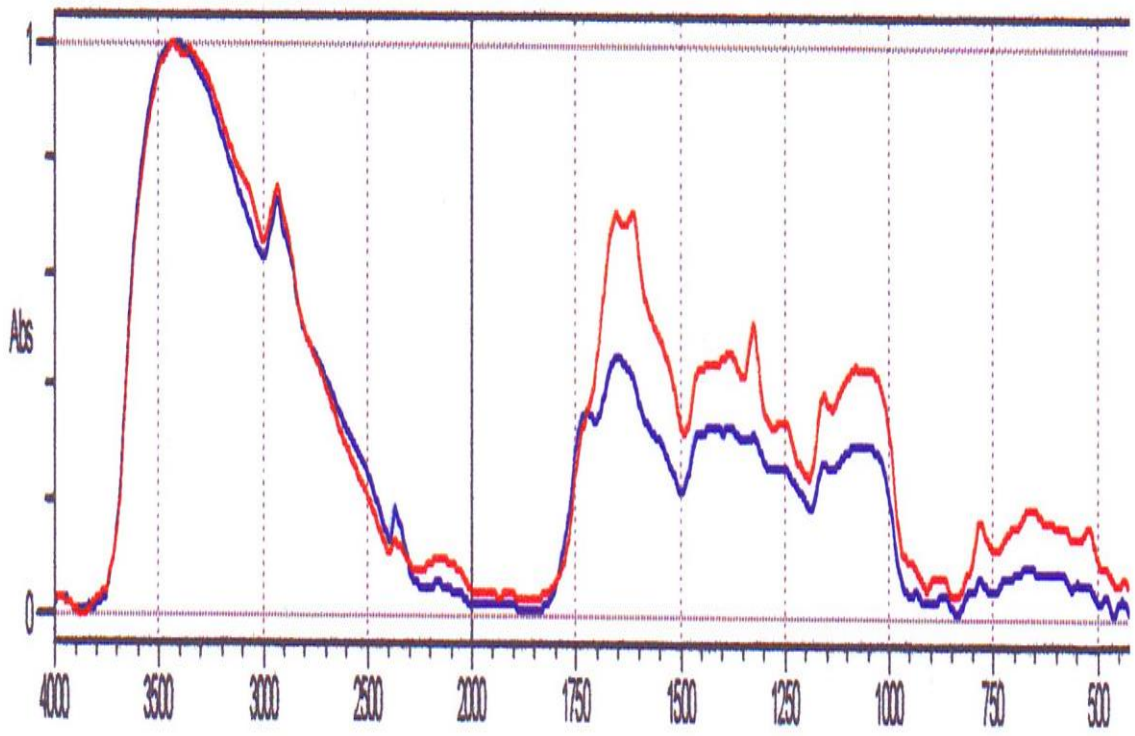

Figure 6. FTIR spectra of 0-day (red line) and 5-day (blue line) of fermented A. oncophyllus by using A. niger at room temperature.

3.9.Chemical Composition

The chemical composition of the sample with the highest prebiotic potential was compared to that of the control (Table 2). The result demonstrated that the fermentation reduced fat content and calcium oxalate, but increased the protein content. Aspergillus niger is generally known as excellent enzymes producer including amylase, cellulase, and β-mananase which work synergistically with other enzymes to degrade polysaccharides including glucomannan and subsequently release reducing sugars (van-Zyl et al., 2010). The concentration of glucose and mannose which are reducing sugars increased significantly after 5-day fermentation, in which glucose and mannose improved to 0.76% and 8.20% respectively. The increase of glucose and mannose could be attributed to τηε β-mannanase activity which improved over fermentation as discussed in section 3.3. This enzyme worked synergistically with other enzymes to hydrolyze glucomannan which led to release more reducing sugars including glucose and mannose. This result was similar with Mikkelson et al. (2013) who found monosaccharides of glucomannan increased after hydrolysis of glucomannan by mannanase.

It was found that fat content reduced 82.6% after the fermentation. This significant decreased in fat content could be due to increase activities of the lipolytic enzymes during fermentation which hydrolyses fat components into fatty acid and glycerol. El-Batal et al. (2015) found incubation time affected lipase activity of A. niger significantly. This trend was also reported by Adebowale and Maliki (2011) who studied fermentation of Pigeon Pea (Cajanus cajan).

In contrast with the other proximate values, the protein content increased 17.6% after 5-day fermentation. The increase in protein values could be attributed to an increase in spore number during the growth of A. niger as reported in Figure 1 (top). This fungal increase resulted in more production of amino acids and other simple peptides (Iqbabul et al., 2014) which in turn improved the protein content. Adebowale and Maliki (2011) found the protein of Pigeon pea (Cajanus cajan) increase 2% after 5-day fermentation, while Iqbabul, Hiikyaa and Amove (2014) observed 5% increase of Mahogany Bean (Afzelia Africana) protein over 3-day fermentation.

Table 2. The chemical composition of 0-day and 5-day of fermented A. oncophyllus by using A. niger at room temperature.

Components	Value (%)	
	0-day	**5-day**
Protein	3.46	4.09
Fat	0.23	0.04
Calcium oxalate	0.35	0.12
Total sugar	87.59	64.15
Glucomannan	62.57	20.19
Total reducing sugar	25.02	43.96
Glucose	<7.34 ppm	0.76
Mannose	<3.94 ppm	8.2

Table 2 also reveals that fermentation effectively reduced more than a half of calcium oxalate content of the native A. oncophyllus tuber. Calcium oxalate has been reported to associate with itchy and irritating effect on the Amorphophallus tubers (Chairiyah et al., 2013) which is a subject of people objection in consuming the tuber. Fermentation successfully decreased this irritating effect 65.7%. This result was in line with Oke and Bolarinwa (2012) who found that calcium oxalate of cocoyam decreased 65% after 2-day fermentation.

4. Conclusions

In general, fermentation of A. oncophyllus flour by using A. niger resulted in the decrease of pH, glucomannan content, fat, and calcium oxalate. In opposite, protein and reducing sugar increased after fermentation. This study showed that the fermentation increased the β-mannanase activity almost triples and led to produce the more oligosaccharides. As a result, the prebiotic potential of the fermented tuber improved significantly. The highest prebiotic potential of the fermented flour was obtained after 5-day fermentation at room temperature. Moreover, the fermentation also effectively reduced 65.7% of calcium oxalate which caused people's objection in consuming the tuber. The first-order sequential kinetic model was in good agreement to predict glucomannan hydrolysis at various temperatures (R^2> 0.939) thus can be used to control the reaction. The model confirmed production of reducing sugars was maximum at room temperature for 4-day fermentation. Thus it is suggested that this fermentation condition was the most suitable condition for producing the highest reducing sugars including the oligosaccharides which supported the prebiotic activity of A. oncophyllus. Moreover, the kinetic constants decreased with increasing temperature due to enzyme denaturation.

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COMPARISON OF THE COMPONENTS OF THE VOLATILE OILS OF THE LEAVES OF TWO VARIETIES OF *OLEA EUROPAEA* L. BY CHANGING THE TYPE OF THE SEPARATION METHOD

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ABSTRACT

Genetic diversity of plant varieties alters the nature of secondary metabolites. In addition, extraction conditions highly affect the quality and quantity of extracted compounds, including secondary metabolites. The purpose of this study was investigating the volatile compounds of the leaves of two varieties of olive, Mission and Conservolea, by varying the extraction method. The Clevenger procedure and the cold percolation method were used. The extracted volatile oils were separated and identified using GC/FID and GC/MS. The number and type of extracted compounds are strongly dependent on the extraction method. The Conservolea variety has more terpene compounds than the Mission variety and this was established in both extraction methods. The results showed that the volatile compounds extracted by the Clevenger extraction method have lower average boiling point and lower average molecular weight in comparison with the volatile compounds extracted by the percolation method.

1. Introduction

Aromatic plants have been known to humanity for thousands of years and the use of them in food and perfume industries have a long history.

Since plant extracts usually are a combination of various types of bioactive compounds, their separation and, identification and characterization of those bioactive compounds still remain a big challenge in the way of the required processes.

In the traditional methods of extracting natural compounds from plants such as water or steam distillation and extraction with organic solvents such as soaking, there are some disadvantages such as loss of volatile components, degradation of compounds and remaining toxic solvents.

Biodiversity is a precious heritage, something that makes sure progress will occur for future generations, and a foundation of tenable agriculture. The extreme reduction in biodiversity has caused a minor genetic variability leading to a resultant reduced capability of plant populations to be able to adapt to new diseases and the ever-changing environmental conditions. Identification of sources of variability in natural populations, cultivars, and varieties must be taken into account for biological characterization, which will allow for an accurate definition of conservation strategies (Campeol et al. 2003; Korir et al. 2012).

Olea europaea L., (Oleaceae) has been widely used in traditional medicine in many

countries. Popular medicine and phytotherapy use olive leaves to treat and prevent hypertension because of their hypoglycemic, antiseptic and diuretic properties (Zarzuelo et al. 1991; Khayyal et al. 2002; Silva et al. 2006).

The leaves also contain important secondary metabolites, such as the secoiridoid derivatives oleacein and oleuropein, the main responsible for their hypotensive activity and the latter also for the hypoglycemic properties (Gucci et al. 1997; Kiritsakis, 1998; McDonald et al. 2001).

There are few reports in the scientific literature about the chemical compounds of leaves of olive.

The Chemical composition of the essential oil of the leaves of three varieties of olive (Cipressino, Frantoio and Leccino) that were obtained from two different harvest times were studied in Italy. The results showed that high amounts of aliphatic aldehydes were present in all the three varieties in both harvest periods, and the amounts of (E)-2-hexenal (an aldehyde with high antimicrobial properties) were highest from July to November (Campeol et al. 2003).

In another report from Italy in the same year, the volatile compounds produced by the leaves and fruit of one variety of olive, namely Olivastra Seggianese, were analysed in two different stages of growth. The results showed that their primary constituents were often aldehydes, especially (E)-2-hexenal. Nevertheless, many terpenoids were also detected in these volatile oils and the major ones were alpha-farnesene (E, E), linalool, beta-caryophyllene, and valencene (Campeol et al. 2001).

In a different study from Tunisia performed in 2012, antibacterial and antifungal properties were detected in volatile oils obtained by the hydrodistillation method from fresh and dried leaves of three varieties of olive (Chemlali, Chemchali and Neb jemel). The main constituents of these essential oils were (E)-3-hexenol, 3-ethenylpyridine, (E)-betadamascenone and phenyl ethyl alcohol, and their percentages were different according to the variety of olive and the method of extraction (Brahmi et al. 2012). In this article, the antioxidant properties of the volatile components were studied and it was concluded that the antioxidant properties were higher in fresh leaves compared to the dried ones. The results of another study on the leaves of Chemlali variety showed that the primary constituents of its essential oil were alpha-pinene, 2,6-dimethyloctane, and 2-methoxy-3-isopropyl piperazine; also medicinal effects of the essential oil of this plant were determined in relieving inflammation (Haloui et al. 2010).

Even though olive oil is consumed all over the world and has an especial place in traditional medicine, browsing in the scientific literature shows that there has not been many studies on the components of the volatile oil of the leaves of this plant. Therefore, due to the importance of the effects of genetic diversity of plant varieties on the nature of the metabolites, and the effects of extraction conditions on quality and quantity of the extracted metabolites, for the first time, this study investigates the influence of extraction method on quantity and quality of the compounds extracted from two varieties of olive.

2. Materials and methods

2.1. Materials

Leaves of two varieties of *Olea europaea* were collected from Qom, one of the central provinces of Iran, from the olive garden of Fadak, during the flowering period in April 2013. The plant materials were authenticated by the Department of Botany of Islamic Azad University, Qom Branch.

Samples were dried and subsequently ground in a blender to obtain fine powder. All reagents and chemicals used in this study were from Merck or Sigma Companies.

2.2. Extraction by percolation method

Ten grams of dried powder were added to 300 ml of solvent in a conical flask at room temperature and the mixture was kept for 48 hours with intermittent shaking. After that, the extract was filtered through Whatman No. 1 filter paper; then the solvent was removed using

a rotary evaporator and subsequently dried, until a constant weight of each extract was obtained. The residues were stored at 4°C for further use (Swami et al. 2008).

2.3. Extraction by hydrodistillation method

The samples were homogenized and turned into fine powder. In order to extract the essential oils, 100 g of the powder were placed in a 1-liter conical flask and then it was connected to the Clevenger apparatus. 500 mL distilled water were added to the flask and were heated to the boiling point. The steam in combination with the essential oils were distilled into a graduated cylinder for 5 hours and then separated from the aqueous layer. The oil was kept in refrigerator until further analysis (Gill et al. 2014).

2.4. Gas Chromatography Analysis

Analytical gas chromatography of the volatile oils was carried out using a Hewlett-Packard 5975B series gas chromatograph with Agilent HP-5 capillary column (30 m×0.25 mm, f.t 0.25 µm); carrier gas, He; split ratio, 1:10, and using a flame ionization detector. The operating conditions were as follows: oven temperature program from rising from 50°C (10 min) to 240°C at 4°C/min heating rate and staying at the final temperature for 15 min. GC/MS was performed on a HP 5975B with a Hewlett–Packard 5973 quadrupole detector, on capillary column HP-**5** (30 m×0.25 mm; f.t 0.25 µm).

The MS operated at 70 eV ionization energy. Retention indices were calculated using retention time of n-alkanes that were injected after the volatile oil at the same chromatographic conditions. Components' relative concentrations were obtained directly from GC peak areas obtained with GC-FID. The components of the oils were identified by comparing their mass spectra and kovats indexes with Wiley library and published books, databases available and credible websites (Adams 1995).

3. Results and discussions

As it is evident in table 1,

Palmitic acid (13.0%), Farnesol (11.8%) and Phenylpropanoic acid (5.3%), respectively are the most prevalent components of the volatile compounds of the Mission variety of olive. In addition, Palmitic acid (14.7%), Phytol (10.2%) and Neryl acetone (5.4%) were the components with the most percentages among the volatile compounds of the Conservolea variety.

Table 1. Comparison of chemical composition of the volatile oils of the leaves of two varieties of olive extracted by hydrodistillation method

NO	Components	Essential oil of Mission	Essential oil of Conservolea	KI
1	Toluene	1.0	2.7	≤800
2	Octane	2.1	4.6	≤800
3	(Z)-Hex-3-en-1-ol	0.3	-	855
4	o-Xylene	-	2.8	869
5	3,4-Cyclobutenopyridine	0.8	0.6	972
6	Decane	1.0	1.6	999
7	Styrene oxide	0.5	-	1050
8	1-Octanol	0.5	-	1074
9	Nonanal	0.8	0.8	1107
10	Dodecane	1.0	0.9	1200
11	Decanal	0.4	0.5	1209
12	Geraniol	1.2	-	1262
13	(E)-2-Decenal	0.6	-	1267

14	Nonanoic acid	2.2	-	1299
15	Theaspirane A	0.5	-	1303
16	2,4-Decadienal	-	0.5	1323
17	α-Copaene	0.6	1.6	1381
18	Ethanone, 1-(4-methylphenyl)-	0.3		1386
19	β-Damascenone	3.9	2.9	1392
20	Tetradecane	-	0. 9	1400
21	Phenylpropanoic acid	5.3	-	1425
22	Dihydro- β -ionol	0.6	-	1454
23	Neryl acetone	2.8	5.4	1459
24	Cetyl alcohol		1.1	1481
25	Butylated hydroxytoluene	0.9		1481
26	trans-β-Ionone	4.4	3.0	1494
27	α-Muurolene	0.4	1.1	1506
28	2-ethyl-2-hexenal	-	0.7	1512
29	2,4-Di-tert-butylphenol	-	0.8	1525
30	2,5-Di-tert-butylphenol	0.6	-	1526
31	Farnesol	11.8	1.1	1574
32	Benzoic acid	3.7	-	1580
33	Lauric acid	-	1.5	1583
34	β -caryophyllene oxide	-	3.2	1592
35	Hexadecane	1.0	1.0	1601
36	2-Octyl octanoate	0.8	1.3	1608
37	(+)-Aromadendrene	0.5		1622
38	Copaene	0.8	1.6	1657
39	Octacosane	-	0.7	1700
40	Myristyl aldehyde	0.3	-	1718
41	trans-Farnesol	0.8	-	1731
42	Myristic acid	2.7	4.2	1786
43	Octadecane	0.2	0.8	1800
44	Hexahydrofarnesyl acetone	2.6	5.2	1850
45	Phthalic acid	0.7	2.0	1877
46	Nonadecane	-	0.5	1900
47	Farnesyl acetone	1.4	2.1	1925
48	Palmitic acid, methyl ester	0.4	0.9	1931
49	Isophytol	-	0.7	1950
50	Dibutyl phthalate	0.9	1.4	1972
51	Palmitic acid	13.0	14.7	1997
52	Hexatriacontane	-	0.6	2097
53	Phytol	5.2	10.2	2120
54	Linolenic acid	3.0	1.1	2161
55	Neophytadiene		0.7	2390
	Total	82.5	87.1	

	Compounds category (%)			
	Non terpenoid oxygenated	43.9	35.1	
	Non terpenoid hydrocarbons	6.3	16.2	
	Mono terpenoid oxygenated	9.0	8.4	
	Di terpenoid oxygenated	5.2	10.9	
	Di terpenoid hydrocarbons	-	0.7	
	Sesquiterpenoid oxygenated	15.6	8.4	
	Sesquiterpenoid hydrocarbons	2.3	7.5	

Regarding the volatile compounds of the pentane extract of the Mission variety, Linolenic acid (26.9%), Palmitic acid (19.8%) and Butyl acetate (19.1%) were the most important components, whereas in the pentane extract of the Conservolea variety, Butyl acetate (20.8%) and Phytol (16.5%) and Palmitic acid (13.2%) were the most important components.

Table 2. Comparison of chemical composition of the volatile oils of the leaves of two varieties of olive extracted by percolation method

NO	Components	Extract of Mission	Extract of Conservolea	KI
1	Methyl Isobutyl Ketone	1.7	4.8	≤800
2	Toluene	3.2	5.5	≤800
3	Octane	1.1	2.4	≤800
4	Butyl acetate	19.1	20.8	810
5	o-Xylene	4.2	5.3	869
6	Ethylbenzene	-	2.8	899
7	α-Copaene	1.0	3.0	1381
8	trans-β-Ionone	1.3	-	1494
9	Dihydroactinidiolide	2.2	3.2	1542
10	Myristic acid	1.9	5.2	1786
11	Butyl 2-ethylhexyl phthalate	1.0	-	1972
12	Palmitic acid	19.8	13.2	1997
13	Phytol	2.3	16.5	2120
14	Linolenic acid	26.9	-	2161
15	Stearic acid	4.7	-	2181
16	Heptacosane, 1-chloro-	1.4	-	2297
17	Squalene	-	6.4	2815
	Total	91.8	89.1	
	Compounds category (%)			
	Non terpenoid oxygenated	75.1	44.0	
	Non terpenoid hydrocarbons	9.9	16.0	
	Mono terpenoid oxygenated	3.5	3.2	
	Di terpenoid oxygenated	2.3	16.5	
	Sesquiterpenoid hydrocarbons	1.0	3.0	
	triterpene hydrocarbons	-	6.4	

As it is demonstrated in table 2 twenty-six substances among the components of the essential oils of Mission and Conservolea varieties were common, and they account for 63.7% of the Mission and 73.3% of the Conservolea. This similarity in the composition of the pentane extracts is comprised of 10 compounds, which account for 56.5% of the Mission's extract and 79.9% of the Conservolea variety's extract. As it is evident, this high rate of similarity in the extracted compounds can be due to the genetic closeness of these two varieties. These two plants, apart from being from the same genus and species and from a botanical perspective are so much alike that are considered varieties of the same plant, they were also cultivated in the same environment with the same weather conditions. According to the results of this study it can be concluded that even though the olive plant varieties share certain similarities in extracted volatile components, differences between cultivars are to the degree that they can be distinguished from one another.

After using the Clevenger method, 43 and 39 volatile compounds were obtained from the Mission and Conservolea varieties respectively; whereas these numbers for these two varieties were 15 and 12 respectively in the pentane extract obtained by the Percolation method. This big difference in the extracted compounds, demonstrates the higher capabilities of the Clevenger method in comparison with the Percolation method in quantitative extraction of volatile compounds. According to the results of this study and similar works (Aghajani and Engashte-Vahed 2016), extraction conditions highly affect the quality and quantity of extracted compounds, including secondary metabolites.

Table 3. Comparison of the compounds extracted from the volatile oils of the leaves of two varieties of olive extracted by hydrodistillation and Percolation methods using three measured parameters

Parameter measured	Essential oil of Mission	Essential oil of Conservolea	Extract of Mission	Extract of Conservolea
Average molecular weight	520.16	545.52	1500.45	1373.9
Average Solubility in water (In 25 ^{o}C , ppm)	2788.27	373.77	576.04	1245.69
Average boiling point (In 760 mm Hg)	406.39	458.49	1161.92	1302.12

As it is demonstrated in table 3, average molecular weights of the extracted volatile compounds by the Clevenger method for the two varieties of Mission and Conservolea are 520.16 u and 545.52 u. These numbers for the Percolation method using the pentane solvent are 1500.45 u and 1373.9 u. In addition, the average boiling points of the extracted volatile compounds by the Clevenger method for Mission and Conservolea varieties are 406.39 °C and 458.49 °C. These numbers for the Percolation method and using the pentane solvent are 1161.92 °C and 1302.12 °C respectively. These results prove this fact that the Clevenger method extracts volatile compounds with lower boiling point and lower molecular weight compared to the Percolation method.

According to tables 1 & 2, the Clevenger method can extract more volatile terpene compounds from both varieties compared to the Percolation method, as 32.2 and 35.8% of all the extracted compounds from Mission and Conservolea varieties by the Clevenger method are comprised of terpene compounds. However, these numbers for the extracted volatile compounds using the Percolation method are 6.8% for Mission and 29.1% for Conservolea varieties. Another interesting point here that is evident in tables 1 & 2 is that the Conservolea variety contains more terpene compounds compared to the Mission variety; this matter is true in both of the extraction methods.

4. Conclusions

Due to the fact that olive oil is eaten all over the world on daily basis, as well as its

importance in modern and traditional medicine, effects of the extraction conditions on quality and quantity of extracted compounds were addressed in this study. Results showed that the volatile compounds extracted by the Clevenger method had lower average boiling points and less molecular weights compared to those extracted by the Percolation method

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NEW GENERATION FUNCTIONAL FOODS-A PROSPECTUS ON PROCESSING TECHNOLOGY ASSISTANCE IN DEVELOPMENT AND PRODUCTION – A REVIEW

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ABSTRACT

Functional foods are important disease curing and prevention agents in monitoring and management of health systems. The development and innovation of functional foods throughout the world is a new conceptual task due to the growing trade of health foods and consumers also showing interest towards health benefits of functional foods/ingredients to keep away maximum of synthetic pharmaceuticals. In pharmaceutical market, several proprietary functional foods are sold under the medical trade name, these proprietary foods are purely health supplements which are purified and extracted from the natural food commodities and their biological function is specific. Several functional foods are proposed to maintain good health and working against to several chronic diseases and disorders. Especially nutrition related disorders can successfully cured with certain bioactive compounds present in foods that is, even can't cure with chemical, synthetic pharmaceuticals. These successful stories in monitoring human health enhance the production of functional foods throughout the world. Several food processing technologies have been identified and implemented their role in formulation of functional food products and ingredients. Encapsulation technology, fortification and vacuum impregnation technology, enzyme technology, edible coating technology, irradiation, and nanotechnologies are some of the prominent technologies fallowed in development of functional foods. The strength and validation of these technologies in production of functional foods were analysed and discussed in this article.

1. Introduction

The innovative concept of healthy food has come from Japan in the 1970's with the term functional foods (FF). From there, this food concept raises the new paths in preparation and production of health foods throughout the world. Several food and nutrition organisations and academies define a functional food as one that encompasses potentially healthy products providing health benefit beyond that of native nutrients it contains. Various terms have appeared worldwide such as nutraceuticals, medifoods, vitafoods and more traditional dietary supplements and fortified foods with the phenomena of functional foods. However, the

term functional food has become the predominant one even though several organisations have attempted to differentiate this emerging food category (Verbeke, 2005). Health Canada defines a functional food as similar in appearance to a conventional food, consumed as part of the usual diet, with demonstrated physiological benefits, and/or to reduce the risk of chronic disease beyond basic nutritional functions' and nutraceuticals as a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with foods (Blades, 2000). However, functional foods are generally considered as those foods which are intended to be consumed as part of the normal diet and that contain functionally active components which exhibits the potential, enhanced health benefits and reduced risk of disease/disorder.

Since the last few years, consumer views in the field of food production and knowledge, behaviour towards processed foods have been changed preferably. Consumers, day by day believe that foods contributed directly to their health wellness and they thought that foods are not only satisfy hunger and also provide essential bioactive compounds for body to prevent nutrition-related diseases, improve physical and mental health (Ortega, 2006). This type of thinking intension towards foods has led to the development of functional foods which are specific and unique in their activity in the body. According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), several dietary patterns along with lifestyle habits constitute major modifiable risk factors in relation to the development of coronary heart disease, cancer, type 2 diabetes, obesity, osteoporosis and periodontal disease. In this concern, functional foods play an outstanding role to combat chronic nutrition related diseases and disorders (Hasler, 1998). In development of functional food products, various technological approaches and concepts realized and tested to prove their efficiency in production of functional foods. Some technologies are adopted by food processors and some are till under laboratory test trials. The research in processing technologies which influence the development of functional food products is a most affordable research area because of the need and importance of new approaches and innovations in the development of functional food products (Mitsuoka, 2014).

2.Food fortification and Vacuum impregnation

Food fortification is a technology which chiefly interferes with the change in nutritional quality, functionality of the specific foods. Fortified foods with biologically active components that deliver potential health benefits in terms of disease prevention are claimed more importance in processed food product trade. Among the technologies used in the development of functional foods, Food fortification and vacuum impregnation technologies are most prominent technologies in which porous food matrices fortified/vacuum impregnated with adequate solutions/suspensions of biologically active compounds (Zhao and Xie, 2004). These techniques have been claimed as a useful way to obtain functional food products, without destroying the initial food matrix, but only occupying its initial porous fraction with a liquid phase (which contain bioactive components) (Alzamora et al., 2005). These techniques are especially useful in the development of fruit or vegetable products that have internal pores in the plant tissue where the biologically active compounds can be introduced. Bioactive compounds may be introduced into food products using this technique without modifying their nature. Several biologically active components which are considered as healing agents can introduce into the food matrix with vacuum impregnation technique as food fortification (Perisee, 2005). Impregnated products can be commercialised as minimally processed fresh functional foods/can be dried in order to obtain more stable products. One of the most interesting fortifiers in the FF development is calcium. Ca content of diet is critical in most stages of human life. This mineral is chiefly found in dairy products, but these are sometimes

the cause of digestive troubles. Ca fortification of fruit or vegetable can be achieved with the special emphasis; Calcium (Ca) impregnating solution can interact with the plant cellular matrix, modifying its structural and mechanical properties. Ca fortified fruit and vegetable products are rich in Ca replaces in those individuals who are allergic to the dairy products (Martin-Diana et al., 2007).

The impregnating solution concentrations of different fortifiers have been developed in terms of the response of the plant tissue to vacuum impregnation process. The impregnation rate is depended on the mechanical and structural properties of the tissue, since, due to the pressure change, VI provokes not only the exchange of internal gas or free liquid in the pores for the external liquid, but also the changes in the pore volume. Vacuum impregnation has been considered as a useful way to introduce desirable solutes into the porous structure of foods (fortification), positively modify their original composition as a new functional product. Robust applications in food fortification/development of functional foods make vacuum impregnation as successful technology in recent years. Especially in the case of fruits and vegetables, vitamins, minerals and other bio molecules were successfully impregnated into the matrix to enrich the whole composition of fruits and vegetables (Fito et al., 2001).

The use of vacuum impregnation to develop functional foods can be orientated in different ways. Several researchers in their studies used the vacuum impregnation technique to modify the original composition of one porous food. Calcium fortification of eggplants, carrots, and oyster mushroom using vacuum impregnation with sucrose solutions, and found that raw material variability induces significant differences in the final impregnation level (Gras et al., 2003). Probiotic-enriched dried fruits using vacuum impregnation technique by applying VI process either with commercial apple juice containing *Saccharomyces cerevisiae*, or with whole milk or apple juice containing 107 or 108 cfu/ml of Lactobacillus *casei* is an evidence to understand the applications of vacuum impregnation in development of probiotic foods. It was reported that dried apple samples could contain about 106cfu/g *Lactobacillus casei*, a similar level to that in commercial dairy products. Following the modelling prediction, experimental validation confirmed that 'vacuum impregnation' could be an effective method for the enrichment of different foods with minerals, vitamins, and other biologically active components (Betoret et al., 2003).

3. Encapsulation technology

Microencapsulation is the envelopment of small solid particles, liquid droplets or gases in a envelop coating. Microencapsulation is based on the embedding effect of a polymeric matrix, which creates a microenvironment in the capsule able to control the interactions between the internal part and the external one. Microencapsulation allows the protection of a wide range of materials of biological interest, from small molecules and protein to cells of bacterial, yeast and animal origin (Benita, 2005). For this reason such versatile technology is widely studied and exploited in the high technological fields of biomedicine, bio-pharmaceutics, and nutra-foods for application ranging from cell therapy to drug and bioactive molecule delivery. The same characteristics make microencapsulation suitable for food industry applications, in particular for the production of high value functional foods. Many encapsulation procedures have been proposed but none of them can be considered as a universally applicable procedure for bioactive food components because, the fact that individual bioactive food components have their own characteristic molecular structure and activity (Desai and Jin Park, 2005). An important requirement is that the encapsulation system has to protect the bioactive component from chemical degradation to keep the bioactive component fully functional. Many food components may interfere with the bioactivity of the added bioactive food component. It is therefore mandatory that the encapsulation procedure protects the bioactive component

during the whole period of processing, storage, and transport (Risch and Reineccius, 1995). How efficient this package load should be depends on the type of molecule that is desired as bioactive component and the specific product that serves as vehicle. When choosing an encapsulation system with high package efficiency, it is always essential to choose a system that can be easily incorporated into the food without interfering with the texture and taste of the food (Nedovic et al., 2011).

The microencapsulation improved the technological characteristics of the powders such as morphology and size, gave long-lasting storage stability and reserved the antioxidant properties (Shahidi and Han, 1993). The most commonly applied bioactive food molecules that are already encapsulated in industrial applications are lipids, proteins, carbohydrates, vitamins, and minerals (Gouin, 2004). They cannot be easily solved in food products because of their extreme low solubility in water and polyunsaturated fatty acids, which are highly susceptible to oxidation, and are now widely applied in powdered products (Gharsallaoui et al., 2007). Therefore many different approaches of encapsulation have been proposed for encapsulation of lipids in order to be able to apply them in a large variety of food products. Bioactive proteins also might require encapsulation. Many food derived peptides act as growth factor, anti-hypertensive agent, antioxidant or immune regulatory factor. Some of these proteins have to reach the site of uptake in the small bowel in an intact conformation in order to exert a beneficial health effect (de Vos et al., 2010).

Carbohydrates that can benefit from microencapsulation are mainly bioactive carbohydrates that are found in dietary fibres. The fibres or its components that would benefit most from encapsulation are the soluble non-digestible polysaccharides (Fox and Allen, 1996). These fibres have been included for cholesterol reduction, reduction of glycemic fluctuations, prevention of constipation, pre-biotic effects, and even for the prevention of cancer (Tungland and Meyer, 2002). The major encapsulation effort in this area is therefore improving the food load of fibres by packing enough fibres in capsules without interfering with the product quality such as changes in texture, mouth feel, or flavor. Encapsulation methods have been also widely applied to enhance viability of pro-biotic bacteria in commercial products. Several authors studied the pro-biotic strain survival under simulated gastrointestinal conditions and similarly for liquid based products such as dairy products (Anal and Singh, 2007). During recent years it has become clearer that pro-biotic effects are determined by the presence of specific bioactive molecules or effect or molecules in the cell envelope of pro-biotic bacteria. These molecules are proteins and have to be preserved in order to achieve functional effects. The survival of these effect or molecules in the product and during passage in the gastrointestinal tract is even more important than the survival of number of pro-biotics (Sultana et al., 2000). Preserving and protecting these effect or molecules will be a major challenge in the near future in development of effective pro-biotic functional foods. The research in this area is limited to the some extend because of technological challenges, consumer perception towards functional foods and lack of knowledge related to the encapsulation technology, and more research is needed to overcome potential challenges raised in encapsulation of bioactive compounds and foods (Kailasapathy, 2009).

4. Microbial and fermentation technology

Fermentation process is a one of the oldest food Pro cessing method, became popular with the dawn of civilization. Ability to increase nutrition value and extended shelf life stands the fermentation as common practice among the ancient communities. Fermentation increases, bio availability of nutrients, taste and functional properties of the foods. With the unique reasons, fermentation technology plays a major role in the development of functional food products for a specific reason (Farnworth, 2008). At present, several fermented foods are being chiefly considered by consumers for their nourishment

and beneficial features. Emergence of a multitude of health promoting fermented milks and other pro-biotic fermented foods have largely advocated the case, due to the health promoting works of certain beneficial microorganisms that are abundantly present in the food. The health-beneficial effect of consuming food with pro-biotic bacteria is a factor contributing to the development of research on the new segment of functional food. Pro-biotic bacteria strains are successfully used in the processed milk products, vegetables and fruit juices; however, their use in dry fermented products is not common yet. Reports on technological aspects of microorganisms in functional foods concentrate almost exclusively on the growth or viability of related microorganisms and on the fermentation performance of microorganisms for specific food materials. Fermentation process consists of the transformation of simple raw materials into a range of value added products by utilizing the growth of microorganisms and their activities on various substrates. Fermentation technology depends on the microbial components and produces different molecules from small laboratory scale to large industrial scale. During this process various bioactive molecules also be produced, that can exert excellent health benefits. For example, various bacteria and yeast have been used to produce conjugated linolenic acid, folate, vitamins and other bioactive components (Hugenholtz and Smid, 2002). Pro-biotic organisms are one of the important groups of such micro-organisms that participates in the fermentation process of various food products i.e. dairy products. Pro-biotics are defined as live micro-organisms that exert health beneficial effects to the host when administered on sufficient amount. Pro-biotic have been considered generally recognized as safe (GRAS) for human consumption and known to exhibit various beneficial health effects i.e. anti-cancer, immunomodulatory, and anti-oxidant, cardio protective and anti-obese/diabetic. There are various mechanisms have been proposed for the bioactivity of the pro-biotics on human health, and one of them is the probiotic mediated production of bioactive components during the fermentation process of foods (Parvez et al., 2006).

Fatty acid rich foods and raw dairy products are the chief sources of saturated fatty acids and if these can be enriched with micro-organisms that can convert saturated fatty acids to unsaturated fatty acids should be definitely one of the optimistic fermentation processes for the foods. Although still there are number of hurdles in inducing microbes in all types of high fat foods, common food products such as ice cream, muffins, doughnuts, dairy products and other fermented foods can be enriched with such microbes that are able to convert most of the saturated fat into unsaturated fat and other bioactive fats (Batish et al., 2004). Bio active peptides are the other type of functional compounds produced by the microbes during the fermentation process. Various probiotics and other microbes have been known for the production of bioactive peptides from milk proteins, wheat protein, and various other plant and meat proteins, which exert various beneficial health effects i.e. anti-hypertensive, immunomodulatory and anti-cancer. In addition, various microbes have been reported to produce short chain fatty acids from dietary fats, carbohydrates, proteins and fibres. The limitation in this direction might be the incorporation of these microbes may change in taste and flavour of the final food products. However, these limitations can also be prevailed by carefully standardizing the amount of microbes, time and temperature of fermentation process.

5. Enzyme processing

Enzymes are well known bio molecules with their potential and unique catalytic activity in preparation and processing of foods. Several enzymatic reactions mediated for the synthesis, separation and extraction of several bioactive compounds in foods. Enzymatic processing enhances the bio availability of various food components and it releases the compounds into the food stream which is further easily absorbed by the human gut. Microbial produced,

enzymatically extracted and modified substances offer significant opportunities for exploiting natural tools to modify and produce specific, potentially functional food ingredients. Enzymes are ideal catalysts to assist the extraction, modification, or synthesis of complex bio-functional substances of natural origin (Kinsella and Whitehead, 1989).

Table 1. Examples of enzymatic modification of food components /ingredients for improved health effects for functional foods. (The table content was adopted from Anne S. Meyer, 2010)

Enzymatic Modification	Bio-functional effect
Phytase: dephosphorylation of phytate	Increased Iron availability (humans)
Lipase: production of 1,3 diacylglycerols	Decreased Plasma triglycerides (humans) Increased energy expenditure (humans) Decreased Body weight and abdominal fat stores (humans)
lipase: enhanced incorporation of conjugated linoleic acid into triglycerides	Decreased Aortic fatty streak (in mice)
Rhamnosidase: de-rhamnosidation of heperetin-7-o-rutinoside (hesparidin)	Increased bioavailability of hesperetin (humans)
Pectinase: modification of potato cell wall material	Increased fermentability of fibre (humans) Decreased weight gain (in rats)

Enzymatic dephosphorylation of phytic acid, enzymatic modification of lipids, enzymatic enhancement of the bioavailability of hesperetin, and enzymatic modification of plant materials to obtain improved dietary fibers.etc, involved potentially in production of various bioactive compounds and their percentile availability in foods. In cereal grains most of the phosphate is stored as phytate, myo-inositol-hexa-kis-phosphate. Phytate is of interest for human nutrition because it forms salts with iron, copper, and zinc, and thus lowers the bioavailability of these minerals and this effect leads to anemia and mineral deficiency in children and adults especially in women. Research studies demonstrated that treatment of wheat bran and pea flour with a microbial phytase from *Aspergillus niger* prior to incorporation of this bran in various preparations resulted in increased iron absorption. Above information showed a clear vision that enzyme-catalyzed dephosphorylation of cereal-based products is an option for development of iron rich functional foods (Gibson et al., 2010; Harland and Morris, 1995).

Certain lipid structures, notably diacylglycerol (DAG) and conjugated linoleic (CLA) acid are natural components of edible plant oils and ruminant fats, respectively, may exert beneficial physiological effects gained as suppression of fat accumulation, reduction of atherosclerosis, and even inhibition of cancer risk in human body. These lipid compounds are produced potentially by the enzymatic reactions. The patented method for production of high-purity 1, 3-DAGs involves a 1, 3-specific lipase-catalyzed esterification of free fatty acids obtained by hydrolysis of triacylglycerols. This process is based on enzymatic glycerolysis, involving lipase-catalyzed triglyceride hydrolysis followed by enzyme-catalyzed acylation of monoacylglycerol formed during the reaction. Hesperetin is a flavonoid compound belonging to the class of flavanones and containing a 3-hydroxyand a 4-methoxy substitution in its B-phenyl ring, and is found abundantly in citrus fruits as hesperidin in glycosilated form. Hesperidin has been claimed to provide health benefits including antioxidant and anti-inflammatory effects and to prevent bone loss. But hesperidin has limited bio availability in human body due to because of rutinoside moiety attached to the flavonoid. However this rutinoside moiety can be removed catalytically by treating orange juice with a hesperidinase enzyme, that is, a fungal α-L-

rhamnosidase which hydrolyzes and removed the rhamnose moiety from hesperidin to obtain hesperetin-7-O-β-D-glucoside (which has more bioavailability than other glycosilated flavonoids) (Guo et al., 2005; Erlund, 2004; Meyer, 2010).

6. Super critical fluid extraction technology

Supercritical fluids (SCF's) are substances where temperature and pressure situated above their critical values. Super critical fluids can be changed in a wide range and it is a characteristic property for the SCF's. Super critical fluid extraction has faster mass transfer than other recoveries. Hence, SCF's extraction considered as more potential recovery method in extractions of biological active components. SCF's extraction method can be achieved in two modes i.e. single/multi stage cross-current extraction and counter current multi stage extraction. Single stage extraction method results high amount of solvent, decreased throughput, higher residual concentration and lower extract concentration. Where Counter-current operation of a separation reduces the amount of solvent necessary, increases throughput, and higher extracts concentrations in the solvent and lower residual concentrations in the raffinate and counter-current operations are useful for separations with high separation factors. Carbon dioxide (CO_2) is the commonly used solvent in SCF's extraction of food materials with wide angle properties like cheap, easily available at high purity, safe to handle, easy removal from the foods and consequently approved for food processing without declaration. Furthermore, it does not involve in the environmental CO_2 problems, when recycled (Brunner, 2005).

Throughout the world, several products possibly produced by SCF technology may be used on every day's life. Essential oils, fatty acids and/or bioactive compounds have been extracted from various foods using supercritical CO2. Soy-isoflavones, fish fatty acid profile has been also widely investigated (Thomson et al., 2008). These compounds as well as other bioactive compounds have been extracted by SFE for the enrichment/fortification of foods based on their need and importance. Super critical fluid extraction plays an important role in improving the functionality of the foods. Separation and extraction of various bio active components, de-alcoholization, de-caffeinization, removal of fat and alkaloids, extraction and separation of various micro and macro molecules, elimination of un desired anti nutritional components, removal of pesticides, Protein purification and to obtain vitamin additives in functional foods can be achieved potentially with the super critical fluid extraction technology (Rizvi et al., 1986).

7. Membrane separation

Membrane separation is a prominent technology in separation and filtration systems associated with unique, selectively permeable membranes. Today, this type of separation technique is a major separation tool in food processing and preservation. Impact of membrane separation in food processing is significant and its applications are widely distributed among the various sub divisions of food industry. The chief applications of membrane processing are in the dairy industry i.e., separation of whey proteins, milk proteins.etc, followed by beverages and egg products. Separation and purification of bioactive compounds i.e. proteins, lipids, and carbohydrates.etc, are important in preparation of functional foods. Separated functional food components through membrane separation have high purity, quantity, and specific in nature. The specificity of molecules/compounds is chiefly depended on the permeability of the membrane, the permeability of membranes is unique and every functional compound has its own suitable membranes for their separation and purification. The percentile value of quantity and purity is better than any other food separation methods. Separation of various bioactive compounds from various food sources may differ from each other. Separated functional compounds are used in fortification and processing of other food products with desired accuracy (Snap and Nakajima, 1996). Vegetable proteins and their derivatives are commonly used for the

manufacture of a variety of functional foods. In the milk processing, to attain removal of bacteria and fat globule fractionation, cross-flow microfiltration is recommended because in cross-flow micro filtration, bulk flow is parallel to the membrane and perpendicular to the permeation flux and relatively cheap in cost when compared to other techniques. Membrane separation technology in production of functional foods is not well documented in literature but some research findings denoted that the importance of membrane separation technology in processing and development of functional food products, especially from beverages (Jost et al., 1999).

8. Edible films and coating technology

Any type of material used for enrobing (coating or wrapping) various food to extend shelf-life of the product that may be eaten together with food with or without further removal is considered an edible film or coating (Baldwin et al., 2011).

Table 2. List of major bio-polymeric compounds used in development of edible films and coatings and their applications in foods.

Type of Coatings	Application in Foods
Chitosan Alginates Gelatin Collagen Edible Wax SPI(Soya Protein Isolate) Cellulose ethers Polyethylene glycol Carrageenan, whey protein isolate Methylcellulose Gellan Guargum Gum Arabica Gum Tragakantha Gum Karaya	Potential coat forming biopolymers to preserve the various food groups for a long time. Good media to carry biosensors and functional compounds to improve the functional properties and safety of foods. Good and unique physical and chemical barrier agents to surrounding environment in food processing and preservation. Used as thickeners, stabilizing agents and bulking agents in preparation of some food products. To make potential composite edible films and coatings for food preservation.

Edible films and coatings are applied on many products to control moisture transfer, gas exchange or oxidation processes. For film-forming materials dispersed in aqueous solutions, solvent removal is required to achieve solid film formation and control of its properties. Edible films can be formed via two main processes: a "wet process" in which biopolymers are dispersed or solubilized in a film forming solution (solution casting) followed by the evaporation of the solvent, and a "dry process" which relies on the thermoplastic behaviour exhibited by some proteins and polysaccharides at low moisture levels in compression moulding and extrusion (Embuscado and Huber, 2009). One major advantage of using edible films and coatings is that they have a high potential to carry active ingredients such as anti-browning agents, colorants, flavours, nutrients, minerals, spices, antimicrobial compounds and other bioactive components that can extend product shelf-life, reduce the risk of pathogen growth on food surfaces and provide specific nutrients that affect beneficially one or more functions of the body (Wu et al., 2002). Researchers have endeavoured to incorporate minerals, vitamins and fatty acids into edible film and coatings formulations to enhance the nutritional value of

some fruits and vegetables, where these micronutrients are present in low quantities.

9. Food irradiation technology

Irradiation of food is an advanced food processing method to preserve foods for a long time without mediation of chemical additives. Few countries approved irradiated foods for consumption and are popular in several areas based on the principles of the codex standards, limited/restricted in other countries due to the conflicts in its safety to the human consumption. Irradiation is a process in which foods treated with ionizing radiation mediated on/below the maximum dose rates i.e.10 kGy (Codex General Standards for Irradiated Foods). The applications of irradiation in development of functional foods is in its initial stage, some findings related to the functional foods has been denoted the faith and possibilities. The colour improvement of plant-derived products without changing their beneficial biological activities was adopted in foods with the application of irradiation technique (Ahn et al., 2004). There is a great potential for application of irradiation as a new processing technology in reduction of undesirable or toxic compounds and has been studied by several researchers to reduce/eliminate unwanted or toxic materials i.e., food allergens (Lee et al., 2006), carcinogenic volatile N-nitrosamines (Ahn et al., 2004), biogenic amines (Kim et al., 2004), and phytic acid with improved antioxidant activity (Ahn et al., 2003). In another way, researchers observed breakdowns in irradiated chlorophyll pigments, which can be used further in bleaching of oils. Based on these experimental results, colourless green tea leaf extract was developed, which claimed potential health benefits (Byun et al., 2002). The complete information regarding irradiation technology is not prominent yet and there is need to discover more findings in radiation technology with special concern of functional foods in future.

10. Food nanotechnology

The National Nanotechnology Initiative has defined nanotechnology as the understanding and control of matter at dimensions of roughly 1-100 nm. Nanotechnology involves imaging, measuring, modelling, and manipulating matter at nanoscale. Nanotechnology applications in agricultural and food industries were first addressed by a United States Department of Agriculture (USDA) bulletin published in 2003 (Joseph and Morrison, 2006). A wide range of nanotechniques and materials are being developed in an attempt to develop greater control over food properties like flavour, texture, rate of processing, heat tolerance, shelf life, traceability, safety, and the bioavailability of nutrients.

Nanotechnology can enable methods to make foods such as soft drinks, ice cream, chocolate, or chips to be marketed as 'health' foods by reducing fat, carbohydrate or calorie content or by increasing protein, fibre or vitamin content and helps to make interactive foods which can allow consumers to modify the food depending on their own nutritional needs. Bioactive compounds are active nutritional constituents that occur in small quantities and play an important role in foods functionality in the body. Nanotechnology has shown greater potential in improving the efficiency of delivery of bioactive compounds in functional foods to improve human health (Shimoni et al., 2009). Enhanced solubility, improved bioavailability, and protected stability of micronutrients and bioactive compounds during processing, storage and distribution has been attained by nanotechnology. The delivery and action specificity of functional food components in gut can be monitored with nanosensors and receptors, which are further developed as Neutraceuticals. Nanocapsulation is one of the advanced techniques used by several food manufacturers, to mask the taste and odour of tuna fish oil (source of omega-3 fatty acids) which is integrated into bread. The nanocapsules break open only when they reach the stomach and hence the unpleasant fish oil taste can be avoided while eating. Nanoencapsulation has

been used for the protection and controlled release of specific functional food molecules and beneficial live probiotic species to promote specificity in release of nutrients and gut function (Champagne and Fustier, 2007). The viability of probiotic organisms including *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus,* and *Bifidobacterium* spp. within freeze dried yogurt can be improved by nanoencapsulation with association of calcium alginate (Sultana et al., 2000). The biological value of lycopene can be increased by incorporation of lycopene nano-structures in tomato juice, sauce, and jam (Tang et al., 2005). Milk protein, casein, was used to make nanosized micelles and has been employed as a vehicle for delivering Neutraceuticals including vitamin D_2 (Livney, 2010). Nanotechnology applications in functional foods are limited and restricted to the certain levels because of conflicts associated with their safety to humans. Application of nanotechnologies are complicated and twisted around the doubts and shades of consumers. Because of above reasons, the nanotechnology applications in development of functional foods must be standardized before the statutory authority, more research findings are needed to justify the safety of nanostructures/materials as a part of consumption (Momin and Joshi, 2015).

11. Other food processing technologies

The development of functional foods takes a diversion with the innovations and possibilities in food processing. The newly emerged technologies in food processing sector has been opened new paths to develop innovative trends, created a new generation functional foods. Ultra sonication, Pulsed electric field technology, High pressure processing, Membrane processing.etc, are aimed to reach new innovations and developments in processing and production of health foods. Among them, few technologies are in the way to introduce more innovations in production of functional food products. Research and development of these technologies towards functional foods are limited yet and several researchers are working on this area throughout the world to overcome the limitations and implications.

12. Conclusion

Functional foods are curative food agents associated with the human health and well being. World industrial personals and researchers focused on the technological influences on development and production of functional foods due to their importance in world consumer market. Technology innovations driving production and formulation of functional foods to maximum levels, at the same time manufacturers and industrial people also looking for innovative and cost effective procedures to implement the better production output and to withstand the competitiveness in the present consumer market. But, some processing technologies are well established and some are at their beginning stage. More inventories in technological aspects are needed to the development, processing, and production of effective functional foods for specific functionality in the body.

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EFFECTS OF FOOD DISINFECTANTS ON VITAMIN C CONTENT OF ROCKETS: CHLORINE, CALCIUM OXIDE AND ACETIC ACID

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ABSTRACT

This study examines the changes in ascorbic acid amounts of the rocket samples with different thickness steeped in different food disinfectants in different concentrations. Rocket samples in different thickness(whole and 3cm-pieces) were steeped in following food disinfectants; distilled water(pH=6.54) and chlorine(50 ppm-pH=7.38, 200 ppm-pH=8.01), calcium-oxide(0.1% Calceramic®-pH=9.14) and acetic acid(0.5%, 2%, 5%, pH=2.35, 2.78, 3.11) for 5 and 15 minutes. *2,6*-Dichlorophenolindophenol and spectrophotometric methods were used in ascorbic acid analysis. Change differences among ascorbic acid amounts of rockets were denominated in percentage(%) in comparison to initial value. Whole rocket samples steeped in distilled water for 5 minutes lost 5.85% of its vitamin C content and 3-cm pieces lost 24.43% of its vitamin C content. After being steeped in 50ppm concentration of chlorine for 5 and 15 minutes, whole rocket samples lost 17.24% and 32.59% of its vitamin C content and 3-cm pieces 36.50% and 38.45%. The rate of loss after steeping in 200ppm chlorine concentration was higher than the loss in 50ppm. Whole rocket steeped in 0.1% calcium-oxide for 5 minutes lost 24.25% of its vitamin C content and the rocket in 3-cm pieces lost 43.96%. Ascorbic acid loss of rockets after being steeped in acetic acid solutions(0.5%,2%,5%) is lower in comparison to other disinfectants. When steeping duration was increased to 15 minutes for all applications, the rates of losses also increased in comparison to 5-minute applications. Acidic feature of food disinfectants, increasing chopping thickness of rockets and shortening steeping duration of rockets in disinfectant solutions enhance ascorbic acid preservation relatively.

1. Introduction

Vitamin C is a water-soluble substance which is essential for human health. Called L-ascorbic acid ($C_6H_8O_6$) in chemistry, vitamin C is a term accepted by Commission on Biochemical Nomenclature of International Union of Pure and Applied Chemistry (International Union of Pure and Applied Chemistry, IUPAC) and its systematic denomination is 2,3-enediol-L-gluconic acid-γ-lactone (Anonymus, 1990). Physiological functions of vitamin C is mostly attributed to its oxidation-reduction features (Naidu, 2003). L-ascorbic acid is active in the synthesis of collagen which is directly related with the etiology of scurvy (Eitenmiller et al., 2007). In addition, L-ascorbic acid is the cofactor of hydroxylase and monooxygenases enzymes which are iron and copper containing metalloenzymes active in amino acid synthesis with carnitine and neurotransmitter (Levine,

1986). As L-ascorbic acid is an electron donor, it has in vivo anti-oxidant effect scurvy (Eitenmiller et al., 2007). For these reasons, it has been claimed that vitamin C has a role in the pathogenesis of numerous neurodegenerative and chronic diseases such as cancer, cardiovascular diseases, diabetics, cataract, immune system dysfunctions as well as scurvy (Peng and Zhang, 2008; Karabayır, 2006). While ascorbic acid is synthesized out of D-glucose or D-galactose by means of glucuronic acid in herbs or mostly liver and rarely kidneys of some animals, it is not synthesized in human body. Therefore, humans have to outsource this vitamin from foods (Naidu, 2003).

Vitamin C is the most vulnerable vitamin. Some preparation, preservation, cooking and storage processes applied to foodstuff cause considerable negative changes on vitamin C molecules. These changes result in remarkable losses of vitamin C (Çakır and Beyhan, 2006). Besides these processes, chosen food disinfectants and disinfection methods have significant impacts on vitamin C losses.

As fresh fruits and vegetables are grown in an epigeal way and not undergone any other process other than washing, they are exposed to several pathogen bacteria such as *Staphylococcus aureus*, *Escherichia coli* O157:H7 and a number of viruses, protozoa and macro-parasites (Beuchat, 1996; FDA, 2001) which cause foodborne intoxication and diseases. The most effective way to ensure food safety and prevent foodborne diseases is considered to be the prevention of microbiological contamination (FAO, 2004). For this reason, it has been a necessity to add food-oriented disinfectants into wash water systems in order to reduce microbial contamination in fruits and vegetables (De RoeveR, 1988).

Chlorinated compounds such as sodium hypochlorite (NaOCl), calcium hypochlorite ($Ca(OCl)_2$) and chlorine dioxide (ClO_2), a number of organic acids such as hydrogen peroxide (H_2O_2), peroxyacedic acid, ozone, citric acid and acetic acid, and electrolyzed water are used to disinfect fruits and vegetables (FDA,1998). The organic acids such as acetic acid, lactic acid and citric acid have also strong anti-microbial effect against psychrophilic and mesophilic bacteria in fresh fruits and vegetables (Bari et al., 2005; Uyttendaele et al. 2004). Moreover, there are some disinfectants containing calcium oxide which are declared as totally natural disinfectants in the markets. Besides the use of these chemical disinfectants, there are various studies suggesting that some flavoring substances such as essential oils, lemon sauce and vinegar have anti-microbial effect (Sengun and Karapinar, 2004; Skandamis et al., 2002; Bornemeier et al., 1997).

A large number of research has determined the effect of different food-oriented disinfectants on microbial load at fruits and vegetables (Caldwell et al., 2003; Rodgers et al., 2004). However, these studies mostly focused on microbiological population and sensory quality of disinfectants while their effect on nutritional values was not examined in details (Vandekinderen et al., 2008; Martínez-Sánchez et al., 2006). On the other hand, since the disinfectants used in vegetables and fruits have oxidant features (Gómez-López et al., 2009; Gómez-López et al., 2013) and change pH levels as they contain acidic and alkali compounds (Gómez-López et al., 2013), they may effect ascorbic acid amounts of vegetables.

As ascorbic acid which has numerous features in organisms is lost for many reasons, it is quite significant to determine loss mechanisms so that bio-efficacy of ascorbic acid in food can be kept high. This research aims to determine the effect of different concentrations of food disinfectants, and application durations on the vitamin C content of rocket by considering preparation methods (whole leaf and chopped) of rocket which is served raw and prone to contamination micro-biologically.

2. Materials and methods

This study was conducted in the Food Chemistry and Analysis Laboratory of Gazi University Nutrition and Dietetics Department between March and October 2014.

2.1. Materials

2.1.1.Sampling

Because of the surface area, rocket is included in the study for it is in the risky group in terms of both microbial contamination and loss of ascorbic acid. Furthermore, rockets usually are served as raw in salads and and prone to have micro-biological load because any heat treatment is applied to rockets before serving.

The rocket samples *(Eruca sativa)* originated from the same geographic area to be used in the study were purchased as they were fresh from a local market before every analysis in at least 5 kg (500±10 g/piece) amounts in accordance with the "Turkish Standards Institution (TSI) TS ISO 874 Fresh Fruit and Vegetables-Sampling" (TSI, 2007) and "TS 1194 Green Salad and Lettuce" (TSI, 2008) standards.

2.1.2.Preparation of the Samples for Analysis

The obtained samples were brought to the laboratory in the cold chain and shortest time possible and were taken into analysis in a dark area isolated from UV beams.

As stated in the "TSI 1194 Green Salad and Lettuce" standards (TSI, 1994), damaged outer leaves, which are ¾ in length of the rocket, and the roots (2 cm) of the rocket are separated. In order to eliminate the length and colour differences of the rocket samples, they are classified as large (dark green colour) and medium (light green colour) in size of their leaves. 100 g of leaf samples of each length and colour were taken. For each disinfectant application protocol, 100-gram vegetable samples to be taken into analysis were washed in 150 mL tap water for 10 seconds and after that they were pre-washed in the same amount of distilled water for the same period. In this stage, distilled water was used for eliminating some substances such as minerals and chlorinated compounds and having neutral pH because these factors might influence the of loss in ascorbic acid amounts.

The excess amount of water on the samples was taken with the help of a rough filter paper. Then some rocket samples were cut on a chopping board in 3 cm width with the help of a stainless steel knife.

2.1.3.Stages of Laboratory Studies

After the rocket samples were prepared for analysis, the laboratory study was conducted in three different stages (Figure 1). They are:

1st stage: making ascorbic acid (preliminary) analysis

Rocket samples cut in different widths without applying any disinfectant solution;

2nd stage: determining the changes in the amount of ascorbic acid after the rocket samples cut in different widths were left in distilled water for different periods.

3rd stage: determining the changes in the amount of ascorbic acid after the rocket samples cut in different widths were made subject to different types of disinfectants in different periods.

2.1.4.Preparing the Disinfectant Solutions

Three different types of disinfectant (acetic acid, chlorine and calcium oxide) were used in the study. According to that, the disinfectant solution composed of the acetic acid of 5% (v/v), 2% (v/v) and 5% (v/v), which was prepared by using concentrated acetic acid (99.8 - 100.5 %, Sigma Aldrich), 50 ppm and 200 ppm (w/v) chlorine (ECOLAB Inc. ®) and 0.1 (w/v) calcium oxide (Calceramic®) was prepared by using 2 L distilled water (Optic Ivymen System Distiller AC-L8 make device) in room temperature. Distilled water was used for preparing the disinfectant solutions because its pH level is close to neutral (pH: 6.54) and for its low level of mineral content.

After the disinfectant solutions were freshly prepared, their pH measurements were made by using a digital pH meter (Selecta®). pH levels of the disinfectants were measured as follows: 50 ppm chlorine: 7.38, 200 ppm chlorine: 8.01, calcium oxide: 9.14, 0.5% acetic acid: 3.11, 2% acetic acid: 2.78, 5% acetic acid: 2.35.

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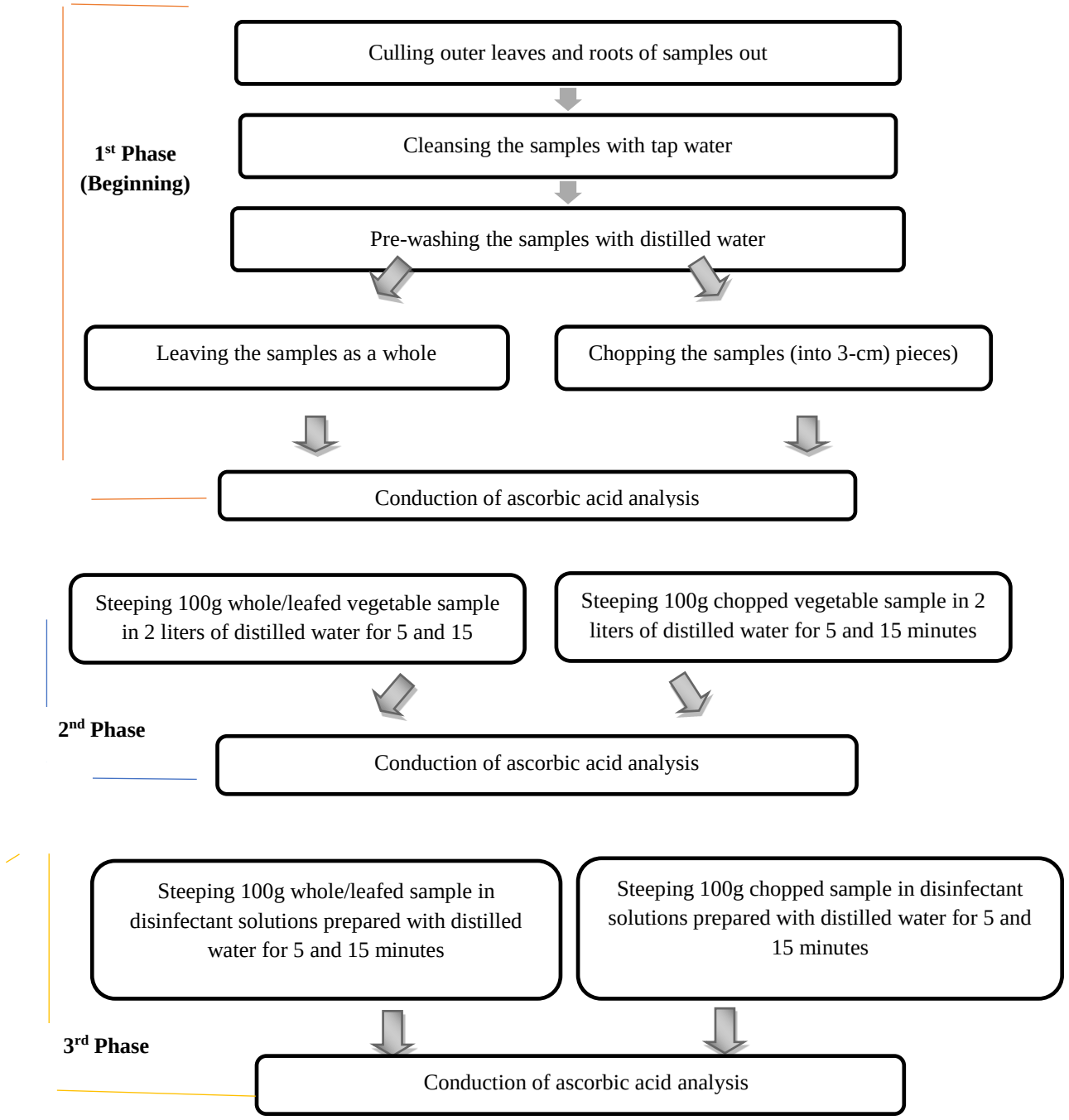


Figure 1. Preparation for analysis and application phases of experiment

2.1.5. Stages of Application of Disinfectants to the Rocket Samples

The rocket samples were left in distilled water and disinfectant solutions for 5 and 15 minutes. Disinfectant solutions were drained after each soaking application. The ascorbic acid amounts of vegetable samples after distilled water and disinfectant applications were defined in mg in 100 mg fresh weight (mg/100 g fresh weight). Change differences among ascorbic acid amounts of vegetables after they were steeped in disinfectant solution were denominated in percentage (%) in comparison to initial value.

2.1.6.Ascorbic Acid Analysis of the Rocket Samples

Spectrophotometric method (Deutsch, 1967) which was based on the reduction of 2,6 dichlorofenolindofenol indicator by ascorbic acid, was used for the ascorbic acid analysis of the samples by adapting it to the matrix of the rocket and to the laboratory conditions. The ascorbic acid analysis of the samples taken in duplicate were made in 525 nm wave length which was maximum absorbance of ascorbic acid in solution with pH:4.54 by using PG Instruments T-80 plus UV-VIS-Spectrophotometer.

Validation tests were made for the method developed and used in the ascorbic acid analysis of the samples. For this reason, linearity (R2), recovery (%), accuracy or coefficient of variation value, which is a % relative standard deviation, limit of detection (LOD) and limit of quantification (LOQ) values were calculated. As a consequence, in the vitamin C analysis method of vegetable samples, linearity value was R2 = 0.9973, recovery value = %80.30, recovery coefficient of variation value =%0.01, LOD value = 0.51 µg/mL and LOQ value = 1.60 µg/mL.

2.2.7.Statistical Evaluation of the Data

The data was analyzed by using SPSS 16.0 programme. arithmetic mean ± standard deviation (±SD) were used as definitive statistics for the measured variables. "Kruskall-Wallis Variance Analysis", "Wilcoxon Test" and "Three Way Analysis of Variance" were used for comparing the rates of the amounts of loss as the result of various disinfectant applications and affecting factors. α=0.05 value was set for level of significance in all analysis.

3.Results and discussions

3.1. Losses caused by Steeping Rockets in Distilled Water

The values of losses (%) occurring in ascorbic acid amounts after steeping rocket samples in distilled water in different durations are shown in Figure 2.

5.85% (13.66±0.007 mg) of loss was determined in initial ascorbic acid amounts (14.51±0.07 mg) of rocket samples steeped in distilled water for 5 minutes as a whole. 3-cm rocket samples steeped in distilled water for the same duration lost 24.43% of its initial ascorbic acid (13.67±0.01 mg) (Figure 2). It was observed that whole rocket samples containing 15.34±0.01 mg of ascorbic acid at the beginning lost 19.62% of ascorbic acid content and the new amount became 12.33±0.007 mg after samples were steepped in distilled water for 15 minutes. After steeping for 15 minutes, the loss in ascorbic acid amounts of 3-cm rocket samples was 21.11% (Figure 2).

There was not a statistically significant difference between the ascorbic acid losses occurring in whole leafed and 3-cm chopped rockets which were steeped in distilled water in the same duration; ($p>0.05$) (Figure 2).

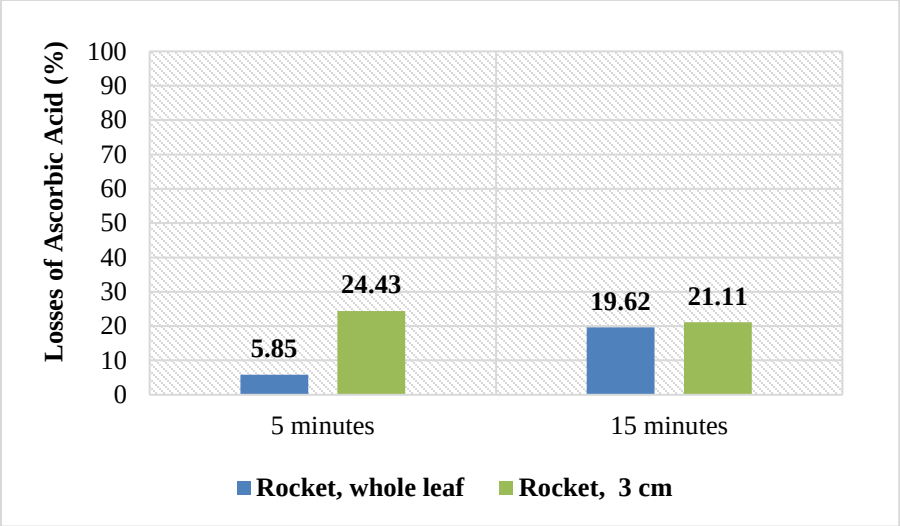


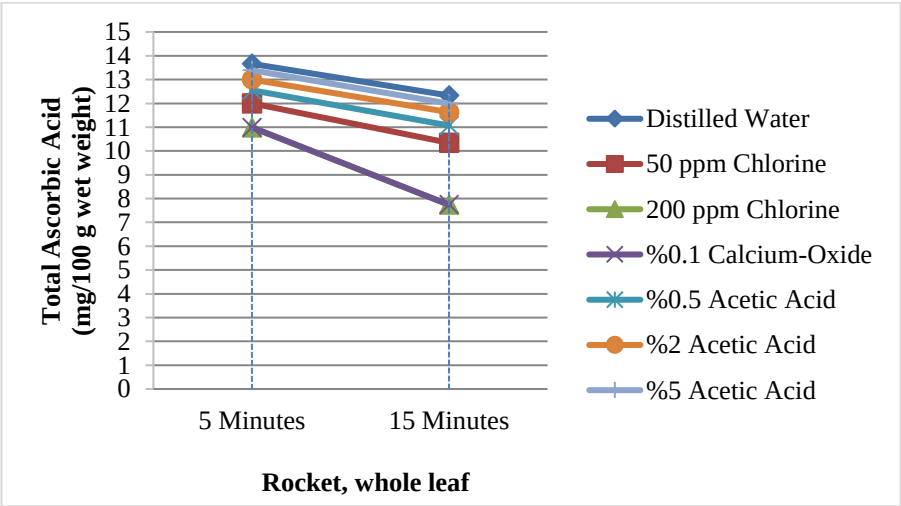
Figure 2. The values of losses (%) occurring in ascorbic acid amounts after steeping rocket samples in distilled water in different durations

3.2.Losses caused by Steeping Rockets in Disinfectant Solutions

3.2.1.Effect of Duration

Figure 3 shows the amount of losses (mg/100g wet weight) in ascorbic acid amounts of whole and 3-cm rocket samples steeped in distilled water and the same disinfectant solution in different durations.

Difference values among ascorbic acid amounts as a result of steeping whole rocket samples in different disinfectant solutions for 5 and 15 minutes: 1.33 mg ($p>0.05$) (%9.73) in distilled water, 1.66 mg ($p>0.05$) (%13.83) in 50 ppm chlorine, 2.08 mg ($p>0.05$) (%19.36) in 200 ppm chlorine, 3.25 mg ($p>0.05$) (%72.11) in 0.1% calcium-oxide solution, 1.48 mg ($p>0.05$) (%11.79) in 0.,5% acetic acid, 1.37 mg ($p>0.05$) (%10.53) in 2% acetic acid, 1.40 mg ($p>0.05$) (%10.45) in 5% acetic acid (Figure 3).



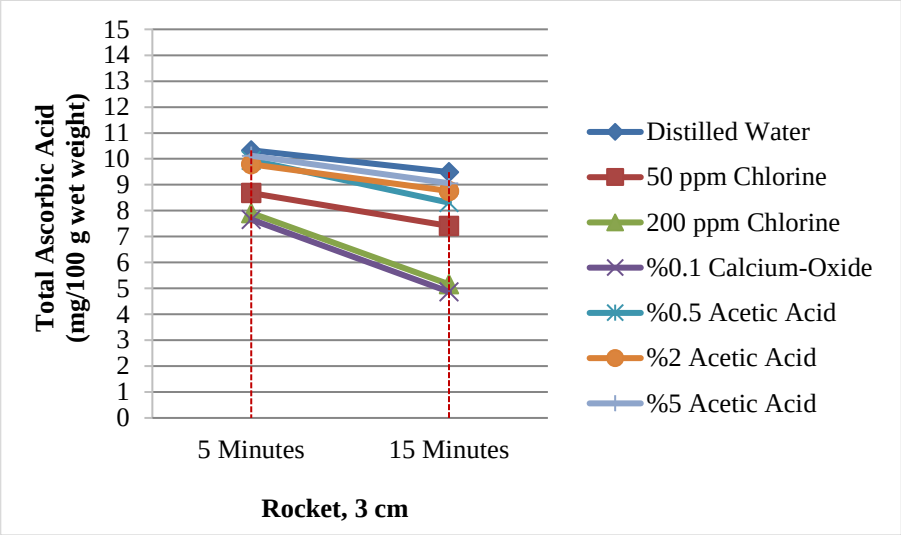


Figure 3. Difference values in ascorbic acid amounts of rocket samples steeped in distilled water, different disinfectant solutions and concentrations in different durations

Difference values among ascorbic acid amounts as a result of steeping 3-cm rocket samples in different disinfectant solutions for 5 and 15 minutes: 0.84 mg (p>0.05) (%8.13) in distilled water, 1.27 mg (p>0.05) (%14.63) in 50 ppm chlorine, 2.75 mg (p>0.05) (%34.76) in 200 ppm chlorine, 2.79 mg (p>0.05) (%36.42) in 0,1% calcium-oxide solution, 1.64 mg (p>0.05) (%16.48) in 0.5% acetic acid, 1.03 mg (p>0.05) (%10.52) in 2% acetic acid, 1.06 mg (p>0.05) (%10.47) in 5% acetic acid (Figure 3).

3.2.2.Effect of Chopping Thickness and Disinfectant

The values of losses (%) occurring in ascorbic acid amounts after steeping rocket samples chopped, in different thickness, in different water in different durations are shown in Figure 4.

It was found out in the steeping applications conducted for 5 minutes in 50 ppm concentration of chlorine that rocket sample lost 17.24% (p>0.05) of its ascorbic acid content while 3-cm rockets lost 36.50%(p>0.05). It was found out in the steeping applications conducted for 15 minutes in 50 ppm concentration of chlorine that rocket sample lost 32.59% (p>0.05) of its ascorbic acid content while 3-cm rockets lost 38,45% (p>0.05). The difference between vitamin C losses of whole and chopped rocket samples steeped in 50 ppm concentration of chlorine for 15 minutes are not statistically significant (p>0.05) (Figure 4).

The difference between steeping whole and chopped rocket samples in 50 ppm concentration of chlorine for 5 and 15 minutes is not statistically significant (p>0.05) (Figure 4).

After steeping whole rocket sample in 200 ppm chlorine concentration for 5 and 15 minutes, it was found out that rocket sample lost 25.96% (p>0.05) and 43.54% (p>0.05) of its ascorbic acid content respectively and 3-cm rocket sample lost 42.13% (p>0.05) and 57.14% (p>0.05) of its ascorbic acid content in the same steeping durations. The difference between whole and chopped rocket samples steeped in 200 ppm chlorine concentration for 5 and 15 minutes are not statistically significant (p>0.05).

Out of the rocket samples steeped in 0.1% calcium-oxide solution for 5 minutes, whole rocket sample lost 24.25% ($p>0.05$) of its ascorbic acid content in comparison to initial value and 3-cm rocket samples lost 43.96% of its ascorbic acid content ($p>0.05$) (Figure 4). Out of the rocket samples steeped in 0.1% calcium-oxide solution for 15 minutes, whole rocket sample lost 49.54% ($p>0.05$) of its ascorbic acid content in comparison to initial value and 3-cm rocket samples lost 59.55% of its ascorbic acid content ($p>0.05$) (Figure 4). The difference between whole and 3-cm rocket samples steeped in 0.1% calcium-oxide solution in different durations is not statistically significant ($p>0.05$) (Figure 4).

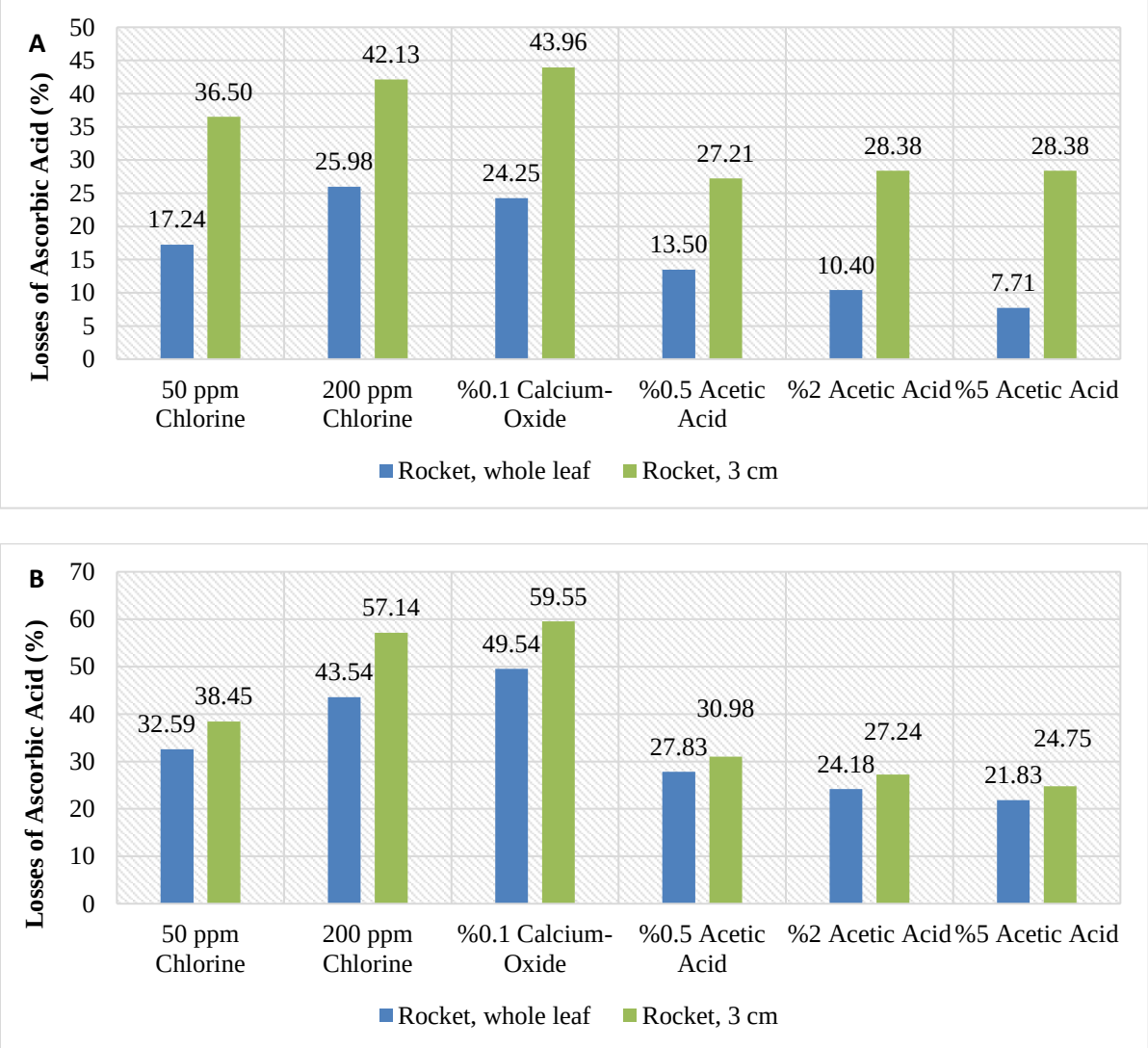


Figure 4. Difference values in ascorbic acid amounts of rocket samples chopped in different thickness and steeped in different disinfectant solutions and concentrations for (A) 5 minutes and (B) 15 minutes

Whole rocket leaf samples steeped in 0.5%, 2% and 5% acetic acid solution for 5 minutes lost 13.50% ($p>0.05$), 10.40% ($p>0.05$) and 7.71% ($p>0.05$) of their ascorbic acid content respectively (Figure 4). The differences among the amount of ascorbic acid losses occurring in whole rocket samples steeped in 0.5%, 2% and 5% acetic acid solutions for 5 minutes are not statistically significant ($p>0.05$) (Figure 4).

In comparison to initial values, 3-cm rocket samples steeped in 0.5%, 2% and 5% acetic acid solutions for 5 minutes lost 27.21% ($p>0.05$), 28.38% ($p>0.05$) and %25.96% of its ascorbic acid content respectively ($p>0.05$) (Figure 4). The differences among the amount of ascorbic acid losses occurring in above-mentioned rocket samples steeped in 0.5%, 2% and 5% acidic acid solutions for 5 minutes are not statistically significant ($p>0.05$) (Figure 4). In comparison to initial values, there was not a statistically significant difference between the ascorbic acid losses occurring in whole leafed and 3-cm chopped rockets which were steeped in the same acetic acid solutions for 5 minutes; ($p>0.05$) (Figure 4).

Whole rocket leaf samples steeped in 0.5%, 2% and 5% acetic acid solution for 15 minutes lost 27.83% ($p>0.05$), 24.18% ($p>0.05$) and 21.83% ($p>0.05$) of their ascorbic acid content respectively (Figure 4). The differences among the amount of ascorbic acid losses occurring in whole rocket samples steeped in different acetic acid solutions for 15 minutes are not statistically significant ($p>0.05$) (Figure 4).

3-cm rocket leaf samples steeped in 0.5%, 2% and 5% acetic acid solution for 15 minutes lost 30.98% ($p>0.05$), 27.24% ($p>0.05$) and 24.75% ($p>0.05$) of their ascorbic acid content respectively (Figure 4) in comparison to initial values. The differences among the amount of ascorbic acid losses occurring in 3-cm rocket samples steeped in 0.5%, 2% and 5% acetic acid solutions for 15 minutes are not statistically significant ($p>0.05$) (Figure 4) compared to initial values. In comparison to initial values, there was not a statistically significant difference between the ascorbic acid losses occurring in whole leafed and 3-cm chopped rockets which were steeped in the same acetic acid solutions for 15 minutes; ($p>0.05$) (Figure 4).

3.3. Discussions

FDA defines disinfection as "exposing foodstuff to cleaning process applied to eliminate or reduce the number of insanitary micro-organisms by means of a treatment which does not affect the quality of product and create any hazard for health. This definition emphasizes the necessity of preserving food quality and assuring food safety and nutritional value preservation by reducing the population of pathogens (FDA, 1998).

It is significant to determine the best method and apply the most suitable disinfectant in the most suitable concentrations in the most suitable durations
to optimize the bio-efficacy of ascorbic acid. Because the ascorbic acid which has significant functions in organisms may incur losses due to various factors (Gamboa-Santos et al., Lešková et al., 2006; Leong and Oey, 2012). The changes occurring in vitamin C amounts after the disinfectant applications conducted in different combinations and vitamin C amount of vegetable rocket which is a member of green-leafy vegetables consumed more especially in winter compared to other seasons (Küçükerdönmez, 2008) should be taken into consideration. Within this context, this study examines the effects of different food disinfectants, concentrations and application durations on ascorbic acid losses of the vegetable rocket considering its preparation methods (as a whole and chopped). In the present study, these disinfectant concentrations and application durations were chosen because they were the most commonly used in the preparation of vegetables.

Vegetables are disinfected before they are chopped at home; however, catering companies usually disinfect the vegetables after they are chopped. The conditions which, cause stress on vegetables such as chopping, increase reactive oxygen species and the transformation of ascorbic acid into dehydroascorbic acid after the

rise in ascorbate oxidase activity. Lamikanra and Watson (2000) indicated that peroxidase activity of melon increased in response to the increase in oxidative stress and degradation of dehydroascorbic acid increased afterwards (Lamikanra and Watson, 2000). These results coincide with the results found out by Zhang et al. 2005. They found out in their studies that when fresh cut celeries were stored at 4oC, oxygen contact surface area expanded and ascorbic acid amount decreased as celeries were chopped (Zhang et al., 2005).

Chopping thickness affects the contact of section surface with water. This effect is significant in terms of ascorbic acid loss. Ascorbic acid is a water soluble vitamin and specific polar level of its water solution is +24 (AOAC, 1966). A study showed that ascorbic acid can easily diffuse into water while peeled and chopped foods are washed (Tapadia et al., 1995). The results of a study determining ascorbic acid levels of raw and cooked sixteen different green-leafy vegetables showed that the samples cooked in higher amount of water lost 18-99% of its ascorbic acid content and the ones cooked in lower amount of water lost 5-97% of its ascorbic acid content (Sreeramulu et al., 1983). The same study proved that removing cooking water may cause up to 80% loss of ascorbic acid (Sreeramulu et al., 1983). In this study, ascorbic acid loss occurring in whole rocket samples was less than the loss in 3-cm chopped rocket samples (Fig. 2). Therefore; chopped rocket samples steeped in disinfectants for a longer span of time lost more ascorbic acid than the whole rocket samples steeped for a shorter span of time (Fig. 2-4). However; there was not a statistically significant difference between ascorbic acid losses of the vegetables steeped in distilled water in different thickness and in the same duration or steeped in distilled water in the same thickness and in different duration ($p>0.05$) (Fig. 3). Although the results of this study were not statistically significant, it was thought that ascorbic acid loss increased as a result of increase in the contact of water and air in relation with the rise in the steeping duration and decrease in chopping thickness of vegetables.

Chlorinated compounds are the most preferred food disinfectants by individuals and catering companies because of their ease of use, wide eradication spectrum and chemically stable, cheap and easily accessible features (Koide et al., 2011). Chlorinated compound are mostly used at 50-200 ppm free chlorine levels and less than 5 minutes of typical contact durations (Francis and O'Beirne, 2002; Watada and Qui, 1999). Therefore; in this study, chlorine solutions prepared at 50ppm (pH:7.38) and 200 ppm (pH:8.01) concentration were applied to rocket samples chopped in different thickness in different durations. In the steeping applications conducted with rocket, it was found out that whole rocket samples steeped in 50 ppm chlorine solution for 5 minutes lost more ascorbic acid. On the other hand, steeping rocket samples for 5 minutes enabled more ascorbic acid preservation than steeping for 15 minutes (Fig. 3, Fig. 4). However; the differences between ascorbic acid losses were not found to be statistically significant ($p>0.05$) (Fig. 3, Fig. 4).

This study showed that 50ppm chlorine added into distilled water increased pH of distilled water from 6,54 to 7,38 and 200ppm chlorine increased pH of distilled water to 8,01. The catabolism of ascorbic acid especially in aqueous disinfectant solutions depends on pH. Ascorbic acid becomes active at different levels at different pH levels (Maniyar et al., 2012). Ascorbic acid is very stable between pH 2 and 4 (Wechtersbach et al., 2011). However; as pH level exceeds pK1 value 4.04 (Gregory, 1996), ascorbic acid loses its stability between pH 4 and 6 at the maximum level and irreversibly and swiftly gets hydrolyzed to 2,3-diketo-L-gulonic acid which has no vitamin activity. A study conducted on chlorine supports above-mentioned data: it is considered that chlorine makes its environment alkali by increasing pH level of tap water and thus steeping spinach in chlorinated water for 30 minutes affects ascorbic acid amount of spinach with minced meat and increased ascorbic acid loss (Çakır and Beyhan,

2006). Another study focused on the effect of chlorine dioxide (3 mg L-1) and sodium hypochlorite (100 mg L-1), used as washing solution, on vitamin C (ascorbic acid and dehydroascorbic acid) content of fresh-cut lettuce and it was found out that vitamin C content of washed lettuce was lower than the one which was not washed independently of washing solution type (López-Gálvez et al., 2010). Another study which examined the effect of washing lettuce in chlorinated and ozonated water on ascorbic acid and total antioxidant activity of lettuce showed that washing lettuce with chlorinated and ozonated water has not any significant effect on ascorbic acid content of lettuce (Kenny and O'Beirne, 2009). Another study was conducted to determine the effect of various decontamination agents such as water, 200mg/L sodium hypochlorite, 250mg/L peroxyacetic acid, neutral electrolyzed water (pH 2.06, 37.5±2.5 mg/L free chlorine) and 1.59mg/L chlorine-dioxide gas on microbial population, sensory quality and nutritional value of leek. The study showed that leeks 22.9% of its ascorbic acid and dehydroascorbic acid amount after being washed with water for 5 minutes and other liquid decontamination agents did not contribute this loss. It was found out that contrary to liquid decontamination agents, chlorine-dioxide gas caused 23.2% loss in ascorbic acid amount and this percentage of loss was considered to be statistically significant (Vandekinderen et al., 2009). In a study, iceberg lettuce was rinsed with tap water, steeped in distilled water or chlorinated water and then kept in propylene bags for 8 days at 4°C in 100% nitrogen environment (Karaca and Velioğlu, 2014). Researchers analyzed vitamin C (ascorbic acid and dehydroascorbic acid) amounts, total phenolic compound and antioxidant amount, polyphenol and carotenoid of the iceberg. As a result, all antioxidant groups gave different reactions to each application; especially ascorbic acid was affected from tap water at the most. After storing process, it was observed that ascorbic acid preservation was highest in the lettuce samples steeped in chlorinated water (Karaca and Velioğlu, 2014). The data of this study revealed that the ascorbic acid loss values obtained at the beginning of chlorinated water application and after storing lettuce can be different. In this study, vegetables were not stored after disinfection application. Therefore, it is significant to consider this in further studies. It is considered in the literature that using chlorine substances in different concentrations and forms may cause these contradicting results.

A commercial disinfectant Calceramic® (%91-98.5 calcium oxide) was used in this research. Calcium oxide is a food additive included in Turkish food codex Notification on food additives with the code E529. Calceramic®, which is produced by baking the shells of scallops called Hokkakai and found in Japan market with the brand names such as "Sörf Sera", "Vegisafe" and "Surfcare (CAO 1000)", is declared as a totally natural disinfectant.

This study proved that the rocket samples steeped in 0,1% concentration of Calceramic® of which active ingredient is calcium oxide, for 5 minutes lost less amount of ascorbic acid (24,25%). On the other hand, steeping rocket samples for 15 minutes caused more ascorbic acid loss than steeping for 5 minutes (Fig. 3, Fig. 4). However; the differences between ascorbic acid losses were not found to be statistically significant ($p>0.05$) (Fig. 3, Fig. 4).

There are limited number or studies in the literature which examine the effect of calcium oxide on the ascorbic acid content of foods (Bodur et al., 2010; Sörf Sera Corporation, 2000). In a study which was conducted at Japan Food Research Laboratories in 2000 and gave similar results with above-mentioned research, Sörf Sera or in other words Calceramic® solution (0.05% and 0.15% disinfectant + tap water) was applied to tomato samples for 10 minutes and the changes occurring in ascorbic acid content (mg/100g) of tomato samples were examined (Sörf Sera Corporation, 2000). As a result of applying 0.05% and 0.15% of Sörf Sera to tomatoes which have 36mg/100g initial ascorbic acid content for 10 minutes, it was found out that ascorbic acid content of tomatoes

decreased to 31mg/100g and 30mg/100g. However; ascorbic acid content of tomato samples exposed to tap water without Sörf Sera was 32mg/100g.

Active ingredient of Calceramic®, calcium oxide transforms to calcium-hydroxide when it is dissolved in water. Therefore; it was suggested in several studies and stated by the seller company that pH level of 0.1% calcium oxide solution exceeds 12 (Bodur et al., 2010). It was indicated that bacteriostatic effect of calcium oxide results from the changes in pH level. In addition, it is suggested that, in another mechanism, hydroxy ions show a strong free radical effect and reacts with some bio-molecules (Bodur et al., 2010). In this study 0.1% calcium oxide, which was added into distilled water while preparing disinfectant solutions, increased the alkalinity and pH level of distilled water from 6.54 to 9.14. It is considered that the changes in pH level distilled water and the increase in the number of hydroxy ions both have bacteriostatic effect on vegetables and plays a significant role in ascorbic acid losses although these losses are not statistically significant. A study supporting this hypothesis showed that 80% of ascorbic acid in the vegetables completely steeped in alkaline water degraded while the rate of loss was 60% in the vegetables half of which were steeped in alkaline water. On the other hand, this rate became 40% when one quarter of the vegetables were steeped (Yurdagel, 1983).

There are several differences between the ascorbic acid values found after Calceramic® application in this study (Fig. 3, Fig. 4) and the results obtained after the limited studies in the literature. The reasons of these differences can be listed as follows; food disinfectants containing calcium-oxide active ingredient cause different changes in pH levels of foods and different pH changes in different parts of foods; initial pH level of the disinfectant water is variable and the solutions with different pH levels are added to the disinfectant water.

The organic acids such as acetic acid, lactic acid and citric acid which are labeled as Generally Recognized as Safe (GRAS) have strong anti-microbial effect against psychrophilic and mesophilic bacteria in fresh fruits and vegetables (Bari et al., 2005; Uyttendaele et al. 2004). Anti-microbial effect of organic acids results from the fact that they deteriorate membrance structure, transport and permeability of cells by causing declines in pH level as they increase the concentration of free hydronium ions (Rico et al., 2007). In this study, it is considered that respectively protective effect of acetic acid on ascorbic acid is activated through its anti-microbial mechanism indirectly. However, there are only a few studies examining the effect of acetic acid on ascorbic acid as is the case with calcium oxide.

It was found out that if vegetables are steeped in all concentrations of acetic acid for 5 minutes they lose less ascorbic acid than 15-minute application. Ascorbic acid loss (7.71%) of whole rocket sample steeped in 5% concentration of acetic acid solution for 5 minutes was found to be less than 15 minutes although this difference was not statistically significant ($p>0.05$) (Fig. 3, Fig. 4).

In a study which produced similar results with above-mentioned study; 2-ppm ozone, 100-ppm chlorinated water and 0,25g/100g citric acid+0,50g/100g ascorbic acid and 10oC of water were applied to lettuce samples and the effect of these applications on the quality of lettuce samples was compared (Ölmez and Akbas, 2009). After storing lettuce samples at 4oC for 12 days, researchers could not observe any significant difference between chlorine and ozone application in terms of ascorbic acid loss. Ascorbic acid preservation was higher in the lettuce samples which were exposed to additional ascorbic acid and citric acid that have more acidic features (Ölmez and Akbas, 2009). Another study which was conducted in a similar method examined the changes in the microbial quality of fresh-cut lettuce samples exposed to organic acids such as citric acid (5 g L-1) and lactic acid (5 mL L-1), chlorine (100 mg L-1) and ozone solutions as well as the quality changes after lettuces were stored for 8 days (Akbas and Ölmez, 2007). As a consequence; there was not any statistically significant change

in the humidity, color, texture, β-carotene and ascorbic acid content of lettuce samples in the all applications until the 8th day (Akbas and Ölmez, 2007).

The studies, in which acetic acid was used, generally focused on microbial and sensory quality of organic acids in different concentrations and durations (Toivonen and Lu, 2013; Artés et al., 2007). These studies showed that sensory quality of the vegetables were affected from the organic acids in high concentration (Toivonen and Lu, 2013; Artés et al., 2007; Van Haute et al., 2013). However, it was suggested that using acetic acid at 2% for 10 minutes caused an increase in the microbial load of salad vegetables without deteriorating their sensory quality (Nastou et al., 2012). In this study, it was observed that rocket leaves grew yellow and brown after the application of acetic acid in all concentrations. Therefore, in the determination the changes to occur in the quality of foods, it is crucial to carry out additional sensory analyses while examining the effect of disinfectants of nutritional value and microbial quality of vegetables.

4. Conclusions

Consequently, this research proved that food disinfectants cause losses in vitamin C content of rocket samples. However, acidic feature of food disinfectants, increasing chopping thickness of rockets and shortening steeping duration of rockets in disinfectant solutions enhance ascorbic acid preservation relatively. In this context, it is necessary to conduct further research which is supported with microbiological and sensory analyses. These studies should also examine more vegetables and disinfectants and the effect of concentrations and durations.

There are some limitations in this research. First of all, statistical analyses showed that the differences between ascorbic acid losses are not statistically significant although there are differences between ascorbic acid losses of vegetables according to chopping thickness and steeping duration ($p>0.05$). In this research, there were numerous variables (number of vegetables, chopping thickness, disinfectant type and concentration, steeping durations) that can affect ascorbic acid content of vegetables; therefore, analyses were made in duplicate and at one sitting. In order to enhance the effect of the study, it is significant to carry out further studies with a team, in duplicate or triplicate and at more than one sitting. Secondly, ascorbic acid content of fruits and vegetables is determined by means of spectrophotometric method based on the degradation of 2,6-dichlorophenolindophenol coloring agent. As ascorbic acid can easily transform into dehydroascorbic acid, it is recommended in some studies and methods to measure both ascorbic acid and dehydroascorbic acid while determining vitamin C content of fruits and vegetables. It is another limitation of this research that dehydroascorbic acid amount has not been determined.

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EFFECT OF FREE AND NANOENCAPSULATED FORMS OF *ZATARIA MULTIFLORA* BOISS. ESSENTIAL OIL ON SOME MICROBIAL AND CHEMICAL PROPERTIES OF BEEF BURGER

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ABSTRACT
Meat products such as burgers are susceptible to microbial and chemical deterioration. Encapsulation of essential oils promotes their stability by protecting them against chemical, enzymatic and environmental changes, as well as covering the unwanted taste and odor. This study was conducted in order to evaluate microbial and chemical properties of beef burger incorporated with different concentration (0, 0.015 and 0.03 % v/w) of *Zataria multiflora* Boiss. essential oil in the free (ZMEO) and nanoliposomal forms (NZMEO) at refrigerated temperature. The dominant compounds of ZMEO in current study were carvacrol (46.40%), thymol (23.61%) and p-Cymene (16.37%). At end of study period, all samples incorporated with free and nanoencapsulated ZMEO showed significantly($P < 0.05$) lower TVC, PTC and mold and yeast count than control samples (approximately more than 1 log CFU/g). Also the result showed, significant reduction of TVB-N and peroxide values of treated samples compared to control samples. Hence, ZMEO and NZMEO can be used as preservative agent to extend shelf life of meat products such as burgers in refrigerator condition.
Keywords: Beef burger, Nanoencapsulation, *Zataria multiflora* Boiss., Essential oil, Shelf life

1. Introduction

Microbial, chemical and enzymatic activities can be decrease the shelf life of foods (Fontana, Schmidt& Labuza; 2008). In the past, most foods were produced and consumed directly from the natural resources, but nowadays, often chemical preservatives were added to food in order to extend its shelf life. This is while, consumers have found greater insight about adverse effects of chemical preservatives (Khanjari et al. 2013; Tajik, et al., 2015). Therefore, in recent years, numerous studies have been done to evaluate antimicrobial and antioxidant activity of the natural preservatives, such as essential oils, in order to extend the shelf life and increase the safety of food (Parsaeimehr, et al, 2010; Khanjari et al, 2013).

"*Za' atar*", is referred to the Arabic word for some herbs like Thyme, Oregano, and Savory that growth in Middle East countries. *Zataria multiflora* Boiss., with the vernacular name of Avishan-e-Shirazi, belongs to the Lamiaceae family (Mousavi et al., 2010; Preedy, 2015). The plant mainly grows in Iran, Pakistan and Afghanistan and uses as a

natural flavor ingredient in food. Carvacrol and thymol are the main phenolic constituents of this essential oil which has FDA approval for using as food additives and antibacterial food packaging (Khanzadi et al., 2006; Misaghi and Basti, 2009; Mousavi et al., 2010).

One of the effective method to increase the physical stability of active compounds and nutritional ingredients as well as protecting active compounds against environmental factors such as oxygen, light, moisture and pH is nanocapsulation (Donsi et al., 2011). Liposomes, single or multiple layers of amphiphilic polymolecular membranes, have been used to encapsulate of essential oils (Taylor and Davidson, 2005; Donsi, 2011).

Beef burgers in Iran prepared by mixing the beef, fat, spices and herbs such as onion and garlic (Sharafati-Chaleshtori, 2015). The aim of this study was to evaluate the effect of free and nanoencapsulated forms of *Zataria multiflora* Boiss. essential oil on some microbial and chemical of beef burger.

2. Materials and methods

2.1.Plant Material

The *Zataria multiflora* Boiss. plant was gathered in the summer (July 2015) from Fars province and its scientific name was confirmed by the Institute of Medicinal Plants, Tehran University of Medical Science, Tehran, Iran.

2.2.EO isolation

The air-dried plant (100g) was hydro-distilled by using Clevenger-type apparatus for 3 h. The analysis of essential oil was done by Gas Chromatography connected to mass spectrometry (GC / MS). The device GC / MS type Thermoquest Finnigan with capillary column 30 meters and inner diameter of 250 micrometers, thickness of the inner layer 0.25 micrometers with a temperature of 50 to 265 ° C for 30 minutes was used. The injection chamber temperature was 250 ° C and the gas was helium at a speed of 1.5 mm per minute. EI detector was with ionization energy of 70 eV and the ionization source temperature was 250 ° C (Basti, et al., 2004; Moosavy et al., 2008).

2.3.Production and preparing nanoliposomes

Nanoliposomes containing the ZMEO were prepared by using thin film evaporation method (Bangham method) as described previously by Khatibi et al. 2016. Soyhosphatidylecoline (10 mg/mL) and cholesterol (2 mg/mL), fat phase, were dissolved in a 500 ml round bottom balloon containing methanol and chloroform (in the ratio 3:1) (grade HPLC). Then, ZMEO was solved in methanol and mixed with phosphatidylecoline and cholesterol. Then thin film was formed on the walls of the balloon by removing the organic solvents by using rotary evaporator. After this step, thin layer of lipid was solved in soluble phosphate buffered saline (PBS) (7.4 = pH) for 15 minutes at 25℃. In the next phase, sonication of final solution was done for 10 min at 4℃ (Liolios et al., 2009; Khatibi et al., 2016).

2.4.Production method of samples

The 3 kg beef and 1.5 kg onion was minced with a steel meat grinder through a plate with 13 mm orifices separately. Then the minced meat and onion were mixed with salt (0.1 kg), fried flour (0.4kg) and different concentration of ZMEO and NZMEO (0, 0.015 and 0.03 %v/w) in the mixer for 5 minutes. Each treatment was formed into patties (100 g portions) by using a meat former, then samples were transferred aseptically to bags and kept under refrigeration temperature (4° C) for 12 days.

2.5.Microbiological analysis

The amount of 10 g of each treatment were homogenized with 90 ml of sterile

Buffered Peptone Water 0.1% (BPW, Merck) solution in a stomacher (Interscience, France) for 2 min. after this step, serial decimal dilutions of each treatment were prepared in BPW 0.1%. Then, appropriate amount of these serial dilutions were transferred to plate count agar (Total mesophilic and psychrotrophic bacteria), and DRBC agar (Mold and yeast). (Patsias et al., 2006; Karabagias et al., 2011)

2.6.Chemical analysis

The measurement of total volatile base nitrogen (TVB-N) and peroxide value (PV) were done according to the method of Reddy and Bhandary, 2015.

2.7.Statistical analysis

All experiments were conducted in independent triplicate. The analysis was done by using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) software package. Microbiological data were transformed into logarithms of the number of colony forming units (CFU/g) and mean and standard deviations were subjected to one way ANOVA test. Tukey's procedure was applied to adjust for multiple comparisons between treatment means, using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) software package. Significance level was statistically considered when $P < 0.05$ in all experimental data.

3.Results and discussions

3.1.Chemical composition of ZMEO

The GC/MS result identified 17 different components which represent 98.06% of ZMEO (Table 1). The dominant compounds of ZMEO in current study were carvacrol (46.40%), thymol (23.61%) and p-Cymene (16.37%). The result of this study was in contrast to the pervious study, (Misaghi and Basti, 2007) that carvacrol was the main constituent (71.12%) of the *Zataria multiflora* Boiss.. In different studies, carvacrol and thymol constituted 30–86% of total ZMEO composition that in accordance with present study. Differences in chemical compositions of essential oils could be attributed to the effects of climate and geographical condition, variety and age of the plant (Valero and Salmeron 2003; Burt, 2004; Bagamboula et al. 2004; Akhila 2009).

Table 1. Chemical constituents of the *Zataria multiflora* Boiss. essential oil identified by gas chromatography-mass spectrometry

Compound	Retention index on the columns *	Amount (%)
Alpha Tujen	924	0.05
Alpha-pinene	930	3.32
Champhen	938	0.11
Beta-pinene	968	0.14
Beta-myrcene	975	0.46
p-Cymene	1003	16.37
Oka Lyptv	1005	0.26
Limonene	1009	0.26
Gamma terpinene	1035	2.38
Thymol methyl ether	1208	0.17

Compound	Retention index on the columns *	Amount (%)
Carvacrol methyl ether	1224	1.19
Thymol	1275	23.61
Carvacrol	1286	46.40
Thymol acetate	1336	0.59
Carvacrol acetate	1348	0.99
Caryophyllene	1414	1.37
Aroma dendrons	1428	0.39
Total	_	98.06

3.2.Microbiological analysis

The initial Total mesophilic bacteria (TVC) of beef burger samples was 4.87 CFU/g. TVC reached to upper microbiological limit (7 log CFU/g) on day 4 for control samples, day 12 for samples containing 0.015 and 0.03% ZMEO, while TVC of burger samples containing 0.015% and 0.03% NZMEO never reached to 7 log CFU/g after a storage period of 12 days. This could be due to the fact that ZMEO is rich of antimicrobial phenolic compounds such as carvacrol and thymol. (Parsaeimehr et al., 2010). These results are in line with other studies reporting a decrease of TVCs of raw meat patties by addition of herbal essential oils or extract. (Sharafati et al., 2015; Hayes et al, 2010; Mohamed et al., 2011; Skandamis and Nychas, 2001; Shahbazi et al., 2015) Also, results of this study showed encapsulated form of ZMEO have stronger antimicrobial activity than its free form that is in agreement with results of Gortzi et al. (2007) that concluded the essential oils of lemon and its main components (β-pinene, γ-terpinene, Neral and α-terpineol) in the form of encapsulated have stronger antimicrobial effects than its pure form, (Fig.1).

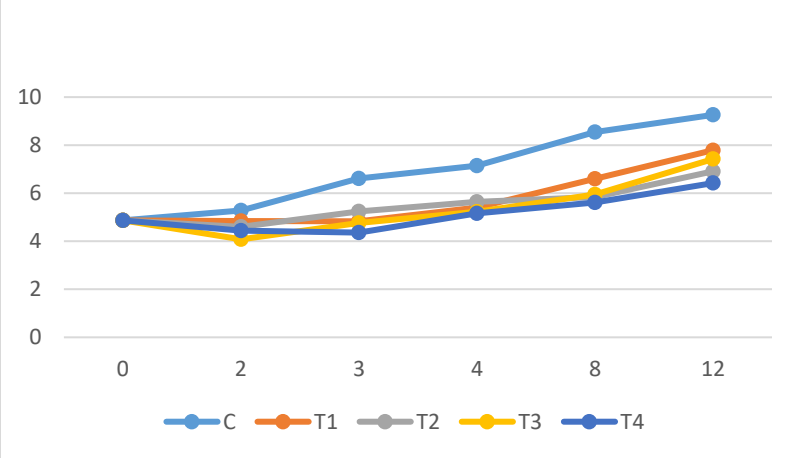

Figure 1. Changes in total viable count (TVC) of beef burgers during refrigerated storage (C: control, T1: treatment with a concentration of 0.015% *Zataria multiflora* essential oil, T2: treatment with a concentration of 0.015% nanocapsulated *Zataria multiflora* essential oil, T3: treatment with a concentration of 0.03% *Zataria multiflora* essential oil, T4: treatment with a concentration of 0.03% nanocapsulated *Zataria multiflora* essential oil)

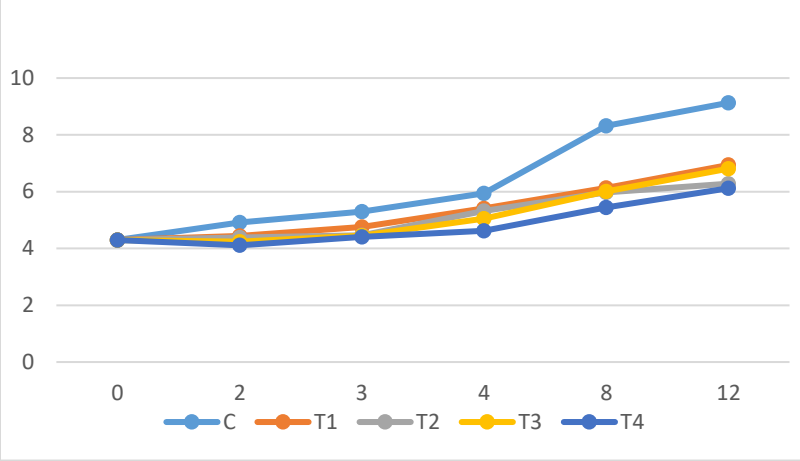

Figure 2. Changes in Psychotropic bacterial count of beef burgers during refrigerated storage (C: control, T1: treatment with a concentration of 0.015% *Zataria multiflora* essential oil, T2: treatment with a concentration of 0.015% nanocapsulated *Zataria multiflora* essential oil, T3: treatment with a concentration of 0.03% *Zataria multiflora* essential oil, T4: treatment with a concentration of 0.03% nanocapsulated *Zataria multiflora* essential oil)

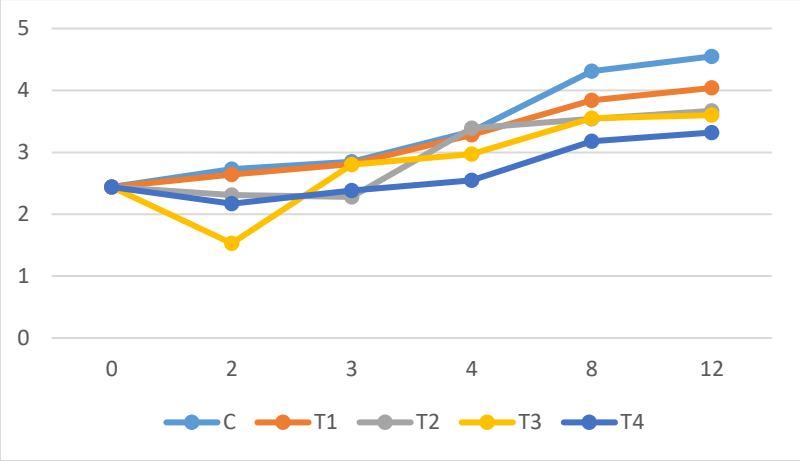

Figure 3. Changes in mold and yeast count of beef burgers during refrigerated storage (C: control, T1: treatment with a concentration of 0.015% *Zataria multiflora* essential oil, T2: treatment with a concentration of 0.015% nanocapsulated *Zataria multiflora* essential oil, T3: treatment with a concentration of 0.03% *Zataria multiflora* essential oil, T4: treatment with a concentration of 0.03% nanocapsulated *Zataria multiflora* essential oil)

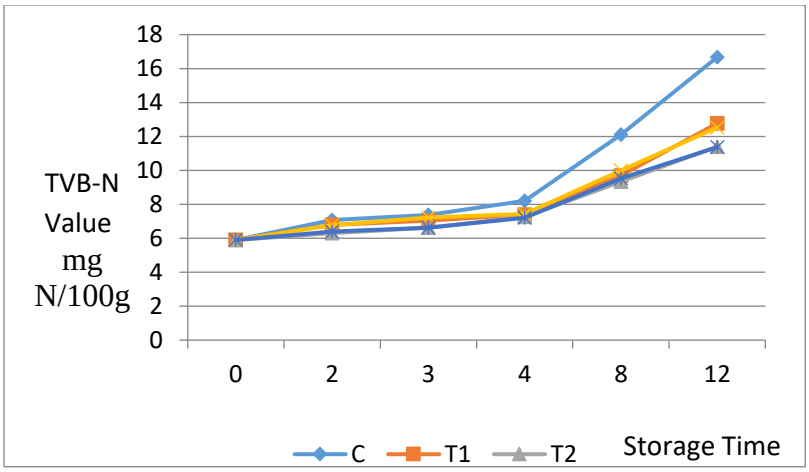

Figure 4. Changes in TVN value of beef burgers during refrigerated storage (C: control, T1: treatment with a concentration of 0.015% *Zataria multiflora* essential oil, T2: treatment with a concentration of 0.015% nanocapsulated *Zataria multiflora* essential oil, T3: treatment with a concentration of 0.03% *Zataria multiflora* essential oil, T4: treatment with a concentration of 0.03% nanocapsulated *Zataria multiflora* essential oil)

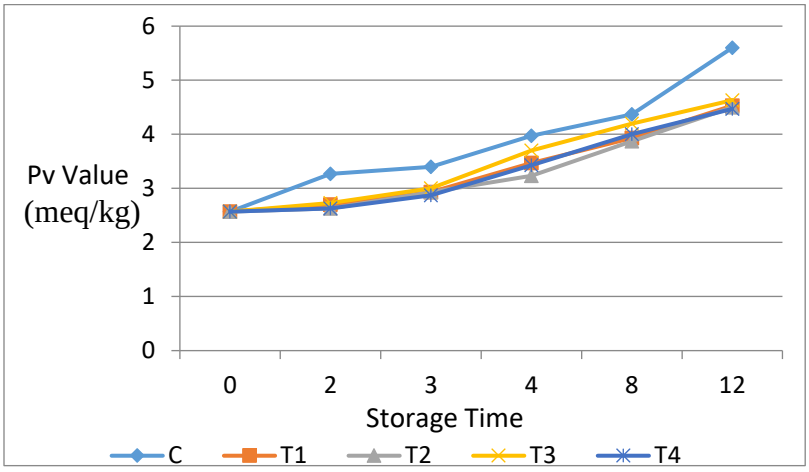

Figure 5. Changes in PV of beef burgers during refrigerated storage (C: control, T1: treatment with a concentration of 0.015% *Zataria multiflora* essential oil, T2: treatment with a concentration of 0.015% nanocapsulated *Zataria multiflora* essential oil, T3: treatment with a concentration of 0.03% *Zataria multiflora* essential oil, T4: treatment with a concentration of 0.03% nanocapsulated *Zataria multiflora* essential oil)

At refrigeration condition, the most important group of microorganisms responsible for spoilage of kept raw meat product are considered psychrotrophic bacteria (PTC). The initial count of psychrotrophic bacteria was found to be 4.30 log CFU/g. At end of study period, all samples incorporated with free and nanoencapsulated ZMEO showed

significantly lower ($P < 0.05$) PTC than control samples (approximately 2-3 log) (Fig. 2). The development of PTC is in accordance with previous studies reported in other studies. (Aytul *et al*. 2004; Giatrakou *et al*.

2010; Karabagias *et al*. 2011; AKSU and Ozer 2012; Shahbazi et al., 2015)

Mold and yeast are able to grow under refrigerator condition. In our study, at first day count of mold and yeast was 2.44 CFU/g. As shown in Fig 3, it reaches 4.55 CFU/g in control samples while their count in samples containing the ZMEO at concentrations of 0.015 and 0.03%, were respectively 4.04 logCFU/g and 3.67 logCFU/g, at the end of study period. The results was in contrast with Zengin and Baysal (2015) results that declare initial numbers of yeasts and molds in Beef patties were almost approximately 6 logCFU/g for samples and reaches to 11.63 CFU/g in control samples. The results found that in treatments containing 0.03% ZMEO and NZMEO were 3.67 logCFU/ g and 3.32 logCFU /g, respectively at the end of study period which is good in agreement with previous studies (Aliakbarlu, 2015).

3.3.Chemical analysis

Total volatile nitrogen (TVB-N)

The TVB-N value at the day 0 was 5.9 mgN/100g and then reached to 16.67mg N/100g in control samples at the end of storage time. As seen in fig 4, compared to control samples, treated samples showed lower TVB-N values. The antibacterial activities of ZMEO and NZMEO resulted to lower TVB-N in treated samples than control samples at the end of storage time ($p<0.05$). (Han, et al., 2014; Raeisi, et al., 2015).

3.4.Peroxide value (PV)

The effect of ZMEO and NZMEO on the changes of PV values are illustrated in Fig. 5. At day 0, the peroxide value initial value was found to be 2.57 meq peroxide/1000 g lipid. At the end of the study, peroxide value in control sample reaches 5.60 meq/kg while peroxide value at twelfth day in samples containing the ZMEO at concentrations of 0.015 and 0.03%, were respectively 4.53 (meq/kg) and 4.50 (meq/kg), also the results found that in the same day, peroxide value in treatments containing NZMEO containing concentrations of 0.015 and 0.03%, were respectively 4.63 (meq/kg) and 4.47 (meq/kg). Moreover off-odors were observed in the control samples probably due to production of molecules especially aldehydes and ketones (Qi, et al., 2013), while no off-odors were found in the treated samples. The result of present study are consistent with Gortzi et al. (2007) and Donsi et. al 2011, that reported increase of antioxidant and antimicrobial activity of oregano and limonene after nanocapsulation in liposomes.

4.Conclusions

Thus, the ZMEO and NZMEO can be used as natural preservatives to extend shelf life of meat products such as burgers in refrigerated condition. The results also showed that the samples of beef burger containing 0.03% NZMEO have the lowest total bacteria, psychotrophic and molds and yeasts count and TVN and PV values. It can be concluded that nanoencapsulation of essential oil can increase of antioxidant and antimicrobial activity of essential oil ($P<0.05$).

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EFFECTS OF SUPERHEATED STEAM DRYING ON THE ANTIBACTERIAL AND ANTI-QUORUM SENSING ACTIVITIES OF SELECTED LABIATAE HERBS

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ABSTRACT

Fresh Labiatae herbs of rosemary, sage, oregano, marjoram, thyme, peppermint and spearmint were analyzed for antibacterial and anti-quorum sensing (anti-QS) activities before and after superheated steam drying (SSD) performed at 150o and 200oC for 10 min. Commercial dried (CD) herbs available from the market were also analyzed for comparison. Among the fresh, CD and superheated steam-dried (SS-D) herbs, only rosemary and sage displayed antibacterial activity against Gram-positive bacteria. No activity was observed in all herbs against Gram-negative bacteria. Tested at extract concentration of 0.5 mg/mL using Chromobacterium violaceum (ATCC 12472), all fresh, CD and SS-D herbs displayed significant quorum sensing inhibition (QSI) compared to the control at $p < 0.05$. All SS-D herbs displayed enhanced QSI that was significantly stronger than fresh herbs but comparable with CD herbs with the exception of SS-D rosemary which had QSI that was significantly stronger than both fresh and CD herbs. At extract concentrations of 0.25, 0.5, 0.75 and 1.0 mg/mL, none of the fresh herbs had any antibacterial effect towards Escherichia coli [pSB401 and pSB1075]. Results of the bioluminescence assay showed that only rosemary had significant anti-QS activity against E. coli [pSB401]. In this study, the effects of SSD on the antibacterial and anti-QS properties of some Labiatae herbs, and the significant anti-QS activity of rosemary against E. coli [pSB401] are reported for the first time.

1. Introduction

Labiatae (Lamiaceae) or the mint family of plants has more than 200 genera with ~4000 species in the Mediterranean region (Naghibi et al., 2005) and ~7000 species worldwide (Raja, 2012). The largest genus is *Salvia* with ~900 species (Venkateshappa and Sreenath, 2013). Labiatae plants are aromatic, densely glandular and rich in essential oils. Leaves are simple and opposite with each pair at right angles to each other. Stems are hairy and quadrangular in cross-section. Flowers are hermaphrodite and inflorescences form whorls arranged in spikes, heads, racemes or cymes. Petals are fused into upper and lower lips (Kokkini et al., 1994; Raja, 2012). Containing essential oils, terpenoids, iridoids, flavonoids and phenolic acids as chemical constituents, Labiatae species are widely cultivated as aromatic plants for use in food flavouring,

perfumery, herbal teas and traditional medicines (Naghibi et al., 2005; Raja, 2012; Venkateshappa and Sreenath, 2013). Among their wide array of biological and pharmacological properties are antioxidant, antimicrobial, analgesic, anti-inflammatory, cytoprotective, chemo-preventive, hypotensive, antispasmodic, antiseptic, sedative, diuretic and cardiotonic activities.

Superheated steam drying (SSD) is an emerging drying technique where moisture from the food product is removed by the temperature difference between the food and the superheated steam in a closed system (Karimi, 2010; Law et al., 2014). When water is heated to its boiling point, saturated steam is produced. Beyond boiling, the steam becomes unsaturated or superheated. At this point, the superheated steam can be used to transfer heat to the product that is being dried and raising the product's temperature to the boiling temperature. SSD offers several advantages over hot-air drying (Karimi, 2010; Law et al., 2014; Mujumdar, 2014). It has been shown that in SSD, energy consumption is lower, smaller equipment may be used, risks of fires and explosions are reduced, and harmful emissions may be eliminated. The system is energy efficient as the exhaust steam can be recycled back to the system and only 30 kJ of heat are needed to convert the saturated steam to superheated steam. A recovery of 60−70% of the exhaust steam is possible and this represents significant energy saving compared to the hot-air drying system. Lacking oxidative reactions, SSD has the ability to maintain colors and nutrients of products with high porosity. Studies have shown that some superheated steam-dried (SS-D) food products such as sugar-beet pulp, potatoes, Asian noodles and spent grains maintain good quality (Pronyk et al., 2004).

Quorum sensing (QS) is a process whereby bacteria communicate among themselves by producing, releasing, detecting and responding to chemical signal molecules called autoinducers (Miller and Bassler, 2001; Waters and Bassler, 2005). Some bacteria use QS to monitor cell densities and regulate gene expression in response to fluctuations in cell-population density. Generally, Gram-negative bacteria use acylated homoserine lactones (AHL) as autoinducers while Gram-positive bacteria use processed oligo-peptides to communicate.

Gram-negative bacteria such as *Chromobacterium violaceum* CV026, *Escherichia coli* [pSB401] and *E. coli* [pSB1075] have been used by researchers in screening for extracts or compounds with anti-QS properties (Koh et al., 2013). These biosensors do not possess the ability to produce any AHL, when supplied exogenously, can induce QS traits such as bioluminescence (*E. coli*) and violacein production (*C. violaceum*), which can be quantified. Much research has been focused on quorum sensing inhibition (QSI) for the development of therapeutic agents to control bacterial pathogenesis (Vattem et al., 2007). QSI offers an alternative to antibiotic treatment, which reduces the risk for development of bacterial resistance and virulence. To date, an increasing number of studies have reported on plant species with QSI against *C. violaceum* and *E. coli* (Koh et al., 2013).

Drying and cooking methods and conditions can impart changes to the biochemical properties of culinary herbs and spices. Such studies on Labiatae (Lamiaceae) herbs are still lacking as noted by Yi and Wetzstein (2011). In our previous studies, the antioxidant, antibacterial and anti-quorum sensing (anti-QS) properties of Labiatae herbs, and the effects of microwave, blanching and boiling were analyzed (Chan et al., 2012a, 2012b). In another study, we reported the antioxidant, anti-tyrosinase, antibacterial and anti-QS properties of

selected spices, and the effects of microwave, blanching and boiling (Chan et al., 2015). In this study, the antibacterial and anti-QS properties of some fresh and commercial dried (CD) Labiatae herbs were analyzed and evaluated. The effects of SSD on their antibacterial and anti-QS properties are reported for the first time.

2. Materials and methods

2.1. Herbs studied

Fresh Labiatae herbs grown in Genting Highlands, Pahang, Malaysia were purchased in local supermarkets in Kuala Lumpur. They included rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), oregano (*Origanum vulgare* L.), marjoram (*Origanum majorana* L.), thyme (*Thymus vulgaris* L.), peppermint (*Mentha piperita* L.), and spearmint (*Mentha spicata* L.). The herbs were screened for antibacterial and anti-QS activities before and after SSD. CD herbs of rosemary, sage, oregano, thyme and peppermint available in the market were also analyzed for comparison.

2.2. Extraction of herbs

Fresh herbs (10 g) and dried herbs (3 g) were ground in a mortar using liquid nitrogen. The powdered herbs were extracted with 100 mL of 70% methanol under continuous swirling at 100 rpm. Extraction was repeated three times for 1 h each time. After filtering, the extracts were oven-dried at 40^{o}C overnight and freeze-dried for 30 min to remove any residual solvent present before they were kept in a chiller for further analysis.

2.3. Superheated steam drying

Fresh herbs were superheated steam-dried (SS-D) at 150^{o} and 200^{o}C for 10 min using a superheated steam oven (Healsio, AX-1600, SHARP). The oven was preheated at the required temperatures for 2 min before the herbs (20 g) were dried. On cooling, the dried herbs were weighed, sealed in an airtight bag and stored in a freezer at −20^{o}C for further analysis.

2.4. Antibacterial activity

Antibacterial activity of herbs was screened using the disc-diffusion method following our previously described procedures (Chan et al., 2012a, 2012b; Tan and Chan, 2014). Agar cultures of Gram-positive *Micrococcus luteus* (ATCC 10240), *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus*, and Gram-negative *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* were prepared. The identity of the bacterial species was verified using the VITEK® 2 microbial identification system. Inoculums (100 µL) were spread evenly onto 20 mL Mueller-Hinton agar set in 90 mm Petri dishes using a sterile cotton swab. Sterilized paper discs (6 mm diameter) were impregnated with 2 mg/disc of plant extract using a micropipette and firmly placed onto the inoculated agar, ensuring even distribution to avoid overlapping of zones. After incubation overnight at 37^{o}C, the mean diameter of inhibition zone represented by a halo around the disc was noted.

2.5. Anti-QS activity against *C. violaceum*

The violacein inhibition assay as described by Blosser and Gray (2000) and Chan et al. (2016) was used to assess anti-quorum sensing (anti-QS) of herbs against *Chromobacterium violaceum* (wild type, ATCC 12472). Herb extracts were dissolved in methanol to produce stock solutions of 5 mg/mL. The stock solutions (0.5 mL) were transferred onto sterile Petri dishes with 4.5 mL of fresh nutrient broth inoculated with *C. violaceum*. Optical density at 720 nm (OD_{720}) was 0.100 A, which corresponded to 1.5 x 10^7 cfu/mL. The transferring of stock solutions would dilute the extracts to a final working concentration of 0.5 mg/mL. The Petri dishes

were incubated for 24 h at 26°C with gentle swirling at 100 rpm before the cell density was measured at OD_{720}. A streak plate of each Petri dish was done to ensure no contamination and that the optical density was a valid representation of cell density. To measure violacein production, 2 mL of broth culture were centrifuged at 13,000 rpm for 15 min to recover the *C. violaceum* cells. Violacein was then extracted using 2 mL of butanol with sonication and absorbance of the extracted violacein was measured at 577 nm (A_{577}) using a Secoman UniLive 9400 UV-vis spectrophotometer against a blank solution of butanol. Violacein production was expressed in terms of violacein units (VU), calculated as the ratio of A_{577}/OD_{720}. Lower ratio value would indicate stronger anti-QS activity.

2.6. Antibacterial activity against *E. coli* strains

The antibacterial activity of fresh herbs against *E. coli* strains was screened using the microtitre broth method based on optical density, following the procedures of Priya et al. (2013). Strains of *E. coli* [pSB401 and pSB1075] were cultured in Luria Bertani (LB) broth consisting of 1% peptone, 0.5% yeast extract and 0.5% NaCl dissolved in 100 mL of distilled water with agitation at 220 rpm. The *E. coli* strains were cultured at 37°C supplemented with antibiotics. Screening for antibacterial activity involved culturing of the *E. coli* strains with the different herb extracts overnight and optical density was read at 600 nm (OD_{600}) for 25 min using the Tecan (Infinite M200) multimode micro-plate reader. The culture without any extract served as control.

2.7. Anti-QS activity against *E. coli* strains

The bioluminescence assay as described by Krishnan et al. (2012) was used to assess the anti-QS activity of fresh herbs against *E. coli* strains. The assay was conducted in 96-well microtitre plates using the Tecan (Infinite M200) multimode micro-plate reader with optical density and luminescence detection. Overnight cultures of *E. coli* [pSB401 and pSB1075] were diluted 1:100 in fresh sterile LB medium, and aliquots (180 µL) with appropriate exogenous *N*-acyl homoserine lactones (AHL) were loaded into each well containing 20 µL of sterile plant extract. The *E. coli* strains [pSB401 and pSB1075] were supplemented with C6-HSL (0.1 µg/mL) and 3-oxo-C12-HSL (0.1 µg/mL), respectively. Bioluminescence based on relative light units (RLU) and optical density at 495 nm (OD_{495}) was determined at 30-min intervals for 24 h, automatically and simultaneously. The inhibition of bioluminescence was expressed as RLU/OD_{495}. The lower the ratio value, the stronger is the anti-QS activity. Experiments were done in triplicate and the data presented as means ± standard deviations (SD) and analyzed by one-way analysis of variance with significance at $p < 0.05$.

3. Results and discussion

3.1. Antibacterial activity

Among the fresh, SS-D and CD herbs, only rosemary and sage displayed antibacterial activity against Gram-positive bacteria of *B. cereus*, *M. luteus* and *S. aureus* (Table 1). At 2 mg/disc, the diameters of inhibition zones (DIZ) were 7−10 mm for rosemary and 8−11 mm for sage. Overall, sage demonstrated slightly stronger antibacterial activity than rosemary, and SS-D and CD herbs displayed weaker antibacterial activity than the fresh samples. The DIZ of SS-D and CD herbs were 7−9 mm except for $SS\text{-}D_{200}$ sage which no longer exhibited any activity. Compared to streptomycin, their antibacterial activity was 1−3 fold weaker. No activity was observed in fresh, SS-D and CD herbs against Gram-negative bacteria of *E. coli*, *S. typhi* and *P. aeruginosa*.

Table 1. Antibacterial activity of SS-D herbs in comparison with fresh and CD herbs based on diameter of inhibition zone

Labiatae herb		Diameter of inhibition zone (mm)		
		B. cereus	*M. luteus*	*S. aureus*
Rosemary	Fresh	7±0	10±1	9±1
	SS-D_{150}	8±1	8±2	9±1
	SS-D_{200}	7±1	7±1	7±1
	CD	9±0	8±1	8±1
Sage	Fresh	11±1	11±2	9±1
	SS-D_{150}	8±0	8±2	9±1
	SS-D_{200}	NA	NA	NA
	CD	8±1	9±1	8±1
Streptomycin		21±2	22±3	17±1

Abbreviations: CD = commercial dried, SS-D = superheated steam-dried at 150 and 200ºC, and NA = no activity. Extract dose was 2 mg/disc. The extracts inhibited the growth of Gram-positive bacteria of *Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus* but not Gram-negative bacteria of *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*.

An early study on the sensitivity of food-borne bacteria (22 Gram-negative and 24 Gram-positive) to sage, rosemary and allspice also reported similar findings (Shelef et al., 1980). At extract concentration of 2%, Gram-positive bacteria were more sensitive than Gram-negative bacteria, and sage had the highest antibacterial activity followed closely by rosemary. Using the agar well-diffusion technique, the essential oil of sage also displayed stronger antibacterial activity than that of rosemary (Bozin et al., 2007). At concentrations of 20% and 50%, both essential oils inhibited the growth of *Shigella sonei*, *Salmonella enteritidis*, *S. typhi* and *E. coli*, but not *P. aeruginosa*. Martac and Podea (2012) reported that the essential oil of rosemary (10 and 20 µL) inhibited Gram-negative *Klebsiella pneumoniae* and *E. coli* but not Gram-positive *Enterococcus faecalis* and *S. aureus*. Against *E. coli*, the strong antibacterial effect of rosemary and mint oil was bacteriostatic, unlike the bactericidal effect of thyme oil (Kalaba and Kalaba, 2014).

Gram-negative bacteria are known to be more resistant to antibiotics than Gram-positive bacteria. This phenomenon has been ascribed to the cell structures. Gram-negative bacteria have an outer membrane as well as periplasmic space in the cell wall, which are not found in Gram-positive bacteria (Bosnic et al., 2006; Shan et al., 2007). Rich in lipopolysaccharides, the outer membrane presents a barrier to the penetration of antibiotics while enzymes in the periplasmic space are capable of breaking down molecules introduced from outside. Gram-positive bacteria do not have such an outer membrane and cell wall structure. Antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane, resulting in the leakage of cytoplasmic constituents.

Collins and Charles (1987) have attributed the antimicrobial activity of rosemary extract to carnosol and ursolic acid. Chemical components of essential oils with antimicrobial properties have been identified as 1,8-cineole, borneol and camphor in rosemary, and as thujone, 1,8-cineole,

borneol and camphor in sage (Tajkarimi et al., 2010). The antibacterial properties of rosemary oil was attributed to 1,8-cineole (24.5%), camphene (13.4%) and α-pinene (12.9%) as major components (Martac and Podea, 2012).

A follow-up of this study would be to find out if there is any antibacterial activity of the other Labiatae herbs at extract doses greater than 2 mg/disc. In our earlier studies (Chan et al., 2012a, 2012b), fresh (rosemary and sage), CD (rosemary, peppermint and spearmint), and oven-dried (rosemary and sage) herbs showed antibacterial activity against the same Gram-positive bacteria. Using the agar well-diffusion method, rosemary, sage and thyme were reported to inhibit *B. cereus* and *S. aureus* but not *E. coli*, and that oregano inhibited all three bacterial species (Shan et al., 2007). Using the microtitre broth method, rosemary has been reported to inhibit the growth of methicillin-resistant *S. aureus* (MRSA) with MIC_{50} of 512 μg/mL (Quave et al., 2008).

Results of this study showed that drying does not eliminate the antibacterial activity of the herbs as reflected by CD and SS-D rosemary and sage. The antibacterial activity of SS-D herbs, along with that of our previous studies on oven-dried herbs (Chan et al., 2012a, 2012b), and on microwave-dried, blanched and boiled spices (Chan et al., 2015), would add some new insights into the understanding of the antibacterial activity of processed herbs and spices.

3.2. Anti-QS activity against *C. violaceum*

The broth culture method was used to assess the QSI of each herb against *C. violaceum*. Inhibition was based on the production of violacein, which was represented in violacein units (VU). Fresh, SS-D and CD herbs were tested at extract concentration of 0.5 mg/mL. Among the fresh herbs, strongest anti-QS activity was observed in rosemary (0.63 ± 0.02), followed by spearmint (0.93 ± 0.04) and marjoram (0.96 ± 0.16) (Table 2).

Table 2. Anti-quorum sensing activity of fresh, SS-D and CD Labiatae herbs based on optical density of inhibition of *Chromobacterium violaceum* growth and violacein production

Labiatae herb		A_{577} (violacein concentration)	OD_{720} (CV cell density)	A_{577}/OD_{720} (violacein unit)
Rosemary	Fresh	0.46±0.01	0.73±0.01	0.63±0.02^{d}
	SS-D_{150}	0.21±0.03	1.28±0.26	0.17±0.06^{a}
	SS-D_{200}	0.33±0.01	1.28±0.03	0.26±0.02^{b}
	CD	1.24±0.57	2.43±1.17	0.52±0.01^{c}
Sage	Fresh	1.18±0.01	0.96±0.07	1.24±0.08^{c}
	SS-D_{150}	0.15±0.04	1.00±0.10	0.15±0.03^{a}
	SS-D_{200}	0.87±0.06	1.78±0.09	0.49±0.06^{b}
	CD	0.55±0.13	0.91±0.13	0.60±0.06^{b}
Oregano	Fresh	1.21±0.13	1.16±0.26	1.06±0.13^{c}
	SS-D_{150}	1.42±0.07	1.95±0.02	0.73±0.03^{b}
	SS-D_{200}	0.72±0.02	1.82±0.20	0.40±0.03^{a}
	CD	0.99±0.04	1.62±0.37	0.63±0.12^{b}
Peppermint	Fresh	0.85±0.14	0.76±0.03	1.12±0.13^{c}
	SS-D_{150}	0.61±0.02	1.98±0.21	0.31±0.04^{b}
	SS-D_{200}	0.34±0.05	1.77±0.16	0.19±0.01^{a}
	CD	0.66±0.03	1.86±0.17	0.36±0.05^{b}

Thyme	Fresh	1.05±0.14	0.86±0.01	1.23±0.17[b]
	SS-D_{150}	0.92±0.17	1.88±0.20	0.49±0.04[a]
	SS-D_{200}	0.62±0.18	2.53±0.14	0.49±0.05[a]
	CD	0.72±0.08	1.64±0.09	0.44±0.08[a]
Marjoram	Fresh	1.26±0.14	1.51±0.26	0.96±0.16[c]
	SS-D_{150}	0.76±0.14	1.30±0.19	0.57±0.18[b]
	SS-D_{200}	0.53±0.07	1.39±0.18	0.38±0.00[a]
Spearmint	Fresh	0.74±0.08	0.80±0.06	0.93±0.04[c]
	SS-D_{150}	0.93±0.06	1.62±0.10	0.57±0.00[b]
	SS-D_{200}	0.87±0.09	2.15±0.31	0.41±0.10[a]
Control		1.30±0.21	0.78±0.02	1.67±0.24

Abbreviations: CV = *Chromobacterium violaceum*, CD = commercial dried, SS-D = superheated steam-dried at 150° and 200°C, *A* = absorbance, OD = optical density, and NA = not available. Values are means ± standard deviations (*n* = 3). Violacein concentration and CV cell density were measured at 577 nm (A_{577}) and 720 nm (OD_{720}), respectively. Violacein production, represented in terms of violacein units (VU), was calculated as A_{577}/OD_{720}. Within the VU column of each herb, different superscript letters (a–d) are significantly different at $p < 0.05$, as measured by the Tukey HSD test.

Values of all fresh, SS-D and CD herbs were significant stronger than the control (1.67 ± 0.24), suggesting that they all possess anti-QS properties. Values of all SS-D herbs were significant stronger than fresh herbs and comparable to CD herbs.

Ranking of fresh herbs based on anti-QS activity was rosemary > spearmint ~ marjoram > oregano ~ peppermint > thyme ~ sage. All SS-D herbs displayed enhanced QSI that was significantly stronger than fresh herbs, the strongest being SS-D_{150} sage (0.15 ± 0.03) and rosemary (0.17 ± 0.06). Of the CD herbs, the strongest activity was displayed by peppermint (0.36 ± 0.05) and thyme (0.44 ± 0.08). Ranking of the CD herbs was peppermint > thyme > rosemary > sage ~ oregano. SS-D herbs with QSI that was significantly stronger than fresh herbs but comparable with CD herbs were sage, oregano, peppermint and thyme. Only SS-D rosemary had QSI that was significantly stronger than both fresh and CD herbs

Possible explanations for the enhanced QSI of SS-D herbs are that drying of fresh herbs using superheated steam at 150° and 200°C for 10 min was sufficient to breakdown the cellular constituents, releasing bound phenolic compounds and forming new compounds with potent QSI properties. These may include the chemical components of essential oils. Among the Labiatae herbs, extracts of oregano, sage, rosemary, basil, thyme and peppermint (Nagy, 2010; Vattem et al., 2007), and essential oils of rosemary, lavender and peppermint (Khan et al., 2009; Szabó et al., 2010) are known to have anti-QS properties.

3.3. Antibacterial activity against *E. coli* strains

At extract concentrations of 0.25, 0.5, 0.75 and 1.0 mg/mL, none of the herbs showed any antibacterial effect towards *E. coli* [pSB401] and *E. coli* [pSB1075]. From the OD_{600} curves of the two strains of *E. coli* over a period of 25 min, the extracts matched those of the controls. This confirms that the different concentrations of extracts did not have inhibitory effects on the growth of both *E. coli* strains used in this study. Figure 1 shows an example of the OD_{600} curves of *E. coli* [pSB401] depicting the non-antibacterial effect of different extract concentrations of rosemary.

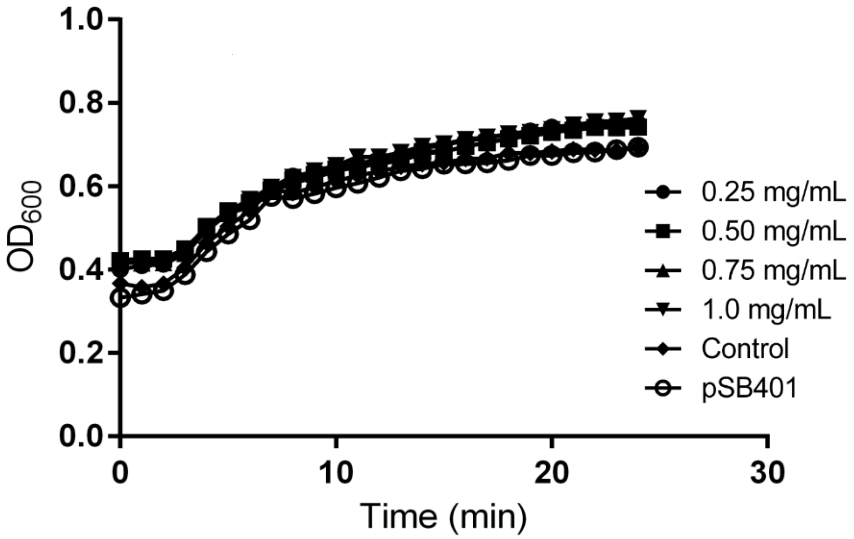

Figure 1. Graph showing the optical density curves at 600 nm (OD_{600}) of *E. coli* [PSB401] cultured with methanol extract of fresh rosemary

3.4. Anti-QS activity against *E. coli* strains

For *E. coli* [pSB401 and pSB1075] cultured separately with each fresh herb extract at concentrations of 0.25, 0.5, 0.75 and 1.0 mg/mL, bioluminescence based on relative light units (RLU) and optical density at 495 nm (OD_{495}) were measured. Results based on the inhibition of bioluminescence (RLU/OD_{495}) showed that none of the herb extracts had any significant anti-QS activity against *E. coli* [pSB1075]. Only the extract of fresh rosemary significantly inhibited the bioluminescence of *E. coli* [pSB401] as shown in Figure 2.

Bioluminescence of *E. coli* [pSB401] is produced in response to short-chain acylated homoserine lactones (AHL) and carries the *luxR* receptor gene that might interfere with the QS-regulated *lux* (Krishnan et al., 2012; Winson et al., 1998). The bioluminescence of *E. coli* [pSB1075] is produced in response to long-chain AHL. Both strains of *E. coli* do not possess the ability to produce any AHL, which have to be supplied exogenously to induce QS traits such as bioluminescence that can be quantified (Koh et al., 2013).

The anti-QS activity of some Labiatae herbs against *C. violaceum* CV026 and CV31532, *P. aeruginosa* PA01 and *E. coli* O157:H7 have been reported by Vattem *et al*. (2007). The study was based on modulation of AHL activity, violacein production and swarming motility. Thyme and rosemary had the ability to interrupt AHL activity and decreased violacein production in CV026 by 60% and 40%, respectively. Oregano did not affect pigment production. When tested with CV31532, slightly different results were obtained. Oregano, rosemary and thyme reduced pigment production by 76%, 68% and 41%, respectively. Experiments on the swarming motility showed that oregano and thyme decreased PA01 swarming by 49% and 48%, and O157:H7 swarming by 19% and 17%, respectively.

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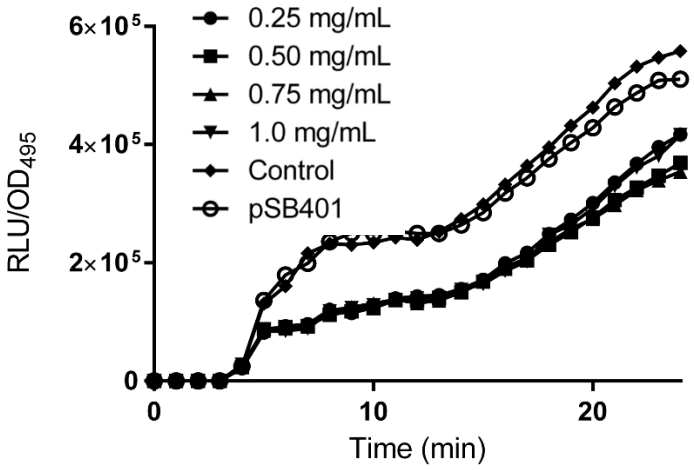

Dunnett's MC test	MD (x10^5)	95% CI (x10^5)	Status
C vs. RM 0.25	1.000	0.074−1.926	Significant
C vs. RM 0.50	1.111	0.186−2.038	Significant
C vs. RM 0.75	1.115	0.189−2.041	Significant
C vs. RM 1.00	1.036	0.110−1.963	Significant

Abbreviations: RLU = relative light units, OD = optical density, MC = multiple comparisons, MD = mean difference, CI = confidence interval, C = control and RM = rosemary.

Figure 2. Graph showing the bioluminescence readings based on RLU/OD495 for *E. coli* [PSB401] cultured with methanol extract of fresh rosemary

4. Conclusions

Among the fresh, SS-D and CD Labiatae herbs, only rosemary and sage displayed antibacterial activity against Gram-positive bacteria with no activity observed against Gram-negative bacteria. All herbs exhibited significant QSI against *C. violaceum* (ATCC 12472). In general, SS-D herbs displayed enhanced QSI that was significantly stronger than fresh herbs and comparable with CD herbs. Only SS-D rosemary had QSI that was significantly stronger than both fresh and CD herbs. Fresh rosemary had significant anti-QS activity but not antibacterial activity against *E. coli* [pSB401]. Overall, SSD sustained the antibacterial activity and enhanced the anti-QS activity of the studied herbs. The effects of SSD on their

antibacterial and anti-QS properties, and the significant anti-QS activity of fresh rosemary against *E. coli* [pSB401] are reported for the first time. As fresh and CD Labiatae herbs are widely sold in the market, the effects of processing on their biological and pharmacological properties warrant further investigations. Drying conditions need to be optimized to maximize the potentials of these herbs for use as pharmaceutical and culinary products.

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EFFECT OF THYMUS DAENENSIS EXTRACT ON OXIDATION STABILITY AND FORMATION OF TRANS FATTY ACIDS IN FRIED PANJEREIE BREAD AND FRYING OIL

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ABSTRACT
The effect of ethanolic Thymus daenensis extract (TE) incorporation, as a natural antioxidant, on oxidation stability as well as the formation of trans fatty acids in used frying oil in preparation of panjereie bread (Iranian traditional cookie) was investigated. The frying was done with a mixture of soybean and palm (1:1) edible oils contained (1, 2 and 3) % TE, in comparison to TBHQ (1 %), at180 °C for 1 hour. According to related results of acid value, peroxide value, conjugated diene value, anisidine value, and total polar compounds analysis; employing the TE or synthetic antioxidant could reduce the formation of oxidation compounds in frying oil in addition to fried panjereie bread. However, by elapsing of time, radical scavenging activity decreased for all samples, the lowest scavenging activity can be correlated to the samples containing 3 % TE. The slowest kinematic viscosity was observed in samples containing 3 % TE. However, the formation of trans fatty acids was increased by elapsing the frying time, the lowest amount of trans fatty acids was observed in prepared panjereie bread with oil containing 3 % TE. Moreover, TE of (1 and 2) % and TBHQ did not have significant difference ($P<0.05$). The TE can be used as a natural antioxidant to reduce the oxidation as well as formation of trans fatty acids in frying oils and fried products.

1. Introduction

The used frying oil in frying as a thermal process for food preparation can act as an environment for transferring the heat to food. The quality of fried food should be correlated with type of food as well as used oil. Due to applied high temperature in frying also in addition to present of the air and moisture in the environment, the physical and chemical degradation process could cause adverse effects on oil performance, quality and safety of fried food (Choe and Min, 2007; Debnath et al., 2009).

However, the produced volatile compounds during the frying can be lost, non-volatile compounds might be accumulated which could stimulate the oxidation and oil degradation and consequent health risks for consumers (Choe and Min, 2007). Such non-volatile products include monomeric triglycerides and polymeric triglycerides that have cyclic fatty acyls and different breakdown products (Urbancic et al., 2014).

According to recent findings, that the correlation between the trans fatty acids and cardiovascular disease were well investigated (Mozaffarian et al., 2006). Although in the past, trans fatty acids were attributed to oil hydrogenation, now the frying can be considered as one of the most important

reasons for the forming of trans fatty acids as function of on frying conditions and the quality of used materials (Liu et al., 2008).

Strong synthetic antioxidants like TBHQ, BHT and BHA are used to prevent the oils degradation during the frying (Zhang et al., 2004).Recently, due to some raised health concerns as result of synthetic antioxidant consumption the application of natural antioxidants attracted considerable attention. One of the natural additives is herbal compounds which could play important role in food industry (Hashemi et al., 2015).

Thymus genus that is known as Avishan in Iran is one of the important aromatic plants which due to biological and pharmaceutical characteristics can be used as a medicinal plant. *T. daenensis* is one of the 4 endemic species out of 14available species in Iran which is widely used as drink and food additives (Mozaffarian, 1996).

Formation of oxidation compounds in frying oil and also in food materials during frying is important, so in this study, panjereie bread, a traditional cookie in Iran produced by the deep frying process, is used as a food model. The aim of this study is to investigate the use of *T.daenensis* extract in the stabilization of frying oil, as well as to study the formation of oxidation compounds and trans fatty acids in panjereie bread.

2. Materials and methods

2.1. Materials

Dried leaves of *T. daenensis* plant (12 % moisture) were provided by a local store in Shiraz city (Fars, Iran) in March 2016. Refined frying oil (soybean oil + palm oil) with no additives was provided by the Narges Oil Company, Shiraz, Iran. All of the chemical materials were purchased from Merck (Darmstadt, Germany) and Sigma Chemical Company (Sigma_Aldrich GmbH, Steinheim, Germany).

2.2. Preparation of ethanolic *T. daenensis* extract

T. daenensis leaves were ground to powder then mixed with ethanol in a ratio of (1:10 w/v). Furthermore, the extraction process was carried out for 48 hours at 40 °C. After filtering, the extract was concentrated with a rotary evaporator (Hahn shin, Korea) at 45 °C and stored at -18 °C until the day of experiments.

2.3. Total phenolic compounds

Phenolic content of TE was determined by Folin Ciocalteu method and absorbance of samples was measured at a wavelength of 765 nm. For preparation of the blank, distilled water (400 µL) was used instead of the sample (Kahkonen et al., 1999).

2.4.Total flavonoids

Flavonoids value in TE was determined by using colorimetric of quercetin method. Quercetin (0-500 mg/L) was used as the standard. Sample absorbance was read at 510 nm and flavonoids value was reported in terms of mg/g (Kim et al., 2003).

2.5. DPPH assay

Different concentrations of TE diluted with ethanol were mixed with 1.0 mL of a 0.3 mM DPPH ethanol solution. Samples absorbance was read at a wavelength of 517 nm after 30 minutes of reaction time (Choi et al., 2002).

2.6. Ferric reducing antioxidant power (FRAP) assay

Briefly, 900 mL FRAP reagent was mixed with 90 mL distilled water then warmed to 37 °C in a water bath. The control reading of the reagent was determined at 595 nm. Subsequently, 30 mL of sample solution (100 mg in 10 mL of n-hexane) was added and absorbance was determined at 595 nm against the control solution. $FeSO_4.7H_2O$ was used as the standard and the results were

reported on the basis of mmol Fe^{2+}/g (Benzie and Strain, 1996).

2.7. Frying procedure

TE at concentrations of (1, 2 and 3) % and TBHQ antioxidants at a concentration of 1% were added to the oil. The frying procedure took place with the help of deep-fat fryer with a 2.5-L-volume vessel at 180°C for 1 hour. Oil and panjereie bread were sampled every 5 minutes. In other words, sampling was performed 12 times for 1 hour. Oil samples were filtered and stored at 4 °C.

2.8.Oil extraction

The oil of panjereie bread was extracted with the use of chloroform/methanol (1:2, v/v) (Bligh and Dyer, 1959).

2.9. Determination of acid value

The acid value was determined according to AOCS method (1993) by titration method using potassium hydroxide.

2.10. Peroxide value (PV)

Acetic acid-chloroform oil samples were mixed with 0.5 mL saturated potassium iodide solution (5± 0.05 g) and were titrated using 0.1 N sodium thiosulphate(AOCS, 1998).

2.11. Conjugated diene value (CDV)

Oil samples were mixed with hexane and their absorbance was read at a wavelength of 234 nm (Shimada et al., 1991).

2.12. Anisidine value (AnV)

Oil samples (0.5–4 ± 0.001 g) were mixed with 1 mL p-anisidine and 25 mL isooctane and then their absorbance were read at a wavelength of 350 nm (AOCS, 1998).

2.13. Determination of radical scavenging activity

50 mg of sample oil was mixed with 4 mL DPPH dissolved in toluene (10^{-4} M) and after 60 minutes; their absorbance was read at a wavelength of 515 nm (Ramadan et al., 2006).

2.14. Determination of total polar compounds

The TPC estimation of oil samples was determined directly in the oil, with a food oil monitor testo 265 (Testo company, Germany), according to the manufacturer`s instructions.

2.15. Measurement of kinematic viscosity

The kinematic viscosity of the oil samples during frying was determined with a Cannon Ubbelohde calibrated capillary viscometer (Cannon Instrument Company, State College, Pennsylvania) by the method reported by Debnath et al. (2012).

2.16. Determination of fatty acid composition by GC-FID

Transesterification of fatty acids into their corresponding fatty acid methyl esters (FAMEs)was done by vigorous shaking of oil in hexane (0.3 g in 7 mL) with 2 mL of 7 mol/L methanolic KOH at 50 °C for 10 min The FAMEs were analyzed using an gas chromatography (GC) HP-5890 (Agilent, Palo Alto, CA, USA) equipped with a CP 88 3400 (Varian Inc., Palo Alto, CA, USA) capillary column of fused silica (120 m in length ×0.25 mm in internal diameter, 0.25µm film thickness) using an flame ionization detector (FID). The used carrier gas was helium with a flow rate of 0.8 mL/min. The oven temperature gradient was 5°C each 5 min from 160 °C to 200 °C; temperatures of the injector and the detector were adjusted as 210 °C and 300 °C, respectively(Sherazi et al. 2009; Asnaashari et al. 2015).

2.17. Statistical analysis

Statistical analysis was performed with one-way ANOVA and significant differences

between samples were determined by Duncan's multiple range tests. The SPSS package program (v. 22.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Differences were considered significant at $P<0.05$

3. Results and discussion

3.1. Extract characterization

Some of the observed antioxidants and antimicrobial characterizations in herbal extracts can be correlated to their phenolic compounds. In this context, with increasing in concentration of phenolic compounds, the antioxidants and antimicrobial will be increased (Hashemi et al., 2015). The measured level of phenolic compounds in TE was reported as 95±2 mg/g which is relativity higher than indicated level for other plant extract such as *Matricaria recutita*(41±2.5 mg/g) (Hashemi et al., 2015)as well as lower in comparison with rosemary (112 mg/g) (Chammem et al., 2015). The flavonoids as herbal polyphenols which has been categorized in more than 4000 types can posse antioxidant and antimicrobial activities. The flavonoids amount in the TE was noted as 44±3 mg/g which is higher than explored concentration in *Matricaria recutita* (26±1.4 mg/g) (Hashemi et al., 2015).

Several factors that could affect the phenolic and flavonoids compounds concentration in herbal extracts, can be summarized as the extraction method, alterations in genetic, the grow climate and harvest conditions (Proestos et al., 2006; Chammem et al., 2015).

In the extraction approaches; although methanol, due to high polarity, is usually used as a solvent, due to observed limitations of methanol application in food industries such as toxicity, ethanol was utilized as a solvent in current study (Nor Aini et al., 2008).

3.2. In vitro antioxidants activity of extract

The result of DPPH assays for evaluation of antioxidant activity of TE showed that IC_{50} of the extract was 32.6±1.4 µg/mL whereas the reported IC_{50} for BHT was 18.8±0.7 µg/mL. In comparison to *Matricaria recutita* extract with IC_{50} =82.3±2.8 µg/mL, much antioxidant power by employing of TE can be obtained (Hashemi et al., 2015).

FRAP as a simple and fast method to evaluate the antioxidant activity which was reported as TE 15.85±0.47 mmol Fe^{2+}/g shows that antioxidant power of TE is very good.

The observed good characteristic of an antioxidant could be attributed to phenolic compounds in TE. Phenolic compounds in herbal extract have multi-functional characteristics and can act as reviving, oxygen radicals neutralizing and metals chelating agents. So, as a result of their activities, oxidation processes will be reduced (Su et al., 2007).

3.3. Antioxidant activity of TE during frying

Free fatty acids are formed during the frying process in frying oil due to the formation of free radicals and break down of double bonds. The recorded changes in fatty acid value are presented in Figure 1. In all of the samples the AV increased by passing the frying time. For example, AV reached 1.75 from the initial value of 0.72 during 1 hour in control sample. Ma et al. (2014) reported that AV for Kizakinonatane oil increases by the increment in the frying time. According to results of current investigation, the TE at concentration of 3 % showed the greatest influence in hydrolysis of fatty acids reduction. Also, by elapsing the frying time the AV increased. Ramadan et al. (2006) reported the increase in free fatty acids was due to the oxidation and cleavage of double bonds to produce carbonyl compounds,

which oxidized to low molecular weight fatty acid during frying.

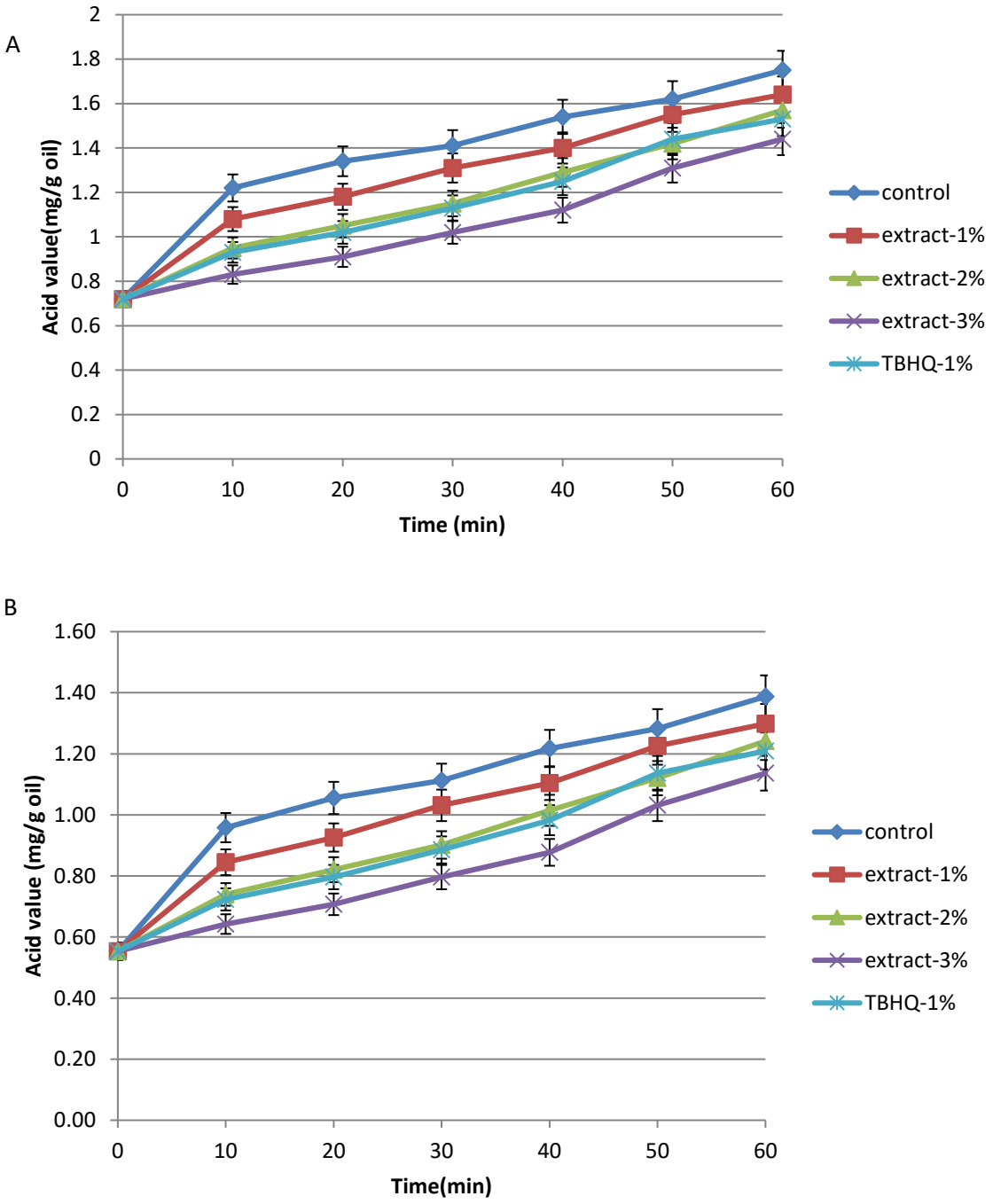

Figure 1. Acid value of fried oil samples (A) and extracted oil from fried panjereie bread samples (B) during frying at 180 °C for 1 h.

Antioxidant activity of TE in frying oil as well as the reduction in oxidation products in panjereie bread was investigated. Peroxide value for frying oil and panjereie bread during frying time over a period of 1 hour is presented in Figure 2.

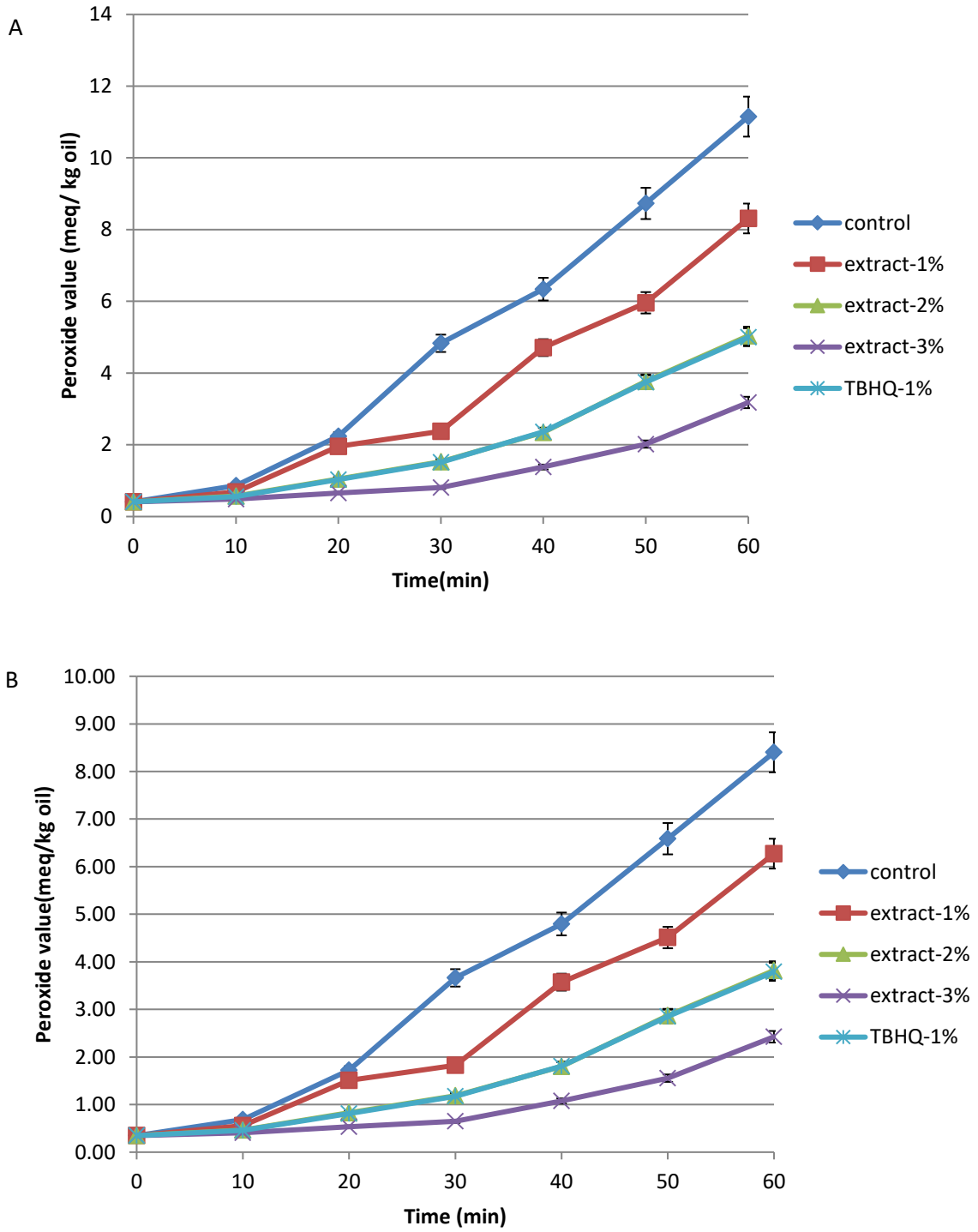

Figure 2. Peroxide value of fried oil samples (A) and extracted oil from fried panjereie bread samples (B) during frying at 180 °C for 1 h.

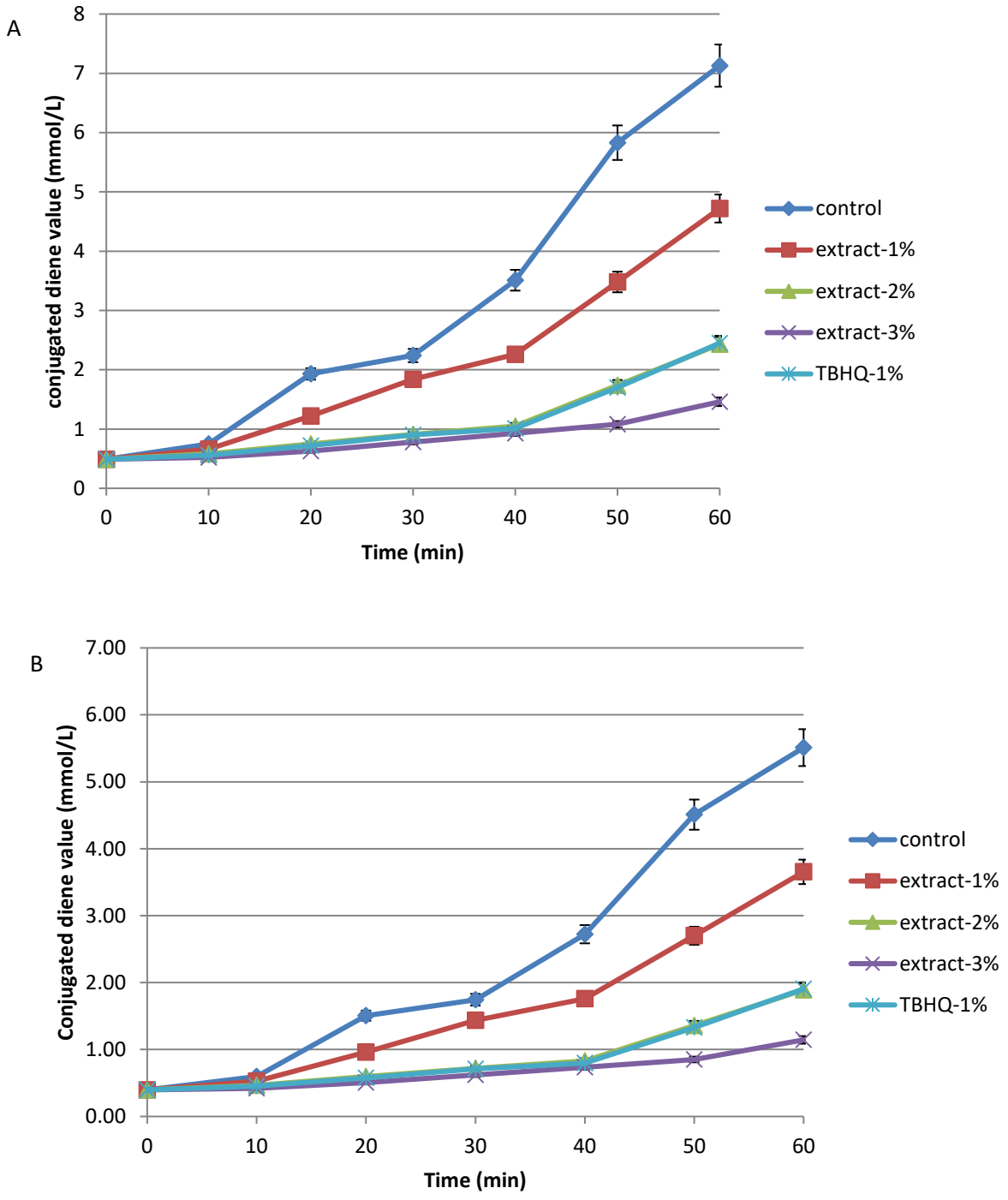

Figure 3. Conjugated diene value of fried oil samples (A) and extracted oil from fried panjereie bread samples (B) during frying at 180 °C for 1 h.

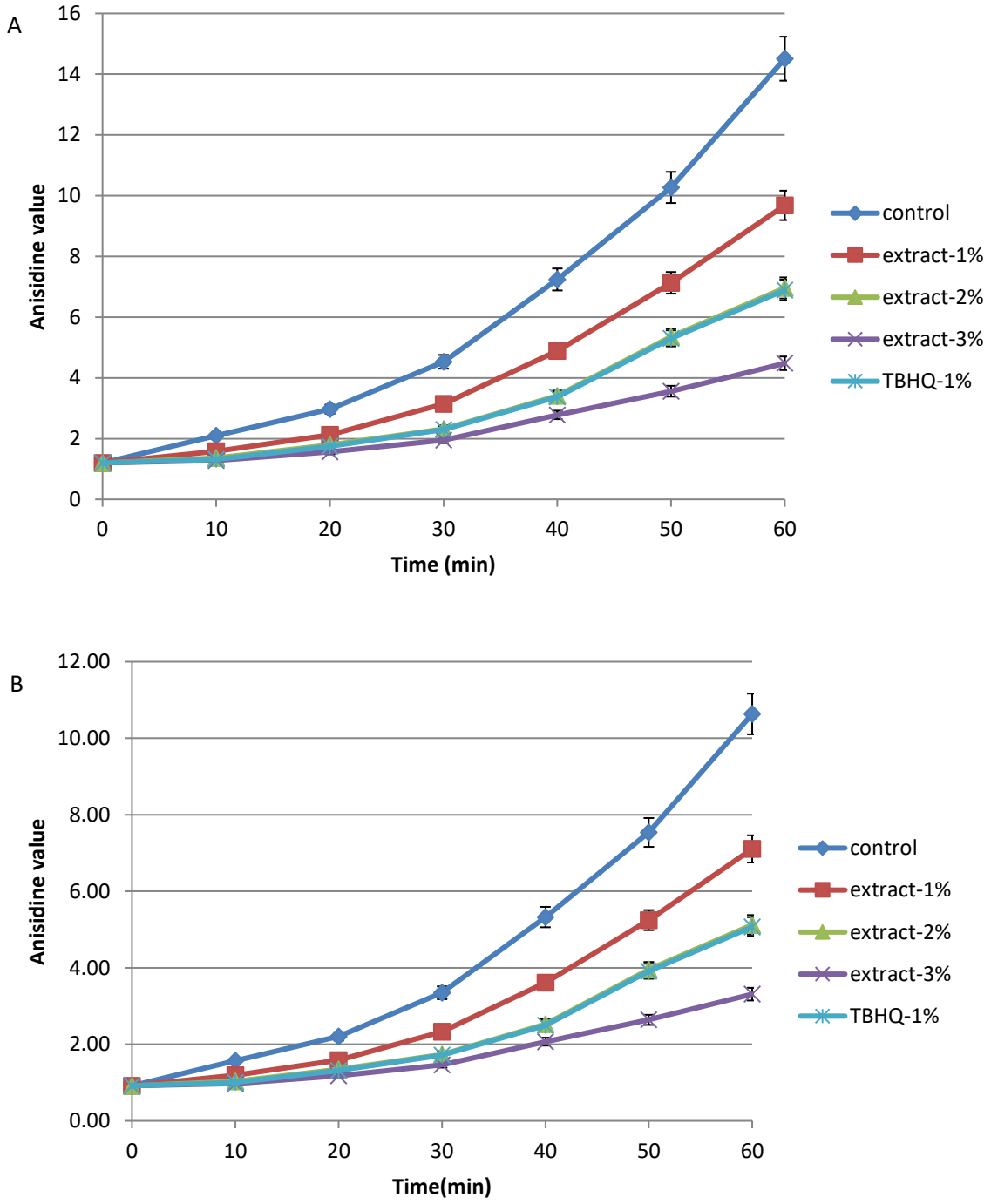

Figure 4. Anisidine value of fried oil samples (A) and extracted oil from fried panjereie bread samples (B) during frying at 180 °C for 1 h.

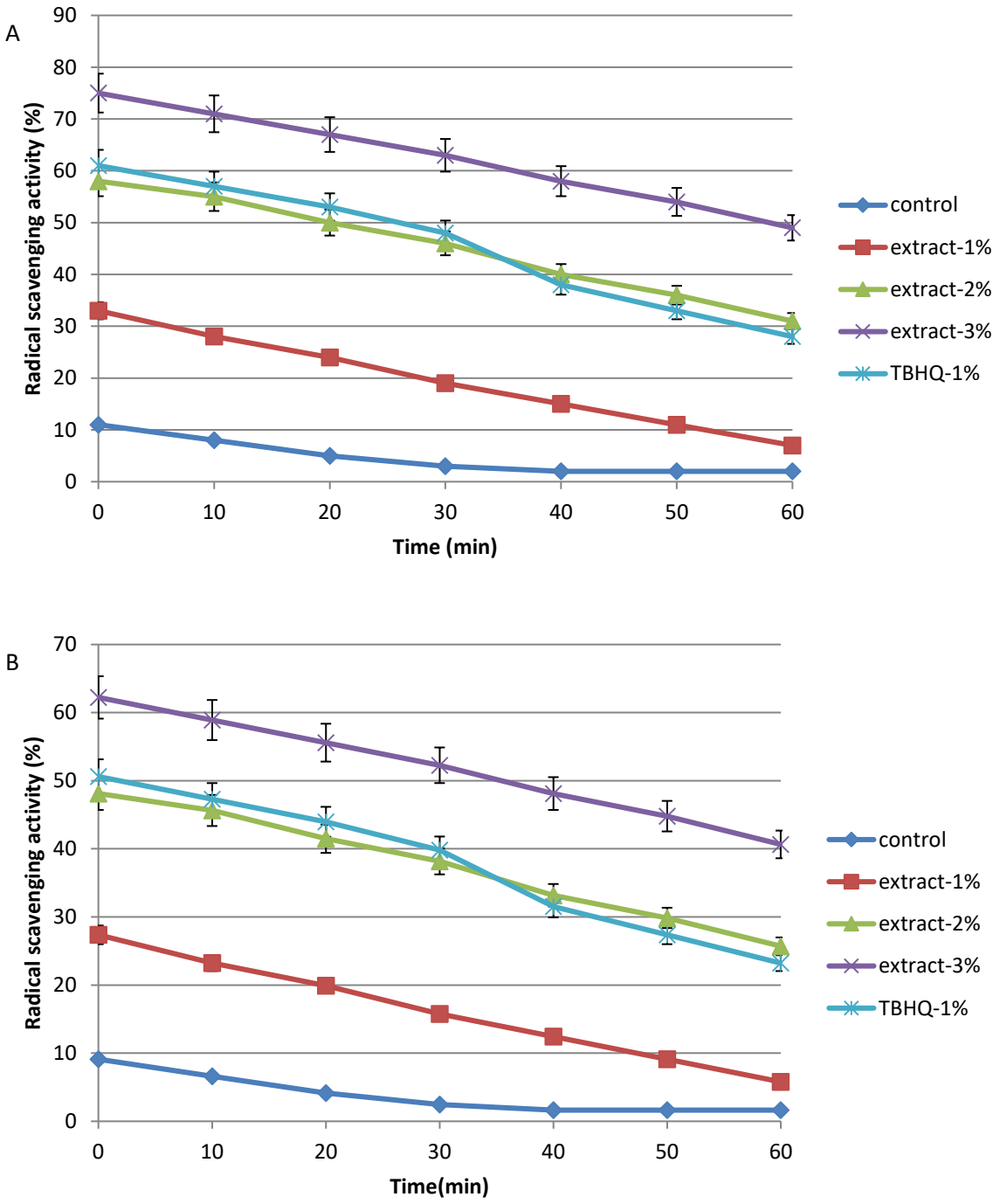

Figure 5. Radical scavenging activity of fried oil samples (A) and extracted oil from fried panjereie bread samples (B) during frying at 180 °C for 1 h.

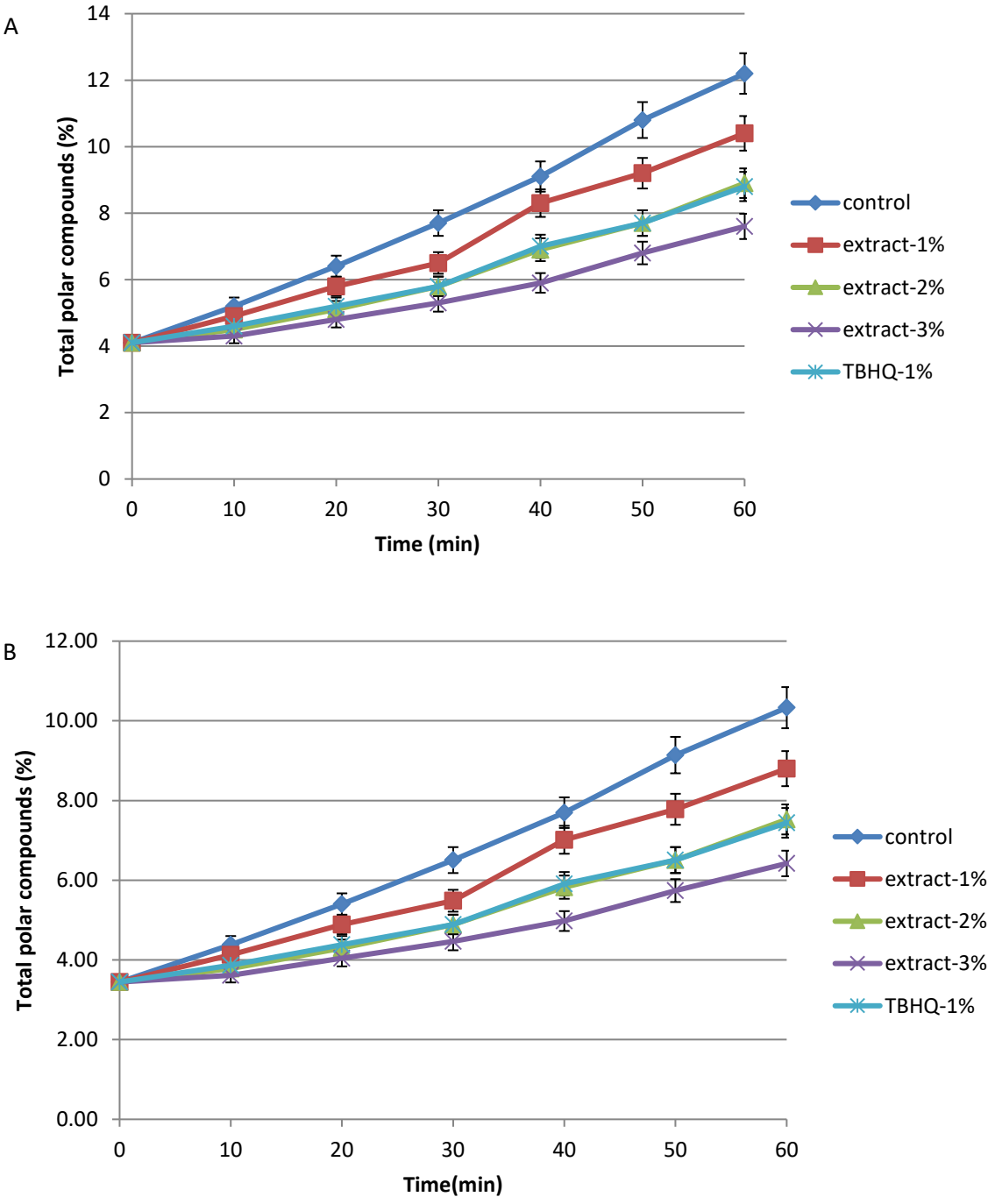

Figure 6. Total polar compounds of fried oil samples (A) and extracted oil from fried panjereie bread samples (B) during frying at 180 °C for 1 h.

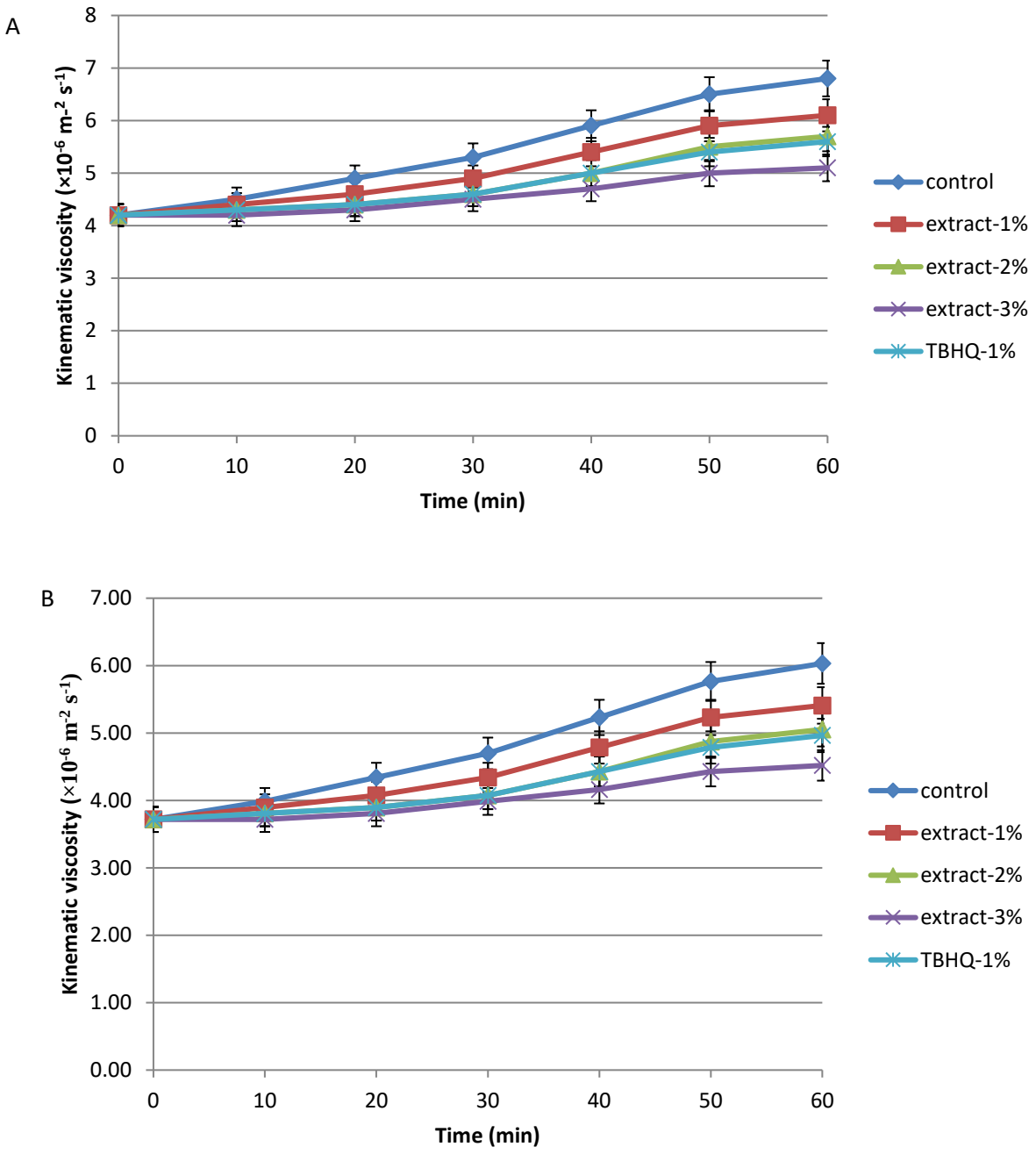

Figure 7. Kinematic viscosity of fried oil samples (A) and extracted oil from fried panjereie bread samples (B) during frying at 180 °C for 1 h.

Peroxide value for frying oil increased by elapsing the frying time. The amount of hydroperoxides in oil was decreased by increasing the concentration of TE. The highest activity in reducing the concentration of hydroperoxide can be achieved with application of TE in concentration of 3%. So that formation of peroxide in panjereie bread during the frying can be reduced by incorporation of TE and TBHQ in different concentrations. Che Man and Jaswir (2000) reported that addition of rosemary extract at a concentration of 0.4 % improved the stability of palm oil and reduced peroxides

formation to 37%. According to Chammem et al. (2015); as result of rosemary extract employing (0.08 %) in conducted frying using the mixture of sunflower oil and soybean oil could reduce hydroperoxides formation to 38 %.

The CD measures the primary oxidation compounds. Although the CD was increased among all of the samples during frying, the lowest level was recorded in the oil samples containing TE of 3 % (Fig.3). After frying for 12 hours, the amount of CD in control sample was higher in comparison with other. Although lower CD was noted for fried panjereie bread, in oil containing antioxidant showed, the lowest can be correlated oil containing 3 % extract. Aachary et al. (2014) reported that addition of crude canolol extract could increase the stability of canola oil during frying; and CD amount after frying was less than other samples. Urbancic et al. (2014) reported that incorporation of rosemary extract into sunflower oil reduces CD formation during frying.

Antioxidant activity of TE was also evaluated by examining the formation of secondary oxidation compounds. The results of anisidine test showed (Figure 4) that AnV was increased for all samples during the frying time. In this study, after 12-hour frying, AnV was measured as 14.51±0.36 for control samples and 4.48±0.13 for a sample containing 3% TE. No notable difference between the sample containing 2 % extract and the sample containing TBHQ was observed. AnV in panjereie bread samples fried by oil containing 3 % TE was less than other samples. Anchary et al. (2014) indicated that phenolic extracts from canola oil deodistillates and crude canolol extract, compared to BHT, had reduced anisidine amount in canola oil during frying.

RSA results showed that potential of oil samples to quenching DPPH radicals reduced during frying (Figure 5). Debnath et al. (2012) reported that RSA was reduced during frying the oil due to the decomposition of the antioxidant and/or the volatilization of antioxidant through evaporation. The extract contains phenolic compounds which can be destroyed by high frying temperature.RSA in oil sample containing 2 % TE, similar to oil sample containing TBHQ 1%, had a decreasing trend. At the end of the frying process, sample containing extract in the concentration of 3 % had the highest RSA. RSA results in samples of panjereie bread were similar to oil samples and the samples fried in oil containing antioxidant had more RSA.

TPC is one of the most important indicators of oil degradation during frying and includes non-volatile cyclic and polymeric compounds. In many countries, when the amount of these compounds in oil rises to 24-25%, consumption of oil is prohibited (Wai, 2007). Variations in TPC for all of the samples during frying are demonstrated in Figure 6. These results show that TPC amount increases by passing the frying time. These results are similar to Houhoula et al. (2002).TPC increase in samples were ordered from highest to lowest as control > 1% TE> 2% TE= TBHQ> 3% TE. These results suggest that the most stable formulation against TPC formation in oil was 3% TE. TPC amount in panjereie bread samples was the greatest amount among samples fried in oil without antioxidant. The existence of TE and TBHQ in frying oil reduced the formation of TPC in panjereie bread. Casarotti and Jorge (2012) reported that when rosemary extract was used as an antioxidant in frying oil, the formation of polar compounds were reduced during frying.

Changes in kinematic viscosity are presented for different samples of oil during frying (Figure 7). Results showed that kinematic viscosity increased with increasing time of frying in all samples. The minimum rate of changes in kinematic viscosity was

observed for oil sample containing 3% TE. Viscosity increase in frying oils is related to polymerization reactions and thermal oxidation. As a result of these reactions, high molecular weight polymeric compounds are formed which increases the viscosity of oil (Al-Harbi and Al-Kahtani, 1993). Teah (1988) reported that as a result of food frying, there is too much water transferred into the oil that will result in oil polymerization and polymeric compounds formation. Debnath et al. (2012) observed that increase in polar compounds in oil made the viscosity of oil to increase. So, there is lower viscosity in oil samples containing 3% TE due to less polar compounds and oxidation of the oil. Quite similar results were obtained in panjereie bread samples and samples containing TE, TBHQ in comparison to control sample had lower viscosity.

Trans fatty acids formation in panjereie bread during frying is showed in Table1. Generally, by increasing the time of frying, trans fatty acids increased in all samples. Most of the isomer trans for fatty acids of samples were of C18:2 type. A number of trans C18:2 after 12-hour frying was measured 0.91±0.06 g/100g and 0.24±0.05 g/100 g for a control sample and sample fried in oil containing 3% extract, respectively. The amount of observed trans fatty acid for control sample was more than other samples. Panjereie bread samples fried in oil containing TE of (1 and 2) % and TBHQ, showed no significant difference in respect of trans fatty acids formation. Panjereie bread samples fried in oil containing 3 % TE showed the lowest level of trans fatty acids among samples. Generally, the results indicated that the extract reduces the formation of trans fatty acids and also degrades polyunsaturated fatty acids. Romero et al. (2000) reported that the formation of trans fatty acids after 20 frying cycles for extra virgin olive oil, high oleic sunflower oil, and the regular sunflower oil is less than 5 mg/g. Another study showed that by increasing frying time, the amount of trans fatty acids increased in soybean and peanut oil (Sebedio et al. 1996). Yang et al. (2014) reported that the formation of trans fatty acids in fried chicken fillets depends on the initial concentration of this type of fatty acids in frying oil and raw chicken fillet.

Table 1. Trans fatty acids formation in extracted oil from fried panjereie bread samples during frying at 180 °C for 1 h.

	Time (min)	**cis-C18:1**	**trans-C18:1**	**cis,cis-C18:2**	**trans-C18:2**	**cis,cis,cis-C18:3**	**trans-C18:3**
Control	**0**	33.52±0.23^{g}	0.07±0^{e}	31.75±0.11^{a}	0.08±0^{e}	3.32±0.04^{a}	0.03±0^{e}
	30	34.82±0.16^{b}	0.43±0.02^{b}	30.12±0.15^{f}	0.53±0.04^{b}	2.8±0.07^{f}	0.18±0^{b}
	60	36.22±0.31^{a}	0.68±0.07^{a}	29.22±0.12^{g}	0.91±0.06^{a}	1.9±0.05^{g}	0.29±0.02^{a}
Extract-1%	**0**	33.52±0.23^{g}	0.07±0^{e}	31.75±0.11^{a}	0.08±0^{e}	3.32±0.04^{a}	0.03±0^{e}
	30	33.92±0.35^{e}	0.16±0.03^{d}	31.18±0.10^{c}	0.22±0.03^{d}	3.12±0.04^{c}	0.09±0^{d}
	60	34.62±0.15^{c}	0.38±0.05^{c}	30.59±0.13^{e}	0.51±0.05^{c}	2.71±0.03^{e}	0.15±0.02^{c}
Extract-2%	**0**	33.52±0.23^{g}	0.07±0^{e}	31.75±0.11^{a}	0.08±0^{e}	3.32±0.04^{a}	0.03±0^{e}
	30	33.82±0.31^{e}	0.15±0.03^{d}	31.10±0.12^{c}	0.20±0.04^{d}	3.19±0.06^{c}	0.08±0.02^{d}
	60	34.54±0.13^{c}	0.35±0.04^{c}	30.47±0.14^{e}	0.47±0.04^{c}	2.76±0.04^{e}	0.13±0.03^{c}
Extract-3%	**0**	33.52±0.23^{g}	0.07±0^{e}	31.75±0.11^{a}	0.08±0^{e}	3.32±0.04^{a}	0.03±0^{e}

	Time (min)	cis-C18:1	trans-C18:1	cis,cis-C18:2	trans-C18:2	cis,cis,cis-C18:3	trans-C18:3
	30	33.22±0.11^{f}	0.09±0.02^{e}	31.54±0.13^{b}	0.11±0.03^{e}	3.26±0.04^{b}	0.05±0.03^{e}
	60	34.07±0.16^{d}	0.19±0.06^{d}	30.87±0.16^{d}	0.24±0.05^{d}	2.93±0.02^{d}	0.09±0^{d}
TBHQ-1%	**0**	33.52±0.23^{g}	0.07±0^{e}	31.75±0.11^{a}	0.08±0^{e}	3.32±0.04^{a}	0.03±0^{e}
	30	33.79±0.30^{e}	0.14±0.03^{d}	31.12±0.11^{c}	0.19±0.03^{d}	3.17±0.05^{c}	0.09±0.02^{d}
	60	34.50±0.12^{c}	0.33±0.05^{c}	30.41±0.13^{e}	0.45±0.05^{c}	2.75±0.03^{e}	0.14±0.02^{c}

4. Conclusions

In this study, TE was incorporated to increase the stability of frying oil against oxidation and to reduce the formation of oxidation compounds as well as trans fatty acids. As result of phenolic compounds existence in the extract the potential to reduce hydroperoxides, secondary compounds of oxidation, polar compounds, and trans fatty acids in frying oil and fried panjereie bread was demonstrated by the TE. Further research is required to evaluate the efficiency of TE extract in combination with other conventional technologies.

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INFLUENCE OF POSTHARVEST TREATMENT BY ANTIOXIDANTS AND CALCIUM CHLORIDE ON THE QUALITY OF PEAR FRUITS DURING STORAGE

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ABSTRACT

Are given of weight loss and assessment of commodity fruits pear variety of Conferencia processed before storage by solutions askorutin 3%, 3% calcium chloride and horseradish root extract.

It is found of advantages of postharvest treatment of pear fruits with 3% ascorutin solution during storage. Treatment with 3% ascorutin solution makes it possible to store pear fruits in the refrigerator at 2 ± 1° C and relative humidity of 90-95% up to 91 days while maintaining quality. In particular, weight loss is 6.2% and the yield of marketable products is 89.9%.

1.Introduction

A pear fruit is a valuable industrial crop due to the presence of sugars, acids, tannins and nitrogenous substances, pectins, flavonoids and vitamins A, C, E, K and B, micro and macro elements. In addition to rich vitamin complex, fruits are considered as a dietary energy-restricted product of 40.2 kcal/ 100g. However, their tender juicy pulp and a thin peel are the unreliable barrier to the growth and penetration of microorganisms into fruits. Therefore, the development of new technologies of protection against damages by microorganisms and preservation of fruit quality is important.

An important indicator of product preservation is the natural weight loss. It occurs as a result of evaporation and respiration of fruits. During the respiration there are processes of oxidation of organic substances with the release of heat and water that enters the air (Murashov S.V., 2012; Serdiuk M.E., 2002).

During storage weight loss depends on several factors: intensity of respiration and allocation of physiological heat, ratio of free and bound liquid in plant tissues, water permeability of cytoplasmic membranes and ground tissues. Moreover, the energy, accumulated in cells, is used for the work of protective systems (Yatsukh O.V., 2005). Therefore, the natural weight loss of products of vegetable origin can be included into integrated indicators characterizing peculiarity of plant raw material to keep quality stability during storage.

Largely, methods and storage conditions affect the quality and duration of storage of fruits. Parameters of these conditions are chosen so as to reduce the intensity of metabolism to the minimum level. Factors of storage are regulated elements of the environment that can help to reduce costs and extend the possible period of storage (Priss O.P., 2000).

One of the most important factors during fruit storage is the postharvest treatment of

fruits with substances that inhibit processes of respiration, ripening and increase resistance of fruits to functional disorders and microbial damage. These substances are calcium chloride, horseradish root extract and ascorutin (Zhhenty M.S., 2006; Paronian V.H., 2003, Pat. 31090).

Treatment of fruits with calcium chloride before storing increases the resistance of fruits to functional disorders and diseases: browning, bitter pit and swelling (Yatsuh O.V., 2005).

The aqueous extract of horseradish root is a natural component with antioxidant, bactericidal and fungicidal properties (Priss O.P., 2000).

The value of ascorutin is that its ingredients are ascorbic acid and rutin. They are biologically active compounds that potentiate the action of each other. Thus, the inhibition of peroxidation processes is achieved that allows maintaining high product quality. Furthermore, ascorbic acid and rutin are antioxidants which are called synergists with respect to each other. Synergy is that rutin inhibits the oxidation of ascorbic acid and increases its antioxidant properties. At the same time, ascorbic acid increases the activity of rutin. As a result of it, the accumulation of peroxide compounds that cause browning of berries is inhibited (Haprindashvili N.A, 2011; Pat.16271)

The research purpose is the influence of postharvest treatment with antioxidants and calcium chloride on quality of pear fruits during storage.

2.Materials and methods

Pear fruits of Konferentsia variety in the Dutch orchard of Uman National University of Horticulture were chosen for the research in 2014-2015. The fruits were harvested in the stage of gathering maturity. Undamaged and clean fruits in good condition were selected under DSTU 01.1-37-162:2004 and placed in boxes number 5 (GOST 10131-93) with a capacity of 15 kg. They were put in the refrigerator compartment of the production fruit store of the refrigerator at 2 ± 1°C and relative humidity of 90-95%. There were such variants as the check variant – untreated fruits, fruits pretreated with aqueous solutions – horseradish root extract, 3% solution of ascorutin and 3% solution of calcium chloride. Number of replications was 3.

During storage the natural weight loss was determined by weighing (Naichenko V.M., 2001). The product analysis was conducted under GSTU 01.1-37-167:2004. Mathematical data processing was performed (Dospiehov B.A., 2001) with the program "Excel 2000".

3.Results and discussions

One of the objective indicators that characterize the quality of fruits after storage is the weight loss arising from the transpiration and use of energy substances in the respiration processes (Figure 1). The results showed that within three months of storage the weight loss of the check variant accounted for 7.8% and after treating with horseradish root extract and calcium chloride weight losses were 6.6 and 6.4%. The weight loss was the least for pear fruits treated with ascorutin solution – 6.2%.

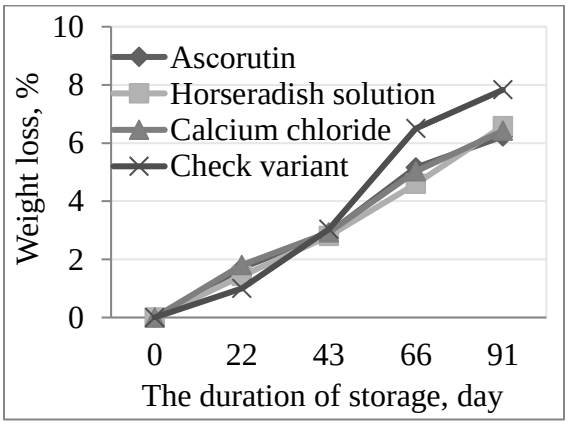

Figure 1. Dynamics of natural weight loss of pear fruits of the Konferentsia variety

during storage, depending on the type of postharvest treatment (NSD_{05} = 0.78)

So, there is a long period of a linear approximation (0.07 ... 0.125% per day) depending on storage conditions (69 days) after relatively large losses (0.05 … 0.1%) per day at the beginning of storage. At the end of the storage period due to activation of metabolism, weight loss increases (0.07 ... 0.1% per day). Our studies have confirmed this trend, as illustrated by the figure. In particular, more intensive weight loss of pear fruits of the check variant was noted within the same period of the storage.

The best results were obtained when treating fruits with ascorutin solution (6.23%). The figure shows that the natural weight loss was less at the end of storage, which is an important prerequisite for selling fruits.

Influence of storage duration was the most effective. The impact of each factor is shown in Figure 2 which shows the influence of treatment method over the weight loss during storage.

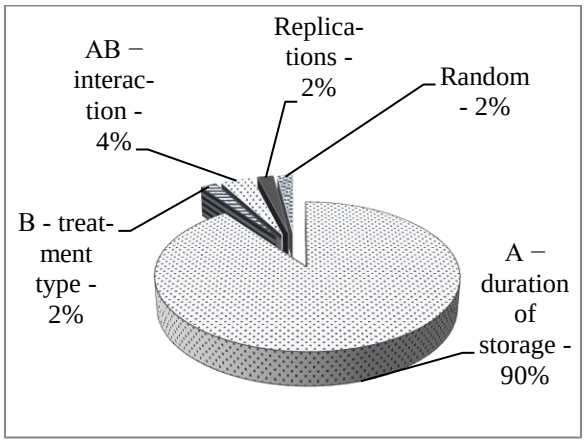

Figure 2. The share of influence of factors A (duration of storage) and B (type of treatment) over the weight loss of pear fruits

The indicator of the weight loss depends on the respiration intensity. According to studies the correlation of these processes is about 3:7 (Yatsuh O.V., 2005). In our studies, the respiration rate of pear fruits at the beginning of storage was 7.3 mg CO_2/ kg hr (Figure 3).

Climacteric respiration rise in the check variant was on day 67 of storage with respiration intensity of CO_2 6.6 mg/ kg hr, while in pre-treated fruits it was lower – 5.4 mg CO_2/ kg hr.

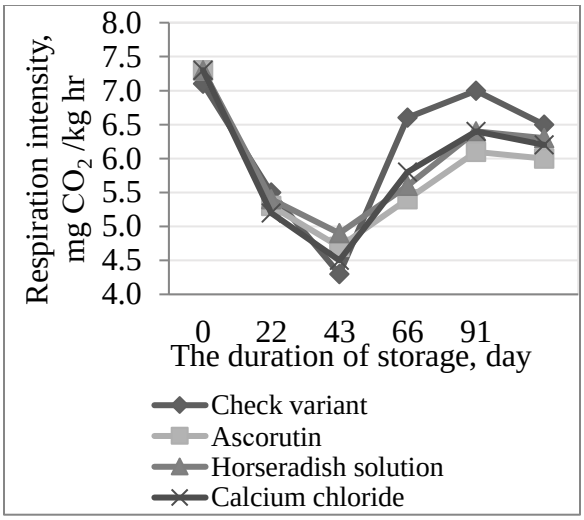

Figure 3. Dynamics of respiration intensity of pear fruits

There is a strong correlation dependence between the loss of weight and respiration intensity of pear fruits and a regression equation is derived (Figure 4).

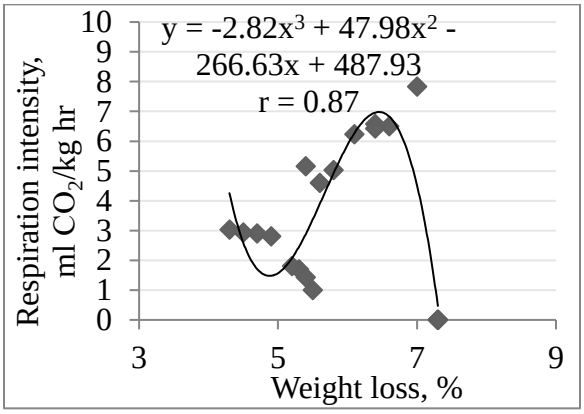

Figure 4. The point chart and the moulded line of regression of straight-line

correlation between weight loss and intensity of respiration of pear fruits

An important indicator of the fruit quality after storage is a commodity-based assessment that determines profit and competitiveness of products in the market.

Figure 5 shows data of the commodity-based assessment of pear fruits after storage.

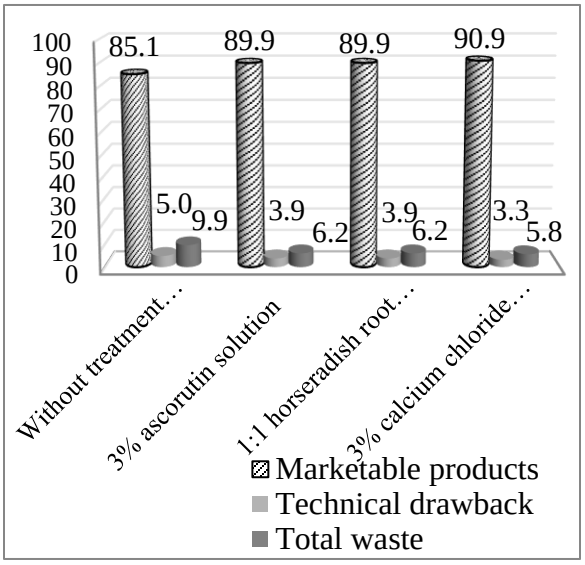

Figure 5. The commodity-based assessment of pear fruits treated with substances of antimicrobial action during storage (average for 2014-2015)

The yield of marketable products amounted to 85.1% after 91-day storage of pear fruits in the refrigerator compartment without surface treatment. 5% of production was the technical drawback because of softening, puckering of fruits and partial change of their color. The total waste was 9.9%.

During 91-day storage period the output of marketable products increased by 3.8% after postharvest treatment of pear fruits with solutions of horseradish and ascorutin compared to the variant without treatment (check variant). The result is directly related to the inhibition of microbial diseases.

Simultaneously, the same treatment of fruits with the solution of calcium chloride increased the yield of marketable products by 4.8%.

4.Conclusions

Thus, the natural weight loss of pear fruits depends on the duration of storage and type of treatment. During 3 month storage of pear fruits the weight loss is the smallest with treatment by ascorutin solution – 6.2%. Also, the intensity of respiration is less. The output of marketable products is 89.9%.

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USING POWDER OF DRY HAWTHORN BERRIES IN BREAD TECHNOLOGY

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ABSTRACT
The appropriateness of additives of non-traditional medicinal raw materials in bread production, in particular hawthorn berries as improvers of natural origin is considered. Study of bread quality indicators for the presence of different number of herbal supplements (hawthorn berries) of multifunctional action in their composition is carried out. Positive changes in physical and chemical parameters and product quality during the preparation of bread by using hawthorn powder are discovered.

1. Introduction

The problem of rational nutrition of population is a priority. Considerable consumption of bakery and flour products by a population suggests it one of the main foods as they are the main source of carbohydrates but the content of important nutrients such as vitamins, minerals and dietary fiber is negligible. According to current views on food, assortment of baking products should be replenished by manufacturing products of high quality and nutritional value, prophylactic and therapeutic purposes.

The desire of enterprises of baking industry to reduce costs of energy, material and human resources, improving technical and economic indices of production better corresponds with accelerated technologies. The use of intensive machining, high doses of yeast, applying acidifiers and improvers in accelerated technologies significantly reduces the duration of dough ripening and the number of necessary equipment. However, according to the market analysis, in the current situation there is a problem of competitiveness of production caused by lack of depth of colloid, biochemical, microbiological processes during its accelerated preparation. Consumers notice decrease in flavor and aroma of wheaten products, rapid pace of hardening, and frequent cases of microbiological spoilage and availability of improvers in the composition of most products (Lebedenko et al., 2014).

The mass consumption of bakery products, on the other hand, gives grounds to nutritiologists to consider them as products of exceptional potential and importance to improve the quality of food of the population, prevention of different diseases and protection of the human body from harmful environmental factors. So the search and development of

activities were important that allow solving problem and tasks of the baking industry to ensure forming quality of finished products that means necessary consumer characteristics and product safety, increasing its nutritional value, physiological properties and their compliance with the requirements of modernity in the economic use of all resources (Lebedenko et al., 2014; Dzhaboeva, 2008).

In Uman National University of Horticulture studies are conducted to determine the chemical composition and nutritional value of a rare medicinal plant (hawthorn berries) which is not widely used in the food industry, especially in bakery as the additive of natural origin. It is known (Dzhaboeva, 2008; Ruzhitska and Lebedenko, 2009) that wild plant raw material, in particular hawthorn, is able to influence biotechnological properties of semi products, technological process, quality of bakery products due to the rich chemical composition, content of food and biologically active substances (BAS). This substantiates the relevance to choose promising raw material from a wide range of herbal supplements to solve problems of bread baking and conducting research to determine its technological properties.

2. Materials and methods

Given the important biological and therapeutic value of hawthorn berries, the aim of the conducted research was to determine the feasibility of using powder of hawthorn berries of Alma-Ata species with a high content of biologically valuable substances in baking production. Also, the purpose was to determine rational parameters of its preparation for the formation of necessary functional and technological properties that provide a positive impact on the technological process and quality of finished products, including by accelerated technologies.

For research we use hawthorn berries of wild Alma-Ata species 2015; powder of hawthorn berries, pulp and peel; high-grade wheat flour made of Favorite wheat variety grown in terms of Right-Bank Forest Steppe of Ukraine with the following quality indicators: humidity – 15%, acidity – 3.0 degrees, mass fraction of gluten – 31.2%, gluten quality – 82 units of ВДК–1 device, ash content – 0.55%; sugar, salt, pressed baking yeast.

Assessment of quality of fresh hawthorn berries was carried out by the conventional method (Naichenko, 2001) and laboratory test bread baking and its organoleptic assessment were carried out by the method of Moscow Technological Institute of Food Technologies (Drobot et al., 2006).

Preparation of hawthorn berry powder, used as an additive, consisted of the following: sorting, washing, inspection, calibration, rinsing, parcooking with live steam at a temperature of 96-100° C during 5-10 minutes which reduces the loss of thermally labile compounds, putting into trays, filling in the warmed dryer, infrared drying at the temperature of the drying agent 80° C which provides high safety of biologically active substances, packaging, packing, grinding to a particle size less than 165 microns. The powder had a light brown color, a pronounced sweet taste, humidity – 8%, inherent smell to hawthorn and storage warranty period of 6-9 months in storage rooms at a temperature of 18-20° C and relative humidity of 65-70%.

To develop formulas and determine the optimal amount of hawthorn powder in bakery products test laboratory baking was performed in recipes of which wheat flour is sequentially replaced with hawthorn powder in the number of 3, 6, 9 and 12% by weight of flour. The effect of powder was studied on the quality and quantity of gluten, as well as quality of finished products (bread). Dough was kneaded at 44.5% moisture and conditions were created for fermentation to final acidity of 3.0 degrees. Dough pieces were put in forms greased with vegetable oil, subjected to maturation and baked at a temperature of 220° C for 30 minutes. Control is bread without additive of hawthorn powder (Drobot et al., 2006).

Mathematical processing of data was carried out with the help of methods of dispersion and regression-correlation analysis using the program Statistica 10. The research program included four analytical replications. With the help of methods of descriptive statistics the results of the research were checked for adequacy and reproducibility. The probability of the absence of the null hypothesis set at the level of 95%. For the formation of the mathematical models the authors used the average values for the four replications that were randomized at the time. Mathematical models were tested for the presence of autocorrelation of residuals using Durbin-Watson theory. Culinary assessment was performed by four experts in three times repetition. Reconciliation of statements of experts were determined by the the value of the coefficient of concordance.

3. Results and discussions

As can be seen from Table 1, the data of the chemical composition of hawthorn berries are within that is given in reference. Hawthorn berries of Alma-Ata species have a high content of dry matter (33.9%), most of which constitute carbohydrates (mono- and disaccharides) which is the main source of energy necessary for life (Naichenko, 2001).

It is found that hawthorn berries contain fiber (1.1%) in their composition which helps to remove cholesterol from the body and prevents atherosclerosis. Mass fraction of pectic substances having the ability to bind and neutralize the action of compounds of some radioactive and heavy metals such as strontium, lead and cobalt is within norms and is 4.7%.

Ascorbic acid plays an important role in the biological value of fruit. As an intermediate catalyst of oxidation-reduction processes, it contains mainly in fruit peel. Its number in Alma-Ata hawthorn species is 18.2 mg/ 100 g. Given the preventive value of hawthorn berries for a man, it is important to examine the possibility of accumulation of a large number of P-active compounds in them (Dzhaboeva, 2008; Naichenko, 2001). The results showed that hawthorn berries contain fairly high amount of polyphenolic compounds (883 mg/ 100 g) and β- carotene (5.0 mg/ 100 g) in their composition which in combination with ascorbic acid have potent antioxidant properties.

Hawthorn berries also contain high amounts of mineral substances, mg/ g: iron – 0.04, potassium – 13.1, magnesium – 1.0, calcium – 3.0, aluminum – 0.03, zinc – 0.07, copper – 0.29 as well as essential oils, resinous substances, fat and tannin components and vitamins of group B. The substances belonging to hawthorn berries dilate coronary and brain vessels, have the ability to reduce the excitability of the nervous system, improve metabolism, reduce blood cholesterol levels and normalize heart rhythms (Kireeva and Gatko, 2008; Systems of food safety management, 2003).

Table 1. Chemical composition of hawthorn berries

Hawthorn species	Dry matter, %	Fibre, %	Pectic substances, %	Ascorbic acid, мг/100 g	Polyphenolic compounds, mg/100 g	β- carotene, mg/100 g
Alma-Ata	33.9	1.1	4.7	18.2	883	5.0
According to references	29.6–47.1	1.4–3.1	0.9–4.8	10–147	230–1980	1.8–5.0

*Values are expressed by mean ± SD of four independent observations, with significance p-value<0.005

Analysis of bread quality was carried out in 14-16 hours after baking. By organoleptic characteristics bread made of flour of Favorite wheat variety meets all specified requirements: surface is smooth, without large cracks and breaks in tin formed bread, flour content of

lower crust is expressed; crumb is baked, elastic, not sticky, not wet to the touch, with developed porosity, without undermixing and dough recovery; taste and smell are characteristic to this name of bread without off-flavour and odor. It is found that physical and chemical properties of the samples were higher than in control (Table 2).

Table 2. Physical and chemical quality indicators of bread

Indicator	White bread made of high-grade flour				
	Control	with the addition of powder of hawthorn berries, % of flour weight			
		3	6	9	12
Humidity of crumb, %	41.0	41.0	41.3	41.5	41.7
Acidity of the crumb, degree	2.8	3.3	3.4	3.5	3.5
Porosity, %	72.0	77.1	76.5	75.8	75.3
Volume of bread	480	490	460	448	432
Specific output of bread, cm^3/ 100 g of bread	376	394	364	355	342
Actual output of bread, g	121.8	127.6	126.3	126.1	126.0
Shape stability, mm	0.50	0.57	0.48	0.46	0.42

*Values are expressed by mean ± SD of four independent observations, with significance p-value<0.005

Table 3. Content and elastic properties of flour gluten

Indicator	Control	Dough samples with the addition of powder of hawthorn berries,% by weight of flour			
		3	6	9	12
Weight fraction of wet gluten, %	32.2	30.1	28.9	27.5	26.6
Weight fraction of dry gluten, %	14.5	13.8	12.4	12.0	11.8
Quality of gluten, units ИДК	82	80	79	77	76
Hydration ability of gluten, %	130.4	131.6	132.5	133.8	135.0

*Values are expressed by mean ± SD of four independent observations, with significance p-value<0.005

The results showed that replacing of flour with powder of hawthorn berries ambiguously affected the indicators of quality of finished products. Moisture content of the samples compared to control changed insignificantly. The highest humidity of bread crumb was in the variant with the addition of 12% powder (41.7%), while in other variants of the experiment this indicator has hardly changed and amounted to 41.0-41.5%. It was found that the presence of hawthorn powder in the recipe contributed to some increase of acidity and lifting force of the dough that in turn has reduced ripening of bakery products for 30 minutes. Thus, the highest acidity (3.5 degrees) was noted in variants with the addition of 9 and 12% powder and the lowest acidity was in variants with the addition of 3 and 6% powder, respectively 3.3 and 3.4 degrees.

Indicator of bread porosity describes the structure, volume as well as digestibility. Low porosity is typical for bread made of badly fermented dough. Minimum value of porosity is specified by standards. The increase in this index indicates greater volume, better

presentation and aerating of the crumb (Drobot et al., 2006).

It was found that the porosity of bread in all variants was satisfactory and amounted to 72.0-77.1% which was by 3.3-4.9% more compared with the control.

Using correlation analysis it was found a strong link between the content of hawthorn, bread volume and its specific output (Fig. 1).

By adding powder of hawthorn berries into the dough in the amount of 3% bread volume increased by 2% compared with control, while putting additive in the amount of 6-12% caused decrease of this indicator by 4.3-10%. The results showed that the specific output of bread with the addition of powder of hawthorn berries was rather high and averaged 366 cm^3/ 100 g of bread. It was the highest in variants with putting additive in the amount of 3 and 6% (394 and 364 g/ 100 g of bread) and the lowest in variants with putting additive in the amount of 9 and 12% (355 and 342 g/ 100 g of bread).

The actual bread output in all variants of test samples has hardly changed and averaged 126.5 g which was by 3.8% more than the control. Shape stability of the tin formed bread was the highest in the variant with the addition of 3% powder (0.57 mm), while in the variant with the addition of 12% powder this indicator was the lowest and amounted to 0.42 mm respectively. This influence of studied additive on the change of physical and chemical indicators of bread quality can be explained by a change in the fermentation activity of yeast cells due to the favorable action of mineral substances of hawthorn berries – potassium and magnesium ions as they have bilateral penetration. Potassium ions have the ability to adjust the osmotic pressure in the yeast cell and magnesium ions intensify effect of almost all yeast enzymes providing energy metabolism of adenylic acids (Kireeva and Gatko, 2008; Systems of food safety management, 2003; Pengelly, 1999).

The influence of additive made of hawthorn berries on the mass fraction, quality and hydration ability of gluten was studied to explain the results of the laboratory test bread baking (Table 3).

The data indicate that when the number of the additive increased from 3 to 12% by weight of flour mass fraction of wet gluten decreased from 10.2 to 17.4% compared with the control but it remained within acceptable norms. The largest content of gluten was in the variant with the addition of 3 and 6% additive and was respectively 30.1 and 28.9%. Slightly lower gluten content was observed in the variants with the addition of 9 and 12% powder (27.5 and 26.6%). Weight fraction of dry gluten in all variants of studies decreased on average by 14%. Its largest number (13.8%) was in the variant with the addition of 3% powder and the lowest number (11.8%) was in the variant with the addition of 12% powder of hawthorn berries.

The results showed that the indicator of gluten deformation also decreased compared to the control sample respectively by 2.4-7.3% regardless of the number of used additive, reflecting the strengthening of elastic characteristics of dough gluten.

Hydration ability of gluten washed out from wheat flour with additives in the amount of 3-12% increased by 0.9-3.5% depending on the variant of the experiment. It was the highest in variants with putting additive in the amount of 3 and 6% respectively 131.6 and 132.5%, while in the variant with 6 and 12% additives this indicator was 133.8 and 135% respectively.

Consequently, replacement of some part of flour by the powder made of hawthorn berries leads to the reduction of gluten and strengthening of its structural and mechanical properties, as evidenced by the decrease of deformation compared with the control. Improving elastic properties of gluten is clearly the result of the formation of protein complexes in the interaction of flour proteins with polysaccharides of used additives. It leads to strengthening the structure of proteic substances due to the additional formation of new bonds (ionic, hydrogen bonds and bonds of hydrophobic action). Polyphenolic

compounds, ascorbic acid and peroxides may also help to strengthen gluten, formed from unsaturated fatty acids, lipids of hawthorn powder and have inhibitory effect on the proteolytic enzymes of flour and contribute to strengthening intra-action of protein (Dzhaboeva, 2008; Ruzhitska and Lebedenko, 2009).

We used mark evaluation of product quality for comparative assessment of consumer properties of bread, taking into account the physical and chemical parameters of bread during laboratory test baking as well as ratio of importance of each indicator, defined on the basis of expert assessments (Drobot et al., 2006).

The statements of the experts were correlated because Coeff. of Concordance were larger than Aver. rank r.

The analysis of the data (Figure 1, 2) showed that by the coefficient of importance and calculations the overall assessment of bread quality of wheat grain flour of Favorite variety in control was 85.5 points, while variants with the addition of 3 and 6% powder of hawthorn berries received the best estimation, respectively 92.5 and 91.0 points, which corresponds to an excellent evaluation. In turn, the variant of the experiment with the addition of 9% powder of hawthorn also exceeded the control variant with the assessment of 87 points and the variant with 12% powder was slightly worse and it was 83 points. Thus, bread of variants of the experiment with the addition of 9 and 12% powder of hawthorn and control was estimated to be good (83-85.5 points) and bread with the addition of 3 and 6% powder of hawthorn was excellent (91-92.5 points).

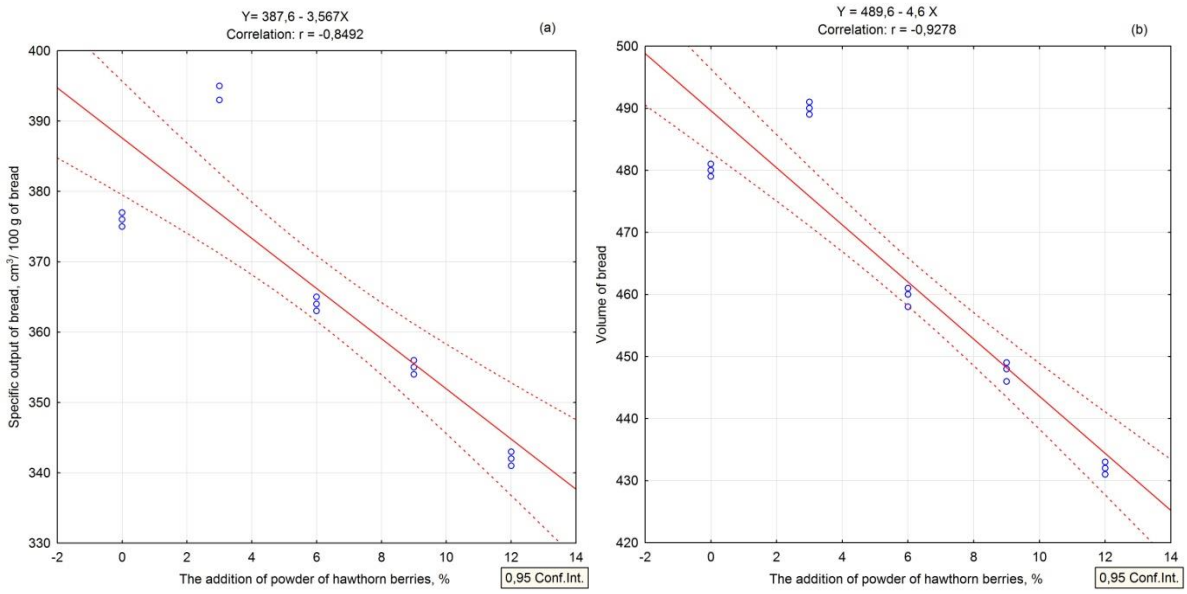

Figure 1. Chemical quality indicators of bread, (a) specific output of bread, (b) volume of bread

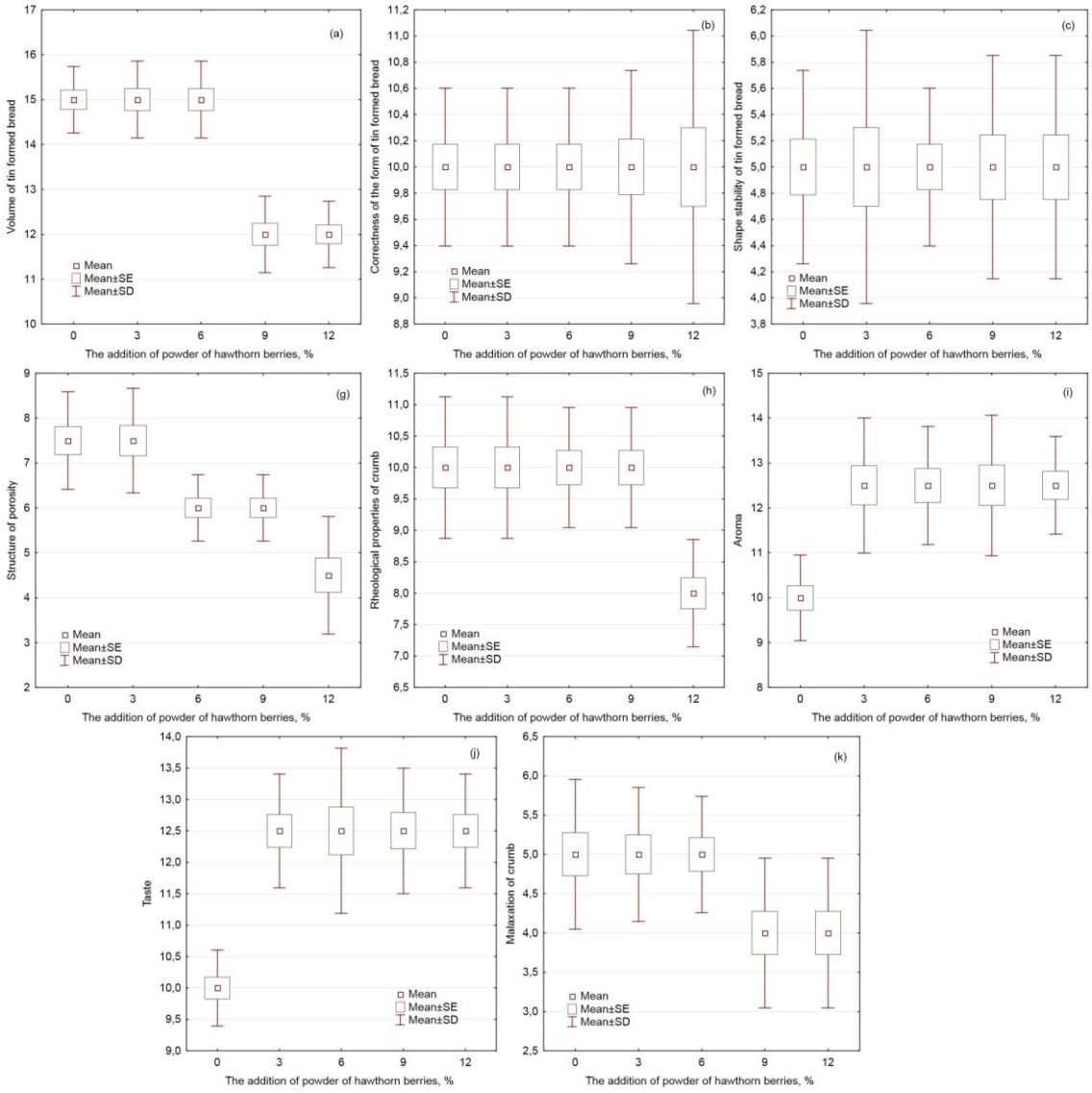

Figure 2. Evaluation of bread quality considering importance ratio, (a) volume of tin formed bread, (b) correctness of the form of tin formed bread, (c) shape stability of tin formed bread, (d) color of crust, (e) condition of crust surface, (f) color of crumb, (g) structure of porosity, (h) rheological properties of crumb, (i) aroma, (j) taste, (k) malaxation of crumb**.**

4. Conclusions

So, as a result of conducted studies it is found that the use of powder made of hawthorn berries in the amount of 3% by weight of flour is fundamentally appropriate in the production of bakery products. Their use makes it possible to intensify maturation of semi products and create better nutrient medium to activate the yeast. The use of the natural additive is quite promising in technology for getting health care products enriched with natural nutrients and will expand the range of functional foods.

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EVALUATION OF NATURALLY OCCURRING BENZOIC ACID LEVEL IN FETA AND CREAM CHEESE DURING FERMENTATION, PRODUCTION PROCESSING AND STORAGE IN REFRIGERATOR

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ABSTRACT

Benzoic acid and its salts are widely used as preservatives in the food industry, but their addition into cheese and other dairy products has been forbidden. However, it was assumed that it was also naturally produced in fermented dairy products and could mistakenly be considered to be adulteration; therefore, it was difficult to interpret the obtained levels during the examination of suspicious samples. In this study, benzoic acid levels naturally occurring in Feta and cream cheese after fermentation, production and storage in refrigerator were assessed by HPLC. After the fermentation stage, benzoic acid was found in all samples. In Feta cheese, benzoic acid content of samples assessed on various days was significantly different ($P < 0.05$) and showed a positive correlation with lactic acid bacteria (LAB) count (r = 0827). By increasing the storage time from 1 to 30 days, the amount of benzoic acid was significantly increased from 24.45 to 41.10 µg/ kg, although its levels on days 40, 50 and 60 did not significantly change. After production, the benzoic acid level of the samples of cream cheese was 8.52 µg/kg, and its concentration did not significantly change during storage. In general, benzoic acid concentration naturally occurring in cheese depends on the type of lactic starter culture used in fermentation stage, LAB growth and storage time. By considering the measurement of analysis method uncertainty, concentrations of naturally occurring benzoic acid was lower than 46.50 mg/kg in Feta cheese and 9.23 µg/ kg in cream cheese.

1. Introduction

One of the most important methods of controlling food product spoilage caused by microorganisms is the use of food preservatives (Panneerselvam *et al.,* 2015). Benzoic acid and sodium benzoate are commonly used as preservatives in the food industry under code numbers E210 and E211. Their undissociated form, created in media with pH values of 2.5–4, shows an inhibiting effect against the growth of yeasts, moulds and a wide range of bacteria (Chipley, 2005). The Joint FAO/WHO Expert Committee on Food established an acceptable daily intake (ADI) for benzoic acid and sodium benzoate of 0–5 mg/kg body weight. Different countries monitor benzoic acid levels in food products in order to ensure the safety of those products, although the intake of benzoic acid differs in various countries. Benzoic acid has no teratogenic or carcinogenic activity (JECFA, 1996). In doses consumed by a person weighing 60 kg (300 mg), it has no toxic effect, although sensitization reactions such as rhinitis, hives and dermatitis have been

observed in some people (Iammarino *et al.*, 2011). Up to now, several methods have been applied to determine benzoic acid levels, including spectrophotometry, gas chromatography with different detectors (FID, MS) or high performance liquid chromatography (HPLC) with a UV detector. Among these methods, liquid/liquid extraction coupled with HPLC-UV is favoured (Yildiz, 2012).

Some foods and commodities contain benzoic acid naturally, and it has been determined that benzoic acid forms in fermented dairy products (Sieber *et al.*, 1995; Iammarino *et al.*, 2011; Amirpour *et al.*, 2015). The addition of benzoic acid into cheese and other dairy products has been forbidden but in some countries acidified milk, yogurt, kefir and buttermilk are except. However, its natural formation in dairy products might also be considered adulteration; therefore, it is difficult to interpret results obtained in examining suspicious samples. In fact, fermented dairy products such as cheese might contain benzoic acid and be considered "non-compliant" even when no benzoic acid has been added as a preservative (Iammarino *et al.*, 2011). Therefore, the measurement of benzoic acid has considerable importance both to determine adulteration and to estimate humans' daily exposure.

Information is scarce regarding changes in concentration of benzoic acid in cheeses during fermentation, production processing and storage. The goal of this study was to discover the change trend of benzoic acid concentration during the production and storage of Feta and cream cheese.

2. Materials and methods

2.1. Chemicals and reagents

Benzoic acid (>99%), sodium salicylate (>99.5%), ammonium acetate (98%), acetic acid glacial (100%) and HPLC-grade acetonitrile, MRS agar and other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Manufacturing of cheese

Samples of ultra-filtrated Feta cheese and cream cheese with milk fat were taken at the Pelareh Dairy Factory (Hamadan, Iran) according to processing system set by Tetra Pak. The initial raw milk samples were clarified and bactofugated to remove microorganism spores, and the fat content of the samples was standardized to 3.3%, followed by pasteurization at 72 °C for 15 s and cooling to 50 °C. The milk-solid-non-fat (MSNF) concentration was obtained at 34% using an ultrafiltration system. To produce the Feta cheese, the retentate was homogenized and pasteurized at 78 °C for 1 min and was fermented by adding a starter culture consisting of *Lactococcus lactis* ssp. *lactis*. The *fermentation time* continued until the pH reached 4.8. The fermented retentate was poured into a cheese container and went through a coagulation tunnel (30 min at 37 °C). In a sealing machine, parchment paper containing edible salt (4% w/w) was placed on top of the cheese curd, and each pack was sealed using aluminium foil. The samples were first stored at 27 °C for 24 h and were then transferred into a refrigerator with a temperature of 4 °C for 60 days. The benzoic acid levels, lactic acid bacteria (LAB) count, acidity and pH of the samples were determined after fermentation, after production and on days 1, 10, 20, 30, 40, 50 and 60 of storage in the refrigerator.

The production process for the samples of cream cheese with milk fat was largely similar to that of the Feta cheese. Briefly, milk fat was added to the pasteurized retentate. After homogenization and cooling to 30 °C, a mesophile starter containing *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* was added, and the mixture was transferred into a tank for

fermentation until the pH reached 4.9. After the fermentation stage, salt and stabilizer were quickly added, and the mixture was pasteurized at 80 °C for 15 s, cooled to 60 °C, poured into cups in amounts of 100 g each, and was stored in a refrigerator for 60 days.

2.3. Analysis of benzoic acid and method validation

2.3.1. Sample preparation

Five grams of the homogenized Feta or cream cheese sample was diluted with deionized water to 50 ml in a volumetric flask and was shaken for 2 min. Then 2 ml of the diluted sample was mixed with 200 µl sodium salicylate (1000 mg/kg) as the internal standard, further diluted by mobile phase to 10 ml and shaken vigorously for 2 min. The mixtures were then filtered through a 0.45 µm cellulose acetate syringe filter, and 20 µl was injected into the HPLC.

2.3.2. Preparation of standard solution of sodium benzoate

Stock solutions of benzoic acid were prepared in distilled water (1000 mg/kg). The working standard solutions in a concentration range from 31.25 to 500 mg/kg were obtained by diluting the stock.

2.3.3.Linearity assay

The linearity of the procedure was determined by injecting the standard solution with concentration ranges from 3.125 to 500 mg/kg. Calibration curves were plotted by least squares linear regression analysis of the ratio of the analyte/sodium salicylate peak areas versus the analyte concentration. The regression equation was Y=0.16086X – 0.03590, and the R^2 was calculated as 0.9996. The limit of detection (LOD) and the limit of qualification (LOQ) were determined as follows: LOD = 3.3σ/S, and LOQ = 10σ/S, where σ is the standard deviation of the response, and S is the slope of the calibration curve.

2.3.4. Determination of accuracy and recovery

The accuracy of the analysis method was identified by using the recovery method. The recovery method was carried out by adding 50, 125, 250, 500 and 1000 µL of benzoic acid solution (1000 mg/kg) into approximately 5 g blank Feta cheese to obtain samples containing 10, 25, 50, 100 and 200 mg/kg benzoic acid, and their recovery percentage and standard deviation were calculated.

2.3.5. Determination of precision

Intra-day precision was determined by injecting the benzoic acid solution at a concentration of 25 mg/kg six times in the same day. The measurement was continued for three days a week to obtain the intra-day precision.

2.4. Measurement of uncertainty

The measurement uncertainty for the benzoic acid was determined according to previous procedures, with some modifies (Iammarino *et al.*, 2011; Golge *et al.*, 2015). We calculated the uncertainty for the benzoic acid solutions and the cheese sample. Mass (m), volume (v), precision (pre), purity of standard (pur) and the standard calibration curve (S) were used for the uncertainty calculation.

By multiplying the combined uncertainty ($U_{combined}$) by 2, based on a confidence level of 95%, an expanded measurement uncertainty of 8.4% was obtained.

2.5. HPLC analysis

A Waters HPLC system (Milford, MA, USA) equipped with a UV detector and C18 analytical column (250 mm × 4.6 mm, i.d., 5 µm) was applied for the benzoate sodium measurement. The mobile phase contained an aqueous ammonium acetate buffer (pH = 4.2) and acetonitrile (80:20 v/v) with a flow rate of

1 ml/min. The detection of benzoate sodium was carried out using the UV detector at a wavelength of 225 nm.

2.6. Assay of lactic acid bacteria

Lactic acid bacteria (LAB) count of the cheese samples was determined both after the fermentation and the production stages and at 1, 10, 20, 30, 40, 50 and 60 days of storage. Twenty-five grams of the cheese samples was diluted in a stomacher (Seward Ltd., London, UK) with 225 ml quarter-strength Ringer's solution at 45 °C. Subsequently, serial dilutions were performed in quarter-strength Ringer's solution. LAB counts were performed on MRS agar in order to obtain isolated LAB colonies. Incubation was done in an anaerobic jar at 30 °C for 72 hours until growth was evident.

2.7. Physicochemical properties analysis

The fat and protein content were also measured using the Gerber's and Kjeldahl methods, respectively (AOAC 2000). Moisture content was identified using the gravimetric method, acidity was measured by titration and expressed as lactic acid percentage, and a pH was determined by pH meter.

2.8. Statistical analysis

The obtained data were analysed using version 16 of the SPSS statistical software package (SPSS Inc., Chicago, IL, USA). All the experiments were repeated five times, and the means and standard deviations were reported for each experiment.

The one-way ANOVA and post-hoc Tukey's test were used to compare the mean concentration of benzoic acid, LAB count, pH, and acidity among the samples. The statistical correlation between the LAB count and benzoic acid concentration was assessed using Pearson's correlation coefficient. $P < 0.05$ was considered a statistically significant difference.

3. Results and discussions

The LOD and LOQ values for benzoic acid in Feta cheese were 0.51 and 1.6 mg/kg, respectively. The recovery data ranged from 96.88% to 105.26%, as shown in Table 1. LOD, LOQ and recovery value of benzoic acid in cream cheese was similar to that of Feta cheese (data not shown). The intra-day and inter-day precision data are exhibited in Table 2. The moisture, fat, and protein contents of Feta and cream cheese are showed in Table 3.

To assess the changes in benzoic acid levels during fermentation, production processing, and cheese storage in the refrigerator, raw milk was analysed for sodium benzoate levels. No benzoic acid was detected in the milk samples used for cheese manufacturing, but benzoic acid was found in all the samples after fermentation. After fermentation stage, Feta cheese (12.15 mg/kg) showed a higher concentration of sodium benzoate than cream cheese (8.49 mg/kg). In addition, the benzoic acid content of the Feta cheese samples significantly differed after storage in a refrigerator. For example, when the storage time was increased from 1 to 30 days, the benzoic acid concentration increase from 24.45 mg/kg to 41.80 mg/kg. However, the benzoic acid level in Feta cheese that was stored for 40, 50, and 60 days in the refrigerator was not significantly different from the acid level on the 30th day of storage in refrigeration. The benzoic acid concentration in cream cheese stored in refrigerator was similar fermented samples

Table 1. Recovery for the determination of sodium benzoate in spiked cheese sample

N	Spiked levels of sodium benzoate (µg/kg)				
	10	25	50	100	200
1	97.26	99.33	106.79	105.21	106.22
2	98.14	98.25	105.32	106.31	104.23
3	95.24	97.21	103.21	103.45	105.32
Mean (n=3)	96.88	98.26	105.11	104.99	105.26
S.D	1.49	1.06	1.80	1.44	0.99
RSD (%)	1.53	1.09	1.7	1.37	0.94

The LAB count in the Feta cheese samples increased based on fermentation time (3.62×10^8 CFU/g) until 30 days in the refrigerator (3.85×10^{10} CFU/g). Their count in the cream cheese after the fermentation was 1.62×10^8 CFU/g and destroyed after production process completion due to sample pasteurization (Table 4).

Table 2. Intra-day and inter-day precision of the method applied for sodium benzoate analysis

Concentration of sodium benzoate (µg/kg)	**Found Concentration**		
	Day 1	**Day2**	**Day 3**
25	24.45	23.45	24.15
25	24.75	23.75	24.35
25	24.85	23.45	24.25
25	23.95	23.85	23.95
25	23.85	23.95	23.85
25	24.15	23.25	24.55
Mean	24.33	23.62	24.18
SD	0.42	0.27	0.26
RSD (%)	1.71	1.15	1.06

Table 3. Chemical composition of feta and cream cheese

Composition	**Cheese type**	
	Feta cheese	**Cream cheese**
Fat (%)	15.21 ± 0.92^b	24.05 ± 1.05^a
Protein (%)	16.12 ± 1.60^a	8.19 ± 0.93^b
Moisture (%)	62.83 ± 1.45^b	65.12 ± 1.85^a

Each value in the table is the mean ± standard deviation of five replications. Mean within the same row followed by different superscripts differ significantly ($P < 0.05$)

In the Feta cheese samples, acidity increment and the pH reduction during storage was significant (Table 4). In cream cheese, acidity and pH showed no significant change.

Cheese is a nutritious dairy product, and different types of cheeses are

produced throughout the world (Gheisari *et al*., 2014; Pakbin *et al*., 2015). The addition of benzoic acid to cheese and other dairy products has been forbidden (Amirpour *et al.*, 2015). However, this study showed the natural production of benzoic acid in cheese samples and the changes in its concentration during storage.

Table 4. Average values of sodium benzoate, lab count, acidity and pH in the analysed samples

Analysis time (days)		Feta cheese				Cream cheese			
		Benzoic acid concentration (mg/kg)	LAB Count (CFU/ g)	Acidity (% lactic acid)	pH	Benzoic acid concentration(mg/kg)	LAB Count (CFU/g)	Acidity (% lactic acid)	pH
After fermentation		12.15±1.15^{b}	3.62×10^{8f}	0.85±0.01^{c}	4.80±0.01^{a}	8.49±0.74^{a}	1.62×10^{8}	0.79±0.01^{a}	4.91±0.01^{a}
After production		18.38±3.05^{b}	1.25×10^{9e}	0.86±0.03^{c}	4.79±0.01^{a}	8.40±0.21^{a}	ND	0.81±0.01^{a}	4.90±0.01^{a}
After storage in refrigerator (day)	1	24.45±1.42^{b}	1.55×10^{10d}	0.87±0.02^{c}	4.77±0.01^{a}	8.52±0.78^{a}	ND	0.82±0.06^{a}	4.90±0.04^{a}
	10	31.57±4.62^{b}	2.67×10^{10c}	0.89±0.01^{c}	4.75±.01^{a}	8.39±0.93^{a}	ND	0.81±0.02^{a}	4.91±0.10^{a}
	20	35.62±3.26^{b}	3.62×10^{10b}	0.90±0.02^{bc}	4.70±.01^{b}	8.44±0.74^{a}	ND	0.82±0.04^{a}	4.90±0.05^{a}
	30	41.80±2.88^{a}	3.85×10^{10a}	0.92±0.02^{b}	4.62±.02^{b}	7.32±1.24^{a}	ND	0.83±0.08^{a}	4.89±0.08^{a}
	40	42.44±3.58^{a}	7.42×10^{8g}	0.95±0.02^{ab}	4.55±.03^{c}	8.54±0.75^{a}	ND	0.83±0.02^{a}	4.88±0.06^{a}
	50	42.54±4.06^{a}	2.64×10^{7h}	0.96±0.04^{a}	4.48±.02^{c}	8.43±1.13^{a}	ND	0.82±0.06^{a}	4.89±0.04^{a}
	60	43.10±3.9^{a}	7.60×10^{6i}	0.99±0.03^{a}	4.46±.011^{c}	8.66±0.80^{a}	ND	0.84±0.06^{a}	4.86±0.02^{a}

Each value in the table is the mean ± standard deviation of five replications. Mean within the same column followed by different superscripts differ significantly ($P < 0.05$).

Benzoic acid formation in dairy product is due to the transformation of hippuric acid to benzoic acid (Iammarino *et al.,* 2011). Hippuric acid naturally occurs in milk, and is converted to benzoic acid by LAB, such as *streptococci* and *lactobacilli* (Sieber *et al.,* 1995). The hippuric acid content of raw milks from different animals varies. For example, Horníčková *et al*., (2015) reported sheep's milk (43.3 ±12.3 mg/kg) had a higher hippuric acid level (15.5 ±8.3 mg/kg) than goat's milk (15.5 ±8.3 mg/kg). Therefore, a significant difference between the benzoic acid levels was found in fermented sheep's milk and goat's milk (29.5 ±16.1 mg/kg and 20.3 ±13.9 mg/kg, respectively) (Horníčková *et al.,* 2015). Other bacteria, such as *Lactococcus luctis*, *Escherichia coli,* and *Pseudomonas fluorescens*, also produce benzoic acid in milk (Amirpour *et al.,* 2015*)*. However, some bacterial cultures used in the fermentation process did not influence the benzoic acid levels (Horníčková *et al.*, 2015). Our results showed that the benzoic acid level found in Feta and cream cheese was different after the fermentation stage. In the current study, a thermophlic and mesophilic starter cultures were used in the production of Feta and cream cheese, respectively. In previous research, higher levels of benzoic acid were found in hard cheeses (28 mg/kg) produced using a thermophilic LAB starter cultures than in semi-hard cheeses (11 mg/kg) produced using mesophilic LAB starter cultures (Garmiene *et al.,* 2011). The results from the current study indicated that benzoic acid levels depended on cheese LAB growth

and type. For example, cream cheese did not show substantial changes in LAB growth during storage because no LAB

positive and significant correlation (r= 0827) between benzoic acid level and LAB count. The LAB are beneficial, naturally occurring microorganisms found in milk, cheese, meat, beverages, and vegetables. These bacteria play an essential role in cheese production (Al Khalaileh and Ajo, 2013).

Different types of cheese made with different production technologies contain different benzoic acid levels. Sieber et al., (1995) reported that the high concentration of benzoic acid in the outer zone of smear-ripened cheeses is more than the stoichiometric transformation of the total amount of hippuric acid available in the milk to benzoic acid. Therefore, benzoic acid might be formed from other natural pathways including phenylalanine degradation, and the auto-oxidation of benzaldehyde. According to results, benzoic acid wasn't produce in cream cheese during storage in refrigerator. Therefore, it seems role of pathway of phenylalanine degradation, and the auto-oxidation of benzaldehyde in benzoic acid production in cheese is low and insignificant. In the current study, the maximum concentration of benzoic acid found in Feta cheese, by considering the measurement of analysis method uncertainty, was 46.50 mg/kg. Our results were similar to other studies. For example, Amirpour et al., (2015) investigated the occurrence of sodium benzoate in cheeses in Iran. The collected UF-Feta cheese, Lighvan cheese, and lactic cheese samples contained mean benzoate concentrations (range) of 50.6 ± 17.4 (27.6–91.2), 34.6 ± 12.3 (16.7–84.1), 17.3 ± 3.7 (11.9–25.6) $mgkg^{-1}$, respectively. In Turkey, the benzoic acid levels in cheese ranged from 3.17–56.77 mg/kg (Yildiz et al., 2011). Iammarino et al., (2011) analysed various cheeses in Italy and reported natural was found in these samples. In addition, the Pearson correlation test showed a

benzoic acid levels below 40.0 mg/kg. Higher levels of benzoic acid in some cheese studies were comparable to the samples assessed in current study. For example, Garmiene et al., (2011) found that the benzoic acid content in hard and semi-hard cheese increased during the ripening stage, reaching 152 mg/kg at 48 months in hard cheeses. However, the authors found that the level of benzoic acid in provolone cheese did not change during the ripening stage. Sieber et al, (1995) reported higher content and wider range of benzoic acid (from traces to 341 mg/kg) in various types of cheese produced in Switzerland. In comparison with our findings, lower value of benzoic acid has been reported in some cheeses. Camembert cheese and processed cheese had the benzoic acid in range of < 0.18–4.2, and 0.18–20.8 mg/kg, respectively (Lim *et al.,* 2013).

The LAB count in Feta cheese increased from fermentation to 30 days of storage in the refrigerator. Our results were similar to previous research. For example, Kamleh *et al*., (2012) found increased lactic acid bacteria levels in Halloumi cheese during storage. The LAB count of 0.9 log CFU/g in freshly-packed Halloumi reached 3.38, 3.64, and 3.43 log CFU/g at 25°C/20 days, 15°C/54 days, and 5°C/170 days, respectively (Kamleh *et al.*, 2012). The changes in cheese acidity depended on the growth of lactic acid and aerobic bacteria (Hasani *et al.,* 2016). The average LAB counts of the Karin Kaymagi cheese samples were 6.11 log CFU/g, and these levels increased for 60 days after production (Yangılar and Ozdemir, 2010). Souza *et al*., (2003) analysed six Serrano cheeses and found 431–484 LAB isolates in these cheese samples. The *lactobacilli* were the most abundant lactic bacteria, followed by *enterococci* and *lactococci*.

The LAB count reduced during a 60-day fermentation process. The growth of *lactococci* and *leuconostoc* was inhibited due to the salt that was added to the cheese (Souza *et al.*, 2003). These discrepancies in the LAB count may also be due to different cheese making and ripening techniques.

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4. Conclusions

In this study, the benzoic acid was produced after the fermentation stage in all the cheese samples. The benzoic acid concentration was dependent on the type of cheese, storage time, and LAB count. An increase in the storage time from 1 to 30 days significantly raised the Feta cheese benzoic acid level and LAB count from 24.45 to 41.80 mg/kg and 1.55×10^{10} to 3.85×10^{10} CFU/g, respectively. The benzoic acid levels in Feta cheese had a positive correlation to the LAB count (r = 0827). After the fermentation stage, the benzoic acid concentration in cream cheese was 8.52 mg/kg, but no significant changes in this level were observed during the 60 days the cheese was stored in the refrigerator. In general, the maximum benzoic acid level (46.50 mg/kg) in cheese occur naturally after 30 days of storage and this concentration of benzoic acid and lower levels were attributed to LAB activity.

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ANTIBACTERIAL ACTIVITY OF VARIOUS EXTRACTS FROM *THYMUS TRANSCAPICUS* AGAINST FOOD PATHOGENIC MICROOGANISMS

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ABSTRACT

Thyme (*Thymus transcapicus*) is a herbaceous perennial plant belonging to the *Lamiaceae* family. Thymus species are considered as medicinal plants due to their pharmacological and biological properties. In the present study, we have investigated the antibacterial capabilities of dichloromethane, ethylacetate and methanol extracts of *Thymus transcapicus*. Antibacterial activities was screening against two Gram-positive bacteria (*Staphylococus aureus*, and *Listeria Monocytogenes)* and two Gram-negative bacteria (*Salmonella enterica*, *Escherichia coli*) by detection of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) method. The highest antibacterial index was observed from methanol extract on *Staphylococus aureus* in MIC-MBC method ($p<0.05$). The results of this investigation indicated that *Thymus transcapicus* was found to possess moderate antibacterial activities. Further research is required to identify the active photochemical responsible for these biological activities.

1. Introduction

Treatment of infectious diseases continues to be problematic in modern time. With the available antiviral and antimicrobial drugs, the treatment often leads to the problem of resistance (Vijayan et al. 2004; Mehrgana et al. 2008). There is little likelihood that available orthodox antiviral drugs can eliminate all or even most viral diseases (Jassim & Naji 2003). The search for new antimicrobial substances exhibiting minimal side effects is warranted because of the severe side effects of some drugs currently in use (Mehrgana et al. 2008; Kandil et al. 1994). So, there is an increasing need for new substances with antiviral and antimicrobial activity. Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases (Vijayan et al. 2004). Therefore, the development of new medicinal plant products is vital in controlling the threats posed by some pathogenic microorganisms (Jassim & Naji 2003).

Lamiaceae (formerly Labiatae) is one of the most important plant families in which *Thymus* with about 215 species, is a significant genus (Mehrgana et al. 2008). *Thymus* species are well known as medicinal plants because of their biological and pharmacological properties. In traditional medicine, the extracts of different species of *Thymus* have been widely used for the treatment of gastritis, diarrhea and enuresis in children, bronchitis and whooping cough (Pertussis) (Ebadi 2002).

Several studies have been shown that *Thymus* species have antibacterial (Mehrgana et al. 2008; Figueiredo et al. 2008; Tohidpour et al. 2010), antifungal (Figueiredo et al. 2008. Bonjar 2004; Sokovic et al. 2009), cytotoxic (Goncalvesa et al. 2010), analgesic (Sokovic et al. 2009), antiparasitic (Goncalvesa et al. 2010), topical anti-inflammatory (Ismaili et al. 2002), antispasmodic (Begrow et al. 2009), mosquitocidal (Pavela et al. 2009) and antioxidant (Zamani et al. 2009; Soares et al. 1997) activities. Antiviral effect of extracts from some plants of the Lamiaceae family against HSV-1 (Vijayan et al. 2004; Nolkemper et al. 2006) and HSV-2 (Nolkemper et al. 2006) has been reported and the extract of *Thymus vulgaris* has been shown antiviral activity (Nolkemper et al. 2006).

Antioxidant (Zamani et al. 2009) and antiemetic (Moallem et al. 2009) effects of *Thymus transcaspicus* have been reported in some studies.

The aim of this study was to evaluate the antimicrobial activities of the dichloromethane, ethylacetate and methanol extracts of *Thymus transcapicus.* To the best of our knowledge, we are the first to report that *Thymus transcapicus* extracts showed antimicrobial activity against the pathogenic microorganisms that were tested.

2. Materials and methods

2.1. Plant material

The Plant material was collected in May 2016 from North Khorasan Province Mountains in Iran. Then, the plant was identified and confirmed by Natural Products & Medicinal Plants Research Centre, North Khorasan University of Medical Sciences (Iran) and Voucher specimen (No: MP 32/4) was deposited in herbarium of the Natural Products & Medicinal Plants Research Centre.

2.2. Preparation of plant extracts

The aerial parts of the plants were dried under shade at room temperature and then cut into small pieces. About 100 g of sample was macerated in methanol, dichloromethane, and ethylacetate at room temperature for 48 h separately. Each solvent was allowed to remain in contact with plant material for 24 h, and replaced with fresh solvent four times. Removal of the solvents under vacuum at 40 °C gave the crude extracts (Boozari et al. 2015).

2.3. Antimicrobial Activity

Determination of the minimum inhibitory concentrations (MIC) antimicrobial activities of methanol, dichloromethane, and ethylacetate extracts of the aerial part of the plant were determined against two Gram-positive bacteria*: S. aureus* (ATCC 6538p), and *L. monocytogenes* (ATCC 35152), two Gram-negative bacteria: *S.enterica* (ATCC 53648), *Escherichia coli* (ATCC 10536).

2.4. Determination of the minimal inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) were determined by broth macro dilution method in 96-well plates by Rios and Duffy methods (Rios et al. 1988; Duffy & Power 2001).

Initial concentration of each extract was prepared with the aid of bath sonicator (0.8 g extract with 4 ml solvent and 30% dimethyl sulphoxide in sterile distilled water and one drop of Tween 80). 1 ml of diluted extract was infused into macro-plate with 1ml of sterile Mueller-Hinton broth (MHB; Hi Media, India) and then diluted (50% with MHB). 0.5 McFarland standard turbidity for microbial suspension equivalent was prepared by suspensions of the growth from brain-heart infusion medium (HiMedia, India). Suspensions were further diluted to obtain a concentration of 10^7 colony-forming units (CFU) per ml for the bacteria. Then, 10 µl of diluted inoculums was added to each well of macro-plate. The sterility of the medium was also tested in two wells and Gentamicin was used as the positive control for bacterial strains. Plates were incubated for 24 h at 37°C for bacteria. The growth of microorganisms was assessed by TTC (2, 3, 5-triphenyl tetrazolium chloride, Sigma, USA) assay. Briefly, 0.5 ml of TTC (5 mg/ml; dissolved in sterile water) was added to each

well and the plates were incubated at 37 °C for bacteria. The results were expressed as the lowest concentration of plant extract that could inhibit any red dye production. MIC values were defined as the lowest concentrations of oil that inhibit bacteria after 24 h. All experiments were done in triplicates.

2.5. Determination of minimum bactericidal concentrations (MBC)

The bactericidal effects of extracts were determined according to the method described by Rios (Rios et al. 1988). 100 µl of clear dilutions in wells of macro-plate were sub cultured on the Mueller- Hinton agar plates and subsequently incubated at 37 °C for 24 h. Minimal bactericidal concentration (MBC) were recorded from the first tube that showed no growth on solid media. All experiments were done in triplicates.

2.6.Statistical analysis

Data for each test were statistically analyzed. The statistical analysis of the data was performed using SPSS statistical software version 18 (SPSS Inc., Chicago, IL). Data recorded as means ± standard deviation of three replicate measurements.

3. Results and discussions

The highest yield of extract was for methanol extract with 2.92 %, dichloromethane and ethylacetate extracts had 1.78 % and 1.58 % yields.

To our knowledge, this is the first report on the antimicrobial activity of *Thymus transcapicus.* Experimental studies carried out in species of *Thymus* have identified phenols and flavonoids as phytochemicals with antimicrobial properties. The *Thymus transcapicus* extracts were tested for antimicrobial activity against 4 different pathogenic microorganisms, including 2 Gram-positive and 2 Gram-negative bacteria.

The Gram-positive strains of bacteria that were tested seemed to be more sensitive to the extracts, which are attributed to the absence of an outer lipopolysaccharide layer in Gram-negative bacteria that provides a resistant barrier (Inouye et al. 2001).

The antibacterial activity of flavonoids and polyphenols has been attributed to inhibition of synthesis of RNA and DNA (Arora et al. 2013). Thus, the antibacterial activity of ethylacetate and methanol extracts of *Thymus transcapicus* could be attributed to the high polyphenolic compounds present in the extract.

The negative control (DMSO) is showed activity against all the microbial strains tested. The positive control was showed activity against all the microbial strains. The extracts can inhibit the growth of microbial strains the growth inhibitory effects of the *Thymus transcapicus* extract were concentration dependent (He et al. 2010).

The standard antibiotic was more potent, having lower MIC values against bacteria. The minimum inhibitory concentrations (MIC) of different extracts were determined by preparing solution of varying concentration (12.5- 100 mg/ml). The methanol and ethylacetate extracts exhibited antibacterial properties against Gram-positive tested bacteria.

According to the present results, these extracts did not show any inhibitory activity against all the Gram-negative microorganisms and Gram-positive bacteria were more sensitive to these extracts than Gram-negative bacteria.

As shown in Figs 1 and 2, the results indicated that among the three extracts, ethylacetate extract has greater antimicrobial activity against the tested microorganisms compared to dichloromethane and methanol extract.

Methanol extract showed good activity against S. aureus, showing the inhibition the lowest MIC values (12.5 mg/ml) and the lowest MBC values (25 mg/ml) ($p<0.05$).

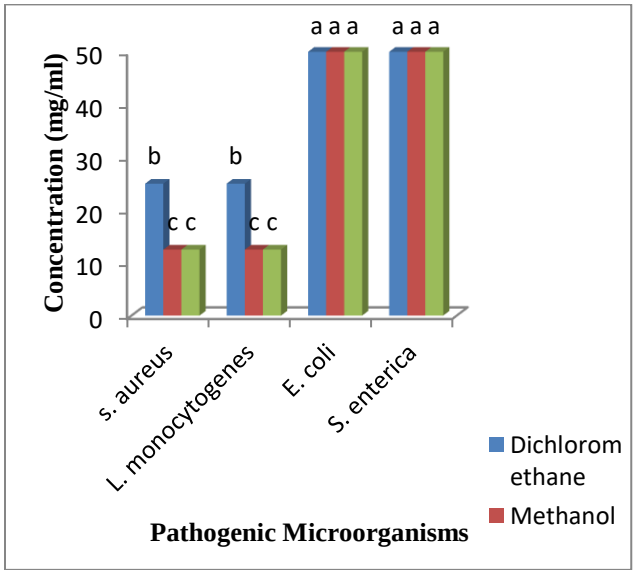

Figure 1. Comparison of minimum inhibitory concentration (MIC, mg/ml) of methanol, dichloromethane and ethyl acetate extracts of *Thymus transcapicus.*
(Each experiment was tested in triplicate.)

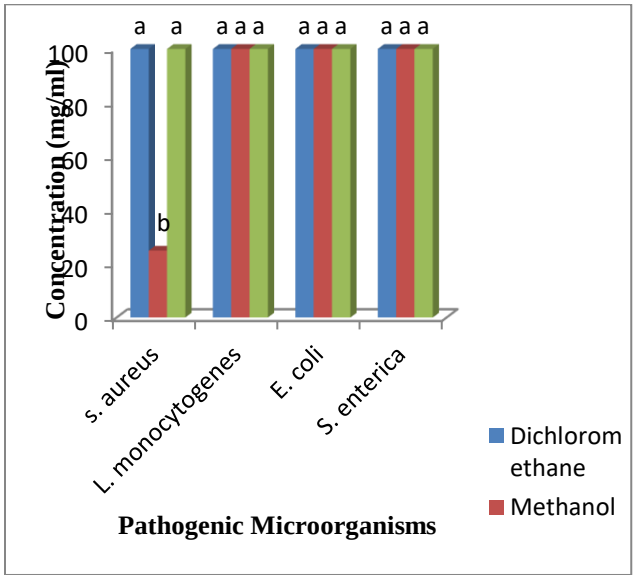

Figure 2. Comparison of minimum bactericidal concentrations (MBC, mg/ml) of methanol, dichloromethane and ethyl acetate extracts of *Thymus transcapicus*
(Each experiment was tested in triplicate.)

Antimicrobial activity of the plants of different areas of the world has been reported (Janovska et al, 2003).

Recent studies have shown that *Thymus* species have antibacterial, antifungal, and antioxidant activities (Rahimmalek *et al.*, 2009; Jordan *et al.*, 2009; Bassam *et al.*, 2004). The anti-bacterial characteristic of *Thymus* spp. is due to the occurrence of thymol in this genus. This substance can be used as a disinfectant. Previous studies showed that the essential oil and extract from *T. daenensis* exhibited antimicrobial activities against *Candida albicans* (Ghasemi Pirbalouti *et al.*, 2009a), *Listeria monocytogenes* (Ghasemi Pirbalouti *et al.*, 2009b), *Campylobacter jejuni* and *Campylobacter coli* (Ghasemi Pirbalouti *et al.*, 2010a), *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (Ghasemi Pirbalouti *et al.*, 2010b), *Escherichia coli* O157:H7 (Ghasemi Pirbalouti *et al.*, 2010c), and *Saprolegnia parasitica* (Ghasemi Pirbalouti *et al.*, 2009c).

Antimicrobial activities of some Thymus species have been shown in other previous studies. *Thymus pubescens* and *Thymus vulgaris* extract demonstrated good antibacterial activity against some drug resistant Gram-positive bacteria (Mehrgana et al., 2008; Tohidpour et al., 2010). The essential oil of the Thymus caramanicus showed high inhibitory activity against Helicobacter pylori (Eftekhar et al., 2009). *T. transcaspicus* essential oil was tested for its antibacterial activity against various Gram-positive and Gram-negative bacteria Standard strains. All the bacteria were inhibited by the essential oil but in variable degrees.

Inhibition of *Staphylococcus aureus* (de Oliveira et al., 2010) and antibacterial effects against E. coli (Pei et al., 2009) by thymol and carvacrol have been reported. Carvacrol also has been reported to exhibit a dose dependent inhibitory effect on Vibrio cholerae in food (Rattanachaikunsopon et al., 2010). Thymol, which is the main component of many Thymus spp. and also in the oil of *T. transcaspicus* (64%), is known as an antiseptic agent (Miri et al., 2002).

Several studies have demonstrated the antimicrobial activity the essential oils and/or the extracts of many species of the genus

Thymus rich in volatile phenols and/or volatile alcohols (Sokovic et al. 2009; Laouer et al. 2009; Mohammed et al. 2009; Reichling et al. 2009; Oh et al. 2009).

The essential oils of *Thymus vulgaris* L., *Thymus tosevii* L. (Sokovic et al. 2009), *Thymus pulegioides* (Pinto et al. 2006) showed antifungal activities against other fungi strains. The most of the species of *Thymus* genus have been investigated for their antimicrobial properties against some bacteria and fungi, depending on thymol and its isomer carvacrol (Laouer et al. 2009; Safaei-Ghomi et al. 2009) and other species depending on thymol as main constituents (Mojab et al. 2008; Cavara et al. 2009).

4. Conclusions

The results of this investigation indicated that *Thymus transcapicus* was found to possess moderate antibacterial activities and its antibacterial activity due to presence of flavonoids. Further research is required to identify the active photochemical responsible for these biological activities.

5. References

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