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Comparative study of molasses produced by traditional and industrial techniques from the viewpoint of furan derivatives, mutagenicity/antimutagenicity, and microbiological safety

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Abstract

Molasses is one of the popular and traditional foods produced and consumed in Turkey for a long time. The present work aimed to evaluate the safety of molasses which is produced traditionally and industrially. Ames/*Salmonella* mutagenicity and the MTT cytotoxicity methods were used for the safety evaluation. The amounts of furan derivatives were comparatively determined. 5-hydroxymethylfurfural (HMF) content of some samples were above the legal limit of 70 mg/kg. Ames/*Salmonella* mutagenicity assay showed that molasses had no mutagenic effect. On the other hand, antimutagenic activity was observed after metabolic activation. The margins of exposure were above the value of 100, thus indicating the safety of samples regarding furan derivatives exposure. The higher HMF content and osmophilic yeast counts of traditionally produced molasses as compared to the industrially produced samples are alarming, and suggest that traditional and industrial food production may need a separate risk assessments by the national authority.

Introduction

Molasses (*pekmez* in Turkish) is traditionally considered as a healthy food, and an integral part of the daily diet of developing children and pregnant women. Molasses is used to protect the body against infections, and to support blood parameters. It is also traditionally known as a healing food among the locals, and consumed to prevent certain diseases such as osteoporosis (Helvacıoğlu *et al.,* 2018). Molasses is commonly produced from fresh or dried grapes; but, there are also molasses obtained from apples, mulberries, apricots, plums, watermelons, figs, and sugar beets. Traditional method is the common method to obtain homemade molasses in rural areas. However, the products currently on the market are mainly prepared by modernised techniques, and at an industrial scale. There are many differences between conventional/traditional and industrial techniques in the production of molasses. Traditionally, after squeezing the grapes, the liquid part is filtered to remove the rape. Then, the filtrate is boiled in an open caldron until reaching pH 3 - 4. At this stage, filtrate becomes blurred and viscous. Marl and white or slightly yellowish soil with high calcium carbonate ($CaCO_3$) content is added to adjust the pH value, and fix the turbidity (Helvacıoğlu *et al.,* 2018). Industrially, $CaCO_3$ is added instead of soil. In addition, boiling and condensation processes of the grape juice are performed in vacuum boilers under reduced pressure to lower the boiling point. Consequently, processing under reduced temperature conditions would possibly prevent the decomposition of grape juice components due to the excessive and long-term application of heat during industrial production. In the traditional technique, however, during high heat treatment, 5 to 10% of the sugar content in the molasses is burned and caramelised to form dark brown chemical substances known as furan derivatives. Therefore, it is expected that the molasses industrially produced using the vacuum method would have a low level of furan derivatives (Koca *et al.,* 2007).

In recent years, there has been a growing concern about the presence of compounds derived from the Maillard reaction produced during food processing at high temperatures. Maillard reaction is a kind of non-enzymatic browning reaction that

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occurs following the interaction between sugars and amino acids, which leads to changes in nutritional value, taste, odour, and colour of the final product (Zhao *et al.,* 2017). It is reported that the most important factor affecting the rate of reaction is high temperature. For every 10°C rise in temperature, the rate of reaction increases by four times (Eskin, 1990). The main compounds derived from this reaction are furan derivatives such as furfural (F) and 5-hydroxymethylfurfural (HMF), and due to the high sugar content, molasses is subjected to Maillard reaction during the production and storage conditions (Tamanna and Mahmood, 2015).

It has also been reported that HMF possesses favourable biological effects including *in vivo* (Mousavi *et al.,* 2016) and *in vitro* antioxidant activity (Li *et al.,* 2011), and prevention against hypoxic injury (Zhao *et al.,* 2013). On the other hand, furan derivatives have also been shown to be carcinogenic in animal laboratory studies (Bakhiya and Appel, 2010). The National Institute of Environmental Health Sciences nominated these derivatives for toxicity testing, such as cytotoxicity, mutagenicity, genotoxicity, and carcinogenicity based on the potential for extensive exposure through the consumption of commonly available beverages and foods, and evidence for the carcinogenicity of members in this class (NTP, 2010).

Regulatory limits exist within the Turkish Food Codex for liquid molasses ingredients, such as heavy metal and HMF (75 mg/kg), and these limits are inspected by the authorities in Turkey. However, molasses produced in rural areas by the traditional methods are usually excluded from such inspections. The present work thus aimed to investigate the safety of molasses, which is produced by both traditional and industrial techniques, in terms of public health. Ames/*Salmonella* mutagenicity test system and the MTT cytotoxicity methods were used for safety evaluation of the molasses. Additionally, the amounts of F, HMF, and 2-furyl methyl ketone (FK) were comparatively determined. Fructose/glucose proportion was evaluated in molasses samples. Microbiological evaluation was also carried out. The antimutagenic activity of the molasses produced by both techniques was also studied. Finally, a risk assessment was conducted regarding the dietary intake of HMF based on molasses consumption and available toxicological data.

Materials and methods

Materials and sample preparation

Grape molasses samples produced by the traditional method were obtained from different regions of Turkey: Çanakkale (north-western), Nevşehir (central), and Malatya (eastern) provinces. The five products obtained from Çanakkale were labelled as C1-5, the three products from Nevşehir as N1-3, and one product from Malatya as M. Industrially produced samples were supplied from markets in Istanbul, Turkey, and labelled as T1-5. The pH range of samples was from 3.7 to 5.1. To minimise the storage time effect on furan derivatives, samples with approximately same production date were included in the present work, and stored at 4°C until analysis. Then, 10 mL of each sample was taken and dissolved in 50 mL of distilled water (Millipore, Simplicity UV, France). After being allowed to cool at -20°C overnight, it was lyophilised (Christ, Alpha 2-4 LD, Germany) to prepare the molasses extract. Then, the samples were stored in a desiccator at 4°C to avoid moisture. The production method, grape origin, other fruit molasses samples (*e.g.,* juniper, mulberry, and apple), and yields of extraction are summarised in Table 1.

Table 1. The production methods, origins, and extraction yields of molasses samples.

Molasses sample	Production method	Origin
Grape		
C1	Traditional	Canakkale
C2	Traditional	Canakkale
C3	Traditional	Canakkale
C4	Traditional	Canakkale
C5	Traditional	Canakkale
N1	Traditional	Nevşehir
N2	Traditional	Nevşehir
N3	Traditional	Nevşehir
M	Traditional	Malatya
T1	Industrial	Market
T2	Industrial	Market
T3	Industrial	Market
T4	Industrial	Market
T5	Industrial	Market
Juniper	Traditional	İstanbul
Mulberry	Industrial	Market
Apple	Traditional	Aydın
Date	Industrial	Market
Fig	Traditional	Aydın
Carob	Industrial	Market
Pomegranate	Traditional	Aydın

Determination of furfural derivatives by HPLC

Stock solutions of HMF, F, and FK (Sigma) were prepared by dissolving in acetonitrile at a concentration of 1,000 ppm. Standard solutions and quality control samples were then prepared as a mixture. The stock solution was diluted with 10 mM phosphate buffer (pH 2.5) to prepare standard solutions at the concentrations of 0.1, 0.5, 1, 5, and 10 µg/mL.

Molasses sample (5 g) was placed into a 50 mL Falcon tube, and added with 25 mL of distilled water. Then, 0.75 mL of Carrez I (zinc sulphate heptahydrate) and Carrez II (potassium ferrocyanide) solutions were added, followed by stirring.

Analyses were performed according to our previous study (Türköz-Acar *et al.,* 2018). Agilent 1260 Infinity HPLC-DAD device and Agilent Poroshell 120 EC-C18 (150 × 3 mm, 2.7 µm) particle diameter column were used for the analysis. To protect the analytical column, Agilent Poroshell (3 × 5 mm) protective column with the same brand and filler material was used. Then, 10 mM pH 2.5 phosphate buffer was used as the mobile phase A, and acetonitrile was used as mobile phase B. During the analysis, these phases were kept at a ratio of A:B (95:5), and running at 0.5 mL/min flow rate. The temperature of the column was set at 35°C, and the injection volume was set at 20 µL. During the analysis, monitoring of the analytes was performed with DAD detector at a wavelength of 284 nm.

Mutagenicity/antimutagenicity assay

Mutagenicity and antimutagenicity assays were performed following the method of Maron and Ames (1983) using *S.* Typhimurium TA98, TA100, TA102, TA1535, and TA97a strains. TA100 and TA1535 were used for the detection of base-pair mutations, TA98 and TA97 for the measurement of frameshift, and TA102 were used for the detection of oxidative mutagens. The bacterial strains were purchased from Moltox, USA. to begin, 1 g of lyophilised molasses samples was weighed and dissolved in 2 mL of sterile distilled water. Afterwards, the samples were diluted to obtain solutions at different concentrations (1, 5, 10, 50, and 100 mg/plate). Both mutagenicity and antimutagenicity assays were performed in the presence and absence of liver S9 (Moltox, USA). For the antimutagenicity assay, the percentage of inhibition was calculated according to our previous method (Charehsaz *et al.,* 2017).

Cytotoxicity assay

The MTT test was performed for the assessment of cytotoxicity using the L929 mouse fibroblast cell line. Briefly, the L929 cells were incubated in 96 culture wells until the formation of a semi-confluent layer (24 h). The cells were then exposed to molasses samples at concentrations of 1 and 2 mg/mL. After 24 h of incubation, formazan formation was determined for both concentrations, and the percentage of viable cells was determined by comparing with the values obtained from control cultures (Charehsaz *et al.,* 2016).

Sugar analysis

Identification and quantification of sugar compounds in molasses samples were determined using Thermo Accela High Performance Liquid Chromatography (HPLC) (Thermo Scientific, England) equipped with a Refractive Index (RI) detector (Thermo Scientific, England) according to Andersson and Hedlund (1983) with slight modification. Chromatographic separation was achieved with a HyperREZ XP Carbohydrate Ca^{++} column (300 × 7.7 mm, 8 µm; Thermo Scientific, England). Column and detector temperatures were maintained at 50 and 35°C, respectively. Elution was performed using 0.005 M H_2SO_4 (Sigma-Aldrich, Germany) as the mobile phase, running at 0.6 mL/min flow rate. Samples were diluted 100 - 400-fold with water before analysis. All samples and standards were filtered through a 0.45 µm RC filter (Minisart Sartorius, Germany) before the injection, and the volume of the injected sample for each run was 10 µL. Standards of D(+) glucose and D(-) fructose (Supelco, USA) were used for both identification and quantification. Quantification was done using external standard calibration graphs, and results was expressed as mg sugar per mL (Ekinci *et al.,* 2016).

Microbiological analysis of molasses samples

Total mesophilic aerobic bacteria (TMAB), coliform bacteria, and osmophilic yeasts in molasses samples were quantified using classical culturing techniques according to ISO 4833, TS ISO 4832, and ISO 21527 standards, respectively. TMAB, coliform bacteria, and osmophilic yeasts were grown on plate count agar (Oxoid, England) at 30°C for 2 d, on violet red bile agar (Merck, Germany) at 37°C for 1 d, and on Dichloran Rose Bengal Chloramphenicol agar (Conda Pronadisa, Spain) at 25°C for 5 d, respectively. Viable counts were performed and expressed in log CFU/mL.

Statistical analysis

Statistical analysis was performed using

SPSS 20 program. Multiple comparisons were made for mutagenicity data. For cytotoxicity assay, statistical analysis was conducted using the same program with paired *t*-test and Wilcoxon signed-ranks test. $p < 0.05$ was considered statistically significant.

Results

Furfural derivatives in molasses samples

The results of the HPLC analysis are given in Table 2. HMF was detected in all samples except juniper and carob. F and FK were detected in two (apple and fig) and three (C1, C2, and C5) of 21 samples, respectively.

Mutagenicity assay

The result of mutagenicity assay revealed that the mutation frequencies for the tested *S.* Typhimurium TA98, TA97a, TA1535, and TA102 strains did not change significantly when compared with the negative control. The number of colonies increased significantly versus negative control at 50 and 100 mg/plate concentrations without S9 activation in C3, N1, N2, apple, date, and fig molasses samples (Table 3). However, this increase was in revertant numbers when compared with the negative control, and was not over ≥ 2-fold as a positive result acceptance criteria (Fowler *et al.,* 2018).

Antimutagenic activity of molasses samples

The percentage of inhibition was below the value of 25% for all investigated samples in five strains without S9 activation, and TA98 strain with S9 activation, thus indicating no antimutagenic

Table 2. Furfural derivative contents of molasses samples (mg/kg).

Sample	Furfural derivative content		
	F	HMF	FK
Grape			
T1	< LOD	8.37 ± 0.10	< LOD
T2	< LOD	8.64 ± 0.07	< LOD
T3	< LOD	30.76 ± 0.21	< LOD
T4	< LOD	13.23 ± 0.34	< LOD
T5	< LOD	45.97 ± 1.20	< LOD
C1	< LOD	50.37 ± 0.48	7.35 ± 0.07
C2	< LOD	43.79 ± 0.58	1.36 ± 0.04
C3	< LOD	110.38 ± 0.26	< LOD
C4	< LOD	17.16 ± 0.09	< LOD
C5	< LOD	63.36 ± 3.89	4.02 ± 0.03
N1	< LOD	39.56 ± 0.09	< LOD
N2	< LOD	15.94 ± 0.73	< LOD
N3	< LOD	21.87 ± 0.25	< LOD
M	< LOD	29.68 ± 0.24	< LOD
Juniper	< LOD	< LOD	< LOD
Mulberry	< LOD	25.77 ± 0.66	< LOD
Apple	58.99 ± 0.48	788.63 ± 2.39	< LOD
Date	< LOD	124.24 ± 0.64	< LOD
Fig	12.80 ± 0.79	850.49 ± 1.49	< LOD
Carob	< LOD	< LOD	< LOD
Pomegranate	< LOD	31.95 ± 0.22	< LOD

LOD = limit of detection; LOD values were 0.0006, 0.002, and 0.002 ppm for F, HMF, and FK, respectively. F: furfural, HMF: 5-hydroxymethylfurfural, and FK: 2-furyl methyl ketone.

Table 3. Results of mutagenicity assay in TA100 strain without S9 activation.

Number of revertant colony/plate[a]					
	Concentration (n = 4)				
Sample	**1 mg/plate**	**5 mg/plate**	**10 mg/plate**	**50 mg/plate**	**100 mg/plate**
Grape					
C1	139.50 ± 15.37	132.25 ± 12.04	138.50 ± 12.50	133.00 ± 12.41	132.25 ± 8.73
C2	139.50 ± 13.70	140.75 ± 21.64	131.00 ± 17.83	144.00 ± 21.12	151.75 ± 16.50
C3	135.75 ± 10.59	132.25 ± 14.38	128.50 ± 19.33	156.75 ± 5.12	185.50 ± 5.45[b]
C4	129.50 ± 9.47	126.50 ± 7.19	133.33 ± 4.04	131.00 ± 11.17	139.50 ± 11.33
C5	131.50 ± 7.94	130.00 ± 11.17	124.25 ± 6.45	142.50 ± 7.77	158.50 ± 12.45
N1	142.25 ± 2.87	136.50 ± 18.16	154.25 ± 11.67	162.00 ± 12.19[b]	176.75 ± 16.94[b]
N2	131.00 ± 6.22	145.50 ± 17.39	139.75 ± 22.50	148.25 ± 8.34	170.00 ± 7.87[b]
N3	122.25 ± 15.46	124.25 ± 11.84	129.25 ± 13.35	130.50 ± 9.29	132.25 ± 14.93
M	141.75 ± 10.40	143.33 ± 10.79	141.50 ± 10.12	139.00 ± 11.17	142.50 ± 12.56
T1	130.00 ± 16.25	134.00 ± 9.66	142.50 ± 24.34	147.33 ± 6.81	135.50 ± 16.58
T2	129.00 ± 11.79	126.67 ± 8.08	126.33 ± 5.51	139.00 ± 6.06	140.00 ± 10.95
T3	128.25 ± 12.71	129.67 ± 18.15	129.67 ± 6.81	135.33 ± 4.51	143.25 ± 16.76
T4	133.75 ± 16.13	137.33 ± 9.07	132.00 ± 10.44	135.75 ± 8.77	131.00 ± 8.76
T5	139.00 ± 10.44	132.67 ± 11.06	121.33 ± 8.33	140.00 ± 16.09	147.67 ± 3.21
Juniper	132.50 ± 6.36	135.00 ± 6.56	133.33 ± 12.34	131.75 ± 13.56	132.00 ± 9.13
Mulberry	129.50 ± 13.20	137.00 ± 13.75	129.75 ± 12.39	129.00 ± 12.99	134.00 ± 13.71
Apple	125.00 ± 8.19	130.00 ± 15.87	126.75 ± 13.07	148.00 ± 12.83	184.50 ± 17.60[b]
Date	135.00 ± 13.64	137.50 ± 12.45	133.00 ± 10.10	162.00 ± 6.98[b]	179.75 ± 16.74[b]
Fig	140.25 ± 17.91	144.25 ± 15.67	147.00 ± 16.59	165.50 ± 17.75[b]	197.50 ± 19.30[b]
Carob	128.50 ± 13.30	138.50 ± 13.87	131.75 ± 25.58	138.00 ± 13.22	139.25 ± 15.84
Pomegranate	136.00 ± 14.73	128.25 ± 10.08	134.50 ± 8.06	133.00 ± 17.20	136.25 ± 15.59

Colony number in negative control: 137.14 ± 14.08; colony number in positive control (1 µg/plate of sodium azide): 1026.50 ± 156.59. [a] mean ± standard deviation; [b] $p < 0.05$ versus negative control.

activity. On the other hand, moderate and strong antimutagenic activity was observed in TA97a, TA100, TA1535, and TA102 strains after metabolic activation (Figure 1). Juniper molasses showed strong antimutagenic activity in TA100, TA97a, TA1535, and TA102 strains. C3, C4, N3, and date in TA100 strain; C4, pomegranate, and carob in TA97a; carob in TA1535; and C2, C4, C5, N1, M, carob, and pomegranate in TA102 strain had strong antimutagenic activity.

Cytotoxicity assay

In the evaluation of cytotoxic effect, the fibroblast cell line (L929) that is frequently used in the general test for acute toxicity and also complies with the ISO 10993 guideline was preferred (Charehsaz *et al.,* 2016). According to the MTT results, molasses samples did not exhibit cytotoxicity on L929 at the concentrations of 1 and 2 mg/mL.

Sugar content of molasses samples

The average total sugar amounts for traditionally produced molasses samples (1,025.13 mg/mL) were higher than industrially produced molasses samples (752.05 mg/mL). Glucose in molasses samples ranged from 160.71 to 833.78 mg/mL, while fructose ranged from 137.58 to 1,009.11 mg/mL. The ratio of fructose to glucose varied between 0.8 and 2.1 among all molasses samples. Among these samples, the highest fructose to glucose ratio (2.1) was found in C2 grape and apple molasses. The representative HPLC-RI chromatograms of standard sugar compounds and molasses sample are shown in Figures 2A and

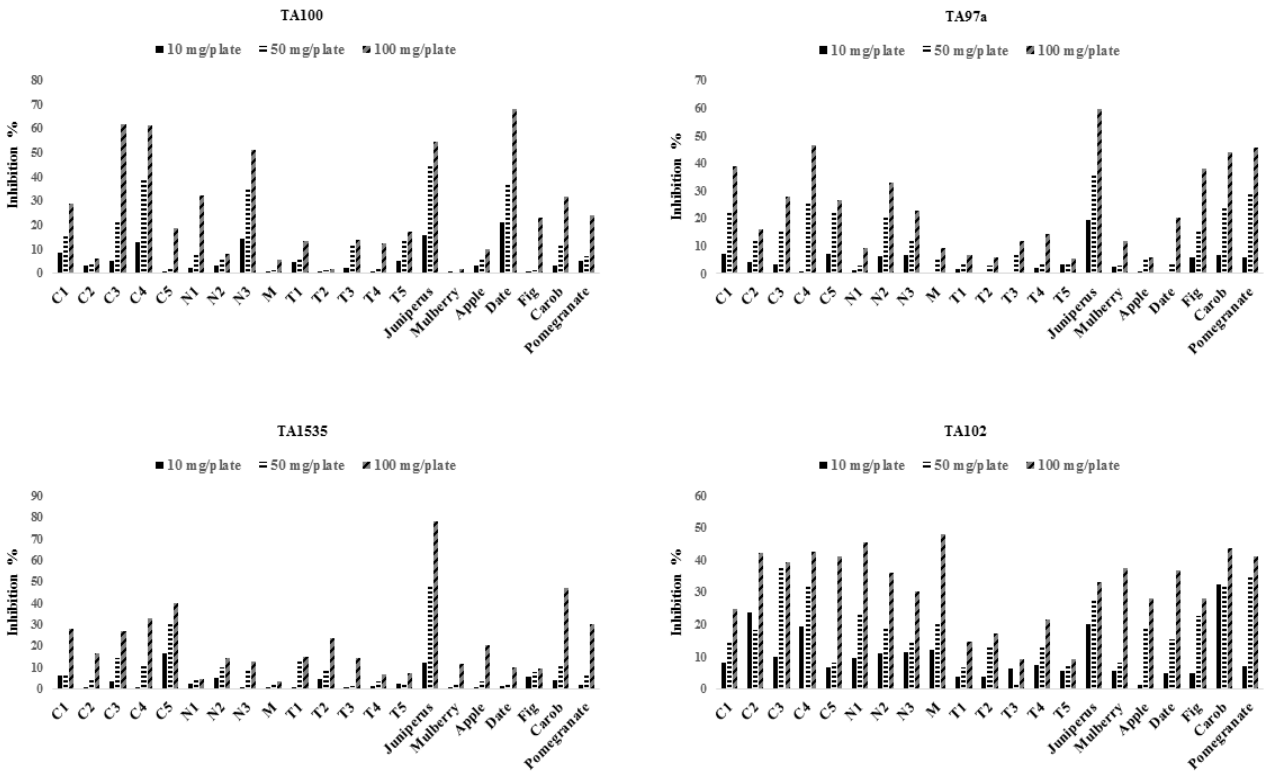

Figure 1. Inhibitory effect of different molasses samples against the mutagenicity of indirect mutagens to *Salmonella* Typhimurium TA98, TA100, TA97a, TA1535, and TA102 strains in the presence of metabolic activation. As an indirect mutagen, 2-aminofluorene (5 µg/plate) was used for TA97a, TA98, and TA100 strains, and 2-aminoanthracene (10 µg/plate) for TA102 and TA1535 strains. Inhibition rate of 40% or more was defined as strong antimutagenicity, and 25 - 40% inhibition as moderate antimutagenicity. Inhibitory effects of less than 25% were considered as weak, and not recognised as a positive result.

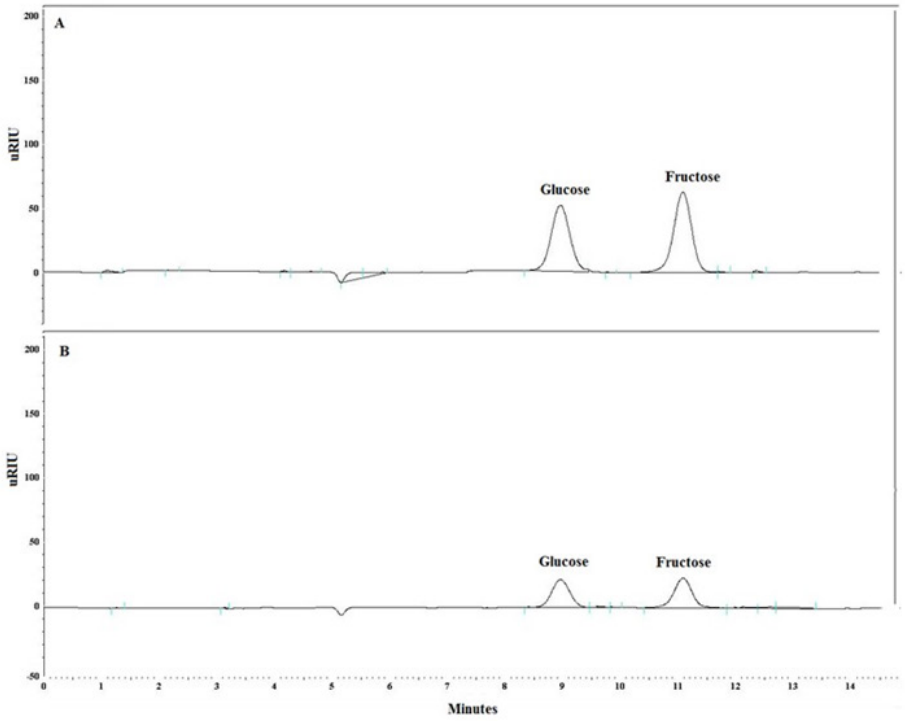

Figure 2. Chromatograms of (A) the mixture of glucose (8 mg/mL) and fructose standards (10 mg/mL), and (B) molasses sample (T1) analysed with HPLC-RI.

2B, respectively.

Microbiological evaluation of molasses samples

While there were no coliform bacteria in any of the 22 molasses samples; in traditionally produced C1, N2, date, and industrially produced T1-T5 grape molasses samples, mesophilic aerobic bacteria were detected with counts ranging between 2.4 and 3.5 log CFU/mL. Osmophilic yeasts were only observed in traditionally produced C4, C5 grape, and pomegranate molasses sample.

Discussion

Furan derivatives content

The HMF levels were highly variable in the analysed samples, ranging from 8.37 to 850.49 mg/kg. It is expected that uncontrolled excess heating during traditional production resulted in more furan derivative formation as compared to industrial technique. The formation of HMF in foods varies with storage and processing conditions, and is especially dependent on temperature (Janzowski *et al.,* 2000). As expected, our results showed that the average HMF content of traditionally produced grape molasses samples was twice higher than those produced industrially. This observation supports the previous report that demonstrated lower quantities of HMF in molasses produced industrially under vacuum condition as compared to the traditionally produced samples using the conventional open-boiling method (Batu, 1991). In addition, HMF levels for C3, apple, and fig samples, which were traditionally produced, and industrially produced date sample, were higher than the maximum acceptable level (70 mg/kg) established by Turkish Standards for molasses (Turkish Food Codex, 2007). According to previous studies, the HMF content of some dried fruits and caramel products can reach up to 1 g/kg because of food processing (Miao *et al.,* 2014). In the literature, the levels of HMF in grape molasses ranged from 7.38 to 801.80 mg/kg (Türkben *et al.,* 2016). The HMF levels of 11.9 - 101.7 mg/kg in grape molasses were found in a study conducted in Ankara, Turkey (Metin, 2014). Similarly, the range of 8.37 - 134.81 mg/kg HMF was found in grape molasses produced in different regions of Turkey in the present work. In addition, apple and fig molasses samples with the highest HMF contents presented high F levels. This observation clearly indicates the high thermal processing in these two samples. It is well established that the F generation is an indicator of intense thermal processing (Bedoya-Ramirez *et al.,* 2017).

Mutagenicity and antimutagenic activity

HMF is a toxic compound because it is converted to sulfoxymethylfurfural by sulfotransferases enzyme, and this compound is considered a mutagenic metabolite (Glatt *et al.,* 2012). Controversial results have been published on the mutagenicity of HMF with *S.* Typhimurium TA98 and TA100 strains in the presence of metabolic activation. Results of mutagenicity tests were negative without metabolic activation (Aeschbacher *et al.,* 1981; Kasai *et al.,* 1982; Kim *et al.,* 1987).

In a study, the genotoxicity of HMF was evaluated using the Ames test, micronucleus, and Comet assays in the metabolically active human HepG2 cell line. HMF did not exhibit mutagenicity in the Ames test, and did not induce micronucleus in the HepG2 cells. In contrast, results of the Comet assay suggested a weak genotoxic effect of HMF in the HepG2 cell line (Severin *et al.,* 2010).

In the present work, the mutagenic potential of molasses was determined using five different mutant *S.* Typhimurium strains according to the OECD guidelines (OECD, 1997). Results showed that molasses samples did not affect bacteria viability, thus suggesting no toxicity in the tested strains at concentrations of up to ≤ 100 mg/plate. The result also revealed that the investigated molasses samples were not mutagenic against the tested *S.* Typhimurium TA98, TA97a, TA102, and TA1535 strains, with or without S9 mix. In TA100 strain, a weak positive response was observed at high concentrations of C3 (100 mg/plate), N1 (50 and 100 mg/plate), N2 (100 mg/plate), apple (100 mg/plate), date (50 and 100 mg/plate), and fig (50 and 100 mg/plate) samples without metabolic activation, but not in the presence of S9 activation. These positive results were obtained in molasses samples, which contained higher HMF amounts than other molasses samples. The HMF content of these samples was in the range of 15.9 - 850.5 mg/kg. Omura *et al.* (1983) found a positive mutagenic effect at 0.2 - 2.0 µg/plate HMF concentrations in TA100 only, most potent without metabolic activation. Also, a weak mutagenic response was reported in *S.* Typhimurium TA100 in the absence of metabolic activation, while no mutagenicity was observed in strains TA97, TA98, TA102, and TA1535 in a range of concentrations between 0.1 - 10 mg/plate (EFSA, 2008). Negative mutagenic results in all *S.* Typhimurium strains with and without metabolic activation were reported in other studies (Aeschbacher *et al.,* 1981; Kim *et al.,* 1987).

HMF is activated to electrophilic sulpho conjugates by sulphotransferases enzyme. Glatt *et al.*

(2012) reported that HMF was inactive in TA100 strain but strongly mutagenic in TA100-derived strains expressing human sulphotransferase. In the evaluation of genotoxicity of foods containing HMF, it can be recommended to use bacterial strains and cells expressing sulphotransferases.

It should be noted that molasses is a complex matrix of different components that may either enhance or inhibit the mutagenic effect of HMF. In our previous study, we showed that molasses has a strong antioxidant activity due to its rich phenolic content (Helvacıoğlu *et al.,* 2018). Wang *et al.* (2011) reported that cane, carob, grape, and sorghum molasses display protective effects against direct mutagens at levels of 1 - 10 mg/plate in TA 100 and TA 98 strains, without S9 activation. However, only carob molasses showed inhibition rate of 36%, which was above the threshold limit of 25% for antimutagenic activity. In the experiment with S9 activation, the antimutagenic activity of all investigated molasses samples in that study increased when compared with the experiment without S9 activation. Similarly, results of the present work revealed that antimutagenic activity of molasses samples against indirect mutagens in TA100, TA97a, TA1535, and TA102 strains increased after metabolic activation when compared with the experiment without S9 activation. This observation indicated the apparent protective effect of molasses metabolites in these strains. The influence of metabolic activation on the antimutagenicity of plant polyphenols has also been stated previously (Charehsaz *et al.,* 2017). This observation may be attributed to the metabolites which directly protects DNA strands from electrophilic metabolites deriving from known mutagens.

Another possibility for the observation of antimutagenic activity only in the presence of metabolic activation is the inhibition of microsomal enzymes such as cytochromes P450 by the plant extracts. Buening *et al.* (1981) postulated that some flavonoids are potent inhibitors of cytochrome P450 enzymes such as CYP1A1 and 1A2. Ashour *et al.* (2017) investigated the potential inhibitory effects of aqueous and methanolic extracts of 57 widely used plants from traditional Chinese medicine on CYP450 3A4, using a luminescence assay. They revealed that many traditional Chinese medicinal plants could inhibit CYP3A4, which might cause potential interference with the metabolism of other concomitantly administered herbs or drugs. It is worthy of note that such inhibition may prevent bioactivation of some pro-carcinogenic/mutagenic chemicals such as benzo(a)pyrene and aflatoxin B_1. Further studies are emerging to clarify these possible mechanisms.

Sugar content of molasses samples

The rate of HMF formation is also dependent on the fructose:glucose ratio, and a high fructose:glucose ratio will accelerate the Maillard reaction (Lee and Nagy, 1990). Although the sugar composition of molasses samples varies depending on the variety or species of fruits, processing techniques, and parameters, sugars in molasses are generally in glucose and fructose forms (Simsek and Artık, 2002). The ratio of fructose to glucose must be between 0.9 and 1.1 in grape molasses (Turkish Food Codex, 2007). In the study of Türkben *et al.* (2016) and Simsek and Artık (2002), the fructose and glucose ratio of traditionally produced grape molasses samples was in the range of 0.9 - 1.1. However, in the present work, the results of the fructose to glucose ratio of T4, C2, C5, N3 grape, and apple molasses samples (1.3 - 2.1) were higher than the maximum limit stated in the Turkish Food Codex.

Microbiological evaluation

Enumeration of TMAB and coliform bacteria is important to show the hygienic quality and microbiological loads in food products (Morton, 2001; Tortorello, 2003). In the present work, the TMAB counts of molasses samples were not higher than the maximum acceptable levels for molasses according to the Turkish Food Codex (2002) on microbiological criteria. However, osmophilic yeast counts of traditionally produced C4 and C5 grape and pomegranate molasses samples were about 2.5 - 3-fold above the maximum acceptable level, thus indicating that hygienic conditions after production were not applied. Osmophilic yeasts can grow in high sugar content food products, and contamination may occur during processing, packaging, or storage of raw materials of the products (Walker and Ayres, 1970). During the traditional production of molasses, generally, modern processing units are not present, and boiling is done in open vessels (Batu, 1991; Türkben *et al.,* 2016). Although osmophilic yeasts do not lead to a significant threat to public health, they usually cause spoilage to high sugar content foods, and decrease the quality of food products, thus resulting in substantial economic losses.

Risk assessment

The risk assessment of the dietary intake of HMF using the consumption of investigated molasses samples was also performed based on the available no-observed-adverse-effect level

(NOAEL) and lowest-observed-adverse-effect level (LOAEL) data.

In a repeated dose toxicity study, rats were given 40, 80, and 160 mg/kg HMF for one year. At 160 mg/kg, there was an increase in the serum gamma globulin and hepatic tributyrase levels. Also, an increase in the spleen weight was observed at the highest dose (Abraham *et al.,* 2011). In a three-month toxicity study in mice, lower weight was reported at the dose of 750 mg/kg when compared with the controls. Histopathological examinations revealed cytoplasmic changes in the kidneys of the 188 mg/kg HMF treated group. At 94 mg/kg and below, no adverse effects were observed (NTP, 2010). The NOAEL value in these two studies was 80 and 94 mg/kg in rats and mice, respectively.

In a two-year carcinogenicity study, groups of 50 males and 50 females rats and mice were given 188, 375, or 750 mg/kg HMF by oral gavage. The olfactory epithelium degeneration was observed in the rats at 750 mg/kg in males, and 188 and 375 mg/kg in females. Respiratory metaplasia increased in the highest dose group. The NOAEL of 375 mg/kg/day and LOAEL of 188 mg/kg/day were derived from the present work. In mice, the highest dose group of both genders showed a significantly lower weight than in the control group. Similar lesions to those observed in the rats were found in mice at 375 and 750 mg/kg dose groups. The NOAEL of 188 mg/kg/day in mice was derived from the present work (Abraham *et al.,* 2011). Hence, the maximum dose observed with no adverse effects (NOAEL) regarding repeated dose toxicity and carcinogenicity in experimental animals is in the range of 80 - 100 mg/kg body weight per day.

Based on the NOAEL of 80 mg/kg/day, the Margin of Exposure (MOE) was calculated to determine human health risk from exposure to the HMF from investigated molasses samples. The MOE is a ratio of the NOAEL to the estimated exposure dose, and low risk is implied when MOE > 100. Considering the consumption of two tablespoons (~30 g/day) molasses by a 50 kg person, the MOE for HMF in investigated samples was at the range of 156 - 16,000, which provided MOE > 100 (Lachenmeier and Rehm, 2015).

The repeated dose toxicity data on F are sufficient for the repeated dose toxicity endpoint. In a study, rats were given microencapsulated F in the diet for 90 days. The NOAEL in that study was established as 53 mg/kg/day (Türköz-Acar *et al.,* 2018). Based on the NOAEL of 53 mg/kg/day, The MOE is 4,492 and 20,703 for apple and fig molasses samples, respectively.

FK is a dietary furan with wide industrial applications, and also found in a variety of food items. Little information is available regarding the health consequences of FK. Sujatha (2008) reported significant variations in the activity of aspartate and alanine aminotransferase in both liver and kidney of mouse after prolonged exposure to FK. The NOAEL of 25 mg/kg/day obtained from a 90-day study in rats (Lough, 1985) provides MOE > 100 in relation to the estimated daily exposure using molasses consumption. Taken together, according to the NOAEL and LOAEL data, the consumption of all investigated molasses samples in the present work was safe in terms of HMF, F, and FK.

Conclusion

Results obtained in the present work suggested that both traditionally and industrially produced molasses samples are safe from the viewpoint of mutagenicity risk, and in fact, they were found to have a protective effect against mutagenicity in the presence of metabolic activation. The margins of exposure for all analysed samples were above the value of 100, thus indicating the safety of samples regarding furan derivative exposure. However, the higher HMF content and osmophilic yeast counts of traditionally produced molasses as compared to the industrially produced samples is alarming, which suggests that traditional and industrial food productions may need separate risk assessments by the national authority.

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