

Validation of HPLC Method for the Determination of 5-hydroxymethylfurfural in Pestil, Köme, Jam, Marmalade And Pekmez

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ABSTRACT

This study represents a high performance liquid chromatography (HPLC) method for the detection of 5-hydroxymethylfurfural in pestil, köme, jam and pekmez samples. The linearity, selectivity, decision limit, detection capability, detection limit, quantification limit, precision, recovery, ruggedness and measurement uncertainty of the method were determined. The developed method, simple and accurate, showed good recovery values (97-108%). The accuracy of the method expressed with the relative standard deviation was below 6%. The detection limit and quantification limit were 0.03 mg/kg and 0.10 mg/kg, respectively. HMF levels in pestil, köme, jam, marmalade and pekmez samples were determined using the validated method.

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INTRODUCTION

5-Hydroxymethylfurfural (HMF) is a furanic compound formed under acidic conditions by the Maillard reaction or sugar dehydration [1]. Maillard reaction is a non-enzymatic browning reaction, occurs when foods including reducing sugars and amino acids are heated. HMF is an intermediate product of this reaction. Moreover, HMF formation takes place during hexoses dehydration at lower pH (< 5) via enolisation, for which the presence of amino acid groups is not needed [2].

HMF and its derivatives have been reported to show toxic properties such as cytotoxic, genotoxic, nephrotoxic, mutagenic and cancerogenic. The presence of HMF in foods has gained interest due to the toxicological concerns about HMF. Although further studies have revealed that HMF does not exhibit a crucial health risk, it has been a matter of debate [3].

Although HMF is nearly absent in untreated foods, it occurs in processed foods containing carbohydrates such as bread, biscuits, jam, marmalade, honey and fruit juice [4, 5, 6]. HMF amount tends to increase during heat treatment and storage. Therefore, the

determination of HMF content can be used to evaluate the effects of food processing industry and storage conditions on the quality of food products [7, 2]. HMF has been used to evaluate the sensorial properties of food products. The changes in the color, flavor and taste of food products during processing and storage are related to the HMF content. Hence it is recognized as an indicator of improper processing and storage conditions [8, 9]. The Turkish Standards [10, 11] state a maximum HMF level of 50 mg/kg in pestil and köme and jam [12]. The established maximum HMF levels are 75 mg/kg, and 100 mg/kg for liquid pekmez, and solid pekmez in accordance with [13].

In the past, a great number of the methods developed for the detection of HMF in foodstuffs were based on spectrophotometric techniques [14, 15]. Although spectrophotometric methods are fast, their sensitivity and specificity are low. Chromatographic methods have been used and developed to detect HMF compounds in food products. UV detection of High performance liquid chromatography (HPLC) is mostly used method used for the determination of HMF in foodstuffs. Accuracy and sensitivity of the

HPLC method are better than that of spectrophotometric methods. Gas chromatography (GC)-mass spectrometry (MS) analysis has been proposed for HMF determination as well [5].

The methods for the determination of HMF have been developed for primarily honey samples. Reliable, sensitive and rapid methods are required to determine HMF in different matrices because of the potential toxic effects of HMF and quality control of food products. The goal of this investigation was to develop and validate a sensitive, reliable and rapid method for the detection of HMF in pestil, köme, jam and pekmez. The proposed method was validated with respect to decision limit, detection limit, quantification limit, selectivity, linearity, precision, recovery and ruggedness. Practising of the developed procedure to real samples was carried out as well.

METHODS

Samples

Pestil, köme, jam and pekmez samples were collected from a local market in Trabzon and Gümüşhane. All samples were stored at 4°C until analysis.

Chemicals

Analytical chemicals and HPLC grade solvents were obtained from Merck (Darmstadt, Germany). HMF standard (99%) was bought from Sigma-Aldrich (St. Louis, MO, USA). Membrane filters (45 µm) were supplied by Millipore (Bedford, MA, USA).

Sample preparation

Specimens were homogenized by an Ultra Turrax mixer (IKA, Germany). 5 g of the sample was dissolved with 25 mL water in a 100 mL flask, 0.5 mL of Carrez I solution and 0.5 mL of Carrez II solution were added, later water was added to the mark. The sample solutions were filtered through 45 µm membrane filters. 100 µL of the sample solution was injected to the HPLC-UV system.

Equipment

Quantitative analysis was carried out using an HPLC-UV system (Agilent 1100 series, USA). The separation of HMF was carried on a C₁₈ column, 250 mm×4.6 mm, 5 µm (Nucleosil, USA). The mobile phase, water-methanol (90:10 v/v), was at a flow rate of 1 mL/min, wavelength at 285 nm.

Method validation

The HPLC method based the method for HMF detection in honey samples was validated and applied to pestil, köme,

jam, marmalade and pekmez samples of the International Honey Commission [16]. The sensitivity, linearity, decision limit, detection capability, detection limit, quantification limit, precision, recovery, ruggedness and measurement obscure of the method were ascertained to validate the method for HMF analysis in the studied samples.

Statistical analysis

Microsoft Excel 2007 (Microsoft Corp., Redmond, WA, USA) was used for data processing. Outliers were checked and removed based on the Cochran test and Grubbs test. Linear regression model was performed using the least squares approach.

RESULTS AND DISCUSSION

Validation

Single laboratory validation was performed according to Regulation 2004/882/EC. Performance characteristics of validated method determined were selectivity, linearity, detection and quantification limits, decision limit, precision, recovery, ruggedness and measurement obscure.

Selectivity

The selectivity of a method describes the ability to detect. Before beginning the validation process, the selectivity of method should be checked against naturally occurring substances. Representative blank samples ($n=20$) were analyzed and their chromatograms were compared with the chromatogram of the spiked samples. As can be seen from Fig. 1, no interference observed at the retention time of HMF indicated that the proposed method was selective for HMF analysis.

Linearity

A calibration curve was obtained by plotting the peak areas of standard solutions which were the three series of five different concentrations. The calibration curve equation described as $y = a(x) + b$, where y is the peak area of standard solution in terms of absorbance, x is the concentration of standard solution in mg/kg. Good linearity was obtained in the studied range, with R^2 value higher than 0.999 (Table 1). Preparation and mass concentration of calibration used for HPLC-UV and some analytical parameters from the developed method.

Limit of detection and limit of quantification

The Limit of quantification (LOQ), lowest content of the analyte which can be measured with reasonable statistical certainty. If both accuracy and precision are

Table 1. Preparation and mass concentration of calibration used for HPLC-UV and some analytical parameters

Main stock solution (mg L ⁻¹)	Volumes V _i (μL) of main stock solution diluted ^b to 25 mL to be used as calibrator					Calibration concentration <i>i</i> (mg L ⁻¹)				
	1 st	2 nd	3 rd	4 th	5 th	1 st	2 nd	3 rd	4 th	5 th
1000 ^a	25	50	100	200	300	1.0	2.0	4.0	8.0	12.0
Calibration range (mg/kg)								1.0-12.0		
Calibration equation								$y = 7036x - 0.3$		
Regression coefficient								0.999		

^a HMF standard was used 99% purified

^b Dilution of V_i of the stock solution to 25 mL with purified water, to produce calibrations *i*

^c *y* is the peak area of standard solution expressed in absorbance, *x* is the concentration of standard solution expressed in mg/kg.

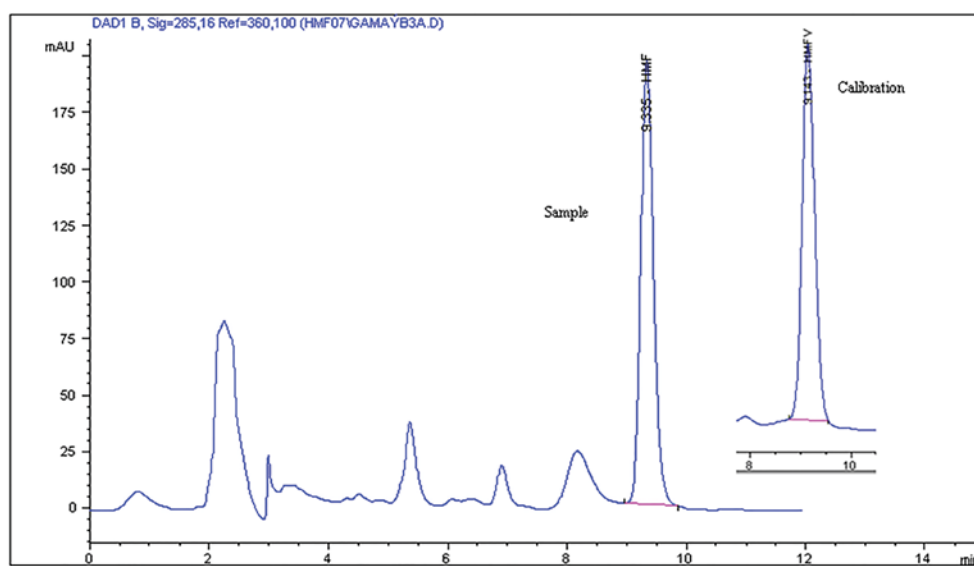


Figure 1. A) representative blank sample B) spiked specimen

constant over a concentration range around the limit of detection, then the limit of quantification is numerically equal to 10 times the standard deviation of the mean of 0.2 mg/kg. The limit of detection (LOD), expressed as the concentration, or the quantity, is derived from the smallest measure, that can be detected with reasonable certainty for a given analytical procedure. The limit of quantification is numerically equal to 3 times the

standard deviation of the mean of 0.2 mg/kg. LOD and LOQ were determined by analyzing ten samples spiked with HMF (0.2 mg/kg) in accordance with Analytical Detection Limit Guidance [17]. In order to estimation of LOD, the standard deviation of the response (*s*) was multiplied by the Student's *t*-test value for ten replicates and nine degrees of freedom. The forecasted LOD values were confirmed according to the guidance [17] as well.

Table 2. The decision limit (CC_α) and detection capability (CC_β) values of the method calculated at the MRL (n=20) for the HMF

Matrix	Added (mg/kg)	Measured ± S.D. (mg/kg)	Error α (1.64 × S.D.)	CC _α (mg/kg)	Added (mg/kg)	Measured ± S.D. (mg/kg)	Error β (1.64 × S.D.)	CC _β (mg/kg)
Jam	0.03 ^a	0.04 ± 0.02	0.03	0.06	0.06	0.05 ± 0.01	0.02	0.08
	50.00 ^b	50.50 ± 0.88	1.44	51.44	51.44	50.65 ± 0.62	1.02	52.46
Liquid pekmez	0.03 ^a	0.03 ± 0.01	0.02	0.05	0.05	0.04 ± 0.01	0.02	0.08
	75.00 ^b	74.50 ± 0.66	1.08	76.08	76.08	75.50 ± 0.79	1.30	77.38
Solid pekmez	0.03 ^a	0.04 ± 0.02	0.03	0.06	0.06	0.04 ± 0.02	0.03	0.09
	100.00 ^b	99.50 ± 1.06	1.74	101.74	101.74	100.50 ± 0.98	1.61	103.35
Kome	0.03 ^a	0.028 ± 0.01	0.02	0.05	0.05	0.048 ± 0.01	0.02	0.07
	50.00 ^b	49.80 ± 0.74	1.21	51.21	51.21	50.80 ± 0.67	1.10	52.31
Pestil	0.03 ^a	0.028 ± 0.01	0.02	0.05	0.05	0.045 ± 0.02	0.10	0.07
	50.00 ^b	49.1 ± 0.91	1.49	51.49	51.49	51.20 ± 0.72	1.18	52.67

^a limit of quantification, ^b maximum permitted limit

Table 3. The repeatability and within-laboratory reproducibility of the method, expressed with the standard deviation and the relative standard deviation.

Analyte	Intra-day (n=6)				Inter-day (n=6)				
	Fortification level mg/kg	Determined level mg/kg	S_r mg/kg	Precision RSD_r (%)	0.66 X Horwitz value (%)	Determined level mg/kg	S_R mg/kg	Precision RSD_R (%)	Horwitz value (%)
Jam	25.00 ^a	25.42	1.05	4.11	6.51	24.54	1.56	6.36	9.86
	50.00 ^b	48.93	1.69	3.45	5.86	49.73	1.17	2.35	8.88
	75.00 ^c	74.35	1.24	1.67	5.51	74.32	1.15	1.55	8.35
Liquid pekmez	37.50 ^a	36.94	1.19	3.22	6.12	37.91	1.22	3.22	9.27
	75.00 ^b	74.80	0.88	1.18	5.51	74.97	1.13	1.51	8.35
	112.50 ^c	111.97	1.08	0.97	5.19	112.24	1.69	1.50	7.86
Solid Pekmez	50.00 ^a	49.63	1.39	2.80	5.86	49.86	1.17	2.35	8.88
	100.00 ^b	99.24	1.26	1.27	5.28	99.84	1.18	1.31	8.00
	150.00 ^c	148.30	0.97	0.65	4.97	149.85	1.72	1.15	7.53
Köme	25.00 ^a	24.02	0.93	3.87	6.51	24.56	1.21	4.93	9.86
	50.00 ^b	48.96	1.07	2.18	5.86	48.87	1.25	2.57	8.88
	75.00 ^c	74.82	1.58	2.11	5.51	74.94	1.89	2.52	8.35
Pestil	25.00 ^a	25.96	0.93	3.58	6.51	23.59	1.21	5.13	9.86
	50.00 ^b	51.23	1.07	2.08	5.86	50.80	1.25	2.46	8.88
	75.00 ^c	74.15	1.58	2.13	5.51	73.90	1.89	2.56	8.35

^a0.5x MRL, ^bMRL, ^c1.5xMRL

LOQ values were evaluated as ten times of the standard deviation. The determined LOD and LOQ values for HMF substances were 0.01 mg/kg and 0.03 mg/kg, respectively.

Decision limit and detection capability

Two new performance characteristic of decision limit (CC_α) and detection capability (CC_β), are submitted by Regulation 2002/657/EC. CC_α refers to the above the limit of samples concluded as non-compliant ($\alpha = 5\%$) and CC_β refers the lowest content of the substances that may be detected, identified and/or quantified in a sample ($\beta = 5\%$). The CC_α and CC_β values were determined to analyze 20 blank samples fortified with HMF at the maximum permitted limit (MRL) regulated by Turkish Food Codex and Turkish Standards. Estimated CC_α and CC_β values according to the following equations of the method were calculated in Table 2.

CC_α = the concentration at MRL + 1.64 x the standard deviation of the fortified samples [1] $CC_\beta = CC_\alpha + 1.64 \times$ the standard deviation of the fortified samples [2]

Precision

Precision was determined by analyzing twelve empty specimen spiked with HMF standard solution at the

concentrations of 0.5, 1 and 1.5 times MRL. For the precision test, specimens were conducted in ten replicates and analyses were fulfilled by the same operator in one day. To determine the intermediate precision, samples (ten replicates) were analyzed by two different operators in three days over a month. The results for the repeatability expressed with the standard deviation (S_r) and the relative standard deviation (RSD_r) and the results for the within-laboratory reproducibility expressed with the standard deviation (S_R) and the relative standard deviation (RSD_R) are presented in Table 3. Both RSD values at the three concentration levels were found to be lower than the reference values (Table 3) calculated from the Horwitz equation.

Recovery

Recovery is a measure of the accuracy. The reclamation of the method was determined instead of trueness since reference material cannot be available. Three different concentrations of the HMF standard were added to samples, 0.5, 1 and 1.5 times MRL. (25, 50, and 75 mg/kg for köme, jam and pestil; 37.5, 75, and 112.5 mg/kg for jam; 50, 100, and 150 mg/kg for solid pekmez; 37.5, 75, and 112.5 mg/kg for liquid pekmez) were analyzed to determine the recovery values. The recovery values obtained ranged from 97.18% to 107.68% (Table 4),

Table 4. The recovery values of spiked samples at three different concentrations (n=6).

Sample	Spiked level (mg/kg)	Mean Determined Level (mg/kg)	Mean Recovery (%)	S (mg/kg)	RSD (%)
Jam	25.00 ^a	24.60	98.40	0.44	1.79
	50.00 ^b	49.11	98.22	0.58	1.18
	75.00 ^c	73.62	98.16	1.05	1.43
Liquid Pekmez	37.50 ^a	37.14	98.80	0.46	1.24
	75.00 ^b	76.33	99.04	0.53	0.69
	112.50 ^c	110.65	98.36	1.47	1.33
Solid Pekmez	50.00 ^a	49.57	99.14	0.68	1.37
	100.00 ^b	99.69	99.69	0.96	0.96
	150.00 ^c	148.44	98.96	0.85	0.86
Köme	25.00 ^a	26.92	107.68	0.64	2.38
	50.00 ^b	49.53	99.06	1.52	3.06
	75.00 ^c	74.12	100.2	0.93	1.25
Pestil	25.00 ^a	23.92	98.83	0.64	2.68
	50.00 ^b	48.59	97.18	1.52	3.13
	75.00 ^c	74.54	99.39	0.93	1.25

^a0.5x MRL, ^bMRL, ^c1.5xMRL

showing good recovery values for the proposed method.

Ruggedness

Ruggedness of the method was evaluated by Youden test. Eight experiments were performed to evaluate the seven selected factors: extraction solution, specimen matrix, specimen preparation, analyst, column temperature, HPLC column and mobile phase. The standard deviation of impacts was evaluated according the following equation.

$$SD = 2\sqrt{\sum \left(\frac{E_i^2}{n}\right)} \quad [3]$$

Where E_i is each of the calculated effect, and n is the number of parameters.

The influence of the factors on method performance has to be checked applying the t -test [18]. The experimental t -values for the factors were calculated according to the formula given below:

$$t = \frac{E_i \cdot \sqrt{n}}{SD \cdot \sqrt{2}} \quad [4]$$

The experimental t -values (Table 5) were found to be lower than the critical value ($t_{crit} = 2.45$ at 95% confidence level), indicating that the method is sufficiently rugged

against the changes in the procedure. As a result, the proposed useful way was validated for the determination of HMF in pestil, köme, jam and pekmez.

Measurement uncertainty

The validation data was used to calculate the measurement uncertainty [19]. Volume, mass, calibration curve, reproducibility and repeatability of the method, preparation of standard, accuracy and reproducibility of the equipment were selected as sources of uncertainty budget. The relative expanded uncertainty of measurement was reckoned using a coverage factor $k=2$, corresponding approximately 95% confidence level. Expanded uncertainty value was 9.1% for HMF in foodstuffs.

Application of the method to real samples

Pestil, köme, jam, and pekmez samples were analyzed using the validated method. Pekmez, a traditional food product in Turkey, is concentrated grape or mulberry juice formed by boiling without the addition of sugar and another ingredients [20]. Pestil and köme in Turkey are made from both fruit juice and concentrated fruit juice [21]. The HMF content of pekmez samples (solid *n:25 and liquid*) was found to be ranged from 1.44 to 66.30 mg/kg, complying with the values set by the Turkish Codex [13]. Available data on the HMF content of pekmez is limited. Our results seemed to be higher

Table 5. Experimental design for the ruggedness study (50.0 mg/kg)

Factor	Original parameters (A-G)	Changed parameter (a-g)
A. Extraction solvent	Water	a. Water / Methanol (90/10 v/v)
B. Sample matrix	Pestil, köme, jam, marmalade and pekmez	b. Blank sample
C. Sample preparation	Addition of carrez I and II	c. No addition of carrez I and II
D. Analyst	Analysts 1	d. Analysts 2
E. Column temperature	22 oC (Room temperature)	e. 27 oC
F. LC column	250 mm'4.6 mm, 5 mm C18	f. 250x4.6 mm, 5µm, ODS 2
G. Mobile phase	Water/ methanol (90/10 v/v)	g. Water/methanol (80/20 v/v)
+a	-b	1 2 3 4 5 6 7 8 Ei Ei*Ei t
A	a	49.3 48.6 49.2 47.9 -48.1 -48.2 -48.9 -51.2 -0.350 0.12250 0.21
B	b	49.3 48.6 -49.2 -47.9 48.1 48.2 -48.9 -51.2 -0.750 0.56250 0.98
C	c	49.3 -48.6 49.2 -47.9 48.1 -48.2 48.9 -51.2 -0.100 0.01000 0.02
D	d	49.3 48.6 -49.2 -47.9 -48.1 -48.2 48.9 51.2 1.150 1.32250 2.32
E	e	49.3 -48.6 49.2 -47.9 -48.1 48.2 -48.9 51.2 1.100 1.21000 2.12
F	f	49.3 -48.6 -49.2 47.9 48.1 -48.2 -48.9 51.2 0.400 0.02286 0.04
G	g	49.3 -48.6 -49.2 47.9 -48.1 48.2 48.9 -51.2 -0.700 0.49000 0.86
		SD 1.06867

^a Original parameters, ^b Changed parameters, Ei is each of the calculated effects.

compared to the literature[22]. The HMF content of jam samples ($n:25$) varied from 12 to 22 mg/kg. These values were in agreement with data found in the literature [2, 8]. The HMF content of Pestil and köme (solid $n:25$ and liquid) was found to be ranged from 1.3 to 45.3 mg/kg. These values were in agreement with data found in the literature [23].

CONCLUSIONS

An method for the extraction of 5-HMF from food samples and its subsequent determination using HPLC with UV detection was validated according to Regulation 2004/882/EC. The validated method provides accurate results and offers quick and economic procedure. The conclusion can be derived that recommended method is suitable for the detection of HMF in the food matrices such as köme, pestil, jam, and pekmez.

REFERENCES

1. Capuano, E. And Fogliano, V. Acrylamide and 5-hydroxymethyl-furfural (HMF), A review of metabolism, toxicity, occurrence in food and mitigation strategies. LWT-Food Sci. Technol., Vol. 44 Pages 793-810. 2011.
2. Teixido, E., Nunez, O., Santos, F.J., Galceran, M.T. 5-Hydroxymethylfurfural content in foodstuffs determined by micellar electrokinetic chromatography. Food Chem Vol. 126, Pages 1192, 2011.
3. Spano, N., Casula, L., Panzanelli, A., Pilo, M.I., Piu, P.C., Scanu, R., Tapparò, A., Sanna, G. A RP-HPLC determination of 5-hydroxymethylfurfural in honey - The case of strawberry tree honey. Talanta Vol: 68 Issue: 4 Pages: 1390-1395, 2006.
4. Ameur LA, Rega B, Giampaoli P, Trystram G, Birlouez-Aragon I. The fate of furfurals and other volatile markers during the baking process of a model cookie. Food Chem Vol.:111, Issue 3, Pages 758-763 2008.
5. Gaspar, EMS., Lopes, J.F. Simple gas chromatographic method for furfural analysis. J Chromatogr A, Vol. 1216, Issue 14, Pages 2762-2767, 2009.
6. Rada-Mendoza, M., Sanz, M.L., Olano, A., Villamiel, M. Formation of hydroxymethylfurfural and furosine during the storage of jams and fruit-based infant foods. Food Chem Vol.85, Issue , Pages 605-609, 2004.
7. Kus, S., Gogus, F., Eren S., Hydroxymethyl furfural content of concentrated food products. Int J Food Properties Vol.8, Issue 2, Pages 367-375, 2005.
8. Rada-Mendoza, M., Olano, A., Villamiel, M. Determination of hydroxymethylfurfural in commercial jams and in fruit-based infant foods. Food Chemistry Vol.79, Issue 4, Pages 513-516, 2002.
9. Teixido, E., Moyano, E., Santos, F.J., Galceran, M.T. Liquid chromatography multi-stage mass spectrometry for the analysis of 5-hydroxymethylfurfural in foods. J Chromatographia A Vol.1185, Issue 1, Pages 102-108, 2008.
10. TS 12677, Spreaded dried mulberry, Turkish Standard, 24.10.2000.
11. TS 12477, Pekmez mass with decorticated fruits, Turkish Standard, 17.11.2015

12. TS 3958, Sour Cherry Jam, Turkish Standard, 09.03.2010.
13. Regulation on Turkish Food Codex, 29 December 2011 - 28157
14. Winkler, O.: Beitrag zum Nachweis und zur Bestimmung von Oxymethylfurfural in Honigund Kunsthonig. Z. Lebensm. Forsch. 102, 160-167, 1955.
15. White, J., Spectrophotometric Method for Hydroxymethylfurfural in Honey. J. AOAC, 509, 1979.
16. Harmonised Methods Of The International Honey Commission <http://www.bee-hexagon.net/en/network.htm>.
17. Analytical Detection Limit Guidance & Laboratory Guide for Determining Method Detection Limits, Wisconsin Department of Natural Resources Laboratory Certification Program, April 1996.
18. Bratinova S., Raffael B., Simoneau C. Guidelines for performance criteria and validation procedures of Analytical methods used in controls of food contact Materials. EUR 24105 EN – 1st edition 2009
19. EURACHEM / CITAC Guide CG 4 Quantifying Uncertainty in Analytical Measurement Third Edition. www.eurachem.org.
20. Kaya, A., Belibagli, KB. Rheology of solid Gaziantep Pekmez. J Food Eng vol.54, Issue 3, Pages 221-226, 2002.
21. Ercişli, S., Orhan, E. Chemical composition of white (*Morusalba*), red (*Morusrubra*) and black (*Morusnigra*) mulberry fruits. Food Chem 103: 1380-1384, 2007.
22. Sengul, M., Ertugay, MF., Sengul, M. Rheological, physical and chemical characteristics of mulberry pekmez. Food Control, Vol. 16, Issue 1, Pages 73-76, 2005.
23. Yildiz, O. Physicochemical and sensory properties of mulberry products: Gümüşhane pestil and köme. Turkish Journal of Agriculture and Forestry, 37: 762-771, 2013.