ORIGINAL ARTICLE

A C G publications

J. Chem. Metrol. 12:1 (2018) 17-25

journal of chemical metrology

Rapid and reliable 25-OH vitamin D2 and 25-OH vitamin D3 measurements by multitasker LC-MS/MS

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(Received February 16, 2018; Revised April 24, 2018; Accepted April 30, 2018)

Abstract: The 25-OH Vitamin D2 and 25-OH Vitamin D3 measurement method was validated using a Zivak Multitasker Fully Automated Sample Preparation and Injection System coupled to a MS detector. The current method incorporates all sample preparations, SPE clean up procedures, LC and MS facilities into an all in one system. This system can report patient data within 6 minutes including the SPE and Sample preparation and mass spectrometry analysis time. The correlation coefficient of the developed method was greater than 0.99 for measurands, 25-OH-vitamin Ds, in the calibration range. The measurement uncertainty of the measurements were evaluated and found as 7.7 % and 7.6 % for 25-OH vitamin D2 and 25-OH-vitamin D3, respectively.

Keywords: 25-OH Vitamin D2; 25-OH Vitamin D3; MS/MS; ZİVAK multitasker; method validation; uncertainty.

1. Introduction

Vitamin D, also known as vitamin sunshine, is a fat soluble vitamin found in the forms of Vitamin D2 and Vitamin D3. Vitamin D2 is usually found in several vegetable based foods while Vitamin D3 is predominantly found in meat. Vitamin D3 is formed naturally by the exposure of our skin to UV radiation found in sunlight. The main function of Vitamin D in human metabolism is defined as regulating calcium and phosphorus levels in the bloodstream. There are an increasing amount of studies and views on the different metabolic functions of Vitamin D. Studies show that its` widespread impact and the need for its reliable and quick measurement is of paramount importance [1-6].

Vitamin D is metabolized in the human body to 25-hydroxyvitamin D3 [25-(OH)D3], 3-epi-25-hydroxyvitamin D3 [3-epi-25(OH)D3] and 25-hydroxyvitamin D2 [25-(OH)D2] and these metabolites are commonly studied in human serums. The total concentration of 25(OH)D2 and 25(OH)D3 in human serums are generally within 16 ng/g - 30 ng/g (40 nmol/L - 75 nmol/L) of which 90% is in the 25(OH)D3 form and

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The article was published by ACG Publications
www.acgpubs.org/jcm © Published January-June 2018 EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.13.18.02.070

the remaining 10% is a combination of all other forms. Due to 3-epi-25(OH)D3 being found only in trace amounts, it is not commonly studied in routine analyses carried out by clinical biochemists. Values below 12 ng/mL (30 nmol/mL) of 25-OH vitamin D are defined as a Vitamin D deficiency and point towards rickets in newly born babies and osteomalacia in adults [4-9]. A value of 12-20 ng/mL (20-50 nmol/mL) is considered inadequate for bone and overall health in healthy individuals. A value of 20-50 ng/mL (50-125 nmol/mL) is deemed adequate for a healthy adult however a value above this is evaluated to be serious and probable evidence for potential side effects.

The metrological examinations of vitamins and metabolites have been well established by metrology institutes and are used in clinical chemistry. The NIST provided service of SRM 972 to clinical laboratories in 2009 [10] and SRM 972a in 2013 [11] while UME CRM 1308 was provided by TUBITAK UME in 2016 [12]. While the various metrology institutes over the world report Vitamin D measurements with low uncertainties in the CCQM level, 2.3% to 6.8% for Vitamin D2 and 2.1% to 12.6% for Vitamin D3, the uncertainty values are generally not only not reported by second tier laboratories but also often report CV values between 10-30% [1-9].

It is seen from reported Vitamin D measurements using the common immunoassay (CLIA, EIA and RIA) method with HPLC methods have a higher range in measurement uncertainties in comparison to LC-MS/MS measurements which have a lower range [7,8]. Therefore the ID-LC-MS/MS is accepted as the primary method of measuring Vitamin D and because of its accurate and precise measurements it is becoming increasingly popular in clinical laboratories [4-7].

The major obstacle in clinical laboratories is the preparation of samples which is time consuming, prone to error and an increase in uncertainty arising from reproducibility which in turn reduces reliability in the measurements. ZIVAK Vitamin D was developed to obtain quick and reliable measurements in the laboratory and 25(OH)D2 and 25(OH)D3 measurements were performed with the ZIVAK Vitamin D analyzer in order to overcome these obstacles. The validation of the instrument and kit was carried out and are reported in this study.

2. Experimental

2.1. Standards and Chemicals

UME CRM 1308 (51.00 ± 2.92 (ng/mL) for 25-OH-vitamin D2 and 49.76 ± 2.65 (ng/mL) for 25-OH-vitamin D3) obtained from TUBITAK UME. 25-OH-vitamin D3 lyophilized serums control produced by ZIVAK coded as ZV-3046-02S1-10, ZV-3046-02S2-10, ZV-3046-02S3-10 (Table 1). The Reagent 1 (ZV-304602R1-10) and Reagent 2 (ZV-304602R2-10) were obtained from ZIVAK Technologies together with the Mobile Phase A (ZV-3046-02-MP-10), SPE Buffer (ZV3046-02-MT-10) and washing solution (ZV3046-02-WB-10). Working lyophilized serum calibrators containing 25-OH Vitamin D2 and D3 were prepared in distilled water (v/v) based on the linearity range and all sample preparation steps, dilution, precipitation, vortexing, centrifuge and injection steps were done by using the ZIVAK Vitamin D Multitasker system with the software of AUTOMASS. The chemicals and reagents were stored in a refrigerator at –20 ^{o}C in glass containers.

Table 1. Lyophilized serum control materials for 25-OH-vitamin Ds

The Code	Control Standard	Lot number	25-OH-VitD2 (ng/mL)	25-OH-VitD3 (ng/mL)
ZV-3046-02S1-10	Calibrator Level 1	VD1702 S1	9.99	14.71
ZV-3046-02S2-10	Calibrator Level 2	VD1702 S2	28.68	25.88
ZV-3046-02S3-10	Calibrator Level 3	VD1702 S3	41.62	32.61
ZV-3046-02S4-10	Calibrator Level 4	VD1702 S4	53.74	39.80

2.2. MS Parameters

Waters Quattro Premier Tandem Quadrupole MS equipped with a heated electrospray ionization source (ESI) was used in positive mode as mass detector in the applied method. The capillary voltage was 3.5 kV.

The details of the applied method were given in Table 2. The Zivak Multitasker Fully Automated Sample Preparation and Injection System were used with the Mobile Phase of the ZIVAK Vitamin D analysis kit.

Table 2. Mass Spectrometry parameters of applied MS/MS method

	Parent ***(m/z)***	**Daughter** ***(m/z)***	**Dwell time (s)**	**Cone (V)**	**Collision (V)**
25-OH-Vitamin D2	413.1	81.6	0.050	24	22
25-OH-Vitamin D3	401.15	383.2	0.050	22	10
25-OH-Vitamin D2-d6 (IS)	407.15	158.75	0.050	22	28

2.3. Sample Preparation

25-Hydroxyvitamin D2-D3 analysis was performed using the Zivak Multitasker Fully Automated Sample Preparation and Injection System connected to an MS system which is a Waters Quattro Premier Tandem Quadrupole MS equipped with a heated electrospray ionization source (ESI). The system was controlled with AUTOMASS software. The SPE purification was done automatically using ZV-3046-CSPE-10, Zivak 25-Hydroxyvitamin D2-D3 Serum LC-MS/MS Trap Column Cartridge via AUTOMASS software and a Zivak 25-Hydroxyvitamin D2-D3 reversed phase column from Zivak Technologies, Turkey with a 50 x 2 mm x 3 µm particle size was used. The Zivak Multitasker Fully Automated Sample Preparation and Injection System consist of a sample tray, vortex, centrifuge, column oven, UPLC pumps and an injection port. The system runs when the samples are placed in the sample tray together with the activation of the AUTOMASS software. 400 µL of the calibrator, control and patient samples are transferred to the 2 mL vials by an automatic injector. After the addition of Reagent 1 and Reagent 2, the mixture is vortexed for 1 min and centrifuged for 3 minutes at 5000 rpm. Then, 50 µL of supernatant is injected to the column through a CSPE system. The results are calculated and sent to the data evaluation system within 2 minutes via AUTOMASS software. A more detailed description of the customized program of the ZİVAK Multitasker Vitamin D system is described in the manual for ZİVAK Vitamin D [15]. The sample chromatograms of the measured CRM (UME CRM 1308) (51.00 ± 2.92) ng/mL and (49.76 ± 2.65) ng/mL values of 25-OH-Vitamin D2 and D3, respectively, are shown in Figure 1.

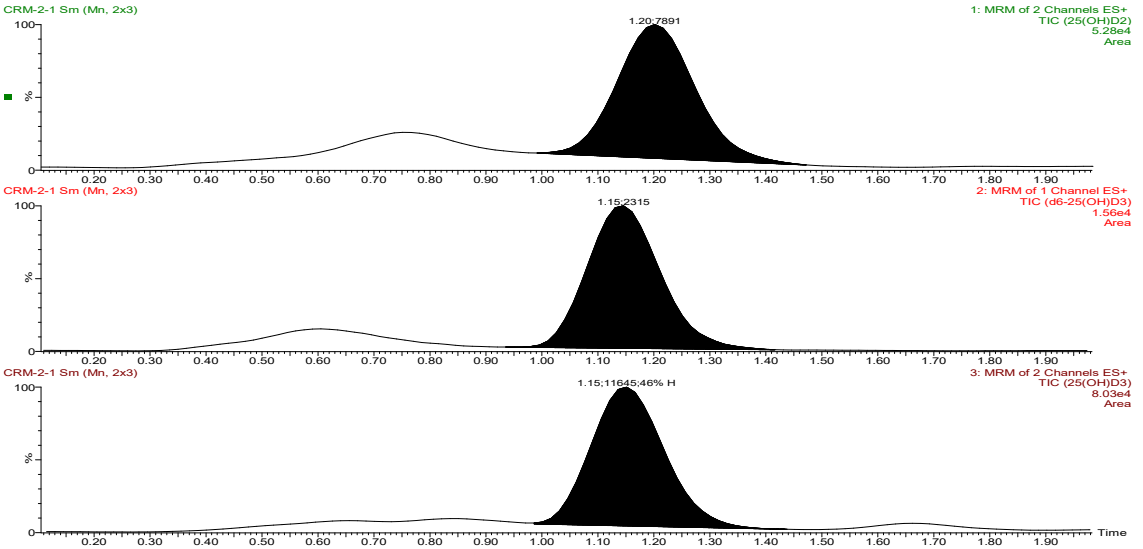

Figure 1. The sample chromatogram of 25-OH-Vitamin Ds by ZIVAK Multitasker coupled MS in UME CRM 1308

3. Results and discussion

3.1. Method Validation

The validation process was carried out based on the EURACHEM guideline [4, 13, 16].

3.1.1. Linearity and working range

The linearity and working range of the method was evaluated by preparing the calibration solutions gravimetrically at four concentration levels and analyzing them in triplicate. Labelled standard was added into the solutions at constant concentration value. The calibration graph was plotted by placing the peak area ratios for native/labeled compounds on -y- axis and concentration of native standard on -x- axis. The linear regression and squared correlation coefficient was used to evaluate the linearity concentration levels and R2 values are shown in Table 3.

3.1.2. LOD and LOQ

The evaluation of limit of detection (LOD) and limit of quantification (LOQ) was accomplished by analyzing the samples contains both D2 at 7 ng/mL and D3 at 10 ng/mL concentration levels. The LOD and LOQ values were obtained by multiplying the standard deviation (SD) of the results by 3 and 10, respectively and were presented in Table 3.

3.1.3. Trueness

The trueness was assessed by using UME CRM 1308. Method was applied in triplicate. The recovery values were calculated as a representative of trueness and presented in Table 3. The obtained results of the measurement of UME CRM 1308 were presented in Table 4.

3.1.4. Precision

Precision consists of repeatability and intermediate precision sub parameters. They were assessed via standard addition method at three different concentration levels which are low, medium and high level of working range. Samples were prepared in triplicate. Repeatability was studied in a day and intermediate precision was studied in three days. The relative standard deviation values (RSD%) were calculated to identify the precision and results were shown in Table 3.

Table 3. Method Validation Results

					Precision			
	Working Range ($ngmL^{-1}$)	Linear Regression Equation	R^2	Recovery (%)	Repeatability RSD%	Intermediate Precision RSD %	LOD ($ngmL^{-1}$)	LOQ ($ng\ mL^{-1}$)
25-OH-Vit. D2	9-45	y=0.074x+0.1645	0.9966	86	5.66	7.85	2.62	8.73
25-OH-Vit.D3	12-35	y=0.092x+0.2016	0.9976	103	4.45	8.32	2.52	8.40

Table 4. Measurement results and expanded uncertainty of UME CRM 1308 in $ngmL^{-1}$

	Certified Value±Uncertainty		Measured Value±Uncertainty	
Replicate Number	D3	D2	D3	D2
CRM-R1	49.76 ±2.65	51.00 ±2.92	52.27 ± 3.97	43.31 ± 3.34
CRM-R2	49.76 ±2.65	51.00 ±2.92	51.71 ± 3.93	42.95 ± 3.31
CRM-R3	49.76 ±2.65	51.00 ±2.92	54.06 ± 4.11	42.24 ± 3.25

3.2. Measurement Uncertainty

The bottom-up approach was applied to obtain measurement uncertainty value [4, 14, 16]. The sources for uncertainty were weighing of sample, calibration graph, trueness and precision.

3.2.1. Weighing of the Sample

Combined standard measurement uncertainty of sample was calculated using Equation 1 where u_{cal} was obtained from certificate of the balance supplied by the manufacturer.

$$u(W) = \sqrt{(u_{Calsample})^2 + (u_{Caltare})^2} \qquad \text{eq.1}$$

where,
$u(W)$: Combined standard measurement uncertainty of weighing sample
$u_{Calsample}$: Standard measurement uncertainty from calibration of balance while measuring sample
$u_{Caltare}$: Standard measurement uncertainty from calibration of balance while measuring tare

3.2.2. Uncertainty of Calibration Graph

The Equation 2 was used to calculate the combined standard measurement uncertainty of calibration graph.

$$u(c_0) = \frac{S}{B_1}\sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_0 - \bar{c})^2}{S_{xx}}} \qquad S_{xx} = \sum_{i=1}^{n} (c_i - \bar{c})^2 \qquad \text{eq.2}$$

where,

$u(c_0)$: Combined standard measurement uncertainty of calibration curve
S: Residual standard deviation
$B1$: Slope
p: Number of measurement to determine c_0
n: Number of measurement for calibration
c_0: Determined concentration of the sample by using calibration curve
c: Average value of the different calibration solution concentrations

3.2.3. Uncertainty of Trueness

The uncertainty value related to assessment of trueness was obtained based on the evaluation of recovery. UME-CRM 1309 produced by Reference Materials Laboratory of TUBITAK National Metrology Institute (UME) was used to evaluate the recovery of method regarding of vitamin D2 and D3. The method was applied on CRM in triplicates in a day and mean was calculated of the results. The uncertainty value of recovery was calculated by the Equation 3.

$$u(R_m) = R_m\sqrt{\left(\frac{\overline{u(C_{obs})}}{\overline{C_{obs}}}\right)^2 + \left(\frac{u(C_{cert})}{C_{cert}}\right)^2} \qquad \text{eq.3}$$

$$R_m = \frac{\overline{C_{obs}}}{C_{cert}} \quad \text{eq.4}$$

Where,
$u(Rm)$:Combined standard measurement uncertainty of recovery
$u(C_{obs})$: Standard measurement uncertainty of observation of CRM or fortified material
$u(C_{cert})$: Standard measurement uncertainty of certification of CRM or fortified material
R_m: Mean recovery
C_{obs}: Obtained concentration in recovery experiments
$Ccert$: Spiked or certified concentration

3.2.4. Uncertainty of Precision

The uncertainty originated from precision was evaluated via repeatability of the method. It was calculated by Equation 5.

$$u(r) = \frac{RSD}{\sqrt{n}} \quad \text{eq.5}$$

where,
$u(r)$: Standard measurement uncertainty of repeatability
RSD: Relative standard deviation
n: Number of sample

The Equation 6 is used for the calculation of combined standard measurement uncertainty of the vitamin D2 and D3 in serum. The expanded measurement uncertainty was obtained by multiplying the combined standard measurement uncertainty value with 2 (coverage factor) at 95% confidence level. The uncertainty budget of vitamin D2 and D3 are presented in Table 5 and Table 6.

$$\frac{u_c(A)}{C_A} = \sqrt{\left(\frac{u(W_{SS})}{W_{SS}}\right)^2 + \left(\frac{u(c_0)}{c_0}\right)^2 + u(R_m)^2 + u(r)^2} \quad \text{eq.6}$$

where,
$u_c(A)$: Combined standard measurement uncertainty of analyte
C_A: Concentration of analyte
$u(W_{SS})$: Combined standard measurement uncertainty of sample intake
W_{SS}: Weight of starting sample
$u(c_0)$: Combined standard measurement uncertainty of calibration curve
c_0: Determined concentration of the sample by using calibration curve
$u(R_m)$: Combined standard measurement uncertainty of recovery
$u(r)$: Standard measurement uncertainty of repeatability

Table 5. Uncertainty budget of 25-OH-Vitamin D2 measurement method

Parameters	Unit	Value (X)	u(x)	u(x)/X
Mass of sample intake	g	10	2.1783E-05	2.18E-06
Calibration	ng/mL	40.0	0.514	1.28E-02
Recovery	%	0.840	0.027	3.26E-02
Repeatability	Ng/mL	42.83	0.6668	1.56E-02
Relative Standard Measurement Uncertainty				0.038
Result (ng/mL)		42.8		
Combined Standard Measurement Uncertainty			1.6	
Expanded Uncertainty (k=2)			3.3	
Relative Measurement Uncertainty (%)			7.7	

Table 6. Uncertainty budget of 25-OH-Vitamin D3 measurement method

Parameters	Unit	Value (X)	u(x)	u(x)/X
Mass of sample intake	g	10	2.1783E-05	2.18E-06
Calibration	ng/mL	40.0	0.405	1.01E-02
Recovery	%	1.059	0.034	3.20E-02
Repeatability	ng/mL	52.68	0.9385	1.78E-02
Relative Standard Measurement Uncertainty				0.038
Result (ng/mL)		52.7		
Combined Standard Measurement Uncertainty			2.0	
Expanded Uncertainty (k=2)			4.0	
Relative Measurement Uncertainty (%)			7.6	

4. Conclusions

In this study it was shown that the ZIVAK Vitamin D analyzer can be successfully attached to a Waters Premier XE LC-MS/MS instrument and through the interface software control the MS. It was proven that the Vitamin D analyzer kit and sample preparation system can make highly accurate and reliable measurements with small uncertainties. Measurements of less than <8% for 25-OH vitamin D2 and 25-OH vitamin D3 with widened uncertainty values were obtained (Table 4 and Table 5). The low LOD and LOQ values obtained with the ZIVAK Vitamin D analyzer are highly suitable and within the decision limit when considering measurements in clinical laboratory applications and their decision limits.

A recent study by NIST showed that, the consensus values of popular and commercially available techniques which are CLIA, EIA, RIA and LC are found to be higher than the NIST value which is defined by LC-ID/MS. Additionally, the applied method can detect 3-epi-25-OH-Vitamin D3 as well. The measurement results of the ZIVAK Vitamin D kit and Multitasker coupled MS agree with the LC-MS/MS measurements consensus value and the NIST expanded uncertainty range in the exercise.

This study clearly shows that, Zivak Multitasker Fully Automated Sample Preparation and Injection System connected to MS system gives the possibility of quick, accurate and reliable measurements of 25-OH vitamin D2 and 25-OH vitamin D3 with an LC-MS/MS in clinical laboratories for routine measurements.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/JCM

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References

[1] M.F. Holick, in W.O.Song, G.R.Beecher, R.R.Eitenmiller and J.D. Winefordner (Eds.) (2000). Modern Analytical Methodoliges in Fat-and Water-Soluble Vitamins,(In Chemical Amalysis: A series of Monographs on Analytical Chemistry and Its Applications,Vol. 154), John Wiley & Sons, Inc., New York, p.51.

[2] R. Belsey, M.B. Clark, M. Bernat, J. Glowacki, M.F. Holick, H.F. Deluca and J.T. potts (1974). physiologic significance of plasma transport of vitamin-d and metabolites, *Am. J. Med.,* **57**, 50-56.

[3] Z. Maunsell, D.J. Wright, S. J. Rainbow (2005). Routine isotope-dilution liquid chromatographytandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D-2 and D-3, *Clin. Chem*., 1683-1690.

[4] a) A.C. Gören, G. Bilse and M. Bilsel (2007). Rapid and simultaneous determination of 25-OH-vitamin D2 and D3 in human serum by LC/MS/MS: Validation and uncertainty assessment, *J.Chem.Metrol.*, **1**, 1-9. b) H.O.Hamad, M. H. Alma, İ.Gulcin, M. A. Yılmaz and E. Karaoğul (2017). Evaluation of phenolic contents and bioactivity of root and nutgall extracts from Iraian *Quercus infectoria* Olivier, *Rec.Nat.Prod*. **11**, 205-210.

[5] E.M. Mineva, R.L. Schleicher, M. Chaudhary-Webb, K.L. Maw, J.C. Botelho, H.W. Vesper and C.M. Pfeiffer (2015). A candidate reference measurement procedure for quantifying serum concentrations of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 using isotope-dilution liquid chromatography-tandem mass spectrometry, *Anal. Bioanal. Chem*., **407**, 5615-5624

[6] K.W. Phinney, M. Bedner, S.C. Tai, V. Vamathevan, L.C. Sander,K.E. Sharpless, S.A. Wise, J.H. Yen, R.L. Schleicher, M. Chaudhary-Webb, J.M. Betz, P.M.Coates and M.F. Picciano, (2002) Development and Certification of a Standard Reference Material for Vitamin D Metabolites in Human Serum, *Anal. Chem*., 8**4**, 956-962.

[7] M. Bedner and K. A. Lippa (2012). NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results Winter 2012 Exercise. P.1-23.

[8] M. Bedner and K. A. Lippa (2013). NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results Summer 2013Comparability Study p.1-23.

[9] S. A. Wise, S.C. Tai, D.L. Duewer, M. Bedner, J.E. Camara, K. A Lippa, L. Qinde, D. Kang, B. Kim, C. Quan, L.Shi, J. Nammoonnoy, V. Vamathevan, A. C. Gören, G. Bilsel and H. Yilmaz (2017). CCQM-K132: low-polarity analytes in a biological matrix: vitamin D metabolites in human serum, *Metrologia,* **54S**, https://doi.org/10.1088/0026-1394/54/1A/08027

[10] Certificate of Analysis, SRM 972 Vitamin D in Human Serum, National Institute of Standards and Technology (NIST), June 9, 2009. https://www-s.nist.gov/srmors/view_cert.cfm?srm=972

[11] Certificate of Analysis, SRM 972a Vitamin D Metabolites in Frozen Human Serum, National Institute of Standards and Technology (NIST), February 20, 2013. https://www-s.nist.gov/srmors/view_cert.cfm?srm=972A

[12] Certification Report, UME CRM 1308, 25-Hydroxy Vitamin D2 and 25-Hydroxy Vitamin D3 in Lyophilized Serum, UME TÜBİTAK, Gebze-Kocaeli, Turkey 9. Certificate of Analysis, SRM 2973 Vitamin D Metabolites in Frozen Human Serum (High Level), National Institute of Standards and Technology (NIST), February 4, 2016. https://www-s.nist.gov/srmors/view_cert.cfm?srm=2973

[13] EURACHEM, The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics, 2014.

[14] EURACHEM/CITAC Guide CG 4 Editors : S L R Ellison, A Williams. Quantifying Uncertainty in Analytical Measurement, 3rd ed., 2012.

[15] ZİVAK Technologies, 25-Hydroxyvitamin D2-D3 Serum LC-MS/MS APCI Analysis Kit User Manual, D. No: ZV-3046-KK-10.R12.
[16] B. Binici, M. Bilsel, M. Karakas, I. Koyuncu , A.C. Goren (2013) An efficient GC-IDMS method for determination of PBDEs and PBB in plastic materials, *Talanta*, **116**,417-426.