# 25-Hydroxy Vitamin $D_2$ and 25-Hydroxy Vitamin $D_3$ in Lyophilized Serum UME CRM 1308

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## SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| $\Delta_m$ | Absolute difference between mean measured value and the certified value |
| ANOVA | Analysis of variance |
| APCI | Atmospheric Pressure Chemical Ionization |
| CRM | Certified Reference Material |
| HPLC | High Performance Liquid Chromatography |
| ID-LCMS/MS | Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry |
| ID-LCMS | Isotope Dilution Liquid Chromatography Mass Spectrometry |
| IS | Internal Standard |
| ISO | International Standards Organisation |
| $k$ | Coverage factor |
| $MS_{between}$ | Mean of squares between-unit from an ANOVA |
| $MS_{within}$ | Mean of squares within-unit from an ANOVA |
| NIST | National Institute for Standards and Technology |
| NIST SRM | Standard Reference Material produced by NIST |
| $RSD_{stab}$ | Relative standard deviation of stability data |
| $s$ | Standard deviation |
| $s_{bb}$ | Between-unit standard deviation |
| SI | International System of Units |
| SRM | Standard Reference Material |
| SS | Sum of Squares |
| $u_{\Delta}$ | Combined measurement uncertainty for the difference between measurement result and certified value |
| $U_{\Delta}$ | Expanded measurement uncertainty for the difference between measurement result and certified value. |
| $u_{bb}$ | Standard uncertainty related to a possible between-unit inhomogeneity |
| $u^*_{bb}$ | Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability |
| $u_{char}$ | Standard uncertainty of the material characterisation |
| $u_{CRM}$ | Combined standard uncertainty of CRM |
| $u_{sts}$ | Standard uncertainty for short term stability study |
| $u_{lts}$ | Standard uncertainty for long term stability study |
| $u_{meas}$ | Measurement uncertainty |
| $U_{CRM}$ | Expanded uncertainty of CRM |
| $\nu_{MS_{within}}$ | $MS_{within}$ degrees of freedom |
| $X_i$ | Time for each repeated analysis |
| $\overline{X}$ | Average of all time periods |
| $x$ | Selected shelf life |

**ABSTRACT**

The Certified Reference Material (CRM) is utilized in chemical measurements, as a useful tool for proving traceability of measurement result and enhances measurement quality. Vitamin D concentrations are frequently measured for treatment and diagnosis purposes. Vitamin D deficiency/excess is evaluated according to the concentration of 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ metabolites.

Number of available CRMs to be used in these measurements is very limited. The purpose of this project is production and certification of UME CRM 1308 "25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ concentrations in lyophilized serum". SI traceability of the related method will be proved by utilization of this CRM in the measurements.

This report includes details for the certification of UME CRM 1308 in accordance with the requirements of ISO Guide 34:2009[1]. The production facilities, chemical analyses, results of homogeneity assessment, stability and characterization studies, statistical evaluation of data and conclusions have been presented and the corresponding uncertainties (Table 1) have been calculated in accordance with the ISO Guide 98-3, Uncertainty Measurement-Part 3: Guide to the Expression of Uncertainty in Measurement-GUM[2].

**Table 1.** Certified values and uncertainties for UME CRM 1308

| Measurand | Mass Fraction [1] [ng/g] | $U_{CRM}$ [2] [ng/g] | Concentration [3] [ng/mL] | $U_{CRM}$ [2,3] [ng/mL] |
|---|---|---|---|---|
| 25-hydroxy vitamin $D_2$ concentration in lyophilized serum | 49.97 | 2.86 | 51.00 | 2.92 |
| 25-hydroxy vitamin $D_3$ concentration in lyophilized serum | 48.76 | 2.59 | 49.76 | 2.65 |

[1] Certified values are the mean of 6 measurement results obtained from two units of the CRM on two different day by ID-LCMS technique. Measurement results and the uncertainties are traceable to International System of Units (SI).

[2] The accompanying uncertainties include the influence of homogeneity, stability and characterization studies. The uncertainty of the certified value is stated as standard uncertainty of the measurement multiplied by the coverage factor k = *2*, which for a normal distribution corresponds to a coverage probability of approximately 95%. The standard uncertainty of measurement has been determined in accordance with GUM "Guide to the Expression of Uncertainty in Measurement".

[3] Certified values and the uncertainties are calculated from the mass fractions (ng/g) using density of the material (1.0206 g/mL) measured at 22 °C.

## INTRODUCTION

The active form of vitamin D helps controlling the calcium level in the body. Calcium is consumed in bone and tooth production together with phosphate ions. Vitamin D can be taken from food (e.g. milk and fish) or synthesized by the effect of sunlight in the body. Both the deficiency (e.g.osteoclasis) and excess (e.g. kidney stone formation) causes several diseases at all ages [3,4]. Additionally, adequate intake of vitamin D was reported as it may prevent several cancer types [5-8]. Measurement of vitamin D metabolite levels in serum is frequently used as a diagnostic tool in clinical chemistry, where measurement quality is of prime importance.

25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ concentrations in lyophilized horse serum was certified in UME CRM 1308. This report presents the details of the production and certification stages including the data, utilized techniques and statistical analysis. UME CRM 1308 was produced to be used as a material in proving the traceability and quality of LC-MS, LC-MS/MS and HPLC-UV measurements.

Systematic description for UME CRM 1308 according to ISO 15194 is "Certified reference material UME CRM 1308 - 25-hydroxy Vitamin $D_2$ and 25-hydroxy Vitamin $D_3$ in Lyophilized Horse Serum".

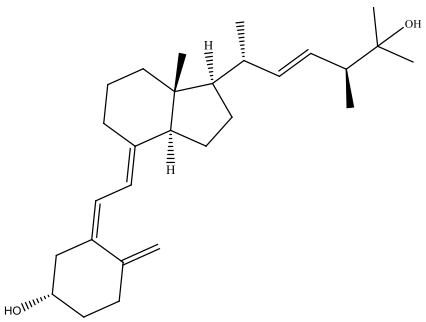
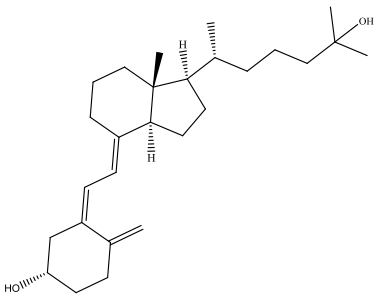
| | | |
|---|---|---|
| Chemical Structure |  |  |
| Short Name | 25-hydroxy vitamin $D_2$ | 25-hydroxy vitamin $D_3$ |
| IUPAC Name | (1S,3Z)-3-[(2E)-2-[(1R,3aS,7aR)-1-[(E,2R,5S)-6-hydroxy-5,6-dimethylhept-3-en-2-yl]-7a-methyl-2,3,3a,5,6,7-hexahydro-1H-inden-4-ylidene]ethylidene]-4-methylidenecyclohexan-1-ol | (1S,3Z)-3-[(2E)-2-[(1R,3aS,7aR)-1-[(2R)-6-Hydroxy-6-methylheptan-2-yl]-7a-methyl-2,3,3a,5,6,7-hexahydro-1H-inden-4-ylidene]ethylidene]-4-methylidenecyclohexan-1-ol |
| Chemical Formula | $C_{28}H_{44}O_2$ | $C_{27}H_{44}O_2$ |

**Figure 1.** Specific properties of vitamin D metabolites

**PARTICIPANTS**

All production and certification stages of UME CRM 1308 have been performed at TÜBİTAK UME, except for material processing, which has been done at İstanbul Pendik Veteriner Kontrol Enstitüsü. Several samples have been sent to competent laboratories (Table 2). Results have been presented in the UME CRM 1308 certification report as an informative value (Annex 4, Table 29).

**Table 2.** Participating institutes and definition of their work.

| **Participant** | **Description of the Work** |
|---|---|
| Acıbadem Labmed Klinik Laboratuvarı<br>Küçük Çamlıca Mah. Ord. Prof. Fahrettin Kerim Gökay Cad.<br>No: 49 Üsküdar<br>İSTANBUL/TÜRKİYE | Participant of Interlaboratory Comparison Study |
| Düzen Laboratuvarları<br>Tunus Cad. No:95 Kavaklıdere<br>ANKARA/TÜRKİYE | Participant of Interlaboratory Comparison Study |
| Ghent University<br>Laboratory of Analytical Chemistry,<br>Faculty Pharmaceutical Sciences, Ghent University<br>Harelbekestraat 72 9000<br>Ghent/BELGIUM | Participant of Interlaboratory Comparison Study |
| İstanbul Üniversitesi Cerrahpaşa Tıp Fakültesi<br>Fikret Biyal Merkez Araştırma Laboratuvarı<br>İstanbul Üniversitesi Cerrahpaşa Tıp Fakültesi Yerleşkesi<br>Kocamustafapaşa Cad. No: 53 Cerrahpaşa 34098<br>İSTANBUL/TÜRKİYE | Participant of Interlaboratory Comparison Study |
| Pendik Veteriner Araştırma Enstitüsü<br>Batı Mah. Erol KAYA Cad. No:1 34890<br>İSTANBUL/TÜRKİYE | Material Processing |
| TÜBİTAK UME<br>TÜBİTAK Gebze Yerleşkesi, Dr. Zeki Acar Cad. No.1, 41470<br>KOCAELİ/TÜRKİYE | Project Management and Certification Studies |

## MATERIAL PROCESSING

UME CRM 1308 was prepared by adding 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ standards into the horse serum containing 25-hydroxy vitamin $D_3$ endogenously. Horse serum was purchased from Biochrom AG (Germany) and pure standards were purchased from Sigma-Aldrich (USA).

The prepared solution was bottled into amber glass bottles as approximately 2 mL for each unit. Filling order was recorded by labelling after filling. A total of 500 units were prepared. All bottles were lyophilized at the same time. The bottles were first lyo-capped in the lyophilizer under vacuum and then screw capped after being removed from the lyophilizer.

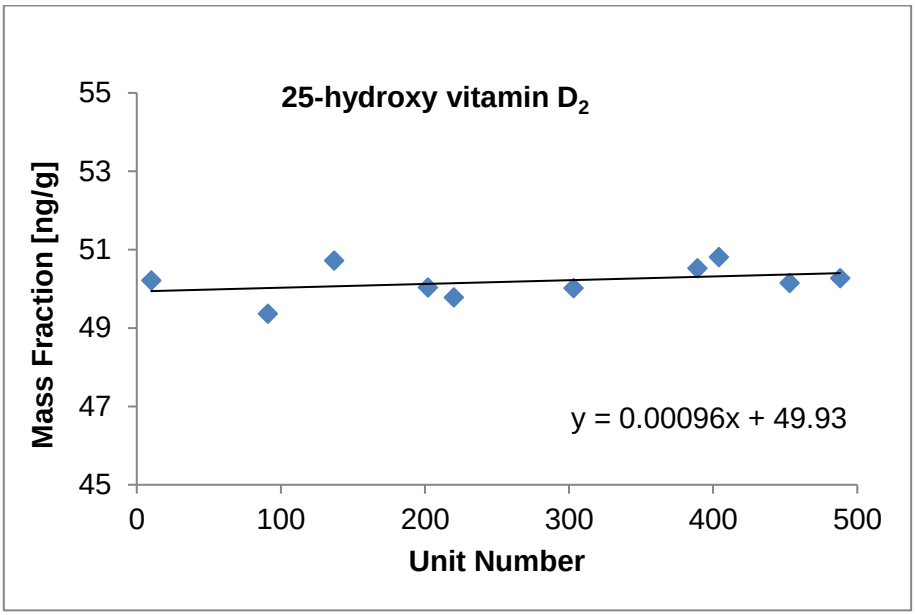
Units were classified in respect to CRM production stages (homogeneity, stability and characterization) with the random stratified sample selection approach by a software developed at TÜBİTAK UME (TRaNS)[9]. After classification into subgroups, samples were stored under selected test conditions.

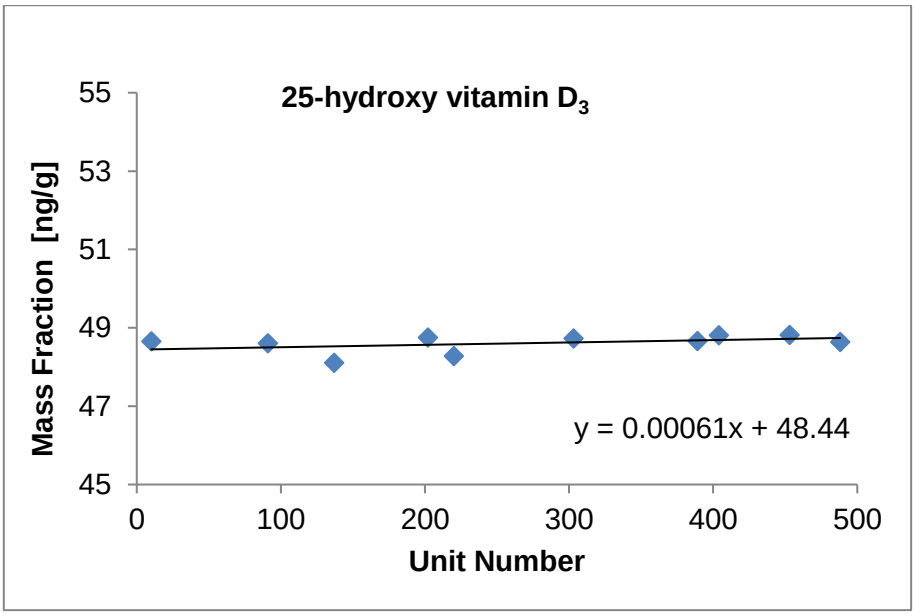
## HOMOGENEITY

In order to determine between unit heterogeneity for certification of 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$, 10 units of UME CRM 1308 have been chosen by utilizing TRaNS[9], since the cubic root of the total number of units is less than 10.

Homogeneity study was done by ID-LCMS/MS technique (Table 3). Measurements were performed under repeatability conditions. Details of the technique were presented in Annex 1. Data has been subjected to regression analysis (Table 4). The slope was tested with t-test in 95 % confidence level. But no statistically significant difference arising from filling or analysis order (Table 5) was detected.

Grubbs' test was applied to detect outliers but none has been identified.




**Graph 1.** Homogeneity study results.

**Table 3.** Results of homogeneity study.

| Unit Number | Mass Fraction [ng/g] | |
|---|---|---|
| | 25-hydroxy vitamin $D_2$ | 25-hydroxy vitamin $D_3$ |
| **10** | 50.22 | 48.66 |
| **137** | 50.73 | 48.11 |
| **220** | 49.79 | 48.28 |
| **303** | 50.02 | 48.74 |
| **404** | 50.81 | 48.81 |
| **453** | 50.15 | 48.82 |
| **488** | 50.28 | 48.64 |
| **91** | 49.37 | 48.61 |
| **202** | 50.04 | 48.76 |
| **389** | 50.53 | 48.67 |

**Table 4.** ANOVA for analyte 25-hydroxy vitamin $D_2$ of homogeneity study.

| Unit Number | Number of Replicates | Average [ng/g] | Variance [$ng^2/g^2$] |
|---|---|---|---|
| **10** | 2 | 50.22 | 0.01 |
| **137** | 2 | 50.73 | 0.86 |
| **220** | 2 | 49.79 | 0.00 |
| **303** | 2 | 50.02 | 0.25 |
| **404** | 2 | 50.81 | 0.03 |
| **453** | 2 | 50.15 | 0.16 |
| **488** | 2 | 50.28 | 0.01 |
| **91** | 2 | 49.37 | 0.00 |
| **202** | 2 | 50.04 | 0.02 |
| **389** | 2 | 50.53 | 0.17 |

| ANOVA | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | ***SS*** | ***df*** | ***MS*** | ***F*** | $F_{crit}$ |
| **Between Groups** | 3.39 | 9 | 0.38 | 2.51 | 3.02 |
| **Within Groups** | 1.50 | 10 | 0.15 | | |

**Table 5.** ANOVA for 25-hydroxy vitamin $D_3$ of homogeneity study measurement.

| Unit Number | Number of Replicates | Average [ng/g] | Variance [$ng^2/g^2$] |
|---|---|---|---|
| **10** | 2 | 48.66 | 0.36 |
| **137** | 2 | 48.11 | 0.67 |
| **220** | 2 | 48.28 | 0.01 |
| **303** | 2 | 48.74 | 0.01 |
| **404** | 2 | 48.81 | 0.87 |
| **453** | 2 | 48.82 | 0.02 |
| **488** | 2 | 48.64 | 0.11 |
| **91** | 2 | 48.61 | 0.05 |
| **202** | 2 | 48.76 | 0.06 |
| **389** | 2 | 48.67 | 0.00 |

**ANOVA**

| Source of Variation | *SS* | *df* | *MS* | *F* | $F_{crit}$ |
|---|---|---|---|---|---|
| **Between Groups** | 0.97 | 9 | 0.11 | 0.50 | 3.02 |
| **Within Groups** | 2.17 | 10 | 0.22 | | |

As $MS_{between} < MS_{within}$, the random variation between units ($s_{bb}$) cannot be estimated. The maximum hidden between bottle heterogeneity ($u^*_{bb}$,) was then evaluated as follows [10]:

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{\upsilon_{MS_{within}}}}$$

In this study, $u^*_{bb}$, is 0.52 ng/g and 0.47 ng/g for 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$, respectively.

ANOVA results confirm the absence of statistical significance (for 25-hydroxy vitamin $D_2$ $F$ = 2.51 < $F_{crit}$ = 3.02 and for 25-hydroxy vitamin $D_3$ $F$ = 0.50 < $F_{crit}$ = 3.02).

**STABILITY**

Two different stability tests, for UME CRM 1308, have been conducted. Short term stability test has been performed to simulate transportation conditions and long term stability test to simulate long term storage conditions.

**Short Term Stability Results**

Short term stability study was performed with 52 units of CRM (where 26 units were replacements) and long term study was conducted by 36 units of CRM (where 18 units were replacements). Samples were selected by TRaNS software.

Short term test temperatures were set as -20 °C, 4 °C, 25 °C and time periods were set as 1, 2, 3, 4 weeks. 4 units per week were placed on the different storage cabinets at different temperatures. In order to set the reference point, 4 units were selected and placed directly to the reference temperature (-80 ± 3 °C). Each week, four units of CRM were transferred to reference temperature. At the end of four weeks, all of the units were analysed together under repeatability conditions. Analyses were performed with ID-LCMS/MS for which the details are presented in Annex 2. Unit numbers for the samples used in this study and corresponding time periods are presented in Table 6 for both of the analytes. Results (Table 7) were evaluated individually for each storage temperature (Table 8 and Table 10). The results were screened for outliers using the Grubbs' test. No outliers detected.

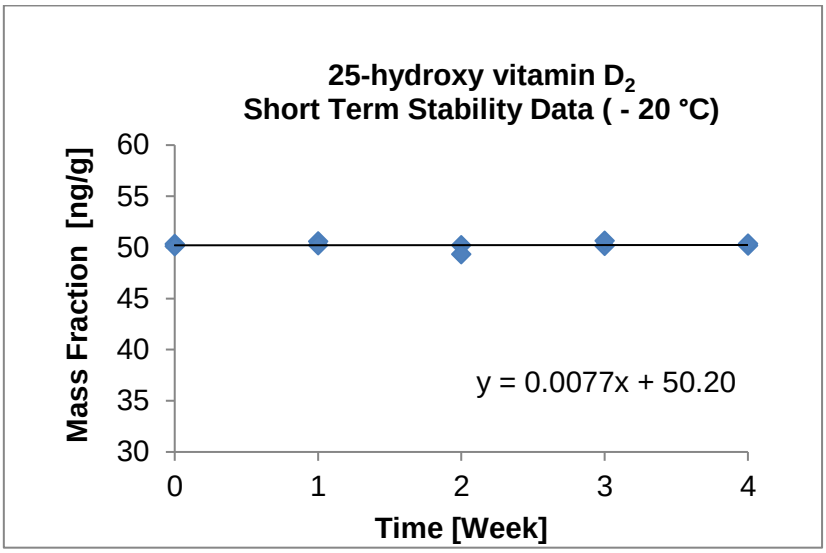
Furthermore, to inspect changes in concentration with temperature, the data was evaluated for each storage temperature separately. When slope of the regression lines were tested for statistical significance, it was observed that the slopes of the regression lines were not significantly different from zero (at 95 % confidence level) at all temperature values selected (Table 9 and Graph 2 for 25-hydroxy vitamin $D_2$ and Table 11 and Graph 3 for 25-hydroxy vitamin $D_3$).

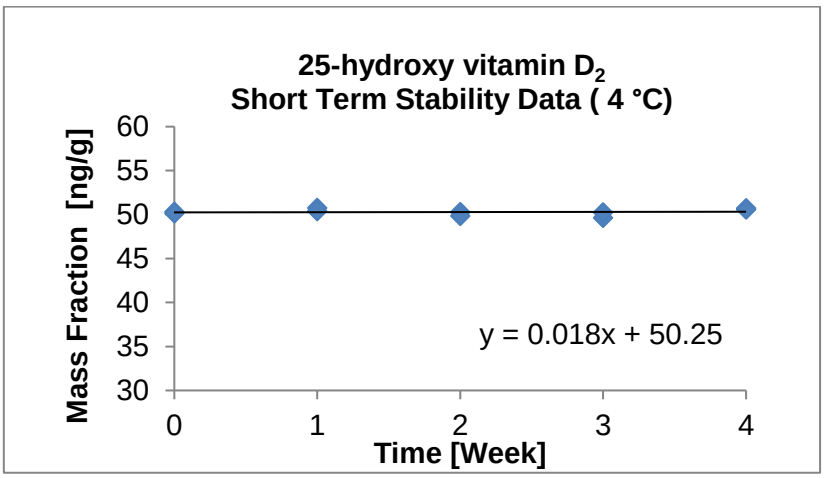
**Table 6**. Isochronous setup for short term stability study.

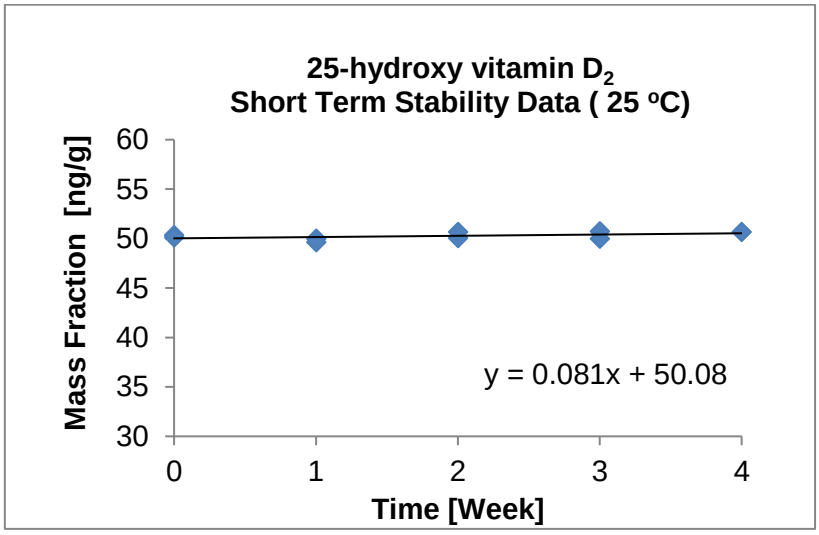
| Unit Number | Temperature [ºC] | Time [Week] |
|---|---|---|
| **402** | -80 | 0 |
| **464** | -80 | 0 |
| **492** | -20 | 1 |
| **6** | -20 | 1 |
| **15** | -20 | 2 |
| **288** | -20 | 2 |
| **301** | -20 | 3 |
| **25** | -20 | 3 |
| **31** | -20 | 4 |
| **305** | -20 | 4 |
| **46** | 4 | 1 |
| **322** | 4 | 1 |
| **61** | 4 | 2 |
| **327** | 4 | 2 |
| **335** | 4 | 3 |
| **69** | 4 | 3 |
| **75** | 4 | 4 |
| **352** | 4 | 4 |
| **90** | 25 | 1 |
| **361** | 25 | 1 |
| **370** | 25 | 2 |
| **103** | 25 | 2 |
| **383** | 25 | 3 |
| **104** | 25 | 3 |
| **388** | 25 | 4 |
| **116** | 25 | 4 |

**Table 7.** Short term stability study results.

| Time [week] | Mass Fraction [ng/g] | | | | | |
|---|---|---|---|---|---|---|
| | 25-hydroxy vitamin $D_2$ | | | 25-hydroxy vitamin $D_3$ | | |
| | -20 ºC | 4 ºC | 25 ºC | -20 ºC | 4 ºC | 25 ºC |
| **0** | 50.33 | 50.33 | 50.33 | 48.95 | 48.95 | 48.95 |
| **0** | 50.14 | 50.14 | 50.14 | 48.28 | 48.28 | 48.28 |
| **1** | 50.59 | 50.76 | 50.01 | 48.82 | 49.01 | 49.03 |
| **1** | 50.23 | 50.39 | 50.19 | 48.14 | 48.24 | 48.49 |
| **2** | 50.23 | 50.29 | 49.65 | 48.95 | 49.03 | 48.43 |
| **2** | 49.33 | 49.83 | 50.07 | 48.23 | 49.21 | 48.75 |
| **3** | 50.17 | 49.61 | 50.66 | 49.14 | 48.37 | 48.26 |
| **3** | 50.66 | 50.24 | 50.75 | 48.75 | 48.21 | 48.80 |
| **4** | 50.17 | 50.71 | 50.00 | 48.66 | 48.69 | 47.82 |
| **4** | 50.37 | 50.59 | 50.67 | 48.92 | 48.34 | 48.78 |




**Graph 2.** Regression lines prepared with short term stability data for 25-hydroxy vitamin $D_2$ at different temperatures.

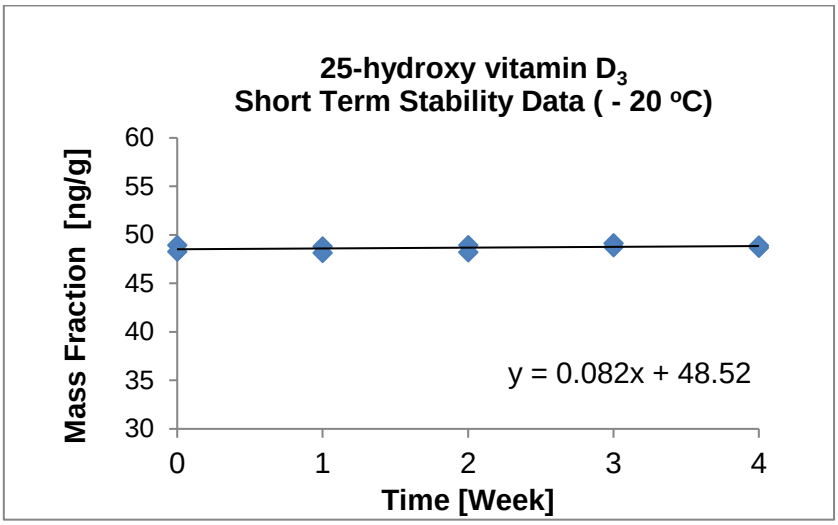
**Table 8.** ANOVA analysis with short term stability data for 25-hydroxy vitamin $D_2$ at different temperatures.

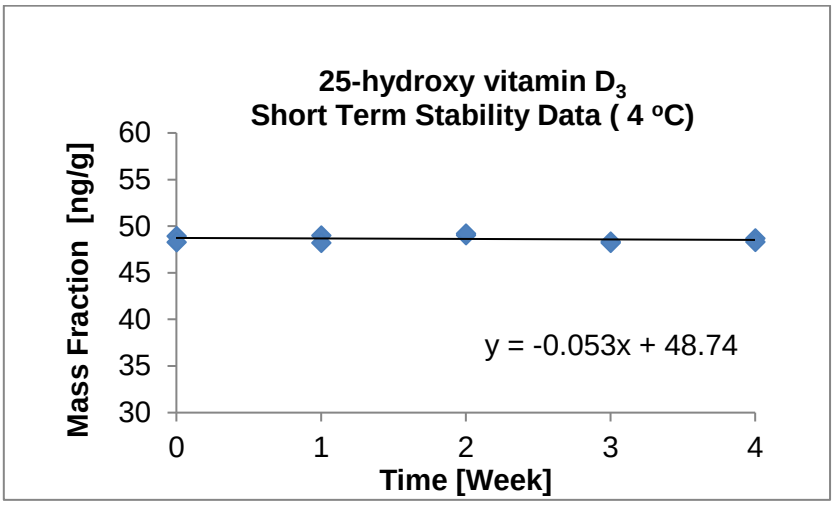
| Storage Temperature [ºC] | Number of Replicates | Average [ng/g] | Variance [$ng^2/g^2$] |
|---|---|---|---|
| -80 | 2 | 50.23 | 0.02 |
| -20 | 8 | 50.22 | 0.16 |
| 4 | 8 | 50.30 | 0.17 |
| 25 | 8 | 50.25 | 0.16 |

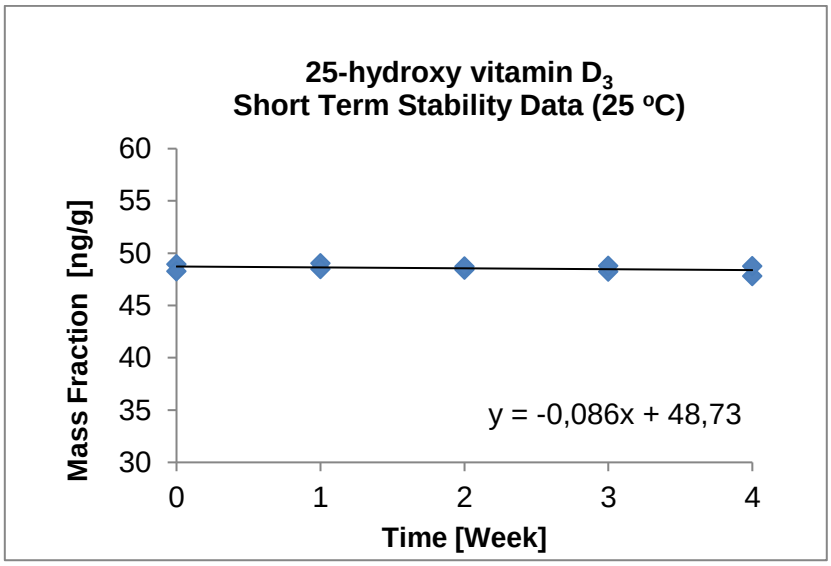
| ANOVA | | | | | |
|---|---|---|---|---|---|
| **Source of Variation** | ***SS*** | ***df*** | ***MS*** | ***F*** | $F_{crit}$ |
| **Between Groups** | 0.03 | 3 | 0.01 | 0.064 | 3.05 |
| **Within Groups** | 3.46 | 22 | 0.16 | | |

**Table 9.** Statistical evaluation of short term stability study results for 25-hydroxy vitamin $D_2$.

| Statistical Parameters | -20 ºC | 4 ºC | 25 ºC |
|---|---|---|---|
| **Slope** | 0.0078 | 0.018 | 0.081 |
| **Slope / $s_b$** | 0.086 | 0.086 | 0.079 |
| **Significance (at 95 % confidence level)** | - | - | - |
| **$u_{sts}$ [ng/g]** | 0.0017 | 0.0017 | 0.0017 |




**Graph 3.** Regression lines prepared by short term stability data for 25-hydroxy vitamin $D_3$ at different temperatures.

**Table 10.** ANOVA with short term stability data for 25-hydroxy vitamin $D_3$ at different temperatures.

| Storage Temperature [ºC] | Number of Replicates | Average [ng/g] | Variance [ng²/g²] |
|---|---|---|---|
| -80 | 2 | 48.61 | 0.22 |
| -20 | 8 | 48.70 | 0.12 |
| 4 | 8 | 48.64 | 0.16 |
| 25 | 8 | 48.54 | 0.15 |

**ANOVA**

| Source of Variation | *SS* | *df* | *MS* | *F* | $F_{crit}$ |
|---|---|---|---|---|---|
| **Between Groups** | 0.10 | 3 | 0.03 | 0.23 | 3.05 |
| **Within Groups** | 3.23 | 22 | 0.15 | | |

**Table 11.** Statistical evaluation of short-term stability study results for 25-hydroxy vitamin $D_3$.

| Statistical Parameters | -20 ºC | 4 ºC | 25 ºC |
|---|---|---|---|
| **Slope** | 0.082 | -0.053 | -0.086 |
| **Slope / $s_b$** | 0.078 | 0.090 | 0.083 |
| **Significance (at 95 % confidence level)** | - | - | - |
| **$u_{sts}$ [ng/g]** | 0.0017 | 0.0019 | 0.0026 |

UME CRM 1308 can be transferred to the end user under dark conditions without additional cooling elements if temperature does not exceed 25 ºC and transportation time does not exceed 4 weeks.

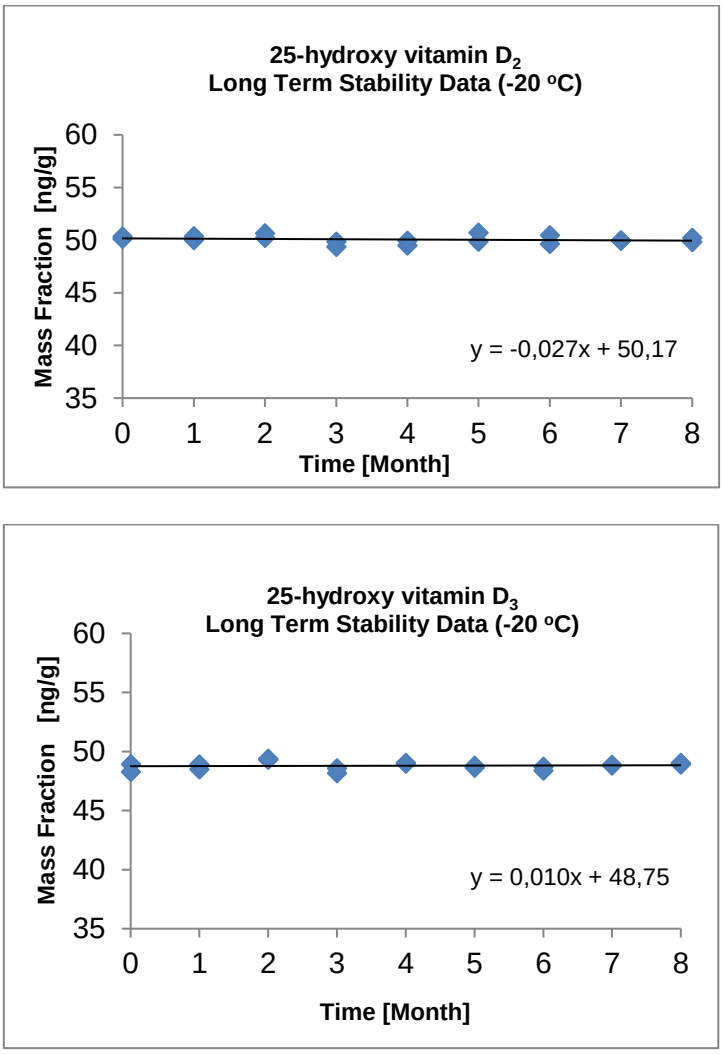
**Long Term Stability Results**

Long term stability study was conducted by 36 units (18 units were replacements) of UME CRM 1308 selected by TRaNS software. In respect to isochronous setup, selected samples were placed to (-20 ± 4) ºC which was the testing temperature for long term stability study. At the end of each time period (every month) 4 units were transferred to reference temperature (-80 ± 3 ºC), and all of the units were analysed under repeatability conditions at the end of 8 months. Measurements were done in an order, different from filling order to address the exact source of variation. Analyses were performed with ID-LCMS/MS for which the details were presented in Annex 2. Two replicates were analysed per unit and the results are presented in Table 12.

Data was subjected to statistical analysis (Table 13). Grubbs' test was applied to detect outliers but none has been identified.

**Table 12.** Data of long term stability study

| Unit Number | Time [month] | Mass Fraction [ng/g] | |
|---|---|---|---|
| | | 25-hydroxy vitamin $D_2$ | 25-hydroxy vitamin $D_3$ |
| 283 | 0 | 50.33 | 48.95 |
| 53 | 0 | 50.14 | 48.28 |
| 380 | 1 | 50.00 | 48.49 |
| 20 | 1 | 50.37 | 48.92 |
| 468 | 2 | 50.23 | 49.40 |
| 336 | 2 | 50.65 | 49.31 |
| 68 | 3 | 49.39 | 48.17 |
| 434 | 3 | 49.87 | 48.59 |
| 119 | 4 | 49.51 | 48.98 |
| 204 | 4 | 49.96 | 49.05 |
| 258 | 5 | 49.86 | 48.64 |
| 310 | 5 | 50.70 | 48.79 |
| 225 | 6 | 49.66 | 48.72 |
| 156 | 6 | 50.47 | 48.38 |
| 351 | 7 | 49.94 | 48.83 |
| 139 | 7 | 49.99 | 48.89 |
| 474 | 8 | 49.83 | 48.92 |
| 56 | 8 | 50.22 | 49.03 |




**Graph 4.** Regression lines prepared with long term stability data

**Table 13.** Statistical evaluation of long-term stability measurement results

| Statistical Parameters | Value | |
|---|---|---|
| | **25-hydroxy vitamin $D_2$** | **25-hydroxy vitamin $D_3$** |
| **Slope** | -0.027 | 0.010 |
| **Slope / $s_b$** | 0.033 | 0.035 |
| **Significance (at 95 % confidence level)** | - | - |
| **$u_{lts}$ [3 years] [ng/g]** | 1.19 | 1.08 |

Slope of the time vs. concentration graph is not significantly different than zero (Graph 4). It is concluded that the change in slope is not statistically significant at 95% confidence level (Table 13).

The extrapolated uncertainty for 3 years was calculated with the equation below [12].

$$u_{lts} = \frac{RSD_{stab}}{\sqrt{\sum (X_i - \overline{X})^2}} .x$$

Results indicate that the temperature at -20 ± 4 °C is suitable for the long term storage of UME CRM 1308. Post certification monitoring studies are going to be carried out with UME CRM 1308 in order to verify the validity of the certified value over longer time.

**CHARACTERIZATION**

Characterization studies for UME CRM 1308 were performed with ID-LCMS which is a primary method producing SI traceable results.

2 units of the candidate CRM was measured with ID-LCMS technique. Isotope labelled 25-hydroxy vitamin $D_2$-$d_3$ and 25-hydroxy vitamin $D_3$-$d_3$ (Medical Isotope Laboratories, Inc., USA) were used as internal standard for ID-LCMS method. The lyophilized sample was dissolved in 2 mL of deionized water. Details about the instrument and the method used are presented in Annex 1. Three replicate measurements were performed from each unit on two different days. Results are presented in Table 14.

**Table 14.** Measurement results obtained with ID-LCMS in the characterisation study

| Unit Number | Mass Fraction [ng/g] | |
|---|---|---|
| | **25-hydroxy vitamin $D_2$** | **25-hydroxy vitamin $D_3$** |
| **100-1** | 49.66 | 49.27 |
| **100-2** | 50.18 | 48.49 |
| **100-3** | 49.93 | 48.51 |
| **70-1** | 50.13 | 48.74 |
| **70-2** | 49.47 | 49.21 |
| **70-3** | 50.44 | 48.36 |
| **Mean** | 49.97 | 48.76 |

**ADDITIONAL MEASUREMENTS**

Additional measurements were also carried out with ID-LCMS/MS and HPLC-UV methods, however these results were not used for value assignment.

With both techniques, 10 units were analysed. Isotope labelled 25-hydroxy vitamin $D_3$-$d_6$ (Medical Isotope Laboratories, Inc., USA) and dodecanophenone (Sigma-Aldrich, USA) were used as internal standard for ID-LCMS/MS and HPLC-UV, respectively. The lyophilized sample was dissolved with 2 mL of deionized water. Details of instruments and the method used are presented in Annex 2 and Annex 3. With both techniques two replicate measurements were done from each sample unit. Results are presented in Table 15 and Table 16.

**Table 15.** Measurement results obtained with ID-LCMS/MS

| Unit Number | Mass Fraction [ng/g] | |
|---|---|---|
| | 25-hydroxy vitamin $D_2$ | 25-hydroxy vitamin $D_3$ |
| **152** | 49.85 | 49.34 |
| **202** | 50.04 | 48.76 |
| **389** | 50.86 | 48.53 |
| **455** | 50.23 | 49.04 |
| **41** | 49.78 | 49.09 |
| **208** | 48.95 | 48.81 |
| **282** | 50.02 | 48.56 |
| **344** | 51.09 | 49.15 |
| **368** | 50.58 | 48.87 |
| **499** | 49.50 | 48.33 |
| **Mean** | 50.09 | 48.85 |

**Table 16.** Measurement results obtained with HPLC-UV

| Unit Number | Mass Fraction [ng/g] | |
|---|---|---|
| | 25-hydroxy vitamin $D_2$ | 25-hydroxy vitamin $D_3$ |
| **379** | 48.73 | 48.49 |
| **71** | 48.52 | 47.76 |
| **142** | 48.57 | 49.05 |
| **377** | 48.50 | 48.74 |
| **334** | 48.68 | 49.22 |
| **19** | 49.17 | 48.56 |
| **180** | 48.64 | 48.75 |
| **470** | 48.18 | 48.58 |
| **130** | 48.66 | 48.47 |
| **426** | 48.23 | 48.64 |
| **Mean** | 48.59 | 48.63 |

The candidate certified reference material was also analysed with an interlaboratory study among competent laboratories. Either an accredited laboratory or a laboratory using a reference method was selected intentionally in accordance with the ISO Guide 34. Results are presented in Annex 4, Table 29.

Density of the reconstituted material was measured at 22 °C using a calibrated pipette and calibrated balance. The result of the density measurement is 1.0206 g/mL.

## VALUE ASSIGNMENT AND UNCERTAINTY CALCULATIONS

The certified value is the mean of the ID-LCMS results, which is a primary method traceable to the SI. Applied method was validated in respect to the quality system set up at TÜBİTAK UME.

The uncertainty for the certified value is the unweighed statistical average of $u_{bb}$, $u_{lts}$ and $u_{char}$ studies. Since the transfer conditions with the lowest uncertainty were selected, the uncertainty contribution of short term stability was neglected. The $U_{CRM}$ was calculated in respect to the equation below.

$$U_{CRM} = k \cdot \sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2}$$

**Table 17.** The uncertainty components for UME CRM 1308

| Measurand | Uncertainty Components [ng/g] | | | |
|---|---|---|---|---|
| | $u_{bb}$ | $u_{lts}$ | $u_{char}$ | $U_{CRM}$ |
| **25-hydroxy vitamin $D_2$ mass fraction in lyophilized serum** | 0.52 | 1.19 | 0.59 | 2.86 |
| **25-hydroxy vitamin $D_3$ mass fraction in lyophilized serum** | 0.47 | 1.08 | 0.53 | 2.59 |

The certified value and uncertainty for 25-hydroxy vitamin $D_2$ in UME CRM 1308 is

**(49.97 ± 2.86) ng/g (*k* = 2, norm)**

The certified value and uncertainty for 25-hydroxy vitamin $D_3$ in UME CRM 1308 is

**(48.76 ± 2.59) ng/g (*k* = 2, norm)**

Systematic description for UME CRM 1308 is "Certified reference material (Horse serum; UME CRM 1308 - 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ in Lyophilized Serum". 25-hydroxy vitamin $D_2$ concentration (reconstituted) c = 49.97 ng/g (U = 2.86 ng/g; k = 2), 25-hydroxy vitamin $D_3$ concentration (reconstituted) c = 48.76 ng/g (U = 2.59 ng/g; k = 2), where U is the expanded uncertainty of the CRM using the coverage factor, k=2.

## TRACEABILITY

In the measurements conducted at TÜBİTAK UME for UME CRM 1308, NIST SRM 2972 "25-Hydroxyvitamin $D_2$ and $D_3$ Calibration Solutions" and NIST SRM 972a Level 3 "Vitamin D Metabolites in Frozen Human Serum" were used.

## COMMUTABILITY

The intended use of this reference material is to check method performance and validation of 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ measurements in serum with LC-MS, LC-MS/MS and HPLC-UV methods.

Commutability of the material with routine *in vitro* diagnostic immunoassay methods has not been assessed. The user would need to assess the commutability, if UME CRM 1308 is going to be used as calibrant for routine *in vitro* diagnostic immunoassay methods.

## INSTRUCTIONS FOR USE

### Intended Use

This material is intended to be used for method performance check and validation purposes of 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ in serum with LC-MS, LC-MS/MS and HPLC-UV methods.

### Scope of Application

UME CRM 1308 is suitable for determination of 25-hydroxy-vitamin $D_2$ and 25-hydroxy vitamin $D_3$ concentrations in human serum using LC-MS, LC-MS/MS and HPLC-UV methods.

UME CRM 1308 is not suitable for the calibration of 25-hydroxy-vitamin $D_2$ and 25-hydroxy vitamin $D_3$ measurements in human serum using immunoassay methods unless the commutability of the material is proven by the user.

### Safety Precautions

Raw material: donor horse serum (origin: Germany) was tested by the manufacturer (Biochrom) for mycoplasma and sterile filtered (0.1 µm). This substance is not classified as dangerous according to Directive 67/548/EEC. Complete toxicity testing was not performed. The usual laboratory safety measures apply. The material is suitable for *in-vitro* use only. Safety Data Sheet (SDS) should be read before use.

### Storage Conditions

The material should be stored at temperatures lower than -20 $^{o}$C in a dry and dark place. Solutions of UME CRM 1308 should not be exposed to direct sunlight or UV light. User should take necessary precautions against evaporation or sublimation of the sample. TÜBİTAK UME cannot be held

responsible for changes that happen during storage of the material at the customer's premises, especially of open samples.

**Reconstitution of the Material**

All content of the unit should be reconstituted at once according to the recommended protocol presented below:

- UME CRM 1308 and the deionized water should be kept at room temperature at least for one hour in the room of balance before weighing.
- Before opening, the bottle should be tapped gently to table or bench top in order to collect all of the content at the bottom of the bottle.
- Screw cap is opened. The bottle with the inner cap is placed to the balance and tare is pressed. Unit is opened at vertical position gently by balancing the inner pressure with outer pressure. Care should be taken in order not to lose any sample on cap or elsewhere. 2 mL of water is then added gently with a calibrated pipette into the sample. Then the inner cap is closed.
- Bottle with its content and inner cap is weighed ($m$).
- The average amount of water added to the analysed units at TÜBİTAK UME was

$$m_{avg} = (1.9787 \pm 0.0200)\ \text{g}\ (k = 2, \text{norm})$$

If $m$ is deviating from $m_{avg}$, then the corrected value of the analyte concentration can be calculated as:

$$\text{Corrected analyte concentration} = \text{Certified value} \times \frac{m_{avg}}{m}$$

The bottle with added water should be gently shaken with its cap and inner cap closed. If material is stacked on to the inner cap, user can gently rotate the bottle upside down several times to dissolve those particles. Solid particles should be inspected by eye and dissolving procedure should be repeated until all of the content is dissolved. User should perform the measurement on the dissolved material as quickly as possible. If it is necessary to keep the dissolved material for later measurements, it should be portioned into the small volumes and stored at temperatures equal to or lower than -20 $^{o}$C in the dark. Subsamples should be stored in the bottles resistive to volatilization and sun light.

**Minimum Sample Intake**

The minimum sample intake suggested for the reconstituted sample is 400 µL.

**Use of the Certified Value**

For assessing the method performance, the measured values of the CRMs are compared with the certified values [13]. The procedure can be described briefly as follows:

- Calculate the absolute difference between mean measured value and the certified value ($\Delta_m$).
- Combine measurement uncertainty ($u_{meas}$) with the uncertainty of the certified value ($u_{CRM}$):

$$u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$$

- Calculate the expanded uncertainty ($U_{\Delta}$) from the combined uncertainty ($u_{\Delta}$) using a coverage factor of two ($k$ = 2), corresponding to a confidence level of approximately 95%.

If $\Delta_m \leq U_{\Delta}$, then it is assumed that there is no significant difference between the measurement result and the certified value at 95% confidence level.

## ACKNOWLEDGMENT

Authors gratefully acknowledge the laboratories participated in the interlaboratory comparison study.

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**REVISION HISTORY**

| Date | Remarks |
|---|---|
| 05.03.2015 | First issue. |
| 31.08.2016 | Material is recertified with a new characterisation study.<br>"Commutability" and "Scope of Application" informations are added.<br>Density information is added, certified values and uncertainties calculated in ng/mL unit is presented. |
| | |

**ANNEX 1: Details of ID-LCMS Technique**

Sample was dissolved with 2 mL of deionized water (≥18 MΩ). 400 µL portion of this solution was transferred into an Eppendorf tube by weighing. 500 µL saturated salt solution and 400 µL, 0.5 mg/L 25 Hydroxy Vitamin $D_2$-d3 (IS) and 25 Hydroxy Vitamin $D_3$-d3 (IS) solution were added respectively. The content was mixed with vortex for 10 s. and was centrifuged for 4 min at 12000xg. 200 µL was taken from the upper layer and diluted by 200 µL deionized water, mixed with vortex and 100 µL of this solution was injected to LC-MS.

**Table 18.** Properties of LC-MS Instrument

| Name of the Component | Producer | Model |
|---|---|---|
| HRMS | Thermo | Q Exactive Orbitrap |
| HPLC | Thermo | Ultimate 3000 |
| HPLC column | Phenomenex | Luna PFP (150 mm x 2 mm i.d., 5.0 µm) |

**Table 19.** LC parameters

| Name | Value |
|---|---|
| **Column Temperature** | 30° C |
| **Mobile Phase** | Methanol: Water (0.1% Formic acid) (82:18) |
| **Flow rate** | 0.35 mL/min, Isocratic |

**Table 20.** MS Parameters

| Analyte | RT [min] | MH+ |
|---|---|---|
| **25-hydroxy vitamin $D_3$** | 7.64 | 383.3302 |
| **25-hydroxy vitamin $D_3$-$d_3$** | 7.64 | 386.3486 |
| **25-hydroxy vitamin $D_2$** | 8.33 | 395.3302 |
| **25-hydroxy vitamin $D_3$-$d_3$** | 8.33 | 398.3484 |

**Table 21.** MS Parameters

| Parameter | Value |
|---|---|
| **Ionization Mode** | APCI + |
| **Sheath Gas Flow Rate** | 35 |
| **Aux Gas Flow** | 10 |
| **Sweep Gas** | 0 |
| **Discharge Current (µA)** | 5 |

## ANNEX 2: Details of ID- LCMS/MS Technique

Sample was dissolved with 2 mL of deionized water (≥ 18 MΩ). 400 µL portion of this solution was transferred into an Eppendorf tube by weighing. 500 µL saturated salt solution and 400 µL, 0.5 mg/L 26,26,26,27,27,27, 25-hydroxy vitamin D3-d6 (IS) solution was added respectively. The content was mixed with vortex for 10 s. and was centrifuged for 4 min at 12000xg. 200 µL was taken from the upper layer and diluted by 200 µL deionized water, mixed with vortex and 100 µL of this solution was injected to LC-MS/MS.

**Table 22.** Properties of LC-MS/MS Instrument

| Name of the Component | Producer | Model |
|---|---|---|
| Triple Quadrupole LC-MS/MS | ZIVAK Technologies® | Tandem Gold |
| Dual HPLC pump | ZIVAK Technologies® | Tandem Gold |
| HPLC column | Phenomenex | Luna PFP (150 mm x 2 mm i.d., 5.0 µm) |
| Degasser | ZIVAK Technologies® | Tandem Gold |
| Autosampler | ZIVAK Technologies® | Tandem Gold |

**Table 23.** LC parameters

| **Name** | **Value** |
|---|---|
| **Column Temperature** | Room temperature |
| **Mobile Phase** | Methanol: Water (0.1% Formic acid) (82:18) |
| **Flow rate** | 0.35 mL/min, Isocratic |

**Table 24.** MS/MS Scanning Parameters

| **Analyte** | **RT [min]** | **MH+** | **MS/MS** | **CE [V]** | **Dwell [s]** |
|---|---|---|---|---|---|
| **25-hydroxy vitamin $D_3$** | 7.09 | 383.2 | 257.1 | 16 | 0.075 |
| **25-hydroxy vitamin $D_3$-$d_6$** | 7.09 | 389.3 | 263.1 | 16 | 0.075 |
| **25-hydroxy vitamin $D_2$** | 7.78 | 395.3 | 269.0 | 18 | 0.075 |

**Table 25.** MS/MS Parameters

| **Parameter** | **Value** |
|---|---|
| **Ionization Mode** | APCI + |
| **API Nebulizer Gas Pressure** | 50 psi |
| **Dryer Gas Temperature, Pressure** | 300 $^{o}$C, 20 psi |
| **Vaporizer Gas Temperature, Pressure** | 350 $^{o}$C, 12 psi |
| **Scan Time** | 0.450 s |
| **Shield Voltage** | + 600 V |
| **Corona Voltage** | 10 V |
| **Capillary Voltage** | 60 V |
| **Detector** | + 1600 V |
| **Collision Gas Pressure** | 2.25 mTorr |
| **Chamber Temperature** | 65 $^{o}$C |
| **Mass Peak width** | 1.5 amu |
| **Quad 1** | 1.5 amu |
| **Quad 3** | 1.5 amu |

## ANNEX 3: Details of HPLC-UV Technique

400 µL of dissolved sample was weighed and transferred into an Eppendorf tube. 500 µL of saturated salt solution and 400 µL dodecanophenone (IS) solutions were added. The sample was mixed with vortex for 10 s. and then it was centrifuged for 4 min at 12000xg and room temperature. 200 µL of upper phase was transferred to the injection vial and 50 µL of this solution was injected to instrument.

**Table 26.** Properties of HPLC-UV Instrument

| Name of the Component | Producer | Model |
|---|---|---|
| Detector | Thermo FINNIGAN® | Surveyor |
| Autosampler | Thermo FINNIGAN® | Surveyor |
| Pump | Thermo FINNIGAN® | Surveyor |
| Column | ZIVAK Technologies® | Vitamin D2/D3 (100 mm x 3 mm, 5.0 µm) |

**Table 27.** HPLC Autosampler Parameters

| | |
|---|---|
| **Autosampler Temperature** | 4 °C |
| **Injection Volume** | 50 µL |
| **Injection Loop Volume** | 100 µL |

**Table 28.** LC-UV Parameters

| | |
|---|---|
| **Column Temperature** | Room temperature |
| **Mobile Phase** | Methanol:Acetonitrile (70:30) |
| **Wavelength** | 264 nm |
| **Flow Rate** | 1 (mL/min), Isocratic |

## ANNEX 4: Reported Results for the Interlaboratory Study

**Table 29.** Measurement results for interlaboratory study obtained by LC-MS or LC-MS/MS.

| Lab. No | Mass Fraction [ng/g] | |
|---|---|---|
| | **25-hydroxy vitamin $D_2$** | **25-hydroxy vitamin $D_3$** |
| **1** | 47.42 | 46.29 |
| **1** | 49.85 | 47.75 |
| **2** | 49.42 | 46.94 |
| **2** | 50.76 | 46.87 |
| **3** | 46.38 | 44.14 |
| **4** | 51.93 | 49.97 |
| **4** | 49.56 | 47.08 |
| **Mean** | 49.33 | 47.01 |