CRM REPORT

A C G publications

J. Chem. Metrol. 18:2 (2024) 165-172

journal of chemical metrology

Development of tocopherols reference material to support quality and authentication control of wheat germ oil

Ibrahim F. Tahoun [*] and Mohamed A.Gab-Allah

National Institute of Standards, Tersa St, El-Matbah, Haram, P. O. Box: 136 Giza, Code No: 12211 Giza, Egypt

(Received June 20, 2024; Revised September 18, 2024; Accepted September 20, 2024)

Abstract: Wheat germ oil has the highest tocopherol content of all-natural vegetable oil. It's used in cosmetics, pharmaceuticals, food, and dietary supplement products due to its high tocopherol content. Thus, the high marketing and nutritional values have led to an adulteration of this valuable oil. The quality and authentication control of wheat germ oil is based mainly on the quantitative determination of tocopherol concentration. Therefore, NIS has developed new candidate reference material for tocopherols in wheat germ oil to support regulatory authorities and food testing laboratories in their efforts to control oil quality and authenticity. The certification was carried out using two independent sample preparation methods in accordance with ISO 17034 and ISO Guide 35 requirements. The results indicate a sufficient homogeneity and stability of CRM for up to 12 months at 4 ℃. The traceability of the CRM to SI units was established using the direct primary method. The certified value of α-tocopherol and β-tocopherol and their corresponding expanded uncertainties (k=2.0) were found to be (2033.9±49.8 mg/kg) and (625.4±38.1 mg/kg), respectively.

Keywords: Wheat germ oil; tocopherols; value assignment; primary method; reference materials.

1. Introduction

Wheat germ oil (WGO) is known to be the richest source in tocopherols, WGO is a valuable product commonly employed in the cosmetics, pharmaceuticals and food industries [1-2]. Furthermore, WGO is widely used as a dietary supplement due to its well-known medical and nutritional values. WGO is considered one of the useful functional food ingredients due to its high α-tocopherol content [3]. The wheat germ oil high tocopherol content improves physical strength, delays aging, and lowers liver and plasma cholesterol levels. Furthermore, it has been shown that WGO affects gene expression in a number of different tissues; these characteristics have been shown to be crucial in safeguarding the liver from several kinds of diseases [4-8]. For its nutritional value, WGO is frequently subjected to be adulterated with other edible oils of lower commercial value. Thus, there is currently a high need for the development of rapid analytical methods, measuring instruments, and reference materials to ensure quality compliance and support in the detection of such adulterations. Each stage of the measurement process, including external and internal quality control, calibration of measuring equipment, method validation, and establishing findings traceability to SI units, are all enhanced by the usage of certified reference materials [9-12]. Matrix reference materials are extremely helpful in assisting laboratories in quality control and validating their

[*] Corresponding author E-Mail: Ibrahim.Tahoun@nis.sci.eg, Tahoun_nis@yahoo.com

The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology July-December 2024 EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.113.2406.3257
Available online: October 23, 2024

analytical processes since they possess the same properties as the real samples [13-17]. In the present work, the method for preparing and characterizing WGO matrix reference material is presented. This reference material was certified for mass fraction of α-tocopherols and β- tocopherol using two independent analytical methods. The measurement results are directly traceable to SI unit by using primary method for assigning values of calibration solutions for tocopherols. Additionally, the prepared reference material's homogeneity, stability, and value assignment were also emphasized.

2. Experimental

2.1. Chemicals and Reagents

Oil samples were procured from markets located nearby in Cairo, Egypt. Potassium hydroxide, hydrochloric acid, HPLC grade solvents hexane, ethanol, isooctane, and isopropyl alcohol were purchased from Merck (Germany), while tert-butylhydroquinone (TBHQ), phenolphthalein, alkali blue, glacial acetic acid, potassium iodide, sodium thiosulfate, α- tocopherol (96%) and β- tocopherol (1 mg/mL) , were purchased from Sigma-Aldrich. The ultrapure water used for sample and mobile phase preparation was obtained from Millipore, Milli-Q, IQ 7005 system (USA).

2.2. Chromatographic Conditions and Equipment

2.2.1 HPLC system

Agilent 1100 HPLC equipped with diode-array detector (DAD), and reversed phase ZORBAX SB-C18 column (3.0 x 250, 5um) was used for analysis oil samples. Isopropanol and water mixture was used as a mobile phase at 20℃ constant temperature and 0.4 mL/min flow rate. The chromatographic data was analyzed using Agilent Chemstation. The identity of tocopherols under study was confirmed by UV spectral properties and peak retention time.

2.2.2 UV-VIS Spectrophotometer

The concentration of α- and β-tocopherols stock solutions was assigned using calibrated Analytikjena Specord 250 Plus UV-VIS Spectrophotometer (Analytik Jena GmbH+Co, Germany). The maximum absorbance of stock solutions was measured in a wavelength range between 280 nm and 320 nm using 10-mm path length cell. The concentration in mg/L was calculated by dividing measured absorbance by 0.0076 and 0.0089 for α- tocopherol and β- tocopherol, respectively [18]. The stock and calibration solutions was stored at 4 ℃, protected from light prior and during measurement, and analyzed on the day of preparation.

2.3. Sample Preparation Methods

Two kilograms of wheat germ oil were mixed with tert-butylhydroquinone (TBHQ), then homogenized by shaking for six hours, and then packed into 20 g brown glass bottles. The bottles were kept at -4 ºC in the dark with a tight seal to prevent degradation or deterioration of the material. In order to determine the concentration of α- and β- tocopherols in the oil samples, two independent sample preparations were applied. In the first method [M1], 0.5 ± 0.01 g of the homogenized samples were accurately weighed in 20 mL brown vials, and then 10 mL ethanol was added. The vials were closed tightly and shaken for 30 minutes. While in the second method [M2], samples were completely dissolved in 10 mL hexane. Then, both solutions were filtered using Agilent 0.45 μm PTFE syringe filters (Part No. 5191-5915) then, analyzed using HPLC.

2.4. Homogeneity Study

To examine the heterogeneity within and between bottles, the material homogeneity experiment was designed in accordance to ISO Guide 35. Ten bottles were selected randomly from the batch using stratified random sampling approach. Every bottle was split into three equal portions, 0.5 gram from each

portions was dissolved in hexane, then measured by HPLC in triplicates. The collected data were analyzed statistically to evaluate between and within variability, in addition to estimation of uncertainty due to heterogeneity of prepared material.

2.5. Stability Study

The wheat germ oil reference was subjected to isochronous stability studies in order to assess the materials' stability during long term storage and dispatch. Twelve bottles were selected from the patch using a stratified random sample technique, and the bottles were divided into three groups. Two groups were stored at 4 ℃ and 40 ℃ for one year, while one group was stored at the reference temperature of -20 ℃. One bottle from each group was transferred to the reference temperature after 3, 6, 9 and 12 months. Then, the collected bottles were dissolved in hexane, and then measured by HPLC in triplicates. The collected data were examined for outliers and analyzed using linear least square regression (LSR) to test the significance of the slopes and estimate the uncertainty in long-term stability.

3. Results and Discussion

3.1. Preparation and Characterization of Reference Material

Polyunsaturated fatty acids are the major composition of germ oil, the presence of double bonds increase sensitivity of oil to oxidation and rancidity, which affect stability and shelf life of oil. Thus, a high amount of TBHQ antioxidant (0.05 g/100g) was added to the bulk material before packaging to minimize matrix and tocopherols oxidation and to increase shelf life of reference material. The efficiency of TBHQ was evaluated by monitoring the physical–chemical characteristics of the oil including peroxide value, and saponification value. The two parameters are normally used to detect change in the composition of oil and effects due to sedimentation and/or decantation. The results of peroxide and saponification values are 8.80 mEq O_2/kg and 189.00 mg KOH/g, respectively. The characterization of the material was based on applying two independent methods for sample preparation, followed by quantitative determination of α-tocopherols and β- tocopherol by RP-HPLC-DAD operated at a wavelength of 296 nm. All operations were performed in the absence of direct sunlight, the samples were stored in amber glassware under controlled temperature to prevent and decrease rate of oxidation. Samples from M1 and M2 were passed through the PTFE syringe filters with the pore size of 0.45 μm and then, loaded to HPLC. The representative HPLC chromatograms Figure 1 and 2 shows very sharp peaks for both α-tocopherols and β- tocopherol. The separation was achieved in 40 minutes with good baseline resolution for α- tocopherol (R=1.8). 10 bottles were selected randomly from the batch, the bottles were measured in ten different days by two methods in triplicate to avoid any systematic errors. The results of characterization were treated to eliminate results which have a specified probability of being outliers or stragglers, using Mandel's h and k statistics at the 5 % significance level. The characterization uncertainties (u_{ch}) are 8.93 mg/kg and 7.81 mg/kg for α-tocopherol and β-tocopherol, respectively. These values are incorporated into the uncertainty of the reference values.

3.2. Homogeneity Assessment

The homogeneity experiment was designed in accordance with ISO Guide 35:2017 requirements [19-23]. In this study, 10 bottles represent 10% of the prepared batch were sampled randomly and samples were tested for homogeneity using direct injection method [M2] to avoid any variability in results due to extraction process. The selected method was previously validated for selectivity, linearity, working range, LOD, LOQ, trueness and precision [13, 17]. All samples were measured in a random order, to differentiate the trend in measurements from the batch filling trend. The results shown in Table 1 indicate good agreement between all tested bottles. Furthermore, the results of the one-way ANOVA show that the $F_{calculated}$ for α-tocopherol and β- tocopherol were lower than the $F_{crtical}$, indicating that homogeneity criteria were met for WGO reference material. The values of $MS_{between}$, MS_{within} are 88.57, 170.54, 361.42 and 201.73 for α-tocopherol and β- tocopherol, respectively.

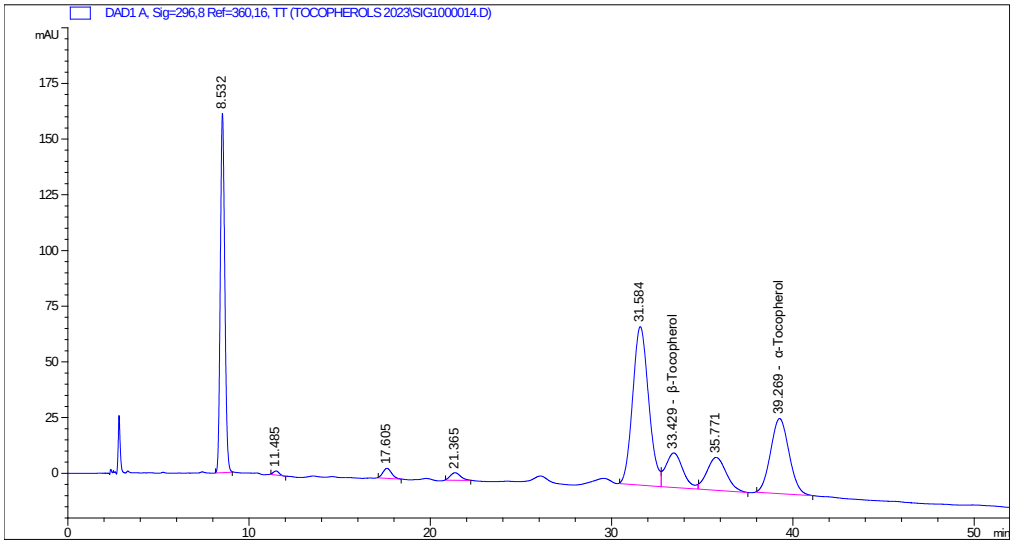

Figure 1. HPLC chromatogram of tocopherols in germ oil extracted by ethanol [Method 1]

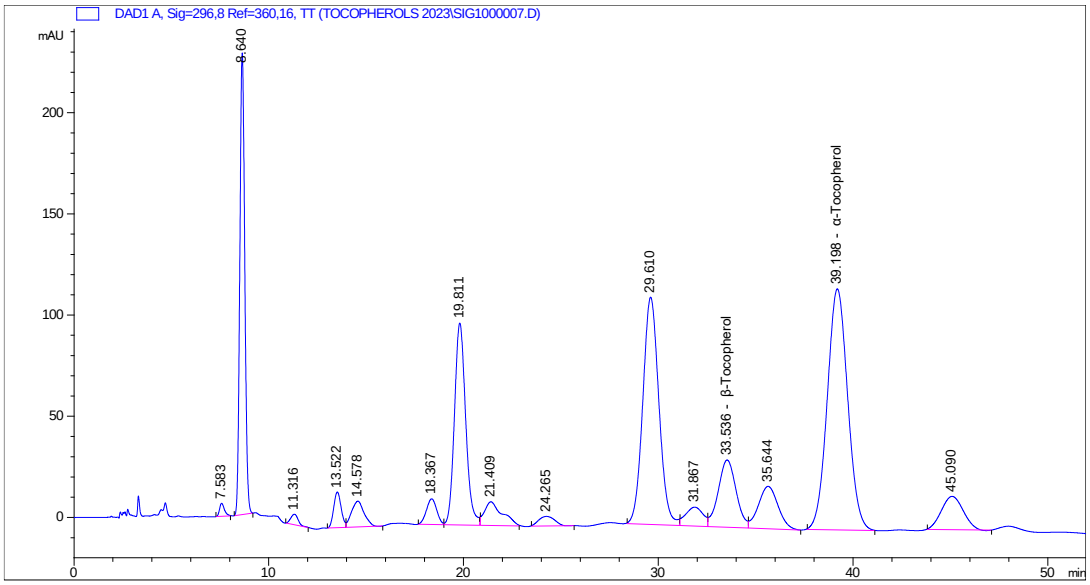

Figure 2. HPLC representative chromatogram of tocopherols in germ oil prepared by Method 2

Based on the ANOVA results, the material homogeneity is better than the test method repeatability because $MS_{between}$ is smaller than MS_{within} for both isomers. Equation 1 was used to determine the uncertainty associated with analytes heterogeneity (u_{bb}) using within-group mean squares.

Where MS_{within} represents within groups mean squares, n is the number of replicate per bottle and N is the number of bottles selected for homogeneity evaluation.

$$u_{bb} = \sqrt{MS_{within}/n}.\sqrt[4]{2/(\nu MS_{within})} \quad (1)$$

The estimated values of material heterogeneity (u_{bb}) are 23.2 mg/kg and 17.3 mg/kg for α-tocopherol and β- tocopherol, respectively. These values are incorporated into uncertainty of reference value.

Table1. Results of homogeneity evaluation of α-tocopherol and β- tocopherol reference material in mg/kg mass fraction

	Bottle Number									
	G1B3	**G2B3**	**G3B3**	**G4B3**	**G5B3**	**G6B3**	**G7B3**	**G8B3**	**G9B3**	**G10B3**
α-tocopherol	2027.1	2009.6	2049.8	2028.1	2009.9	2034.1	2031.2	2039.4	2054.2	2004.8
	2017.6	2049.8	2009.6	1999.0	2039.2	2041.3	2054.8	2017.1	2029.7	2039.9
	2029.8	2033.2	2009.1	2042.4	2049.7	2024.9	2029.3	2049.5	2014.4	2059.1
Mean	**2024.8**	**2030.9**	**2022.8**	**2023.2**	**2032.9**	**2033.4**	**2038.4**	**2035.3**	**2032.8**	**2034.6**
StDev	**6.4**	**20.2**	**23.4**	**22.1**	**20.6**	**8.2**	**14.2**	**16.6**	**20.1**	**27.5**
β- tocopherol	624.3	646.2	630.9	599.5	638.1	621.3	603.0	623.7	612.9	634.7
	623.3	632.4	617.4	622.8	620.6	647.6	615.9	647.1	631.3	618.3
	585.5	608.0	639.9	616.1	613.6	623.3	622.4	617.4	622.8	620.6
Mean	**611.0**	**628.9**	**629.4**	**612.8**	**624.1**	**630.7**	**613.8**	**629.4**	**622.3**	**624.5**
StDev	**22.1**	**19.4**	**11.3**	**12.0**	**12.6**	**14.6**	**9.9**	**15.6**	**9.2**	**8.9**

3.3. Stability Assessment

In order to assess the stability of tocopherols in WGO reference material, an isochronous experimental design technique was applied to investigate the stability of tocopherols in WGO reference material over 12 months.

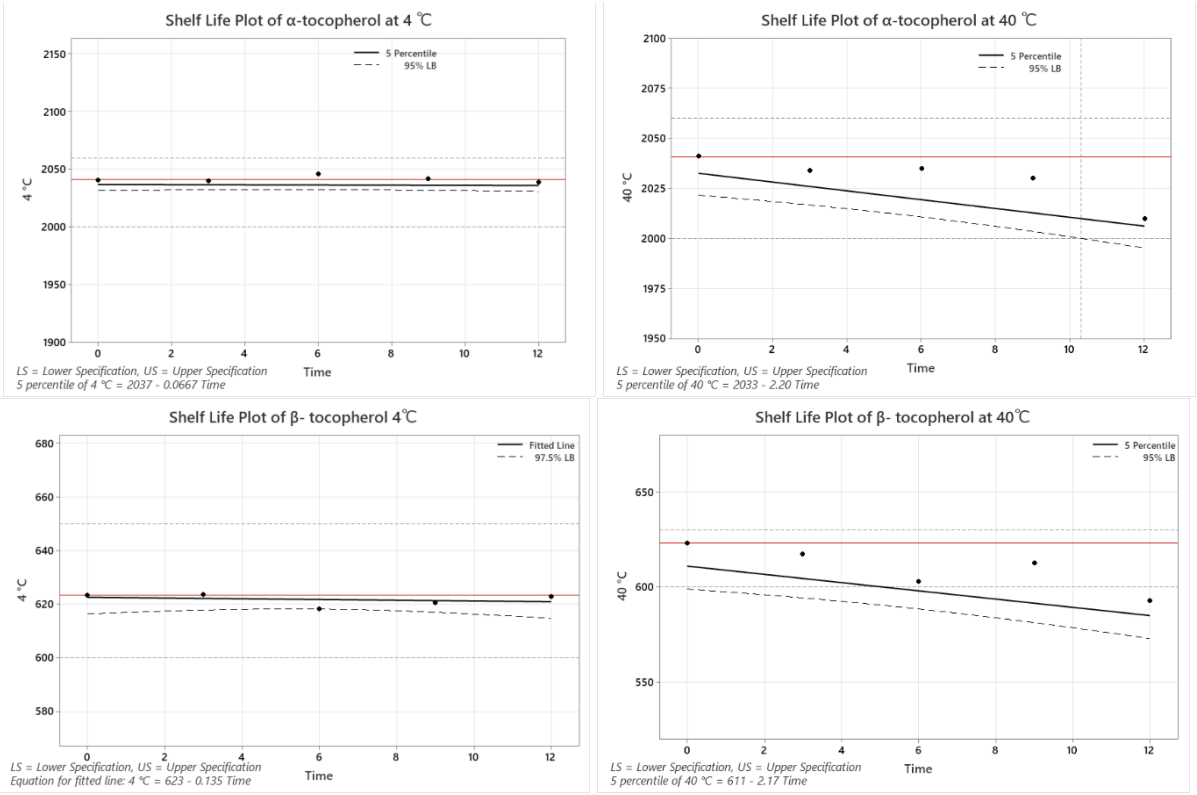

Figure 3. LSR plots for long-term stability study of α-tocopherol and β- tocopherol in WGO during 12 months of storage at 4 ℃and 40℃

The bottles were stored at 4 °C, 40 °C, and the reference temperature for 4 weeks and 12 months, respectively. After the predetermined storage periods, the samples were brought back and stored at the reference temperature, all samples were analyzed after completing study in the same time by the direct injection method [M2], and each bottle was measured three times to ensure repeatability and to eliminate any

error in the measurements. The slope of the fitted straight line was calculated by linear least square regression by plotting data as a function of storage time using the statistical packages: Minitab 20.2 (Minitab Inc. Brandon Court, Progress Way, Coventry, UK), The measured slopes were then assessed for significance using a two-tailed t-test at a 95% confidence level (α = 0.05) [19-23]. Figure 3 shows slopes of the stability evaluation and regression parameters. Based on statistical stability data analysis, it is advised to store WGO reference material for long term storage at 4 °C. Uncertainties of stability measurement have been estimated from errors in the regression slopes, the estimated values are 0.33 mg/kg and 0.26 mg/kg for α-tocopherol and β- tocopherol, respectively. These values contributed to combined uncertainty of the assigned values [21-23]. Further stability monitoring will be performed throughout the duration of reference material availability to maintain confidence in the status of certified values.

3.4. Traceability and Value Assignment

Primary calibrators for α-tocopherols and β- tocopherol were used to establish the metrological traceability of reference values to the SI units. The value assignment of tocopherols concentration was based on the extinction coefficients, masses, and purity evaluations [24-25]. The standard uncertainties for the purity measurements (1.57 mg/kg) represent the standard deviation of the mean values of stock solutions concentrations measured by UV/VIS spectrophotometer using Beer's Law with purity corrections for impurity separated by liquid chromatography and absorbed at 292 and 296 nm for α-tocopherols and β-tocopherol, respectively.

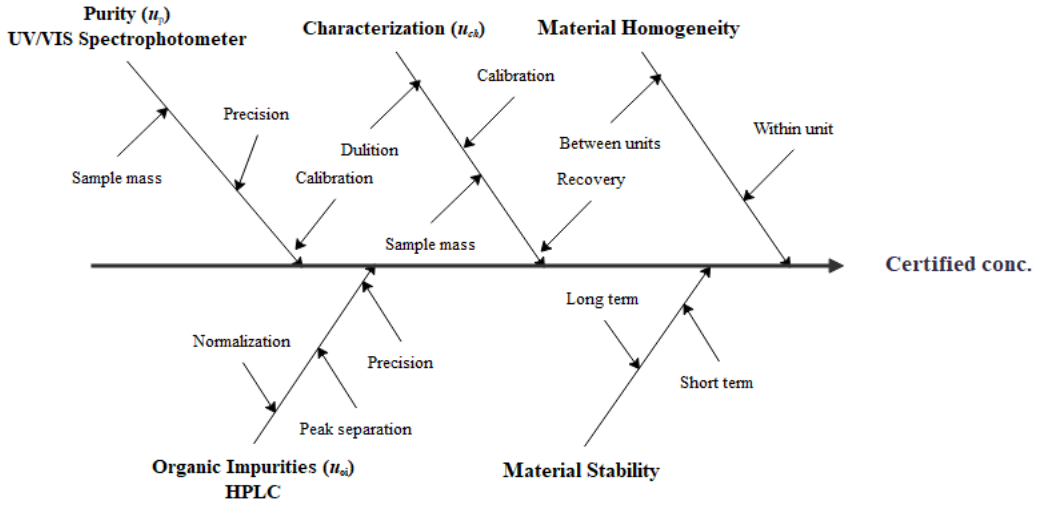

Figure 4. Fishbone diagram for uncertainty sources of WGO reference material

Expanded uncertainty is expressed as two times the root of the sum of the squares of the different components shown in Figure 4 at a confidence level of roughly 95%. After treating outliers using Grubb's statistical test, the certified values are computed by summing the values from methods 1 and 2 using Paule and Mandel's weighting algorithm [26-30]. The certified value of α-tocopherols and β- tocopherol and their expanded uncertainties (k = 2.0) were found to be 2033.9±49.8 mg/kg) and (625.4±38.1 mg/kg), respectively. These concentrations have been considered to be suitable for assessing the analytical procedures in relation to the levels specified in ISO 9936:2016 [18]. Furthermore, since the relative expanded uncertainties are substantially lower than the values mandated by ISO 9936:2016, the provided certified reference materials will be helpful to many food testing laboratories.

4. Conclusions

In this study, the development of reference material for the determination of tocopherols in wheat germ oil was presented. The homogeneity and stability evaluation confirm homogeneity and stability of the material for one year under the storage condition 4 °C. The quantification and value assignment processes

are free from systematic errors, as confirmed by the good agreement between the results of methods M1 and M2. The certified values and their corresponding expanded uncertainties (k = 2.0) were found to be 2033.9±49.8 mg/kg) and (625.4±38.1 mg/kg) for α-tocopherols and β- tocopherol, respectively. This reference material is regarded to be useful for validating test methods, quality assurance, and authentication control of wheat germ oil.

ORCID
Ibrahim F. Tahoun: 0000-0002-0482-6198
Mohamed A.Gab-Allah: 0000-0002-0993-5991

References

[1] X. Liu, C. Chi, S. Zhou and Y. Jiang (2024). Comparison of wheat germ and oil characteristics and stability by different stabilization techniques, *LWT Food. Sci. Tecnol.* **191**,115664.

[2] K.S. Aslan, I. Karabulut and T.B. Koc (2021). Changes in oxidative stability and phytochemical contents of microencapsulated wheat germ oil during accelerated storage, *Food Biosci.* **44**, 101415.

[3] D. Bartolini, R. Marinelli, A.M. Stabile, T. Frammartino, A. Guerrini, S. Garetto, J. Lucci, A. Migni, L. Zatini and G. Marcantonini *et al.* (2022). Wheat germ oil vitamin E cytoprotective effect and its nutrigenomics signature in human hepatocyte lipotoxicity, *Heliyon* **8**, e10748.

[4] G.C. Türkoglu, M. Sarıısık, S.Y. Karavana and F.A. Kose (2021). Production of wheat germ oil containing multilayer hydrogel dressing, *Carbohydr. Polym*. **269**, 118287.

[5] Y. Zou, Y. Gao, H. He and T. Yang (2018). Effect of roasting on physico-chemical properties, antioxidant capacity, and oxidative stability of wheat germ oil, *LWT-Food Sci. Technol*. **90**, 246–253.

[6] M. Karadeniz, S. Sahin, and G. Sumnu (2018). Enhancement of storage stability of wheat germ oil by encapsulation, *Ind. Crop. Prod*. **114**, 14–18.

[7] M. Karabacak, M. Kanbur, G. Eraslan and Z.S. Sarıca (2011). The antioxidant effect of wheat germ oil on subchronic coumaphos exposure in mice, *Ecotoxicol. Environ. Safety* **74**, 2119–2125.

[8] S. Rebolleda, N. Rubio, S. Beltrán, M. T.Sanz and M.L. González-Sanjose (2012). Supercritical fluid extraction of corn germ oil: Study of the influence of process parameters on the extraction yield and oil quality, *J. Supercrit. Fluids*. **72**, 270–277.

[9] I.F. Tahoun and M.A. Gab-Allah (2022). Development of ledipasvir and sofosbuvir pure certified reference materials for improving quality of pharmaceutical analysis, *J. Chem. Metrol*. **16**(**2**), 68-77.

[10] H. Moustafa, I.F. Tahoun, A.I. Abou-Kandil and N.A. Darwish (2021). Characterization of low-density polyethylene reference material for melt flow rate by collaboration of NIS and a network of competent laboratories, *J. Chem. Metrol*. **15**(**2**),102-112.

[11] I.F. Tahoun, E.A. Rend, M.A. Gab-Allah (2021). Preparation and value assignment of parabens and phenoxyethanol in cosmetic cream certified reference material, *J. Chem. Metrol*. **15**(**1**), 1-10.

[12] A.B. Shehata, A.R. Alaskar, M.A. Alrasheed, A.S. Alosaimi, F.A. Alkharraa and A.M. Alzahrani (2020). Certification of sodium benzoate solution reference material by HPLC-UV, LC-MS/MS and UV-VIS-NIR spectrophotometry for food and drug analysis, *J. Chem. Metrol*. **14**(**2**), 88-105.

[13] A.B. Shehata, M.S. Rizk, A.M. Farag and I.F. Tahoun (2014). Certification of three reference materials for α- and γ -tocopherol in edible oils, *Mapan-J. Metrol. Soc. I*. **29**,183-194.

[14] I.F. Tahoun and A.B. Shehata (2016). Preparation of honey reference material for water content by karl fisher and refractometric methods, *Mapan-J. Metrol. Soc. I*. **31**, 25–29.

[15] A.B. Shehata, R.N.Yamani and I.F.Tahoun (2019). Intra- and interlaboratory approach for certification of reference materials for assuring quality of low-alloy steel measurement results, *Mapan-J. Metrol. Soc. I*. **34**, 259–266.

[16] I.F. Tahoun and A.B. Shehata (2016). Development of four parabens reference materials certified for purity mass fraction by mass balance approach, *Mapan-J. Metrol. Soc. I*. **31**, 145–152.

[17] A.B. Shehata, M.S. Rizk, A.M. Farag and I.F. Tahoun (2015). Development of two reference materials for all trans-retinol, retinyl palmitate, α- and γ-tocopherols in milk powder and infant formula, *J. Food. Drug. Anal*. **23,** 82-92.

[18] ISO 9936 (2016). Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography, International Organization of Standardization (ISO), Geneva.

[19] T.P.J. Linsinger, J. Pauwels, A.M.H. van der Veen, H. Schimmel and A. Lamberty (2001). Homogeneity and stability of reference materials, *Accred. Qual. Assur*. **6**, 20–26.

[20] A.M.H. van der Veen, T.P.J. Linsinger, A. Lamberty and J. Pauwels (2001). Uncertainty calculations in the certification of reference materials 3. Stability study, *Accred. Qual. Assur*. **6**, 257–263.

[21] ISO Guide 35(2017). Reference materials—guidance for characterization and assessment of homogeneity and stability. International Organization for Standardization (ISO), Geneva.

[22] ISO/IEC 17034 (2016). General requirements for the competence of reference material producers. International Organization for Standardization (ISO), Geneva.

[23] A.M.H. van der Veen, T. Linsinger and J. Pauwels (2001). Uncertainty calculations in the certification of reference materials.2. Homogeneity study, *Accred. Qual. Assur*. **6**, 26-30.

[24] J.B. Thomas, D.L. Duewer and I.O. Mugenya (2012). Preparation and value assignment of standard reference material 968e fat-soluble vitamins, carotenoids, and cholesterol in human serum, *Anal. Bioanal. Chem*. **402**, 749e62.

[25] M.M. Schantz, J.B. Thomas, D.L. Poster, L.C. Sander and S.A. Wise (2007). New solution standard reference materials for organic constituents. *NIST*. **39**, 30e4.

[26] BIPM, IEC, IFCC, ISO, IUPAC, IUPAP, OIML. Guide to the expression of uncertainty in measurement. 1st ed. Switzerland: ISO: Geneva; 1993. ISBN 92-67-10188-9 corrected and reprinted 1995.

[27] S.L.R. Ellison and A. Williams (Eds) (2012). Eurachem/CITAC guide: Quantifying Uncertainty in Analytical Measurement, 3rd Edition.

[28] S.B. Schiller, Statistical aspects of the certification of chemical batch SRMs, *Natl. Inst. Stand. Technol. Spec. Publ*. **260**–125 (1996).

[29] S.B. Schiller and K.R. Eberhard (1991). Combining data from independent chemical analysis methods, *Spectroch. Acta*. **46B**, 1607–1613.

[30] W. May, R. Parris, C. Beck, J. Fassett, R. Greenberg, F. Guenther, G. Kramer, S. Wise, T. Gills, J. Colbert, R. Gettings and B. MacDonald (2000). Standard Reference Materials : Definitions of terms and modes used at NIST for value-assignment of reference materials for chemical measurements, *NIST Special Publication*, **260**–136.