

Quantitation of Fat Soluble Vitamins in Different Walnut Genotypes and Commercial Cultivars; a Comprehensive and Robust LC-MS/MS Method Validation

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ABSTRACT: Walnut, being both nutritious and delicious, has been regarded as a health food due to its preventive and therapeutic effects. In the present study, a robust LC-MS/MS analytical method was developed to simultaneously quantify five fat soluble vitamins (D₂, D₃, K₂, E, K₁) in eleven walnut genotypes and two commercial cultivars. Linearity, accuracy, inter- and intra-day precision, detection limit (LOD), quantification limit (LOQ) and uncertainty (U at confidence level of 95% (k = 2)) were the validation parameters of the developed LC-MS/MS method. The established linearity ranges for the studied vitamins were between 50 and 1200 mg L⁻¹ for vitamins D₂, D₃, K₁, between 100-2400 mg L⁻¹ for vitamin E and between 200-4800 mg L⁻¹ for vitamin K₂. The coefficients of determination for the vitamins D₂, D₃, K₂, E, K₁ were between 0.9990, 0.9991, 0.9995, 0.9997, 0.9991, respectively. Vitamins D₃, K₂ and K₁ were not present in the analysed walnut samples. On the other hand, vitamins E and D₂ were in high quantities in all studied walnut samples. D₂ and E were the most abundant fat soluble vitamins in MDE32 (29.1 mg g⁻¹ dry plant) and MDE5 (15.2 mg g⁻¹ dry plant) genotypes. Despite the fact that the walnut was selected as real life sample, the validated LC-MS/MS method is enforceable to a broad range of food species.

Keywords: Fat soluble vitamins, analytical method validation, LC-MS/MS, walnut.

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INTRODUCTION

Being the most prevalent tree nut throughout the world, walnut global production has ramped from 1.6×10^6 tons (2004) up to 3.5×10^6 tons (2014) (FAO, 2018). Although its origin is central Asia, cultivation of walnut has latterly reached lands as far as Mexico, Egypt, Ukraine, Romaine or France. Nevertheless, Turkey, Iran, US and China are still the main production countries (FAO, 2018). The improvement of novel walnut genotypes has been innervated by the agriculture of walnut tree in the mentioned different places, their adaption to the environment of cultivating area and the local markets' predilection. Those cultivars embody an enormous genetic inventory of the species that ought to be studied with the aim to understand their particular features and make the most of it.

The interest in walnut consumption and production depends on the health promotion characteristics of the walnut. Consumption of walnut builds up the lipoprotein profile in blood, demonstrates antiatherogenic and anticancer effects, conduces to the adjustment of inflammatory activity and response immunological activity (Yang et al., 2009; Awad and Fink, 2000). Furthermore, walnut consumption isn't affiliated with weight rise (Ibarrola-Jurado et al., 2013). The positive benefits of walnuts in lowering blood cholesterol should not be ignored (Fraser et al., 1992). One reason for these advantages is the tocopherol content of walnut. Experiments have shown that certain foods such as nuts reduces low density lipoprotein and total plasma cholesterol, which in turn reduces the potential risk of coronary heart disease. (Sabaté et al., 1993; Abbey et al., 1994).

Walnut is evaluated as a fine vitamin E source, with tocopherol concentrations betwixt

194 and 297 $\mu\text{g/g}$ (Amaral et al., 2005). Alpha and delta tocopherols are the main ones detected in walnut oil (Crews et al., 2005; Martínez et al., 2006), while high concentration of γ -tocopherol has been found in partly defatted flours (Santos et al., 2017). Mentioned fat-soluble molecules take part in pivotal functions, acting not only as antioxidants but also as membrane stabilizers (Amaral et al., 2005).

Taking into account the nutritional importance of walnuts and important role of fat soluble vitamins, investigating their content in walnuts is of great importance. Nevertheless, the complex and labile ingredients of walnuts have created hassles for detection methods used to determine its vitamin content. Vitamins in nuts have been investigated by various analytical techniques such as UV-Vis, GC-MS, HPLC and LC-MS/MS (Rabadán et al., 2018; Bakkalbaşı et al., 2012; Stuetz et al., 2017;

In the present study, the development and validation of a comprehensive and robust LC-MS/MS method was aimed for simultaneous and quantitative determination of five fat soluble vitamins (D_2 , D_3 , K_2 , E, K_1) in eleven walnut genotypes and two commercial cultivars.

MATERIALS and METHODS

Chemicals and Standard Solutions

Reference standards of fat soluble vitamin A acetate (used as internal standard), vitamin D_2 (99.7%), vitamin D_3 (98.9%), vitamin K_2 (100%), vitamin E (99.5%) and vitamin K_1 (97.7%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Formic acid, hexane and chromatographic grade methanol were obtained from Merck (Darmstadt, Germany). Type 1 pure water was acquired from Sartorius (Goettingen, Germany) Arium ro Ultrapure Water System.

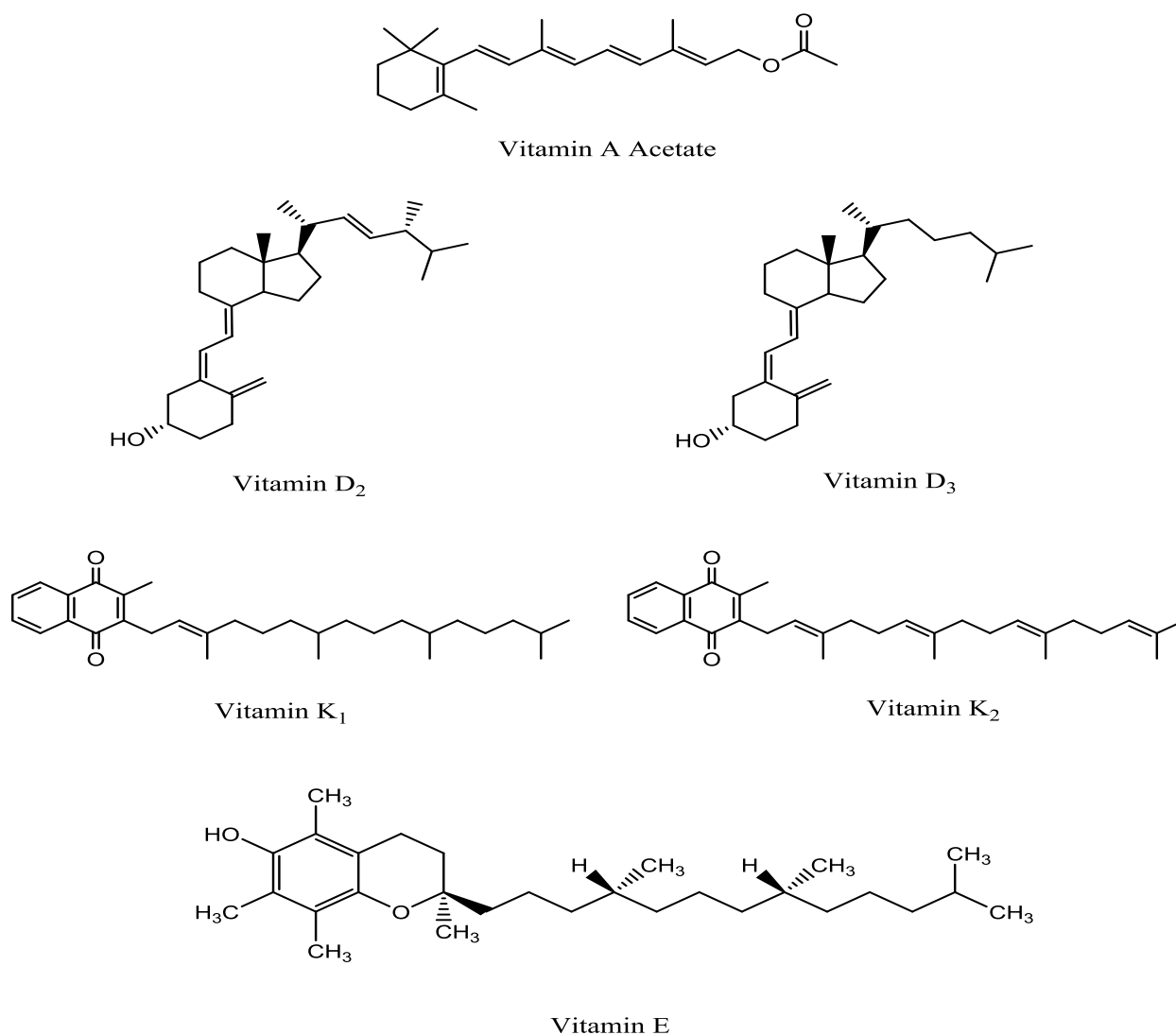


Figure 1. Molecular structures of the vitamin standards used in the LC-MS/MS method

Main stock solutions of vitamin A acetate (2000 mg L^{-1} -IS), vitamin D₂ (80 mg L^{-1}), vitamin D₃ (80 mg L^{-1}), vitamin K₂ (320 mg L^{-1}), vitamin E (160 mg L^{-1}) and vitamin K₁ (80 mg L^{-1}) were prepared from their purchased standards (Figure 1). The prepared standard solutions were kept at -20°C until to be studied. Then, solutions for the calibration points were got ready by diluting the standard stock solutions. Each calibration point and sample solutions were spiked with $50 \mu\text{L}$ of 5 mg L^{-1} internal standard solution in order its concentration to be $250 \mu\text{g/L}$.

Plant material

Eleven walnut genotypes (MDE2, MDE5, MDE18, MDE24, MDE32, MDE39, MDE42, MDE45, MDE50, MDE56, MDE67) were selected from the Derik district of Mardin province in the Southeast part of Turkey in 2014 and two walnut cultivars (Chandler and Franquette) were collected from commercial plantations of Sanliurfa province, Southeast Turkey. To determine their fat soluble vitamins in their fruits, walnut samples were immediately dried and stored in their shells at room temperature until analysis.

Extraction of walnuts for LC-MS/MS

The air dried walnut fruits were removed from their shells. Afterwards, they were grounded and 0.25 grams of the fruit samples were placed in a 15 mL centrifuge tube. Then 50 μL of 5 mg L^{-1} internal standard solution was added to the sample. Then, 1.5 mL water and 1.5 mL methanol were put in and the mixture was shaken vigorously for 1 min. The centrifuge tube containing the mixture was kept in the ultrasonic bath for 15 minutes. Then, the tube was filled up to 10 mL with hexane. The mixture was shaken for 5 minutes. Then the mixture was centrifuged for 10 minutes (4400 rpm). Then, 1 mL of the supernatant was put into a glass tube and the solvents were evaporated under N_2 gas stream. The extract was dissolved in 1 mL of methanol and filtered through 0,2 μm syringe filter stored in the fridge ($-20\text{ }^\circ\text{C}$) prior to LC-MS/MS analysis.

LC-MS Instrumentation and chromatographic conditions

Quantitative investigation of five fat soluble vitamins was performed by using a Nexera model Shimadzu-UHPLC combined with an LCMS 8040 model tandem mass spectrometer. The equipment of the liquid chromatograph was; SIL-30AC autosampler, DGU- 20A3R degasser, CTO-10ASvp column oven and LC-30AD binary pumps. The chromatographic separation was carried out with a Restek Ultra PFPP (150 $\text{mm}\times 4.6\text{ mm}$, 5 μm) column. Reversed-phase liquid chromatograph was optimized to achieve satisfactory separation of the vitamins under study. Temperature of the column was fixed at $25\text{ }^\circ\text{C}$. The elution gradient comprised mobile phase A (water, 0.15 % formic acid) and mobile phase B (methanol, 0.15 % formic acid). The following gradient program was applied: 80-100% B (0-20 min), 100% B (20-25 min), 80% B (25-30 min). Flow rate of

the solvent and the injection volume was optimized as 0.6 mL/min and 1 μL , respectively.

The triple quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source that operated in positive mode. LabSolutions software (Shimadzu) was used for LC- MS/MS data processing. The multiple reaction monitoring (MRM) mode was applied to quantify the vitamins under study following two or three transitions per compound, first one to quantify and the latter to qualify. The mass spectrometer operating conditions were determined as DL temperature, $250\text{ }^\circ\text{C}$; heat block temperature, $400\text{ }^\circ\text{C}$; interface temperature, $350\text{ }^\circ\text{C}$; drying gas flow (nitrogen); 15 L/min and nebulizing gas flow (nitrogen), 3 L/min.

LC-MS/MS analytical method validation studies

In this study, validation and development of a robust and comprehensive LC-MS/MS method was carried out for the quantification of five fat soluble vitamins (D_2 , D_3 , K_2 , E, K_1) in eleven walnut genotypes and two commercial cultivars. The performance features of the analytical method were established by using internal and external standard solutions, spiked and non-spiked samples. Within this in mind, the developed LC-MS/MS method was validated in terms of linearity, limits of detection and quantification (LOD/LOQ), inter-day and intra-day precision (repeatability), accuracy (recovery) and relative standard uncertainty (U% at 95 % confidence level ($k=2$)). The validation parameters of the LC-MS/MS method were given in Table 1.

RESULTS AND DISCUSSION

Linearity

The linearities of the analytes were determined by using internal standard calibration with eight concentration levels for each vitamin analyte, and each concentration level was analyzed in triplicate. Internal standard was used to correct the the analyte loss during sample

preparation. The developed method determined to be linear for the studied vitamins. The correlation coefficients for the vitamins D₂, D₃, K₂, E, K₁, between the ranges of tested concentrations were 0.9990, 0.9991, 0.9995, 0.9997, 0.9991, respectively. The linear regression equations of the calibration curves were shown in Table 1.

Table 1. Analytical parameters belong to the validated LC-MS/MS method (^aRT: Retention time (min), ^bMother ion(m/z): Parent ions of the vitamin compounds (m/z ratio), ^cR²: Coefficient of determination, ^dRSD: Relative standard deviation, ^eLOD/LOQ (µg L⁻¹): Limits of detection and quantification, ^f U (%): percent relative uncertainty at 95% confidence level (k = 2), ^g IS: Internal Standard)

No	Analytes	RT ^a	Mother ion (m/z) ^b	Daughter ions	Ion. mode	Equation	R ^{2c}	RSD% ^d		Linearity Range (µg L ⁻¹)	LOD/LOQ (µg L ⁻¹) ^e	Recovery (%)		U ^f
								Interday	Intraday			Interday	Intraday	
1	Vitamin A acetate (IS) ^g	12.9	329.3	269.3/254.3/145.2	Poz	-	-	-	-	-	-	-	-	-
2	Vitamin D ₂	13.9	397.5	69.2/107.2/105.0	Poz	y=1422000x+38095.6	0.9990	0.0247	0.0364	50-1200	5.3/17.7	0.9943	1.0195	0.075
3	Vitamin D ₃	14.0	385.5	259.4/367.4/107.1	Poz	y=821541x+20012.6	0.9991	0.0187	0.0215	50-1200	10.1/33.7	1.0132	1.0323	0.089
4	Vitamin K ₂	17.9	445.4	187.2/227.0	Poz	y=215092x+9826.5	0.9995	0.0353	0.0512	200-4800	25.6/85.3	1.0213	1.0451	0.204
5	Vitamin E	18.0	431.5	165.2/137.2	Poz	y=122530x+19271.7	0.9997	0.0182	0.0388	100-2400	8.3/27.7	1.0254	1.0568	0.164
6	Vitamin K ₁	20.4	451.5	187.2/185.0	Poz	y=431340x+7885.3	0.9991	0.0546	0.0763	50-1200	6.4/21.3	1.0342	1.0587	0.097

Accuracy (recovery) and precision (repeatability)

Precision and accuracy studies of the developed method were carried out by standard addition to a chosen walnut sample. For intraday precision evaluation, fortified samples were analyzed for six replicates within a single day, whereas fortified samples were examined in six replicates per day for three consecutive days to conduct inter-day precision assay. As a result of the intra-day and inter-day studies, RSD % and recovery values were used to assess the precision and accuracy (Table 1). The following equation

was used to calculate the recovery: recovery (%) = (detected concentration – original concentration)/spiked concentration × 100. Interday/intraday RSD % values were determined to be 0.0247/0.0364, 0.0187/0.0215, 0.0353/0.512, 0.0182/0.0388 and 0.0546/0.0763 for vitamins D₂, D₃, K₂, E and K₁, respectively. As for recovery studies, the measured percent recoveries were found to be 0.9943/1.0195, 1.0132/1.0323, 1.0213/1.0451, 1.0254/1.0568 and 1.0342/1.0587 for vitamins D₂, D₃, K₂, E and K₁, respectively.

Limits of detection and quantification (LOD/LOQ)

To evaluate the LOD and LOQ values for the vitamin compounds used, ten identical solutions that contains the lowest concentration signaled by each vitamin standard were prepared and injected to the instrument. The determined limits of detection and quantification values for the studied analytes were as; 5.3/17.7, 10.1/33.7, 25.6/85.3, 8.3/27.7 and 6.4/21.3 for vitamins D₂, D₃, K₂, E and K₁, respectively. LOD and LOQ values were reckoned by the following equations (Equations 1 and 2):

$$\text{LOD} = \text{Mean} + 3 \times \text{Standard Deviation} \quad (1)$$

$$\text{LOQ} = \text{Mean} + 10 \times \text{Standard Deviation} \quad (2)$$

Standard relative uncertainty (U⁹⁵)

Standard uncertainties of the analytes were determined by the precision and accuracy studies in agreement with the EURACHEM Guide (EURACHEM CITAC Guide, 2004).

Simultaneous quantitation of WSVs in walnut samples by LC-MS/MS

The developed and validated LC-MS/MS method was applied to simultaneously quantify five fat soluble vitamins (vitamins D₂, D₃, K₂, E and K₁) in eleven walnut genotypes (MDE2, MDE5, MDE18, MDE24, MDE32, MDE39, MDE42, MDE45, MDE50, MDE56, MDE67) and two commercial walnut cultivars (Chandler and Franqueete) from the Southeast part of Turkey. According to the quantitative results of the LC-MS/MS analysis, amounts of vitamin D₂ and vitamin E found to be quite high in the studied genotypes and commercial cultivars of walnut samples. However, other studied vitamins (D₃, K₂ and K₁) were not present in any of the walnut samples (Table 2). Figure 2 and Figure 3 shows the TIC chromatogram for the LC-MS/MS analysis of vitamin standards and MDE 2 genotype. Figure 4 shows the LC-MS/MS TIC chromatograms of other walnut samples.

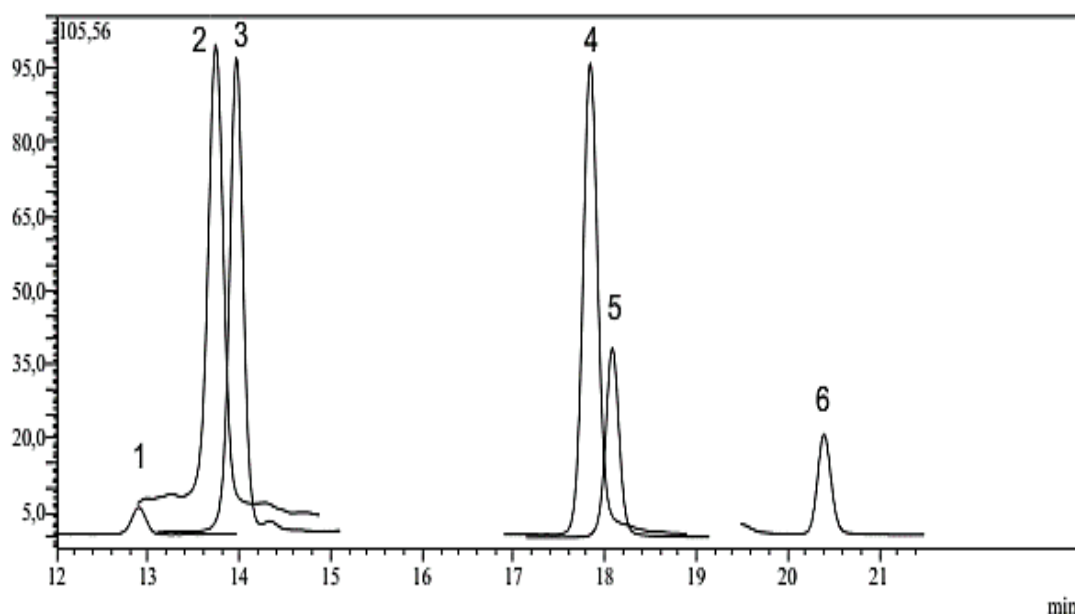


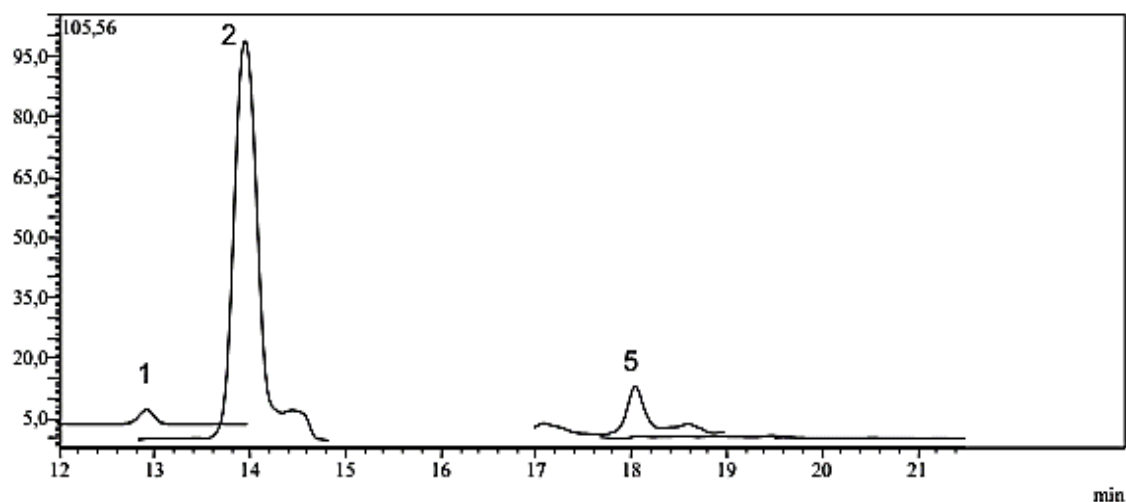
Figure 2. TIC chromatogram of fat soluble vitamin standards (1: vitamin A acetate (IS), 2: vitamin D₂, 3: vitamin D₃, 4: vitamin K₂, 5: vitamin E, 6: vitamin K₁) analysed by LC-MS/MS

Table 1. Quantitative results of fat soluble vitamins in walnut samples by LC-MS/MS (mg analyte/kg dry fruit)

Sample	Vitamin D ₂	Vitamin D ₃	Vitamin K ₂	Vitamin E	Vitamin K ₁
MDE2	28.4	N.D.	N.D.	9.2	N.D.
MDE5	19.8	N.D.	N.D.	15.2	N.D.
MDE18	22.0	N.D.	N.D.	6.2	N.D.
MDE24	21.2	N.D.	N.D.	7.6	N.D.
MDE32	29.0	N.D.	N.D.	7.0	N.D.
MDE39	24.2	N.D.	N.D.	13.6	N.D.
MDE42	28.2	N.D.	N.D.	9.2	N.D.
MDE45	26.8	N.D.	N.D.	6.8	N.D.
MDE50	28.8	N.D.	N.D.	9.2	N.D.
MDE56	21.0	N.D.	N.D.	6.6	N.D.
MDE67	20.6	N.D.	N.D.	6.6	N.D.
Chandler	23.4	N.D.	N.D.	8.8	N.D.
Franquette	26.4	N.D.	N.D.	8.6	N.D.

Being a fat-soluble vitamin, vitamin D (ergocalciferol-D₂, cholecalciferol-D₃, alfacalcidol) helps the human body absorb phosphorus and calcium. Calcium, and phosphorus is important for building and maintaining bone strength having the right amount of vitamin D. Vitamin D₂ was the most abundant fat soluble vitamin in all of the studied walnut samples, followed by vitamin E. Considering the fact that vitamin D₃ is only found in animal-sourced foods and vitamin D₂

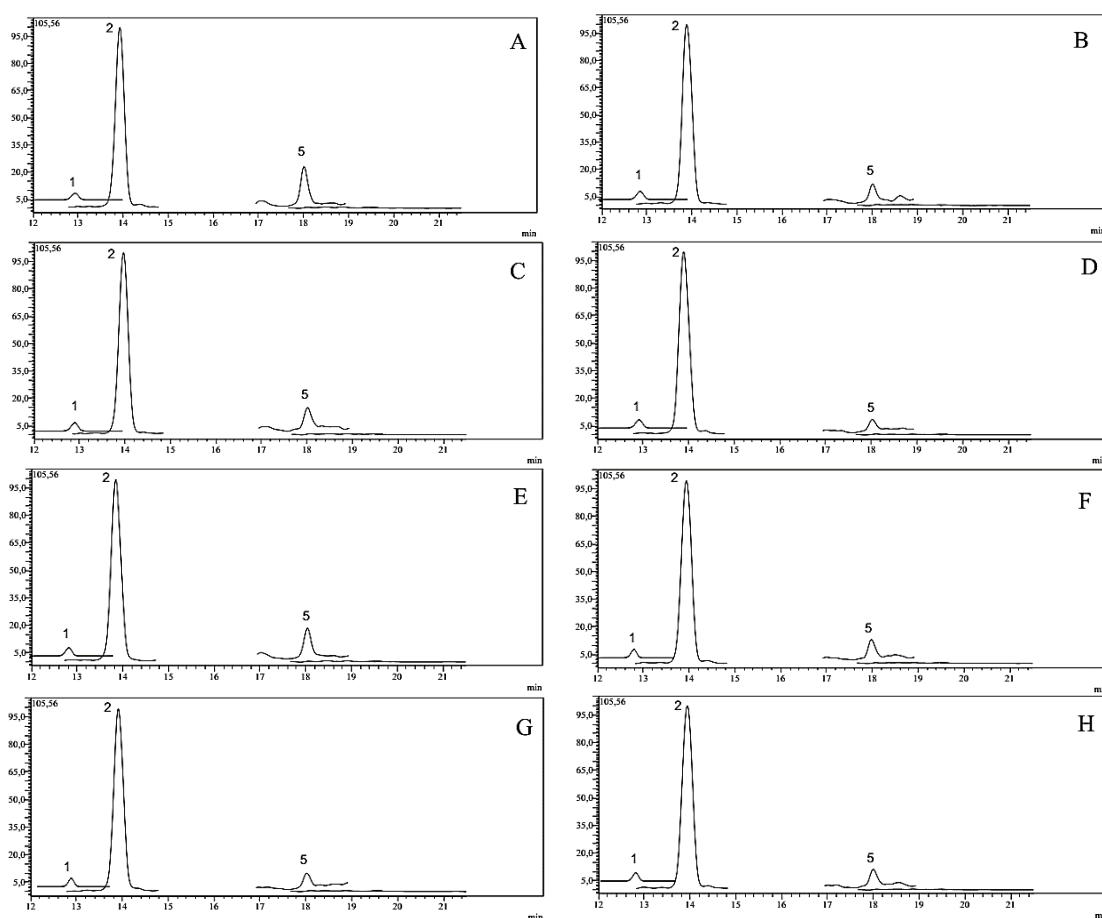
mainly comes from plant sources and fortified foods, it is expected walnuts do not contain vitamin D₃. Being the predominant vitamin in all walnut samples, vitamin D₂ content of the MDE32, MDE50, MDE2 and MDE42 genotypes (29.0, 28.8, 28.4 and 28.2 mg/kg plant, respectively) were the most abundant among the other samples. On the other hand, MDE5 and MDE67 genotypes had the lowest amount of vitamin D₂ (19.8 and 20.6 mg/kg plant, respectively).

**Figure 3.** TIC chromatogram of the genotype MDE2 analysed by LC-MS/MS

Vitamin E is a key component for strong immunity, healthy eyes and skin. MDE5 and MDE39 contained remarkable quantities of vitamin E (15.2 and 13.6 mg/kg dry plant, respectively), while MDE18, MDE56 and MDE67 contained the lowest amount of the vitamin (6.2, 6.6 and 6.6 mg/kg dry plant, respectively). From a general perspective, it is noteworthy that, local walnut genotypes were richer than the commercial cultivars (Chandler and Franquette) in terms of vitamins D₂ and E.

Several recent literatures mentioned some fat soluble vitamins in nuts. In a former study, the impact of roasting on vitamin contents of nuts was investigated and α - and γ - tocopherol (2.1, 1.4 and 0.9 mg α -tocopherol/100 g walnut, respectively) contents of walnut found to be

decreased (Stuetz et al., 2017). In another study, tocopherol contents of seven walnut varieties from Turkey were studied. In this study α -, γ - and δ -tocopherol amounts of walnuts ranged from 9.69 to 14.13, 298.75 to 470.02 and 10.06 to 25.98 mg/kg walnut, respectively (Bakkalbaşı et al., 2014). Savage et al. evaluated the tocopherol contents of different walnut cultivars from Europa, US and New Zealand (Savage et al., 1999). They found that α -, β -, γ - and δ -tocopherol amounts ranged between 14.8-28.7, 1.0-8.2, 206.9-355.0 and 28.0-62.1 μ g/g oil, respectively. A recent study demonstrated that, Lake and Combe varieties of Persian walnuts contained 267.87 and 205.45 μ g/g of γ -tocopherol, respectively (Tsao et al., 2007).



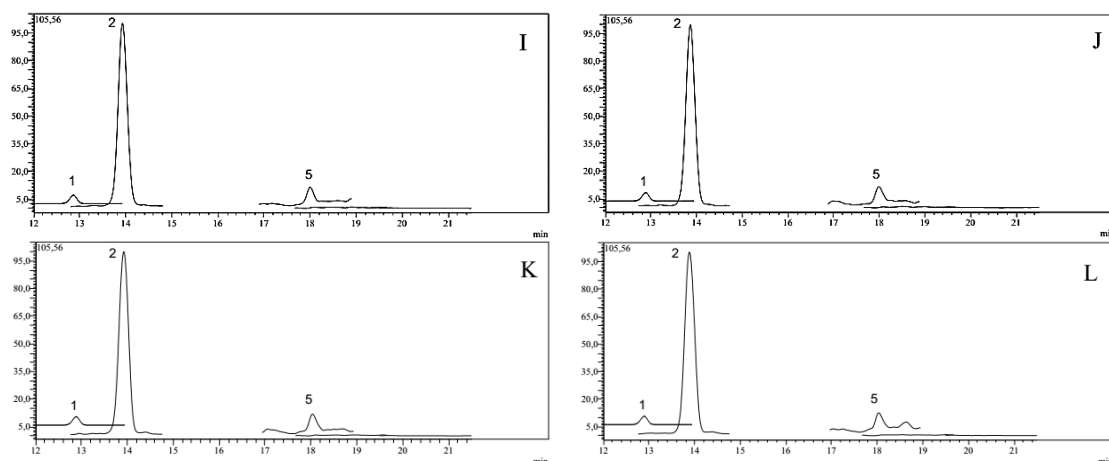


Figure 4. TIC chromatogram of the walnut samples analysed by LC-MS/MS (A: MDE 5, B: MDE 18, C: MDE 24, D: MDE 32, E: MDE 39, F: MDE 42, G: MDE 45, H: MDE 50, I: MDE 56, J: MDE 67, K: Chandler, L: Franquette)

CONCLUSION

To conclude, in the present study a comprehensive and robust LC-MS/MS method was developed and validated for simultaneous quantitation of fat soluble vitamins D₂, D₃, K₂, E and K₁ in eleven walnut genotypes and two commercial walnut cultivars from the Southeast part of Turkey. According to the quantitative results of the LC-MS/MS analysis, amounts of vitamin D₂ and vitamin E found to be quite high in the studied genotypes and commercial cultivars of walnut samples. However, other studied vitamins (D₃, K₂ and K₁) were not present in any of the walnut samples. Additionally, MDE5 and MDE39 were the richest samples in terms of vitamin E. The results of the present study show that walnuts can provide valuable amounts of vitamin D₂ and vitamin E. The data confirm that of walnut genotypes and cultivars are a rich source of significant vitamins that would be very beneficial to human health. The results will be useful to know about the nutritional properties of the walnut genotypes and cultivars, may guide us in designing strategies, might contribute to

breeding studies of walnut genotypes and their commercial production in future.

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