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Vitamin K (phylloquinone and menaquinones) in foods – Cost-effective quantification by LC-ESI-MS/MS

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ABSTRACT

Further research on vitamin K is necessary as growing evidence of vitamin K's importance in human health beyond blood coagulation and bone health is emerging. We present a cost-effective LC-ESI-MS/MS method for quantification of phylloquinone (PK), and menaquinones (MK) 4–10 in food using deuterium labelled (d7) compounds (d7-PK, d7-MK-4, d7-MK-7 and d7-MK-9) as internal standards. The validation of the method included assessment of matrix effect, limit of quantification (LOQ), precision, and trueness. The LC-ESI-MS/MS method runtime is 9 min. The method was compared to a validated LC-FLD method (CEN 14148), for quantification of vitamin K in broccoli, cheese, natto, liver, and microalgae. LOQs of the LC-ESI-MS/MS method were ≤4 μg/100 g food. The intra- and inter-assay precision was <15% for PK, MK-4, MK-7 and MK-9; <20% for MK-5, MK-8, and MK-10, and ≤25% for MK-6. No significant differences between the quantified content by the LC-ESI-MS/MS and LC-FLD methods were observed.

1. Introduction

The Danish scientist Henrik Dam first discovered vitamin K as a coagulation factor in chicken (Dam, 1935). It is now evident that vitamin K describes a group of fat-soluble vitamers namely phylloquinone (PK) and menaquinones (MKs). PK is produced by plants and phototrophic cyanobacteria, as it functions as an electron transporter situated in photosystem I (Nowicka & Kruk, 2016). MKs, especially long chain MKs, are produced in many archaea and bacteria where they are part of the anaerobic respiratory and photosynthetic electron transport chain in the membrane (Nowicka & Kruk, 2010). MK-4 is only to a limited extent synthesised in archaea and bacteria but found in great quantities in animal tissue where it is synthesised through degradation of phylloquinone (Shearer & Newman, 2014).

Vitamin K plays a role in the protein-metabolism if the glutamate (Glu) residues of the proteins needs to be ɣ-carboxylated into ɣ-carboxyglutamate (Gla) residues by the vitamin K dependent ɣ-glutamyl carboxylase (Shearer & Okano, 2018).

The estimated adequate intake and recommended nutrient intake of 1 μg PK/kg body weight/day corresponds with the estimated average intake of 72–196 μg/day (≥18 years old) in Europe. This recommendation is, however, solely based on PK and the plasma thrombin (a Gla protein) level in the blood, without taking into account any of the MKs or other biomarkers (EFSA, 2017; FAO & WHO, 2004).

An intake higher intake than 1 μg PK/kg body weight/day is needed to overcome the concentration of uncarboxylated Gla proteins associated with a higher risk of bone fracture (uncarboxylated osteocalcin) and vascular complications (uncarboxylated matrix Gla protein) (EFSA, 2017; Halder et al., 2019).

Limited data of the content of vitamin K, especially the MKs, in food is at present available. The lack of knowledge surrounding vitamin K might be due to many reasons, for instance, lack of analytical methods and high costs of analytical standards.

Reversed-phase liquid chromatography (LC) coupled with a post-column reduction of the vitamin K vitamers to the corresponding hydroquinones followed by fluorescence detection is the most commonly

Abbreviations: PK, of vitamin K vitamers phylloquinone; MKs, menaquinones; MK-4, menaquinone-4; MK-5, menaquinone-5; MK-6, menaquinone-6; MK-7, menaquinone-7; MK-8, menaquinone-8; MK-9, menaquinone-9; MK-10, menaquinone-10; *d7*-PK, $^{2}H_{7}$-phylloquinone; *d7*-MK-4, $^{2}H_{7}$-menaquinone-4; *d7*-MK-5, $^{2}H_{7}$-menaquinone-5; *d7*-MK-6, $^{2}H_{7}$-menaquinone-6; *d7*-MK-7, $^{2}H_{7}$-menaquinone-7; *d7*-MK-8, $^{2}H_{7}$-menaquinone-8; *d7*-MK-9, $^{2}H_{7}$-menaquinone-9; *d7*-MK-10, $^{2}H_{7}$-menaquinone-10.

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used method for quantification of vitamin K vitamers in food (Booth, Davidson, & Sadowski, 1994; Elder, Haytowitz, Howe, Peterson, & Booth, 2006; European Committee for Standardization, 2003; Faria, De Arruda, Araújo, & Penteado, 2017; Ferreira, Haytowitz, Tassinari, Peterson, & Booth, 2006; Jakob & Elmadfa, 2000; Kamao et al., 2007; Koivu-Tikkanen, Ollilainen, & Piironen, 2000; Peterson et al., 2002; Schurgers & Vermeer, 2000; Vermeer, Raes, Hoofd, Knapen, & Xanthoulea, 2018).

Another widely used method is reversed-phase LC coupled to a mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (LC-APCI-MS) (Fu et al., 2017; Fu, Shen, Finnan, & Haytowitz, 2016; Gentili et al., 2016; Jäpelt & Jakobsen, 2016; Karl, Fu, Dolnikowski, Saltzman, & Booth, 2014; Tarvainen, Fabritius, & Yang, 2019). Few have utilized reversed-phase LC coupled to an MS equipped with an electrospray ionization (ESI) source (LC-ESI-MS) (Campillo et al., 2019; Jensen, Ložnjak Švarc, & Jakobsen, 2021; Xu et al., 2020).

At present only standard reference material (SRM) is available for PK, and not for any of the MKs. Method comparisons therefore serves as an alternative to determine the accuracy of a method when SRM is unavailable. As far as we are aware, no broad comparison between different method principles has been included in the validation of analytical methods for vitamin K, which would be of high interest for the assessment of accuracy of the quantification of vitamin K vitamers in food.

Another important factor in regards to accuracy of the quantification of vitamin K vitamers in food is the choice of internal standards (IS). Due to the high costs of standards, it is a common practice to utilize one IS to quantify more than one compound. Karl et al., 2014 utilises d7-PK as IS for quantification of PK, and MK-4 to MK-13 in baby food and faeces using LC-APCI-MS. They attain recoveries between 76.5% and 94.7% and relative standard deviations (RSD) between 2.4% and 22.6%. Tarvainen et al., 2019 quantified PK, and MK-6 to MK-11 using MK-4 as IS, where recoveries of PK, MK-4, MK-7, and MK-9 ranged between 85.6% and 109.8% with RSD between 6.2% and 19.1%.The use of APCI sources can result in matrix dependent effects which may, however, be compensated for by using an appropriate IS such that the accuracy and precision can be upheld for the method (Zhou, Yang, & Wang, 2017). The LC-MS/MS method in this study was set up with an ESI source, which compared to APCI is more prone to display matrix effects (Ložnjak Švarc, Barnkob, & Jakobsen, 2021; Zhou et al., 2017). It is therefore of the outmost importance to assess the matrix effect of each vitamer analyte. For the vitamers where the IS is not a deuterium-labelled version of the respective vitamer the IS with lowest matrix interference available should be selected.

A further approach to circumvent high costs of standards is to use a calibration curve of one compound and a calibration factor to construct a calibration curve for a second compound. This has been an approach when analysing other compounds using mass spectrometry (Zhou et al., 2008), and when analysing different vitamin K vitamers (Tarvainen et al., 2019). However, Tarvainen et al., 2019 did not include a calibration factor when quantifying MK-6 using the calibration curve for MK-7, and when quantifing MK-8 using the calibration curve of MK-9. Tarvainen et al., 2019 assumed that the peak area in relation to concentration would decrease as the prenyl-tail of the MK increased and they therefore concluded that they could only report a minimum content of MK-6 and MK-8 in the food, and hereby have a low accuracy. To ensure the accuracy of a method it is therefore of utmost importance to assess the matrix effect.

Our study aimed to develop and validate an LC-ESI-MS/MS method for quantification of the 8 vitamin K compounds PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10 using only four standards (PK, MK-4, MK-7, MK-9) and the respective internal standards (d7-PK, d7-MK-4, d7-MK-7, d7-MK-9). The validation included establishment of calibration curves for all MKs using small quantities of MK-5, MK-6, MK-8, and MK-10, assessment of the matrix effect, and comparison to results obtained by a LC-fluorescence detection (LC-FLD). The food matrices used for the comparison study were broccoli pulp, cheese (cow and goat), natto, liver (veal and lamb), and microalgae. These food matrices varied in origin (bacteria and eukaryote (plant, animal)), and content of PK, MKs, fat, carbohydrates, and proteins.

2. Materials and methods

2.1. Samples

The samples used were broccoli pulp, cheese (goat and cow), natto, liver (veal and lamb), and microalgae. The central stalk was cut from broccoli and the broccoli was thereafter juiced. The resulting broccoli pulp was utilised for this study. Parts that were non-edible were removed from the cow cheese that was then cut into smaller pieces. A lamb liver was cut into smaller pieces. Broccoli pulp, cow cheese, natto and lamb liver were each frozen in liquid nitrogen and grounded in a coffee grinder (EGK 200, Rommelsbacher, Germany) for 20 s and stored in 50 mL plastic containers at −20 °C. The goat cheese and veal liver were homogenised in a Braun 3210 kitchen machine. The microalgae (90% Nannochloropsis oculate strain CCMP525 and 10% Tettraselmis chui, strain PLY429 from Reed Mariculture; https://reedmariculture.com/) was freeze-dried 72 h and then homogenised in a Retsch RM200 automatized grinder. All samples were stored at −80 °C.

2.2. Method validation

A method comparison to evaluate differences in precision and accuracy in quantification of PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10 was performed according to the Nordic Committee on Food Analysis – Procedure 4 (NMKL, 2009). The following criteria to evaluate the LC-ESI-MS/MS method were set up: a matrix effect with no influence on the result, accuracy of the calibration curve between 80% and 120%, LOQ at 0.7 μg/100 g for all vitamin K vitamers, intra- and inter-assay precision ≤15%, acceptable comparison to the results obtained by the method using LC-FLD.

See section 2.3 for analytical procedure for LC-MS/MS, section 2.4 for analytical procedure for LC-FLD, and section 2.5 and 2.6 for accuracy (precision and trueness) and statistical analysis, respectively.

2.3. LC-ESI-MS/Ms

2.3.1. Reagents and standards

All chemicals were of analytical grade quality. The following vitamin K standards (PK, MK-4, MK-7 and MK-9) used at DTU were from Sigma Aldrich (Darmstadt, Germany). Standards used at the Institute of Marine Research (IMR) were MK4 and PK from Sigma-Aldrich (Darmstadt, Germany), MK5, MK6, MK8 and MK9 from Biosynth Carbosynth (UK), MK7 from Kappa Bioscience (Oslo, Norway) and MK10 from Toronto Research Chemicals (Canada)). The vitamin K mixed standard solution (vit-K mix solution) was prepared at IMR and shipped to DTU containing: PK (74.3 ng/mL), MK-4 (60.1 ng/mL), MK-5 (59.7 ng/mL), MK-6 (86.5 ng/mL), MK-7 (97.0 ng/mL), MK-8 (44.0 ng/mL), MK-9 (48.0 ng/mL) and MK-10 (85.5 ng/mL) dissolved in methanol. The subsequent deuterium ($^2H_7$) labelled IS (d7) were used at DTU: d7-PK, d7-MK-4, d7-MK-7, and d7-MK-9 (IsoSciences, PA, US).

Ten calibration solutions were made containing 250 ng/mL IS (d7-PK, d7-MK-4, d7-MK-7, and d7-MK-9), and 1, 2.5, 5, 10, 25, 50, 100, 250, 375, 500 ng/mL of unlabelled standards (PK, MK-4, MK-7 and MK-9) dissolved in methanol. For establishment of a calibration curve for each vitamer, eight calibration solutions were made using the labelled IS and the vit-K mix solution. The content in these were 250 ng/mL IS (d7-PK, d7-MK-4, d7-MK-7, and d7-MK-9), and 0.6, 1.2, 2.4, 6.0, 7.2, 12, 36 and 84 ng MK-4/mL and the corresponding concentrations of PK, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10 dissolved in methanol. Additionally, a working IS solution containing 2.5 μg/mL of each of the IS dissolved in *n*-heptane was made. The vitamin K standards were stored in

amber vials at −20 °C. The enzymes, Lipozyme® TL 100L (100 Kilo Lipase Unit (KLU)/g) and Lecitase™ Ultra (10 KLU/g) from Novozymes A/S (Bagsværd, Denmark) were Stored in 4 mL aliquots at 5 °C.

2.3.2. Analytical procedure

Vitamin K was extracted and analysed according to previously described procedures (Jensen et al., 2021) with small adaptations described in Supplementary Online Material (SOM 1).

Quantification of vitamin K vitamers was done using LC-MS/MS with multiple reaction monitoring (MRM) mode using nitrogen as collision gas as previously described (Jensen et al., 2021) and described in details in SOM 1. Specific MRM parameters for each vitamer and IS are depicted in Fig. 1. Qualification of each vitamer was done by comparison of the response ratio between the specific qualifier ion and the corresponding quantifier ion. The quantification was done by comparing the response ratio between the quantifier ion of the vitamer and the corresponding ion of the IS.

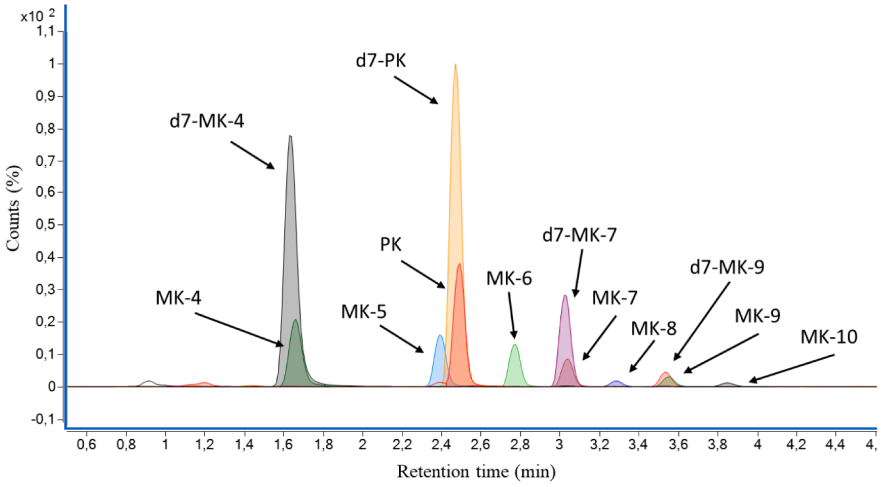
2.3.3. Calibration curves

The prepared standards for the calibration curve (see section 2.3.1) were analysed in duplicates on three different days (n = 6).

2.3.4. Matrix effect

The matrix effect was studied in cow cheese (Klovborg), natto and lamb liver using the post-extraction IS normalised matrix effect protocol (De Nicolò, Cantù, & D'Avolio, 2017; Matuszewski, Constanzer, & Chavez-Eng, 2003; Zhou et al., 2017). Before the samples were analysed using the LC-ESI-MS/MS method, all samples were exposed to the daylight for 48 h and under UV light for another 48 h, in order to photodegrade all vitamin K vitamers in the samples. Each sample was extracted and purified in quadruplicates according to Section 2.3.2. One sample of each matrix was then analysed to confirm that no vitamin K vitamers were present in the samples (<LOD). From the remaining three samples of each matrix, 50 µl were added into vials and spiked with 2.6 ng IS using the working IS solution (section 2.2) and 2.2 ng MK-4 and the corresponding concentrations of PK, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10 using the vit-K mix solution (section 2.2). All samples were

A



B

Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Fragmentor (V)	Collision energy (eV)
Phylloquinone	451.71	187.0	2.361	148	25
		185.1	2.361	148	17
d7-Phylloquinone	458.71	194.1	2.331	168	17
Menaquinone-4	445.71	187.1	1.485	120	25
		109.1	1.485	120	21
d7- Menaquinone-4	452.71	194.1	1.462	128	21
Menaquinones-5	513.8	187.1	2.389	135	25
		109.1	2.389	135	25
Menaquinone-6	581.9	187.1	2.775	158	25
		109.1	2.775	158	25
Menaquinone-7	650.01	187.1	3.211	168	25
		109.1	3.211	168	25
d7-Menaquinone-7	657.01	194.1	3.197	164	33
Menaquinones-8	718.1	187.1	3.288	175	35
		227.1	3.288	175	35
Menaquinone-9	786.31	109.1	3.784	184	40
		187.0	3.784	184	40
d7-Menaquinone-9	793.31	194.1	3.770	188	40
Menaquinones-10	854.3	187.1	3.851	195	40
		227.2	3.851	195	40

Fig. 1. Chromatogram of vitamin K vitamers (PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, and MK-10) and the four IS (d7-PK, d7-MK-4, d7-MK-7, and d7-MK-9) (A), and MRM parameters for all compounds optimized for the Agilent 6470 triple quadrupole instrument (B).

hereafter analysed at two different days using the LC-ESI-MS/MS method (section 2.3.2), then the response factor and the RME (De Nicolò et al., 2017) with confidence intervals were computed.

The response factor and RME were calculated based on the following equations (Eqs. (1)–(3)), where PA describes the peak area of the compound:.

$$Response\ factor_{Matrix} = \frac{PA_{Vitamin\ K\ vitamer\ in\ spiked\ matrix}}{PA_{ISinspikedmatrix}} \times \frac{[IS]_{Matrix}}{[Vitamin\ K\ vitamer]_{Matrix}} \quad (1)$$

$$Response\ factor_{MetOH} = \frac{PA_{Vitamin\ K\ vitamer\ in\ spiked\ MetOH}}{PA_{IS\ in\ spiked\ MetOH}} \times \frac{[IS]_{MetOH}}{[Vitamin\ K\ vitamer]_{MetOH}} \quad (2)$$

$$RME = \frac{Response\ factor_{Matrix}}{Response\ factor_{MetOH}} \quad (3)$$

2.3.5. Limit of quantification

Based on nutritionally relevant levels, the acceptable LOQ was assessed as the level for foods, which contribute with 1% of the recommended dietary intake if eaten in an amount of 100 g food. The acceptable LOQ was therefore determined to be 0.7 µg/100 g food. The calibration standard with the lowest concentration of vitamin K vitamers (PK, MK-4, MK-7, and MK-9), which had a signal to noise (S/N) $>$ 10 combined with an accuracy between 80% and 120% was determined to be the LOQ. The LOQ of MK-5, MK-6, MK-8, and MK-10 was determined to be the LOQ of the corresponding calibrant vitamer multiplied by the calibration factor (see section 3.2.3.).

2.4. LC-Fluorescence detection

2.4.1. Reagents and standards

All chemicals were of analytical grade quality. The standards were from Sigma-Aldrich (MK4 and PK), Biosynth Carbosynth (MK5, MK6, MK8 and MK9), Kappa Bioscience (MK7) and Toronto Research Chemicals (MK10). The vitamin K standards were stored at −20 °C in amber vials. They were dissolved in ethyl acetate in first stock solution and further solutions were made in methanol, resulting in 99.9% methanol in working solutions. Lipase type VII from Candida Rugosa were bought from Sigma-Aldrich.

2.4.2. Analytical procedure

Vitamin K was extracted according to CEN/EN 14148:2003 (European Committee for Standardization, 2003) for samples not containing chlorophyll bound vitamin K. In brief, 1 g of sample was weighed in a tube and vortexed together with 15 mL of water at 40 °C, where after 5 mL of phosphate buffer (0.8 M, pH 7.9) was added together with 1 g of lipase (1000 U/mg) and then the sample was mixed for 2–3 min. The sample was then incubated at 37 °C for 2 h, while being manually shaked every 20 min. The sample was then cooled to room temperature and 10 mL of ethanol:methanol (95:5 v/v) and 1 g of K_2CO_3 was added and the sample was mixed followed by addition of 30 mL hexane, mixing and centrifugation at 2000 g for 10 min. The hexane phase was then transferred to an amber vial, evaporated to dryness and redisolved in methanol:ethyl acetate (1:1). Quantification was done with external standard curves for all the vitamin K vitamers using LC-FLD, after online electrochemical reduction of vitamin K.

For samples containing chlorophyll bound vitamin K (broccoli and microalgae) extraction was done according to the previously published procedure (Jensen et al., 2021) with the following modifications: the lipase and SPE clean-up steps were not included and hexane was used instead of heptane as the extraction solvent. After evaporation of hexane the samples were dissolved in methanol:ethyl acetate (1:1). Quantification was done with external standard curves for all the vitamin K vitamers using LC-FLD, after online electrochemical reduction of vitamin K.

2.4.3. Calibration curves

A ‘standard mix’ with the following concentrations were made: PK 12.4 ng/mL, MK4 10.0 ng/mL, MK5 9.95 ng/mL, MK6 14.4 ng/mL, MK7 16.2 ng/mL, MK8 16.4 ng/mL, MK9 17.6 ng/mL, MK10 14.3 ng/mL. The standard curve was made by injecting 0.2, 0.6, 1.8, 5.4, 16.2 and 48.6 µl of the ‘standard mix’ at the beginning and at end of each analytical serie. The calibration range for each vitamer is shown in SOM2.

2.4.4. Limit of quantification

The LOQ is defined as the level of the standard with the lowest concentration of the vitamin K vitamer that had S/N > 10. For practical reasons the LOQ is adjusted for four conditions in every sample: amount of sample weighed, aliquot of hexane phase, volume of final solvent and amount injected on the autosampler. For samples containing low amounts of vitamin K the lowest LOQ’s ranging from 0.02 to 0.05 ug/100 g can be achieved by: weighing 1 g sample, using maximum aliquot of hexane phase, using 0.5 mL of final solvent solution and injecting 20 µl sample. In the matrices analysed herein the concentrations are on high levels for one or several vitamin K forms. This increases the LOQ for the other vitamers by demanding higher solution volumes or decreased injection volumes of the samples due to the technical features of the FLD.

2.5. Precision and trueness

The intra- and inter-assay precision and trueness of the method were assessed by quantifying the endogenous content of PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10 in broccoli pulp, cheese (cow and goat), natto, liver (veal and lamb), and microalgae by analysing at least triplicates at three different days both at DTU (LC-MS/MS) and at IMR (LC-FLD).

2.6. Statistical analysis

The average and 95% confidence intervals were calculated for the RME.

To test for significance (p-value $<$ 0.05) between days, and to determine intra- and inter-day standard deviations (SD) when testing

the precision and trueness of the method, one-way ANOVA tests were performed. Statistical work was performed using Excel (Microsoft® Excel® 2016 (16.0.5188.1000)) and RStudio (© 2009–2019 RStudio, Inc., Version 1.2.1335). Results are given as mean and relative standard deviation (RSD) calculated as in Eq. (4), when n ≥ 3.

$$RSD(\%) = \frac{SD}{mean} * 100 \quad (4)$$

The pooled SD was calculated based on Eq. (5) with n_k describing the number of samples in group k.

$$SD_{pooled} = \sqrt{\frac{((n_1 - 1) * RSD_1)^2 + ((n_2 - 1) * RSD_2)^2 + \ldots + ((n_k - 1) * RSD_k)^2}{n_1 + n_2 + \ldots + n_k - k}} \quad (5)$$

A Bland-Altman test (Bland & Altman, 1999) was performed to determine the difference of the quantified vitamin K content in the seven different samples between the LC-ESI-MS/MS method and the LC-FLD method.

3. Results and discussion

3.1. LC-ESI-MS/MS conditions

The chromatogram of the calibration standard (100 ng/ml) including the eight different vitamin K vitamers and the four IS obtained by the optimised analytical method described in section 2.3 is shown in Fig. 1A. The total run time of the LC-ESI-MS/MS method is 9 min allowing baseline separation of all vitamers.

Previously reported LC-MS methods for quantifications of phylloquinone and a varying range of menaquinones have varied from 9 to 22 min (Gentili et al., 2016; Goncalves et al., 2014; Jäpelt & Jakobsen, 2016; Jensen et al., 2021; Karl et al., 2014; Tarvainen et al., 2019; Xu et al., 2020).

3.2. Quantification of vitamin K vitamers by LC-MS/MS

3.2.1. Matrix effect to identify optimal IS

For the present method deuterium labelled standards of four different vitamin K vitamers were chosen, to have deuterium labelled PK, and MKs with varying prenyl chain length.

The response factor and the RME with confidence intervals were calculated based on the peak areas of each vitamer in each food matrix (cow cheese (Klovborg), natto and lamb liver) with methanol serving as the reference. The samples were analysed in triplicate at two different days. The resulting RME are depicted in Fig. 2. An RME value equal to 1 means that the matrix effect, if any, for the respective vitamer was compensated for by the IS used.

From Fig. 2A it is seen that by using d7-PK as IS for PK it is possible to correct for the matrix effect observed in the food matrices whereas the

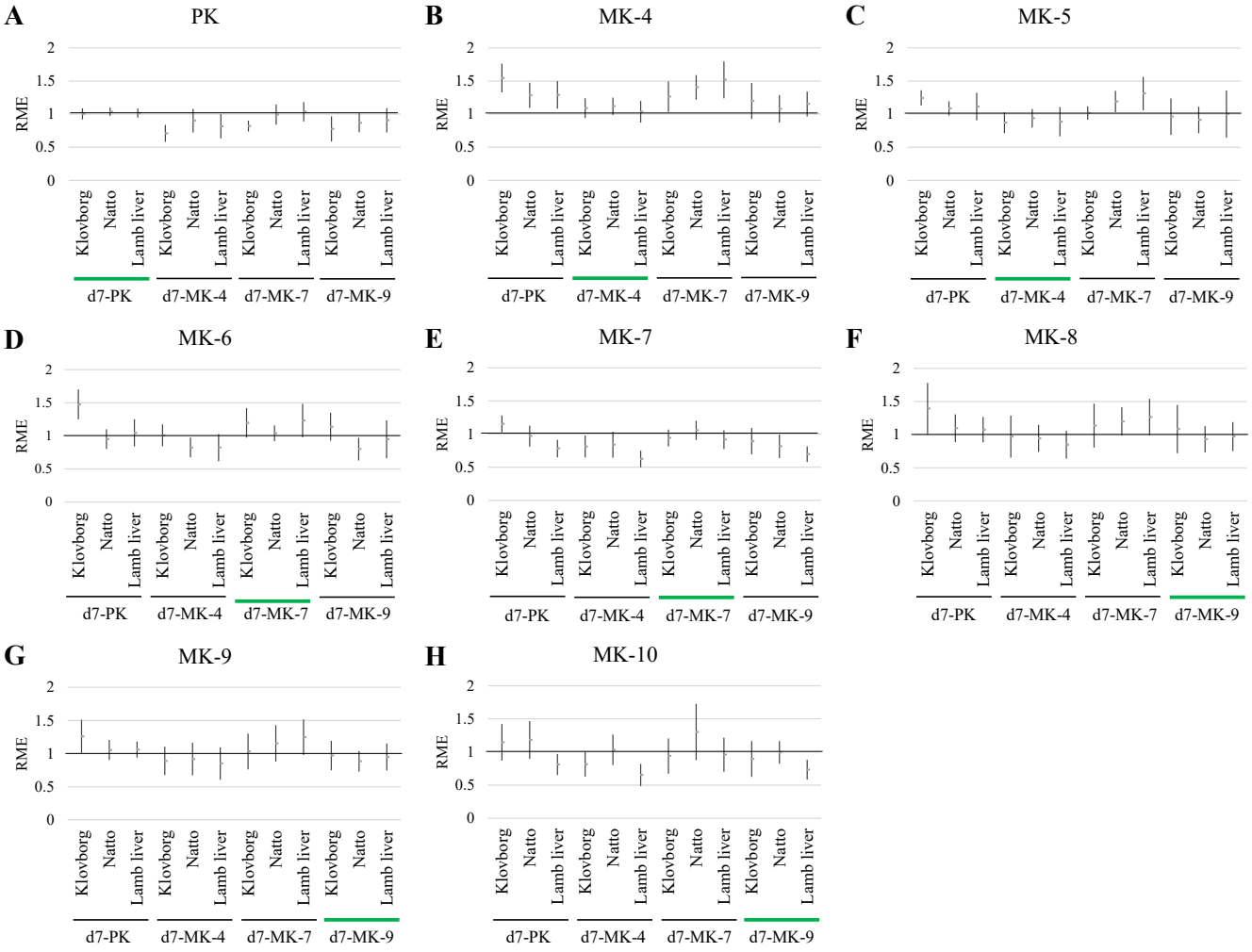


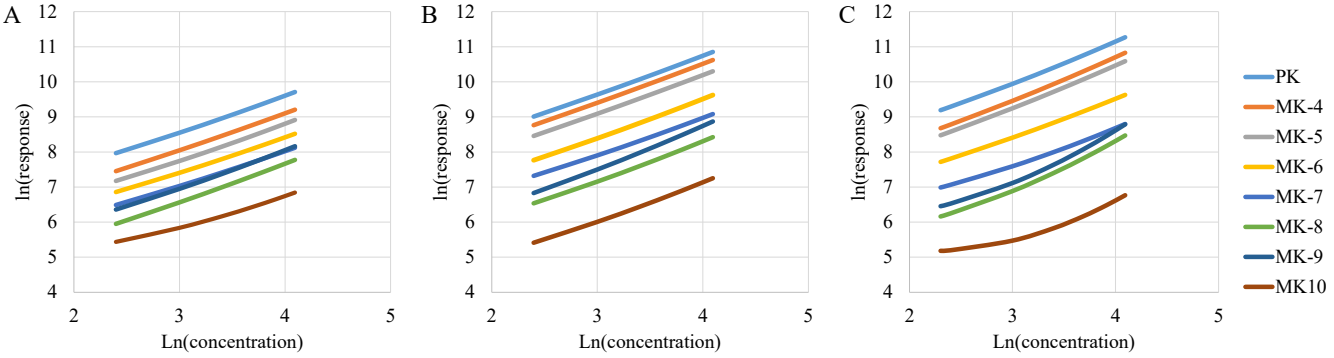
Fig. 2. The relative matrix effect (RME) of vitamin K vitamers; PK (A), MK-4 (B), MK-5 (C), MK-6 (D), MK-7 (E), MK-8 (F), MK-9 (G) and MK-10 (H) and all IS (d7-PK, d7-MK-4, d7-MK-7 and d7-MK-9). Green indicates the IS that was chosen for the specific vitamers as the best IS to correct for matrix effect of the specific vitamer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

use of d7-MK-4, d7-MK-7 and d7-MK-9 are not able to correct for the matrix effects to the same extent. For MK-4 it is seen that d7-MK-4 can correct for the matrix effect (Fig. 2B). Likewise, d7-MK-7 can correct for matrix effects of MK-7 and d7-MK-9 can correct for matrix effects observed for MK-9 (see Fig. 2E and 2G). This is in correspondence with what was found by Jensen et al., 2021. It is further observed that d7-MK-4 and d7-MK-9 were able to correct for matrix effects of MK-5, that d7-MK-7 were able to correct for matrix effects of MK-6, that d7-MK-4, d7-MK-7 and d7-MK-9 could correct for matrix effects of MK-8 and that d7-MK-7 could correct for matrix effects of MK-10. To our knowledge no detailed study of the matrix effect correction abilities of other IS for vitamin K vitamers have been conducted except for what was done by Jensen et al., 2021 and Huang et al. 2016. Both these studies investigated matrix effect if identical deuterium labelled standards were used. The goal of utilising an IS is to correct for both loss during sample clean-up and changes in ionisation efficiency during LC-MS/MS quantification (Zhou et al., 2017). When choosing the IS it is important to take into account the structural and chemical properties, preferably being very similar to the analyte of interest and hereby the IS would exhibit the same loss during sample clean-up and the same relative matrix effect as the analyte of interest (Matuszewski et al., 2003; Zhou et al., 2017). Karl et al., 2014 emphasized the importance of studying matrix effects to ensure trueness and precision of quantification of vitamin K vitamers, but did assess the matrix effect themselves. Matrix effects are especially observed when utilising ESI, as opposed to APCI. ESI was used in the present method, which further emphasises the need for assessment of the matrix effect (Matuszewski et al., 2003; Zhou et al., 2017). Other IS could also be taken into consideration, such as ^{13}C-labelled standards as these are superior to deuterium labelled standards in the quantification of vitamin D (Ložnjak Švarc et al., 2021). Deuterium labelling will change the RT as the lipophilicity of the compounds change slightly, which is not the case for ^{13}C-labelled standards and these may therefore be able to correct for matrix effect to a higher extent (Ložnjak Švarc et al., 2021; Zhou et al., 2017). However, due to costs deuterium labelled standards were used in the present method.

3.2.2. Calibration curves

To determine which IS would be the most optimal to utilise for quantification of MK-5, MK-6, MK-8 and MK-10, a comparison of the response at different concentrations of mentioned MKs, and PK, MK-4, MK-7 and MK-9 at different days was performed. It was shown that the response ratios between PK and d7-PK, MK-4 and d7-MK-4, MK-7 and d7-MK-7, and MK-9 and d7-MK-9 are constant. If the peak area ratio between MK-5, MK-6, MK-8 or MK-10 and either PK, MK-4, MK-7 or MK-9 is constant it is assumed that the peak area ratio between the MK-5, MK-6, MK-8 or MK-10 and the corresponding IS will also be constant.

As the calibration curves for PK, MK-4, MK-7 and MK-9 are second order models with a weighing of $\frac{1}{x}$ of the natural logarithm (ln) of the peak area depending on the ln of the concentration (ng/mL) (Jensen et al., 2021), it was assumed that such similar model would be the best fit for MK-5, MK-6, MK-8 and MK-10. For each vitamer and day the "ln" of the peak area and the "ln" of the concentration (ng/mL) was used to construct second order models with a weighing of $\frac{1}{x}\left(\frac{1}{concentration}\right)$. The



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Vitamer with no IS	Vitamer with corresponding IS	Peak area ratio (average)	RSD (%)
MK-5	PK	0.93	1.7
	MK-4	0.97	0.6
	MK-7	1.15	4.1
	MK-9	1.19	6.0
MK-6	PK	0.87	1.6
	MK-4	0.90	1.7
	MK-7	1.07	2.1
	MK-9	1.10	4.1
MK-8	PK	0.75	4.7
	MK-4	0.78	4.9
	MK-7	0.93	2.4
	MK-9	0.96	0.9
MK-10	PK	0.63	8.5
	MK-4	0.64	5.4
	MK-7	0.78	5.5
	MK-9	0.81	3.8

Fig. 3. Second order models describing the ln(peak area) depending on the ln(concentration (ng/mL)) of each vitamin K vitamer (PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, and MK-10) with weighing of $\frac{1}{x}$ at day one (A), day two (B), and day three (C). Average predicted peak area ratios between the models for each vitamer in the concentration range 11–60 ng/mL (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resulting models for day 1, day 2 and day 3 are shown in Fig. 3, A-C.

To determine which peak area ratios between the vitamers were the most stable in average of the results from the three days, the predicted peak area determined by the different models were compared at the concentrations: 11 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, 50 ng/mL and 60 ng/mL (Fig. 3D).

It was observed that the predicted peak area ratio between MK-5 and MK-4 (0.97 ± 0.6%) was more stable over a three-day period than the ratio between MK-5 and PK, MK-7 and MK-9, respectively (Fig. 3D). The predicted peak area ratio between MK-6 and PK (0.87 ± 1.6%) was more stable over a three-day period than the ratio between MK-6 and MK-4, MK-7 or MK-9 (Fig. 3D). The predicted peak area ratio between MK-8 and MK-9 (0.96 ± 0.9%) was more stable over a three-day period than the ratio between MK-8 and PK, MK-4 or MK-7, respectively, while the predicted peak area ratio between MK-10 and MK-9 (0.81 ± 3.8%) was more stable over a three-day period than the ratio between MK-10 and PK, MK-4 or MK-7, respectively (Fig. 3D).

Three factors were taken into consideration when choosing the specific IS for MK-5, MK-6, MK-8 and MK-10: the structure of the vitamer compared to the different IS, the RME with the 95% confidence intervals, and the stability of the predicted peak area ratios at specific concentrations over time. Thus d7-MK4 was chosen as IS for MK5, d7-MK-7 was chosen as IS for MK-6, and d7-MK-9 was chosen as IS for both MK-8 and MK-10.

3.2.3. Calibration curves correction factor

Second order calibration curves were now constructed using the ln of the peak are ratio between the vitamer and the chosen IS, and the ln of the concentration ratio between the vitamer and the IS, with a weighing of $\frac{1}{concentration\ \ ratio}$. The calibration model was based on the best fit with a weighing based on the data being heteroscedastic (Desharnais, Camirand-Lemyre, Mireault, & Skinner, 2017). The resulting calibration curves at each of the three days are shown in Fig. 4A-C.

Using the constructed calibration curves the concentrations at different peak area ratios were found. The calibration curve for MK-4 will be used to quantify MK-5, thus the differences in the determined concentration at specific peak area ratios were compared. It was observed that at all peak area ratios based on the three days injection, the difference in determined concentration was 1.30 ± 0.06. The MK-5 concentration in a sample can be quantified using the calibration curve for MK-4 and correct the result by multiplication with the calibration factor of 1.3. The calibration factor was similarly calculated for MK-6, MK-8 and MK-10 based on the calibration curves of MK-7, MK-9, and MK-9, respectively. The resulting factors are shown in Fig. 4D.

It is expected that by utilizing another standard curve and a calibration factor to quantify the content of another compound will result in a decreased precision and hereby a greater SD. However, when high cost of standards is making them unavailable the extrapolation of calibration curves is an acceptable alternative in our aim to estimate the content of the vitamin K vitamers in foods.

3.3. Limit of quantification for LC-MS/MS

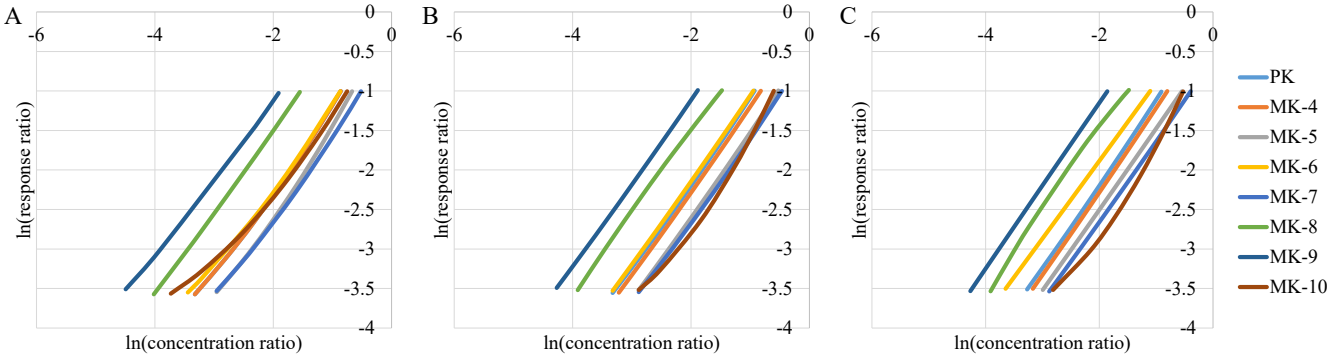
The LOQ was defined as the level of the standard with the lowest concentration of the vitamin K vitamer that had a S/N > 10 and an accuracy between 80 and 120%. The LOQ for MK-5, MK-6, MK-8, and MK-10 was defined as the calibrant vitamer times the calibration factor if the calibration factor is >1, otherwise the LOQ of the calibrant vitamer was utilised. The resulting LOQs are 0.5 μg/100 g for PK, MK-4, MK-6 and MK-7, 0.7 μg/100 g for MK-5, 1.0 μg/100 g for MK-9, 1.4 μg/100 g for MK-8, and 4.0 μg/100 g for MK-10.

The above mentioned LOQs were in accordance with the validation criteria for PK, MK-4, MK-5, MK-6, and MK-7, but for MK-8, MK-9, and MK-10 the LOQ estimated was above the aimed level.

Other methods analysing vitamin K have reported LOQs between 0.04 and 1.4 μg/100 g for PK, between 0.05 and 4 μg/100 g for MK-4, 0.5 μg/100 g for MK-5, 0.6 μg/100 g for MK-6, 0.1–2.6 μg/100 g for MK-7, 0.1–4.3 μg/100 g for MK-8, 0.1–5 μg/100 g for MK-9, and between 0.1 and 0.4 μg/100 g for MK-10 (Gentili et al., 2016; Karl et al., 2014; Koivu-Tikkanen et al., 2000; Tarvainen et al., 2019; Walther et al., 2021). Our results are comparable to others, except for MK-10 which show a higher LOQ.

3.4. Precision and trueness

Content of the vitamin K vitamers (PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10) quantified in foods by the LC-MS/MS-method were compared to results obtained by the LC-FLD-method (described



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Vitamer of interest	MK-5	MK-6	MK-8	MK-10
IS	D7-MK-4	D7-MK-7	D7-MK-9	D7-MK-9
Exploited calibration curve (calibrant vitamer)	MK-4	MK-7	MK-9	MK-9
Calibration factor	1.30	0.58	1.42	4.03
RSD of calibration factor (%)	4.7	16	7.6	21

Fig. 4. Calibration curves of all vitamin K vitamers utilising the chosen IS for the specific vitamers constructed at day one (A), day two (B), and day three (C). (D) Vitamer of interest (MK-5, MK-6, MK-8 and MK-10) with assigned internal standard (IS), exploited calibration curve and calibration factor with relative standard deviation.

Table 1
Quantified content (μg/100 g) of vitamin K (PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, and MK-10) in broccoli pulp, cheese (cow and goat), natto, liver (lamb and veal), and microalgae.

FoodMatrix			Broccoli pulp	Cow cheese	Natto	Lamb liver	Veal liver	Goat cheese	Microalgae
PK	(μg/100 g)	LC-ESI-MS/MS	308	1.7	18	2.9	<0.5	6.1	1 180
		LC-FLD	257	1.4	24	2.9	0.2	4.8	985
MK-4	(μg/100 g)	LC-ESI-MS/MS	<0.5	7.3	2.2	1.1	2.9	3.8	2 255
		LC-FLD	<0.8	6.0	2.1	0.78	3.6	4.6	2 105
MK-5	(μg/100 g)	LC-ESI-MS/MS	<0.7	<0.7	6.2	<0.7	<0.7	<0.7	16
		LC-FLD	<1	0.5	11	<0.1	<0.05	0.5	18
MK-6	(μg/100 g)	LC-ESI-MS/MS	<0.5	<0.5	46	<0.5	<0.5	<0.5	1 363
		LC-FLD	<2	0.5	67	<0.1	<0.9	0.7	1 212
MK-7	(μg/100 g)	LC-ESI-MS/MS	<0.5	1.5	784	<0.5	<0.5	2.2	202
		LC-FLD	<0.8	1.2	980	0.4	0.9	1.7	216
MK-8	(μg/100 g)	LC-ESI-MS/MS	<1.4	6.4	7.0	<1.4	<1.4	12	24
		LC-FLD	<1	7.4	7.3	0.2	1.0	16	81
MK-9	(μg/100 g)	LC-ESI-MS/MS	<1.0	19	<1.0	<1.0	<1.0	85	28
		LC-FLD	<2	24	<5	0.2	0.6	99	35
MK-10	(μg/100 g)	LC-ESI-MS/MS	<4.0	<4.0	<4.0	<4.0	<4.0	8.7	405
		LC-FLD	<3	0.9	<8	0.6	1.5	6.4	436

Table 2
Precision estimated as pooled intra- and inter-assay RSD from quantification of vitamin K vitamers in broccoli, cheese (cow and goat), liver (lamb and veal), natto, and microalgae.

Precision		Intra-assay RSD	Inter-assay RSD
PK	LC-ESI-MS/MS	*6.0*	*7.5*
	LC-FLD	*2.8*	*2.8*
MK-4	LC-ESI-MS/MS	*9.8*	*15*
	LC-FLD	*3.1*	*3.7*
MK-5	LC-ESI-MS/MS	*8.9*	*17*
	LC-FLD	*5.5*	*7.7*
MK-6	LC-ESI-MS/MS	*7.7*	*25*
	LC-FLD	*4.8*	*5.1*
MK-7	LC-ESI-MS/MS	*11*	*14*
	LC-FLD	*2.6*	*3.1*
MK-8	LC-ESI-MS/MS	*17*	*19*
	LC-FLD	*3.8*	*5.5*
MK-9	LC-ESI-MS/MS	*9.2*	*9.4*
	LC-FLD	*3.9*	*5.7*
MK-10	LC-ESI-MS/MS	*6.9*	*8.4*
	LC-FLD	*3.0*	*5.1*

in section 2.4). The content of vitamin K vitamers in the food matrices is shown in Table 1 with the associated pooled intra- and inter-assay (Eq. (5)) RSD (Eq. (4)) shown in Table 2.

No significant differences between the days of analysis was observed (*P-value* > 0.05). Some differences between the content of vitamin K vitamers quantified in the food matrices were observed between the two analytical methods investigated without any clear tendencies. A mean difference between the methods were determined to be 6.6% based on the Bland-Altman analysis which was not significantly different from 0 (*P-value* > 0.05). The average quantified vitamin K vitamer ratio between the two methods $\left(\frac{Content(\mu g/100g)_{LC-ESI-MS/MS}}{Content(\mu g/100g)_{LC-FLD}}\right)$ was 0.97 ± 0.2 with the ratio ranging from 0.3 to 1.4. The vitamers that gave rise to the most pronounced differences between the quantification between the two methods were MK-4 in lamb liver (1.1 μg/100 g vs 0.78 μg/100 g by LC-ESI-MS/MS and LC-FLD, respectively) and MK-8 quantified in microalgae (Table 1). However, the obtained difference between the two methods of 20% was considered acceptable. To estimate the trueness for an analytical method the content of a given compound it is recommended to analyse a SRM. Until now, there only exist SRM that harbour quantified PK content. Earlier it was shown that the above described LC-ESI-MS/MS method quantified the PK content in the SRM kelp powder (*Thallus laminariae*) NIST3232 (SRM 3232, www.nist.gov), with no significant difference from the stated content of the SRM (Jensen et al., 2021). No SRM with stated content of any menaquinones exist and this exchange of samples is therefore of significant improvement of the assessment of trueness of the menaquinones. Furthermore, the content of vitamin K in broccoli pulp, cheese, natto and liver is comparable with what was found by others (Jensen et al., 2021; Tarvainen et al., 2019; Vermeer et al., 2018).

From Table 2 it is observed that the intra- and inter-assay RSD for the LC-ESI-MS/MS method are at the acceptable level being ≤15%, except for MK-5, MK-6 and MK-8 that showed a precision for inter-assay of 17%, 25% and 19% respectively. Lower precision is expected for MK-5, MK-6, MK-8 and MK-10 due to the matrix effects that might not be fully corrected for and therefore create uncertainties of the calibration factor and hereby increase the standard deviation of these vitamers. Under the given circumstances, we find the LC-ESI-MS/MS method able to quantify the content of vitamin K vitamers in food with an acceptable trueness and precision. Due to the precision, it is not recommended to perform single determination for MK-5, MK-6, MK-8 and MK-10 but at preferable run triple determination. The precision observed for the LC-ESI-MS/MS methods in this study is comparable with what was found by other LC-MS methods (Huang et al., 2016; Karl et al., 2014). The inter-assay precision at <8% for the LC-FLD method described in this study, is comparable with what was found by others using LC-FLD (Ferreira et al., 2006; Vermeer et al., 2018).

4. Conclusion

An LC-ESI-MS/MS method for quantitatively determination of vitamin K vitamers (PK and MKs) in different foods was established and results compared to those obtained by a validated LC-FLD method performed at a different laboratory. The food matrices included in the validation were broccoli pulp, cheese (cow and goat), natto, liver (lamb and veal), and microalgae, which were chosen to resemble a broad range of foods. The optimised LC-ESI-MS/MS method was able to quantify PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, and MK-10 utilising only d7-PK, d7-MK-4, d7-MK-7, and d7-MK-9 as IS, and generally an inter-assay precision <20%. The total run time was 9 min with LOQs between 0.5 and 4.0 μg/100 g food. The contents of vitamin K vitamers in the different food matrices were in agreement with results from the LC-FLD method with a quantified content ratio of 0.97 ± 0.2 with the ratio ranging from 0.3 to 1.4, and Bland-Altman test showed a mean difference of 6.6% which was not significant different from 0% (*P-value* > 0.05). This method poses as an alternative cost-effective method for quantification of a broad range of vitamin K vitamers with a short run time and without the need to buy additional expensive standards.

CRediT authorship contribution statement

Marie Bagge Jensen: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Kari Elin Rød:** Methodology, Validation, Investigation, Writing – review & editing. **Petra Ložnjak Švarc:** Investigation, Writing – review & editing. **Eystein Oveland:** Methodology, Investigation, Validation, Writing – review & editing. **Jette Jakobsen:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132672.

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