In vitro bioaccessibility of vitamin K (phylloquinone and menaquinones) in food and supplements assessed by INFOGEST 2.0 – vit K

Marie Bagge Jensen [a,*], Anja Pia Biltoft-Jensen [b], Jette Jakobsen [a]

[a] Research Group for Bioactives - Analysis and Application, National Food Institute, Technical University of Denmark, Kemitorvet, 2800, Kgs. Lyngby, Denmark
[b] Research Group for Nutrition, Sustainability and Health Promotion, National Food Institute, Technical University of Denmark, Kemitorvet, 2800, Kgs. Lyngby, Denmark

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ABSTRACT

Vitamin K describes a group of fat-soluble vitamers namely phylloquinone and menaquinones. The growing evidence for vitamin K's role beyond blood coagulation, and the possible differences between the vitamers are emerging. Knowledge of the content of menaquinones in different food matrixes and the potential differences in bioaccessibility between the vitamin K vitamers and food matrixes are limited. In this study, the bioaccessibility was assessed using the INFOGEST 2.0 static in vitro digestion model optimised by including a Danish standard meal. The presence of the standard meal was crucial to obtaining a robust and stable digestion model. The bioaccessibility of the Danish standard meal, water, vitamin K standards, vitamin K supplements, broccoli, spinach, natto, pasteurised whole egg and canola oil was assessed by three replications. The bioaccessibility was in the range 30%–102%. The lowest bioaccessibility was observed in broccoli while the highest bioaccessibility was found in egg and canola oil. No competition in the bioaccessibility between vitamin K vitamers and vitamin D was observed.

1. Introduction

Vitamin K, first discovered by the Danish scientist Henrik Dam in 1935 (Dam, 1935), describes a group of fat-soluble vitamers namely phylloquinone (PK) and menaquinones (MKs). PK is primarily found in green vegetables whereas MKs are primarily found in animal products and fermented food (Nowicka and Kruk, 2016; Palmer et al., 2020; Shearer and Newman, 2014).

All vitamin K vitamers harbours a 1,4-naphthoquinone group with a polyisoprenoid side chain that varies in length (MKs) or a phytyl side chain (PK), with PK having a Log P value > 9 and MK-7 having a Log P value > 14 (Shearer and Okano, 2018).

It has been established that vitamin K is a coagulation factor and are important for bone health (Braam et al., 2004; EFSA, 2017; Halder et al., 2019; Knapen et al., 2013; Palmer et al., 2020). It is further suggested that MKs might play a role in prevention of, cardiovascular disease, type 2 diabetes, cancer and have immunosuppressive effects (Fan et al., 2018; Halder et al., 2019).

At present only an adequate intake of 1 µg PK/kg body weight/day (70 µg/day) exist, which is corresponding to the estimated average intake of 72–196 µg/day (for people ≥18 years old) (EFSA, 2017; FAO,

& WHO, 2004). No average requirement and recommended intake is set for MKs as there is a lack of knowledge about the MK content in food, the bioaccessibility, and the bioavailability of both PK and MKs, hence the actual intake of vitamin K cannot be assessed satisfyingly to be utilised in epidemiological studies (Palmer et al., 2020).

The bioavailability of a compound is defined as the bioactive fraction that reaches the target tissue in the body, and is dependent on the bioaccessibility, absorption, distribution, metabolism and excretion (McClements and Peng, 2020; Wu and Chen, 2021). The bioaccessibility of a compound is defined as the fraction that is released from the food matrix and available for absorption in the body, and is often the rate limiting factor for the bioavailability of lipophilic compounds (McClements and Peng, 2020; Wu and Chen, 2021).

Lipophilic compounds such as fat-soluble vitamins (A, E, D and K) needs to be incorporated into mixed micelles in order to enter the mucus layer for absorption in the intestinal epithelial cells (McClements and Peng, 2020; Yuan et al., 2018).

The bioaccessibility of carotenoids have been assessed in humans (Tyssandier et al., 2003). However, *in vivo* assessment of bioaccessibility and bioavailability is time consuming, expensive and can be ethically challenging, which pose a need for *in vitro* models for assessment of

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bioaccessibility (Brodkorb et al., 2019; McClements and Peng, 2020). *In vitro* digestion models can be divided into static and dynamic models, and *in vivo* correlation have been shown for both (Brodkorb et al., 2019; Dupont et al., 2019; Minekus et al., 2014; Reboul et al., 2006). These models have proven to be a good tool for screening of bioaccessibility in a broad range om food and as a tool for assessment for important factors influencing bioaccessibility and digestion of different compounds and food (Brodkorb et al., 2019; McClements and Peng, 2020).

The aim of this study was to validate the INFOGEST 2.0 model in regards to stability and robustness when assessing the bioaccessibility of vitamin K in food and supplements. The food samples assessed for vitamin K bioaccessibility was chosen to represent a broad range of food and content of the vitamin K vitamers. The bioaccessibility of eight vitamin K vitamers (PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10) were assessed using a recently published LC-ESI-MS/MS method (Jensen et al., 2021; Jensen et al., 2022).

2. Materials and method

This study consisted of three parts. The first part of the study determined the precision of the INFOGEST method of cheese and broccoli samples. The second part aimed at optimising the INFOGEST method for assessment of bioaccessibility of vitamin K in food and supplements, by digesting spinach followed by fractional factorial experiments to test different conditions. The third part determined the bioaccessibility of vitamin K vitamers in food, supplements and standards.

2.1. Samples

All samples for the experiment were bought in local shops in the Copenhagen area.

2.1.1. Samples for INFOGEST 2.0

Fresh broccoli and cow cheese (Klovborg, Arla Foods Amba, Denmark). Non-edible parts were cut from fresh broccoli and cow cheese. The broccoli was juiced (Moulinex centrifugal juicer, GROUP SBE Brand, France) and the broccoli pulp (broccoli) used for the experiment. Each sample were then frozen in liquid nitrogen and homogenized for 20 s in a coffee grinder (EGK 200, Rommelsbacher, Germany), mixed thoroughly, divided into 50 mL plastic containers, and stored at −20 °C.

2.1.2. Samples used in the optimisation and validation of INFOGEST 2.0 for vitamin K (INFOGEST 2.0 – vit K)

Spinach were mixed and divided in 9 portions, each 25 g. For one night, three of the portions were stored at −20 °C and the remaining six portions were stored at 5 °C. The three portions stored at −20 °C and three portions stored at 5 °C were boiled in water at 100 °C for 5 min. Each portion was hereafter divided in two, one for digestion according to section 2.3.1., and the other for quantification of vitamin K content analysed according to section 2.4.

The standard meal was prepared by adding 479 g chicken breast fillet, 388 g potatoes (washed and cut into pieces), 190 mL cream (38% fat), 280 g corn (canned), 288 g carrots (washed and cut into pieces) and 20 mL canola oil into a pot with a lid on, and baked in the oven at 200 °C for 1 h. The meal was hereafter mixed (1094 homogeniser, Tecator, Sweden) and divided into 50 mL amber Eppendorf tube and stored at −80 °C.

2.1.3. Samples used to assess bioaccessibility of vitamin K by INFOGEST 2.0-vit K

Pasteurised whole eggs (eggs) was constructed by mixing egg white (631 g) and egg yolk (315 g), both pasteurised, which were then divided in 15 mL amber Eppendorf tubes and stored at −80 °C. Natto was frozen in liquid nitrogen and homogenized in a coffee grinder for 20 s, divided

in 50 mL amber Eppendorf tubes and stored at - 20 °C. Canola oil was divided in 12 mL amber glass bottles and stored at - 20 °C. Dietary supplements of PK (Naturdrogeriet, Hørning, Denmark) and MK-7 (dansk farmaceutisk industri a-s, Stenløse, Denmark). Ten tablets were homogenized using a mortar, and stored in amber 15 mL Eppendorf tubes at −20 °C.

2.2. Bioaccessibility models

2.2.1. INFOGEST 2.0 - procedure

The static *in vitro* digestion using the INFOGEST 2.0 method was performed according to the published protocol (Brodkorb et al., 2019). In brief, all enzyme activity was assessed, solutions prepared and pH tests performed according to (Brodkorb et al., 2019).

All nine samples of spinach were "chewed" using a mortar and pestle, and samples were weight off to be digested using the INFOGEST method. All other samples had previously been homogenized into small pieces or was liquid, and no further "chewing" step was therefore incorporated.

The *in vitro* static digestion model INFOGEST 2.0 consist of three phases; an oral phase, a gastric phase, and an intestinal. For the oral phase 1 g of food sample was weighed of into a 15 mL amber Eppendorf tube where 1 mL of simulated salivary fluid was added. The sample was then incubated in an overhead shaker at 37 °C for 2 min. The sample now entered the gastric phase where 2 mL of simulated gastric fluid was added together with pepsin (2000 U/mL) and gastric lipase (60 U/mL) and the pH was adjusted to pH 3.0. The sample was then incubated in an overhead shaker at 37 °C for 2 h. The sample now entered the intestinal phase where 4 mL of simulated intestinal fluid was added together with bile (10 mM bile salt) and pancreatin (trypsin activity of 100 U/mL) and the pH was adjusted to pH 7.0. The sample was then incubated in an overhead shaker at 37 °C for 2 h. The sample was then centrifuged at 5500 *g* for at least 30 min at 37 °C to separate the solid phase from the micellar phase. The micellar phase was transferred to another tube and the total weight of the micellar phase was noted.

2.2.2. INFOGEST 2.0 optimisation and validation studies for vitamin K

Initially spinach (fresh, boiled and frozen boiled) was digested using 1 g of each. Furthermore, digestions of 0.8 g raw spinach, 0.4 g boiled spinach, and 0.4 g frozen boiled spinach with added water to a total of 1 g was digested.

Following these studies, four fractional factorial (2^{3-1}) experiments (NMKL, 2009) were performed as shown in supplementary material (SM) Table SM 1.

The first experiment tested the effect on the bioaccessibility of the following factors: 1) type of spinach (raw/frozen boiled), 2) oil (oil or no oil), and 3) amount of bile (normal or the double amount of normal).

The second experiment tested the effect on the bioaccessibility of the following factors: 4) amount of vitamin K (200 ng of PK, MK-4, MK-7 and MK-9 or 100 ng of PK, MK-4, MK.7 and MK-9), 5) vitamin D3 (100 ng or 0 ng), 6) amount of bile (normal or the double amount of normal).

The third experiment tested the effect on the bioaccessibility of the following factors: 7) amount of standard meal (0.8 g or 0.4 g), 8) amount of raw spinach (0.1 g or 0.2 g), and 9) amount of bile (normal or the double amount).

The fourth experiment tested the effect on the bioaccessibility of the following factors: 10) amount of standard meal (0.8 g or 0.6 g), 11) amount of vitamin K (500 ng PK, MK-4, MK-7 and MK-9 or 1000 ng PK, MK-4, MK-7 and MK-9), and 12) amount of vitamin D3 (500 ng or 1000 ng).

The assessment of significant was performed as described by NMKL (NMKL, 2009) and in section 2.4.

2.2.3. INFOGEST 2.0 for vitamin K (INFOGEST 2.0-vitK)

The sample digested was prepared by weighing in 60% (w/w) standard meal and 40% (w/w) food matrix of interest. Hereafter the procedure was as described in section 2.2.1.

2.3. Analytical methods

2.3.1. Procedure for quantification of vitamin K in food

The extraction, detection and quantification of the eight vitamin K vitamers (PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10) using four internal standards (d7-PK, d7-MK-4, d7-MK-7, and d7-MK-9) were performed as previously described (Jensen et al., 2021; (Jensen et al., 2022)). See SM2 for a chromatogram of a natto sample.

2.3.2. Procedure for quantification of vitamin K in supplements

The extraction of the vitamin K vitamers from supplements where done according to the pharmacopeia (U.S. Pharmacopeia, n.d.) with some modifications. Ten tablets were weighed and powdered in a mortar. In a 50 mL amber Eppendorf tube, 100 mg of the tablet powder was added together with 10 mL dimethyl sulfoxide (DMSO) and 15 mL n-heptane. The sample was then incubated in a shaking water bath for 45 min at 60 °C. The sample was then centrifuged at 3000 *g* for 10 min at 10 °C where after the heptane layer was transferred to a 100 mL volumetric flask. To the DMSO phase another 15 mL of *n*-hepaten was added and the mix was shaken for 5 min on a table shaker followed by centrifugation at 3000*g* at 10 °C for 10 min. The heptane phase was transferred to the same 100 mL volumetric flask. The addition of 15 mL *n*-heptane to the DMSO phase, shaking for 5 min and centrifugation at 3000*g* at 10 °C for 10 min and transfer of the heptane phase to the 100 mL volumetric flask was repeated another 3 times. Heptane was then added up to the 100 mL mark on the 100 mL volumetric flask and mixed thoroughly on a magnetic stirrer. In a 2 mL amber vial 50 μL of the 2.5 μg/mL IS solution was added together with 200 μL of the tablet solution and evaporated to dryness and hereafter redissolved in 500 μL methanol by ultrasonication for 15 min. The sample was hereafter analysed using the LC-ESI-MS/MS method described in (Jensen et al., 2021; (Jensen et al., 2022)).

2.4. Calculations and statistical analysis

Mass Hunter Workstation Software (LC/MD data Acquisition version B08.00, Quantitative Analysis version B0701, Qualitative Analysis version B07.00.) was used to perform LC-ESI-MS/MS instrument control and data acquisition and analysis.

In this study, the bioaccessibility (B%) was defined according to equation (1).

$$B\% = \frac{m_M}{m_I} * 100 \qquad [1]$$

Where mM described the amount of vitamin K in the mixed micellar phase and mI describes the amount of vitamin K ingested. To determine if the effect of the factors in the fractional factorial (2^{3-1}) experiments were significant, the following equations were used:

$$D_a = \frac{D+E}{2} - \frac{F+G}{2}$$

$$D_b = \frac{D+F}{2} - \frac{E+G}{2}$$

$$D_c = \frac{D+G}{2} - \frac{E+F}{2}$$

The resulting bioaccessibilities of vitamin K in the four different samples in each fractional factorial experiments with varying factors are denoted D, E, F and G. D_a then describes the difference in bioaccessibility caused by factor a/A, D_b describes the difference in bioaccessibility caused by factor b/B, and D_c describes the difference in bioaccessibility caused by factor d/D. If these differences are higher than 2*SD, the factors has a significant (*p-value* < 0.05) effect on the bioaccessibility.

One-way ANOVA test and Tukey honest significant difference tests were performed to test for significant differences (*p-value* < 0.05)

between the bioaccessibility of the vitamin K vitamers between and within samples. One-way ANOVA tests were further performed to determine the intra- and inter-assay SD of the bioaccessibility of broccoli and cow cheese for precision assessment.

Linear regression analyses to determine if the fat had a significant (*p-value* < 0.05) effect on the bioaccessibility was performed.

All statistical work was performed using Excel (Microsoft® Excel® 2016 (16.0.5017.1000)) and RStudio (© 2009–2019 RStudio, Inc., Version 1.2.1335). Results are in general given as mean ± standard deviation (SD) when n ≥ 3.

3. Results and discussion

3.1. INFOGEST 2.0

The precision of determination of the bioaccessibility of vitamin K using INFOGEST 2.0 was assessed by digestion and analysis of broccoli and cow cheese in triplicated at three different days (n = 9). An acceptable precision was achieved with an intra-assay SD ranging from 1.9% to 3.1%, and an inter-assay SD ranging from 2.2% to 3.3% (Table 1).

3.2. INFOGEST 2.0 optimisation and validation studies for vitamin K

3.2.1. Effect of amount of vitamin K

Initially the bioaccessibility was assessed using the original INFO-GEST 2.0 method i.e. digesting 1 g of either raw spinach, boiled spinach or frozen boiled spinach. The highest bioaccessibility was found in raw spinach (Fig. 1A). Afterwards the amount of spinach digested was adjusted so the amount of phylloquinone was the same in all digests (0.8 g raw spinach, 0.4 g boiled spinach and 0.4 g frozen boiled spinach). A higher bioaccessibility was observed in boiled and frozen boiled spinach compared to raw spinach (Fig. 1B). This indicated that the bioaccessibility of vitamin K assessed by INFOGEST 2.0 is concentration dependent (Fig. 1).

As the goal of this study was to compare the bioaccessibility of different food matrices with a varying content and number of vitamin K vitamers the INFOGEST 2.0 was optimised in order to achieve a concentration independent bioaccessibility determination over the vitamin K range of interest.

3.2.2. Effect of standard meal

The observed concentration dependent bioaccessibility where thought to originate from non-representative formation of mixed micelles when digesting only spinach. Others have found that the hydrophobic domains within the mixed micelles must be large enough to accommodate the hydrophobic bioactive compound (McClements and Peng, 2020; Yuan et al., 2018). The size of the mixed micelles are

Table 1
Precision of determination of vitamin K bioaccessibility using INFOGEST 2.0 assessed in broccoli and cow cheese (Klovborg) by digesting the samples in triplicate at three different days (n = 9) and analysing for vitamin K.

Food matrix		Broccoli	Cow cheese (Klovborg)
Phylloquinone	Bioaccessibility (%)	32	–
	SD Intra-assay (%)	1.9	
	SD Inter-assay (%)	2.2	
Menaquinone-4	Bioaccessibility (%)	–	6.0
	SD Intra-assay (%)		3.1
	SD Inter-assay (%)		3.0
Menaquinone-7	Bioaccessibility (%)	–	–
	SD Intra-assay (%)		
	SD Inter-assay (%)		
Menaquinone-9	Bioaccessibility (%)	–	5.3
	SD Intra-assay (%)		2.7
	SD Inter-assay (%)		3.3

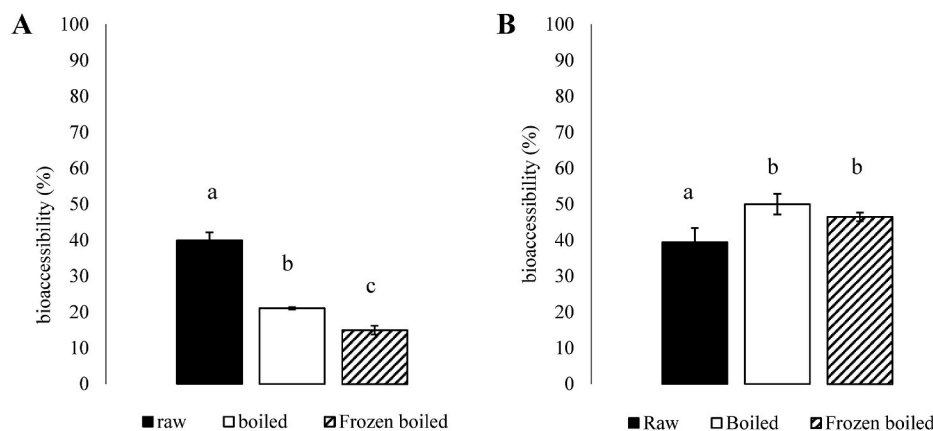- The content was < LOQ (0.5 μg/100 g).

Fig. 1. Bioaccessibility of spinach. A: bioaccessibility of 1 g of spinach (raw, boiled or frozen boiled). B: Bioaccessibility of approximately 2 µg originating from spinach (raw, boiled or frozen boiled). Error bars depicts the sd. Different letters indicate significant difference (P-value < 0.05). Every mean was based on three samples (n = 3).

dependent on the food and especially the fat content in the ingested food (Yuan et al., 2018). In general it is expected that food matrices like spinach would seldom be consumed alone but rather be a constituent of a meal consisting of a mix of many food components (Arla Foods, 2018; Pedersen, 2015). Others have also introduced a standard meal in combination with the food of interest (Reboul et al., 2006).

To partly mimic a meal, and partly to enable the static digestion model, INFOGEST 2.0, to become a stable and robust model that can be used at different concentrations of vitamin K, a standard meal was incorporated in every digest. Furthermore, the updated version of INFOGEST 2.0 (INFOGEST 2.0-vitK) was validated for assessment of vitamin K based on four three fractional factorial (2^{3-1}) experiments (Table 2 and SM 1).

These experiments showed that with the addition of the standard meal the variation in bile amount and vitamin K did not significant effect the bioaccessibility.

The standard meal was based on a typical meal in Denmark (Arla Foods, 2018; Pedersen, 2015), with the nutritional information stated in Table 3.

It was concluded that addition of the standard meal was necessary to be able to compare different food matrices with a different content of vitamin K as the INFOGEST 2.0 – vit K was independent of the added amount of vitamin K in the studied range (Table 2 and SM 1).

It was further observed that the bioaccessibility of vitamin K was unaffected by the presence of vitamin D3 (Table 2 and SM1), indicating that no competition for incorporation in the mixed micelles are taken place at the studied amounts of vitamin D and vitamin K. Others have found carotenoids may compete for incorporation into mixed micelle (Poulaert et al., 2012; Tyssandier et al., 2001). And other studies have found no competition for incorporation of carotenoids in mixed

Table 2
The result of the four fractional factorial (2^{3-1}) experiments. For each experiment 3 factors were studied and the resulting P-values are shown.

Experiments	Factors tested	Significance
Experiment 1	Processing and amount of vitamin K in sample	P-value < 0.05
	Presence of oil	P-value < 0.05
	Amount of added bile	P-value < 0.05
Experiment 2	Amount of vitamin K ingested	P-value > 0.05
	Presence of vitamin D	P-value > 0.05
	Amount of added bile	P-value > 0.05
Experiment 3	Amount of standard meal ingested	P-value < 0.05
	Amount of spinach ingested	P-value > 0.05
	Amount of added bile	P-value > 0.05
Experiment 4	Amount of standard meal ingested	P-value < 0.05
	Amount of vitamin K ingested	P-value > 0.05
	Amount of vitamin D ingested	P-value > 0.05

Table 3
Nutritional information of the standard meal.

Energy/Nutrient	Content pr. portion (100 g)
Energy (KJ)	576
Protein (g)	6.8
Fat (g)	7.5
Saturated fat (g)	2.6
Monounsaturated fat (g)	2.8
Polyunsaturated fat (g)	1.4
Carbohydrates (g)	11
Dietary fibre (g)	1.4
Phylloquinone (µg)	6.8
D-vitamin (µg)	0.1

micelles, but found that the bioaccessibility of carotenoids were concentration dependent (Jeffery et al., 2012). More studies on the potential competition between different vitamin K vitamers for incorporation into mixed micelles are needed.

3.3. Bioaccessibility of vitamin K in food (INFOGEST 2.0 – vit K)

The optimised INFOGEST 2.0 model (INFOGEST 2.0 vitK) were utilised to assess the content and bioaccessibility of vitamin K vitamers in different food and supplements (Table 4).

Significant differences in the bioaccessibility between the different food matrices and between different vitamin K vitamers were observed. The highest bioaccessibility of PK was observed for pure MK-7 standard, MK-7 supplement powder, pasteurised eggs where the main contributor of PK is the standard meal, and in canola oil where the biggest contributor of PK is the oil. The lowest bioaccessibility of PK was found in broccoli with broccoli being the biggest contributor of PK. The bioaccessibility of PK in canola oil or pasteurised egg was higher than in PK supplement powder, with the lowest bioaccessibility for PK found in broccoli. This is in agreement with what was found by others when the bioavailability of PK was studied in humans (Booth et al., 2002; Schurgers and Vermeer, 2000).

The bioaccessibility of MK-4 varies significantly between the food matrices. The highest bioaccessibility is found in pasteurised egg and in canola oil, whereas the lowest bioaccessibility of MK-4 was found in broccoli and water, where, for both, the major contributor of MK-4 was the standard meal. Here it is again observed that the bioaccessibility in canola oil or pasteurised egg was higher than in supplement powder, and the lowest bioaccessibility was found in broccoli and water.

No significant difference in the bioaccessibility of MK-7 was observed between pure MK-7 standard, MK-7 supplement powder and natto. This is in agreement with what was found by others when

Table 4

Content (mean ± sd) and bioaccessibility (mean ± sd) of vitamin K vitamers in different food matrices, supplements and standards. nd: not determined. Different letters indicate significant differences (P-value < 0.05). Different superscripted numbers indicate significant differences (P-value < 0.05). -: was not calculated due to < LOQ (0.5 μg/100 g–4 μg/100 g) in the mixed micelle phase.

Content	μg/100 g ± SD							
Food matrix	PK	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10
Standard meal	2.3 ± 0.2	10 ± 3.3	< 0.7	< 0.5	< 0.5	< 1.4	< 1	< 4
Broccoli	290 ± 2.6	< 0.5	< 0.7	< 0.5	< 0.5	< 1.4	< 1	< 4
Egg	0.2 ± 0.07	8.2 ± 2.8	< 0.7	< 0.5	< 0.5	< 1.4	< 1	< 4
Canola oil	61 ± 3.3	< 0.5	< 0.7	< 0.5	< 0.5	< 1.4	< 1	< 4
Natto	32 ± 1.0	3.3 ± 0.08	12 ± 0.3	100 ± 3.3	930 ± 19	< 1.4	< 1	< 4
PK supplement	55 800 ± 5200	< 0.5	nd	nd	< 0.5	nd	< 1	nd
MK-7 supplement	< 0.5	< 0.5	nd	nd	5900 ± 5.5	nd	< 1	nd
Bioaccessibility	**-INFOGEST 2.0-VitK (%) ± SD**							
Food matrix	**PK**	**MK-4**	**MK-5**	**MK-6**	**MK-7**	**MK-8**	**MK-9**	**MK-10**
Broccoli	31 ± 3.0[e2]	46 ± 5.0[c1]	–	–	–	–	–	–
Egg	83 ± 4.2[a2]	102 ± 8.1[a1]	–	–	–	–	–	–
Water	63 ± 5.4[cd1]	43 ± 3.6[c2]	–	–	–	–	–	–
Canola oil	88 ± 9.5[a1]	97 ± 9.6[a1]	–	–	–	–	–	–
Natto	52 ± 3.2[d1]	57 ± 3.2[bc1]	–	68 ± 8.4[1]	55 ± 2.1[a1]	–	–	–
PK supplement	70 ± 5.1[bc1]	62 ± 4.0[b1]	–	–	–	–	–	–
MK-7 supplement	82 ± 6.5[ab1]	64 ± 5.8[b2]	–	–	65 ± 7.1[a12]	–	–	–
PK standard	56 ± 2.6[d1]	65 ± 5.3[b1]	–	–	–	–	–	–
MK-7 standard	87 ± 1.5[a1]	65 ± 3.4[b2]	–	–	57 ± 1.4[a3]	–	–	–

assessing the bioavailability in humans (Møller et al., 2016).

Significant differences in the bioaccessibility were observed for the different vitamin K vitamers within the same food matrices. This difference is thought to be dependent on the source of the vitamers. In the water it is assumed that the PK is originating both from the chicken meat, vegetables, oil and cream, whereas the MK-4 is thought to only originate from the chicken in the standard meal and due to the difference in source a difference in bioaccessibility of PK and MK-4 is observed. The same difference in bioaccessibility between PK and MK-4 is seen when pure MK-7 standard and MK-7 supplement is digested. No significant difference is observed between the vitamers when natto is digested, where natto is the predominant source of all the vitamers except for MK-4.

The bioaccessibility of PK and MK-4 increased between the digest of water and MK-7 supplement or pure MK-7 standard. This indicates that instead of competition between the vitamin K vitamers there may be synergistic effects effecting the incorporation of the vitamers in the micelle, that the presence of more lipophilic vitamers may increase the solubilisation of less lipophilic vitamers. The same pattern was not observed between digests of water and PK supplement or pure PK standard, indicating that the lipophilicity may be important for the synergistic effect.

It was observed that the food matrix had a significant effect on the bioaccessibility (Table 4). Based on regression analysis it was stated that the significant effect could not be explained by the fat content (Table 5) in the samples (p-value = 0.34) or the presence of a cell wall.

It is thought that vitamin K along with other fat-soluble vitamins, and fatty acids are incorporated in micelles in the intestinal lumen and by these are transported into the enterocytes (Shearer et al., 2012). Two factors are thought to affect the incorporation of fat-soluble vitamins into mixed micelles where they are bioaccessible: (1) the release of the vitamin from the food matrices, (2) their solubilisation within the mixed micelle (Tan et al., 2020).

The food matrices included in this study is of varying origin and their physicochemical properties differs greatly in regards to the position of the vitamin K vitamers, which may cause the observed differences in bioaccessibility.

In broccoli, and natto vitamin K is thought to be situated in the intracellular membranes behind the cell wall and the plasma membrane in either bacteria or plant tissue (Nowicka and Kruk, 2016). The vitamin K in these food matrices is therefore thought to be less bioaccessible than food matrices like canola oil where the vitamin K is solubilised in the oil or in pasteurised eggs, which does not harbour a cell wall.

The oil content further differs in the food matrixes (Table 5) which may have an effect on the composition of fatty acids in the mixed micelle, which may influence solubilisation of vitamin K in these and hereby effect the bioaccessibility of vitamin K.

Other factors like preparation of the food matrix (Netzel et al., 2011), the length and type of the fatty acids present in the food matrix (Yuan et al., 2018), and the presence of other compounds (Margier et al., 2019; Riethorst et al., 2018), may affect the solubilisation of vitamin K in the mixed micelle. Further studies of these factors effect on the bioaccessibility is therefore urgently needed to further understand these mechanisms.

Others have found that the bioavailability of PK and MK-7 differs when a meal consisting of natto and cooked spinach was eaten by people however, it is not studied if the observed difference is originating from differences in bioaccessibility, absorption, distribution in the body, etc. (Schurgers and Vermeer, 2000).

By assessing the bioaccessibility based on equation (1) without filtration of the mixed micelle phase we are at risk at overestimating the bioaccessible fraction of vitamin K as there might be lipid droplets containing vitamin K present, which are not incorporated into the mixed micelles and would not be able to enter the mucus layer and therefore is not a bioaccessible fraction of vitamin K (McClements and Peng, 2020; Riethorst et al., 2018). As it is unsure whether the absolute vitamin K bioaccessibility assessed in this study can be compared to other studies, we find it more relevant to focus on the relative bioaccessibilities of the foods investigated.

Table 5

Fat content in the food matrices of interest (Arla Foods, 2018; Food DTU & Nutrition Division for Risk Assessment and, 2019; Hu et al., 2010).

Food matrix	Fat content (g/100 g)
Canola oil	100
Natto	~17
Pasteurised egg	8.6
Standard meal	7.6
Broccoli pulp	0
Supplement[a]	0

[a] Fat content declared both for PK, MK-4 and MK-7 supplements.

4. Conclusion

The precision of the determined bioaccessibility of vitamin K vitamers in broccoli and cow cheese using the INFOGEST 2.0 model was assessed acceptable with standard deviations of 1.9%–3.3%. However, the INFOGEST 2.0 is concentration dependent when the bioaccessibility of vitamin K in spinach is determined. When a standard meal was incorporated into the digestion, the *in vitro* model became robust. The results obtained were stable when assessing the bioaccessibility of vitamin K in food matrices with different concentrations of vitamin K. The optimised INFOGEST 2.0 model containing the standard meal was named INFOGEST 2.0 – vit K. Using this method it is possible to compare bioaccessibilities of different food matrices containing different amounts of vitamin K vitamers.

In this study we have assessed the bioaccessibility of eight vitamin K vitamers (PK, MK-4, MK-5, MK-7, MK-8, MK-9 and MK-10) using the INFOGEST 2.0 – vit K method in food matrices representing a broad range of food. The bioaccessibility ranged from 30% to 102% varying both between and within the different food matrices. A general tendency for the order of PK and MK-4 bioaccessibility was observed where the bioaccessibility in canola oil and pasteurised eggs was higher than the bioaccessibility in supplement powder. The lowest bioaccessibility was observed in broccoli. No significant difference in the bioaccessibility of MK-7 between natto and MK-7 supplement powder was observed. The fat content of the samples could not alone explain the differences in bioaccessibilities.

CRediT authorship contribution statement

Marie Bagge Jensen: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Anja Pia Biltoft-Jensen:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Jette Jakobsen:** Conceptualization, Methodology, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.01.018.

References

Booth, S.L., Lichtenstein, A.H., Dallal, G.E., 2002. Phylloquinone absorption from phylloquinone-fortified oil is greater than from a vegetable in younger and older men and women. J. Nutr. 132 (9), 2609–2612. Retrieved from. http://ovidsp.ovid.com/ovidweb.cgi?T=JS&CSC=Y&NEWS=N&PAGE=fulltext&D=med4&AN=12221218%5Cnhttp://resolver.ebscohost.com/openurl?issn=00223166&Volume=132&issue=9&spage=2609&title=Phylloquinone+absorption+from+phylloquinone-fortified+oil+is+greater+than+from.

Braam, L.A.J.L.M., Hoeks, A.P.G., Brouns, F., Halmuyák, K., Gerichhausen, M.J.W., Vermeer, C., 2004. Beneficial effects of vitamins D and K on the elastic properties of the vessel wall in postmenopausal women: a follow-up study. Thromb. Haemostasis 91 (2), 373–380. https://doi.org/10.1160/th03-07-0423.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Recio, I., 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 14 (4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Dam, H., 1935. The antihæmorrhagic vitamin of the chick.: occurrence and chemical nature. Nature 135, 652–653. https://doi.org/10.1038/135652b0.

Dupont, D., Alric, M., Blanquet-Diot, S., Bornhorst, G., Cueva, C., Deglaire, A., Van den Abbeele, P., 2019. Can dynamic in vitro digestion systems mimic the physiological reality? Crit. Rev. Food Sci. Nutr. 59 (10), 1546–1562. https://doi.org/10.1080/10408398.2017.1421900.

EFSA, E.F.S.A., 2017. Dietary reference values for vitamin K. EFSA J. 15 (5) https://doi.org/10.2903/j.efsa.2017.4780.

Fan, X., Chen, J., Duan, L., Li, S., 2018. Research progress on the anticancer effects of Vitamin K2 (Review). Oncol. Lett. 15 (6), 8926–8934. https://doi.org/10.3892/ol.2018.8502.

FAO & WHO, 2004. Vitamin and mineral requirements in human nutrition, Second edition. World Health Organization.

Food DTU, & Nutrition Division for Risk Assessment, 2019. Frida from. https://frida.fooddata.dk/?lang=en. (Accessed 5 May 2019).

Foods, Arla, 2018. Ny Undersøgelse: Danskerne Er Enige - Her Er Vores Favoritter from. https://www.arla.dk/om-arla/nyheder/2018/pressrelease/ny-undersoegelse-danskerne-er-enige-her-er-vores-favoritretter-2781089/. (Accessed 6 September 2021).

Halder, M., Petsophonsakul, P., Akbulut, A.C., Pavlic, A., Bohan, F., Anderson, E., Schurgers, L., 2019. Vitamin K: double bonds beyond coagulation insights into differences between vitamin K1 and K2 in health and disease. Int. J. Mol. Sci. 20 (4), 1–15. https://doi.org/10.3390/ijms20040896.

Hu, Y., Ge, C., Yuan, W., Zhu, R., Zhang, W., Du, L., Xue, J., 2010. Characterization of fermented black soybean natto inoculated with Bacillus natto during fermentation. J. Sci. Food Agric. 90 (7), 1194–1202. https://doi.org/10.1002/jsfa.3947.

Jeffery, J.L., Turner, N.D., King, S.R., 2012. Carotenoid bioaccessibility from nine raw carotenoid-storing fruits and vegetables using an in vitro model. J. Sci. Food Agric. 92 (13), 2603–2610. https://doi.org/10.1002/jsfa.5768.

Jensen, M.B., Ložnjak Švarc, P., Jakobsen, J., 2021. Vitamin K (phylloquinone and menaquinones) in foods – optimisation of extraction, clean-up and LC–ESI-MS/MS method for quantification. Food Chem. 345 https://doi.org/10.1016/j.foodchem.2020.128835.

Jensen, M.B., Rød, K.E., Ložnjak Švarc, P., Oveland, E., Jakobsen, J., 2022. Vitamin K (phylloquinone and menaquinones) in foods – cost-effective quantification by LC-ESI-MS/MS. Food Chem. Submitted to.

Knapen, M.H.J., Drummen, N.E., Smit, E., Vermeer, C., Theuwissen, E., 2013. Three-year low-dose menaquinone-7 supplementation helps decrease bone loss in healthy postmenopausal women. Osteoporos. Int. 24 (9), 2499–2507. https://doi.org/10.1007/s00198-013-2325-6.

Margier, M., Antoine, T., Siriaco, A., Nowicki, M., Halimi, C., Maillot, M., Reboul, E., 2019. The presence of pulses within a meal can alter fat-soluble vitamin bioavailability. Mol. Nutr. Food Res. 63 (11) https://doi.org/10.1002/mnfr.201801323.

McClements, D.J., Peng, S.F., 2020. Current status in our understanding of physicochemical basis of bioaccessibility. Curr. Opin. Food Sci. 31, 57–62. https://doi.org/10.1016/j.cofs.2019.11.005.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food-an international consensus. Food Funct. 5 (6), 1113–1124. https://doi.org/10.1039/c3fo60702j.

Møller, M., Gjelstad, I.M.F., Baksaas, I., Grande, T., Aukrust, I.R., Drevon, C.A., 2016. Bioavailability and chemical/functional aspects of synthetic MK-7 vs fermentation-derived MK-7 in randomised controlled trials. Int. J. Vitam. Nutr. Res. 1–15. https://doi.org/10.1024/0300-9831/a000258.

Netzel, M., Netzel, G., Zabaras, D., Lundin, L., Day, L., Addepalli, R., Seymour, R., 2011. Release and absorption of carotenes from processed carrots (Daucus carota) using in vitro digestion coupled with a Caco-2 cell trans-well culture model. Food Res. Int. 44 (4), 868–874. https://doi.org/10.1016/j.foodres.2010.10.058.

NMKL, 2009. NMKL Procedure No. 4 (2009) Validation of chemical analytical methods. Nordic Comm. Food Anal. 4 (3), 1–45.

Nowicka, B., Kruk, J., 2016. Powered by light: phototrophy and photosynthesis in prokaryotes and its evolution. Microbiol. Res. 186–187, 99–118. https://doi.org/10.1016/j.micres.2016.04.001.

Palmer, C.R., Blekkenhorst, L.C., Lewis, J.R., Ward, N.C., Schultz, C.J., Hodgson, J.M., Sim, M., 2020. Quantifying dietary vitamin K and its link to cardiovascular health: a narrative review. Food Funct. 11 (4), 2826–2837. https://doi.org/10.1039/c9fo02321f.

Pedersen, A.N., Christensen, T., Matthiessen, J., Knudsen, V.K., Rosenlund-Sørensen, M., Biltoft-Jensen, A., Hinsch, H.-J., Ygil, K.H., Kørup, K., Saxholt, E., Trolle, E., Søndergaard, A.B., Fagt, S., 2015. Dietary Habits in Denmark 2011-2013. Main Results. Retrieved from. http://www.food.dtu.dk.

Poulaert, M., Borel, P., Caporiccio, B., Gunata, Z., Dhuique-Mayer, C., 2012. Grapefruit juices impair the bioaccessibility of β-carotene from orange-fleshed sweet potato but not its intestinal uptake by caco-2 cells. J. Agric. Food Chem. 60 (2), 685–691. https://doi.org/10.1021/jf204004c.

Reboul, E., Richelle, M., Perrot, E., Desmoulins-Malezet, C., Pirisi, V., Borel, P., 2006. Bioaccessibility of carotenoids and vitamin E from their main dietary sources. J. Agric. Food Chem. 54 (23), 8749–8755. https://doi.org/10.1021/jf061818s.

Riethorst, D., Mitra, A., Kesisoglou, F., Xu, W., Tack, J., Brouwers, J., Augustijns, P., 2018. Human intestinal fluid layer separation: the effect on colloidal structures & solubility of lipophilic compounds. Eur. J. Pharm. Biopharm. 129 (May), 104–110. https://doi.org/10.1016/j.ejpb.2018.05.026.

Schurgers, L.J., Vermeer, C., 2000. Determination of phylloquinone and menaquinones in food effect of food matrix on circulating vitamin K concentrations. Haemostasis 30, 298–307. https://doi.org/10.1159/000054147.

Shearer, M.J., Newman, P., 2014. Recent trends in the metabolism and cell biology of vitamin K with special reference to vitamin K cycling and MK-4 biosynthesis. JLR (J. Lipid Res.) 55 (3), 345–362. https://doi.org/10.1194/jlr.R045559.

Shearer, M.J., Okano, T., 2018. Key pathways and regulators of vitamin K function and intermediary metabolism. Annu. Rev. Nutr. 38 (1), 127–151. https://doi.org/10.1146/annurev-nutr-082117-051741.

Shearer, M.J., Fu, X., Booth, S.L., 2012. Vitamin K nutrition , metabolism , and requirements: current concepts and future research. Adv. Nutr. 3 (2), 182–195. https://doi.org/10.3945/an.111.001800.182.

Tan, Y., Li, R., Liu, C., Muriel Mundo, J., Zhou, H., Liu, J., McClements, D.J., 2020. Chitosan reduces vitamin D bioaccessibility in food emulsions by binding to mixed micelles. Food Funct. 11 (1), 187–199. https://doi.org/10.1039/c9fo02164g.

Tyssandier, V., Lyan, B., Borel, P., 2001. Main factors governing the transfer of carotenoids from emulsion lipid droplets to micelles. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1533 (3), 285–292. https://doi.org/10.1016/S1388-1981(01)00163-9.

Tyssandier, V., Reboul, E., Dumas, J.F., Bouteloup-Demange, C., Armand, M., Marcand, J., Borel, P., 2003. Processing of vegetable-borne carotenoids in the human stomach and duodenum. Am. J. Physiol. Gastrointest. Liver Physiol. 284 (6 47-6) https://doi.org/10.1152/ajpgi.00410.2002.

U.S. Pharmacopeia. (n.d.). Oil and Water-Soluble Vitamins with Mineral Tablets. from. http://www.pharmacopeia.cn/v29240/usp29nf24s0_m88698.html#usp29nf24s0_m88698. (Accessed 8 September 2021).

Wu, P., Chen, X.D., 2021. Validation of in vitro bioaccessibility assays — a key aspect in the rational design of functional foods towards tailored bioavailability. Curr. Opin. Food Sci. 39, 160–170. https://doi.org/10.1016/j.cofs.2021.03.002.

Yuan, X., Liu, X., McClements, D.J., Cao, Y., Xiao, H., 2018. Enhancement of phytochemical bioaccessibility from plant-based foods using excipient emulsions: impact of lipid type on carotenoid solubilization from spinach. Food Funct. 9 (8), 4352–4365. https://doi.org/10.1039/c8fo01118d.