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Identification of *cis/trans* isomers of menaquinone-7 in food as exemplified by dietary supplements

Arkadiusz Szterk*, Adam Zmysłowski, Katarzyna Bus

National Medicines Institute, Department of Spectrometric Methods, 30/40 Chełmska, 00-725 Warsaw, Poland

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ABSTRACT

For the first time, the *cis/trans* isomer content of menaquinone-7 in food products has been identified and marked. A novel method of marking isomers of vitamin K_2MK-7 in dietary supplements was developed and validated. Five different isomers of *cis/trans* vitamin K_2MK-7 were identified. Identification of *cis/trans* isomers was performed by HRMS-QTOF, whereas their quantities were determined by using CAD and DAD detectors. In the majority of cases, the content of biologically active all *trans* vitamin K_2MK-7 was below its declared content. The content of all *trans* K_2MK-7 was in the range between 5.5 and 49 μg in pills. In one of the studied supplements, this vitamin was not found, regardless of the claim on the product label. The content of *cis/trans* isomers in certain dietary supplements exceeded the content of all *trans* K_2MK-7 by up to 3.7 times.

1. Introduction

Vitamin K is a fat-soluble vitamin. The term vitamin K applies to a number of different chemical substances that contain a 2-methyl-1,4-naphthoquinone group and different lateral hydrocarbon chains at C_3 (Shea & Booth, 2007). The two naturally existing forms of vitamin K are vitamin K_1 (phylloquinone or phytomenadione), composed of a long phytyl lateral chain, and vitamin K_2 (menaquinones), a major feature of which is a long polyprenyl lateral chain (Fig. 1).

Vitamin K_2 consists of a group of chemical compounds, the composition of which can be expressed as K_2MK-n, where n indicates the number of isoprenoid unsaturated units in a chain (usually, n is 4–13). One additional form of vitamin K is known as vitamin K_3 (menadione); however, this is a synthetic form and is a provitamin. Provitamin K_3 is not recommended for humans because it has certain toxic properties and may lead to haemolytic anaemia as well as allergic reactions (Hamidi & Cheung, 2014; Jinghe, Mizuta, & Ozaki, 2015).

The most important biological function of vitamin K in humans is its indispensable role in producing a haemostatic agent in the liver (first and foremost, prothrombin). However, there are different, new and important functions of vitamin K, such as its participation in forming osseous tissue, carboxylation of proteins, nucleic acid metabolism, and preventing certain types of haemophilia. Moreover, vitamin K has antibacterial and antimycotic properties and can be used to fight inflammation and pains (Geleijnse et al., 2004; Cockayne et al., 2006; Beulens et al., 2009, 2013; Vermeer, 2012). The most recent research indicates that vitamin K, mainly K_2MK-7, participates in the modification of proteins consisting of the gamma carboxylation of glutamic acid (GLU), the result of which is the formation of gamma carboxyglutamic acid (GlA), which has affinity to calcium ions. The best-known proteins of GLA include osteocalcin, MGP (MatrixGla Protein), and S-proteins. The largest affinity to calcium ions is that of a carboxylated form of osteocalcin, which has 3 residues of gamma carboxyglutamic acid (γ-carboxylated osteocalcin, Gla-OC). Osteocalcin binds calcium ions in the form of hydroxyapatite, constituting 70% of the total bone mass. A high level of non-carboxylated osteocalcin is connected with the risk of bone fractures.

An excessively low intake, mainly of vitamin K_2MK-7, may reduce bone mineralization and result in an increase in the risk of fractures and occurrence of osteoporosis (Gundberg, Lian, & Booth, 2013; Plaza & Lamson, 2005; Yamaguchi, 2014). Moreover, it was demonstrated that menaquinones are a significantly more active form of vitamin K than vitamin K_1 with regard to biological activities other than participating in the blood coagulation process (Beulens et al., 2009; Spronk et al., 2003; Weber, 2001). Because vitamin K_2MK-7 has a very important role in the bone-forming process and also reduces the risk of the occurrence of cardiovascular diseases (CVD), as well as calcification of blood vessels (Beulens et al., 2009; Gast et al., 2009; Geleijnse et al., 2004), it is important to provide humans with vitamin K_2MK-7 as part of their diet.

It needs to be emphasized that the food sources of vitamin K for humans are mainly plants and algae. A high concentration of vitamin K_1 is found in green vegetables (e.g., broccoli, iceberg lettuce and spinach) and also in vegetable oils (e.g., soya oil, olive oil) (Booth, 2012). For

* Corresponding author.
E-mail address: szterkarkadiusz@gmail.com (A. Szterk).

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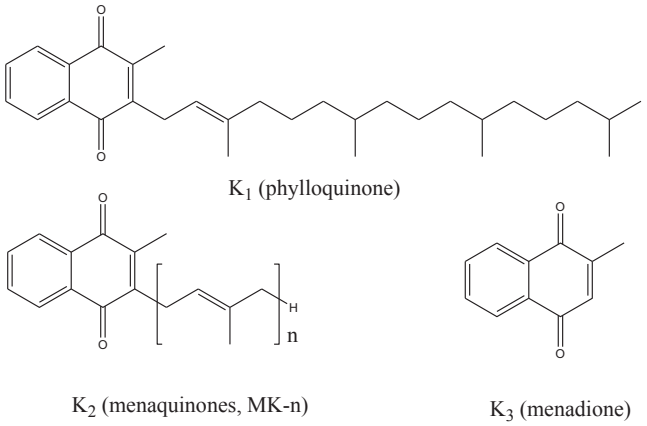

Fig. 1. Chemical structure of vitamin K.

humans, the most important form of vitamin K_2 is vitamin K_2MK-7, which is present at low concentrations in animal products, such as eggs and meat, as well as at high concentrations (1200 μg 100 g^{-1}) in fermented products, such as natto, cheese, curd and sauerkraut (Hamidi & Cheung, 2014; Scheiber et al., 2015; Weber, 2001). However, this vitamin is found in few food products in comparison with vitamin K_1, such that its daily and long-term supply is limited. There is another problem with human's demand for this vitamin. At the moment, there are no standards for the human daily demand for vitamin K_2MK-7. However, by analysing the global literature concerning the influence of menaquinones on humans and the dietary intake of vitamin K_1 and K_2, it can be concluded that a daily intake of vitamin K_2 for an adult woman should not be greater than 90 μg d^{-1} and for men greater than 120 μg d^{-1}, which means that the daily demand for vitamin K should not be exceeded (Beulens et al., 2013). On the basis of the current state of knowledge, it is thought that menaquinones in a human diet should constitute approximately 25% of all forms of vitamin K (K_1 and K_2) (Nimptsch, Rohrmann, & Linseisen, 2008; Schurgers & Vermeer, 2000). On the basis of the recommendations of the European Food Safety Authority (Bresson et al., 2008), the daily intake of menaquinones for adult women should be approximately 30 μg d^{-1} and for adult males approximately 50 μg d^{-1}. Providing the intake of such quantities of vitamin K_2 in a diet is not easy, because this vitamin is not as common in food as vitamin K_1. It seems that the best method of complementing the human diet with vitamin K_2 may be dietary supplements that are commonly available on the global market. There is a major problem with dietary supplements in Europe and all over the world in terms of their quality. Essentially, the sole requirement of food law, in European and other countries, is that a product is safe for human consumption. Less attention is paid to the chemical quality of dietary supplements. The chemical quality of dietary supplements is very important from the point of view of vitamin K_2MK-7. This vitamin is obtained either from natural sources, mainly natto (Sato et al., 2001), or in a significantly more economical way, by chemical synthesis (Baj et al., 2016; Daines, Payne, Humphries, & Abell, 2003; Sato, Inoue, & Saito, 1973; Snyder & Rapoport, 1974). K_2MK-7 preparations obtained from natto contain 100% of the *trans* form of K_2MK-n, whereas K_2MK-7 constitutes more than 95% of all menaquinones (Kamao et al., 2007; Sato et al., 2001; Schurgers & Vermeer, 2000; Walther, Karl, Booth, & Boyaval, 2013). There are no reports in the literature ascertaining the presence of the *cis* form in natural preparations of K_2MK-7. The *cis/trans* isomers of menaquinones are ascertained during chemical synthesis (Baj et al., 2016; Daines et al., 2003; Sato et al., 1973; Snyder & Rapoport, 1974). Only *trans* menaquinones demonstrate biological activity (Beulens et al., 2013; Shearer & Newman, 2008; Yamaguchi, 2014). The studies conducted by Lowenthal and Rivera (1979) demonstrated that the *cis* forms of vitamin K have 1% of the biological activity of the *trans* form. The results of this research were confirmed by other researchers (Huang et al., 2012; Knauer, Siegfried, Willingham, & Matschiner, 1975; SCCS., 2010). Moreover, in accordance with the opinion of the FAO issued in 2008, it is only the *trans* form of menaquinones, mainly including K_2MK-7, that is important for humans from the point of view of its biological functions, and it can be found in different preparations, including dietary supplements, facilitating compensation of the deficits of this vitamin in the diet. In the global literature, there are no scientific reports concerning the content of the *cis/trans* form of menaquinones in dietary supplements. Moreover, there are virtually no studies of supplements in terms of the content of this vitamin, which is currently very fashionable and frequently found in supplements.

The objective of this dissertation was to study a few of the most popular dietary supplements containing vitamin K_2MK-7 in terms of the presence of the *trans* form as well as the *cis/trans* form. In this study, a new analytical method that is selective for *cis/trans* isomers of vitamin K_2MK-7 was developed. An additional objective of this dissertation was to verify whether the declaration concerning the content of vitamin K2MK-7 that is placed on it is compatible with the actual composition of the supplement.

2. Materials and methods

2.1. Research materials

The research material consisted of 8 different dietary supplements of vitamin K_2MK-7 in the form of hard pills purchased from 10 different chemists. Three of the supplements were produced in Poland (Polish producer 1: Natural Supplements "naturalna witamina K_2 + D_3 forte"; Polish producer 2: "Molekin D_3 + K_2"; Polish producer 3: Polski Lek "witamina D_3 + K_2"), three in Norway (Norway producer 1: "Menna Q7 vitamina 30 caps." Norway producer 2: "Menna Q7 vitamina 30 caps. forte" Norway producer 3: "Menna Q7 vitamina 60 caps."), one in Sweden ("Kinon"), and one in the USA ("natural vitamin K_2 from natto"). All supplements were preparations with labels that declared the content of vitamin K_2MK-7 or a mixture of vitamin K_2MK-7 and vitamin D_3.

2.2. Chemicals

Tetrahydrofuran (THF) for analysis EMPARTA® ACS, methanol anhydrous, 99.8%, methanol hypergrade for LC–MS LiChrosolv®, 2-propanol hypergrade for LC–MS LiChrosolv®, *n*-hexane hypergrade for LC–MS LiChrosolv®, ammonium acetate for LC–MS LiChropur®, acetic acid 100% for LC–MS LiChropur®, menaquinone-7 (vitamin K_2MK-7) United States Pharmacopeia (USP) Reference Standard, vitamin K_2MK-7 100 μg ml^{-1} in acetonitrile, and certified reference material (check standard) were purchased from SIGMA Aldrich, Poland, Gliwice.

2.3. Sample preparation

After determining the average mass of a pill (by means of weighing 30 different pills), the pills were crushed in a mortar. Appropriate mass of crushed pills, which corresponds to the determinated average mass of pills was placed into a 30 ml glass centrifuge test tube, and 20 ml of THF was added. The test tube was plugged with a Teflon cork and placed into an ultrasonic cleaning device, in which the vitamin was extracted for 30 min (22 kHz and 30 W). Afterwards, the sample was centrifuged (speed: 4000 rpm) for 10 min at 4 °C. The supernatant was poured into a round-bottom laboratory flask and evaporated until dry in a vacuum evaporation device at 25 °C, while protecting the sample against light (the laboratory flask was wrapped in aluminium foil). The dry remnant was dissolved in 1 ml of the mixture of methanol: THF at a proportion 7:3 v/v. Five microliters of the sample was placed into a chromatography column.

2.4. Chromatographic analysis and detection: DAD, CAD and QTOF

A UHPLC Ultimate 3000 (Dionex Thermo Fisher Scientific, Sunnyvale, California, USA) system consisting of a pump, degasser, autosampler, column heater detector (analytical λ = 268 nm) and pulse damper coupled with an ESA Corona CAD (Charged Aerosol Detectors) instrument was used. The CAD response range was 100 pA, with no filter selected. Nitrogen from a nitrogen generator regulated at 0.24 MPa was introduced to the detector. Data processing was carried out with Chromeleon 6.8 and Chromeleon Validation ICH software (Dionex) and the chromatographic conditions were optimized with DryLab 2000 Plus software (Molnar Institute). To determine the peak elution order, a mass spectrometer maXis 4G from Bruker Daltonic (Billerica, Massachusetts, USA) was used. The chromatographic conditions for MS analysis were the same as for UHPLC–DAD-CAD. The QTOF settings were electrospray ionization (ESI) in positive ion mode, dry gas (nitrogen) flow rate of 8.0 l min^{-1}, dry heater at 180 °C, capillary voltage of 4500 V and end plate offset of -500 V. MS data were recorded in full scan mode (from 50 to 3000 *m/z*). The mass spectrometer was used in high resolution mode (R = 60,000), and an internal calibrant (1 mM aqueous solution of sodium formate) was used to make a precise mass measurement. Chromatographic separation was conducted with the use of a COSMOSIL cholester column (2.0 mm AND.D. × 150 mm) with a pore diameter of 3.0 μm. The work was conducted in a gradient system (phase A: 6.5 mM ammonium acetate and 6.5 mM acetic acid in the 10% solution of water in methanol). Phase B also contained 6.5 mM ammonium acetate and 6.5 mM acetic acid in the following solution: 2-propanol:*n*-hexane in the proportion: 2:1 (v/v). For preparing phase B, ammonium acetate and acetic acid were dissolved for 10 min in an ultrasound cleaning device before combining with *n*-hexane. The following gradient system was applied: 0–5 min 18% B, 5–30 min 40% B, 30–32 min 40% B, 32–33 min 18% B, 30–35 min 18% B (balancing columns until the initial conditions are restored). Chromatographic separation was conducted with a constant flow of mobile phase (350 μl min^{-1}) at a temperature of 40 °C ± 0.1 °C.

2.5. Marking vitamin K_2MK-7 in dried natto

Marking vitamin K_2Mk-7 in natto was conducted in accordance with Schurgers and Vermeer (2000).

2.6. Method validation

Quantitative analysis was performed by a standard-addition method. In this way, besides estimating the unknown amount of the analytes occurring in the different dietary supplements, it was possible to evaluate the sensitivity (LOD and LOQ) and linear dynamic range in the various matrices. The recoveries, matrix effect, linearity, accuracy (repeatability and reproducibility), limits of detection (LOD) and limits of quantitation (LOQ) were calculated after determining the levels of all *trans* vitamin K_2MK-7 in the specific food.

2.6.1. Preparation of stock, spiking solutions, LOD and LOQ calculation

A primary stock solution of all *trans* vitamin K_2MK-7 (5.0 mg ml^{-1}) was prepared in a methanol: THF (7:3 v/v) solution. Spiking solutions of the analyte (10, 20 and μg ml^{-1}) were prepared with the mixed intermediate solution of the analyte, and all of these solutions were stored at −20 °C, while protecting it against light. Twelve calibration curve points (0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, 102.4, 204.8 and 250.0 μg ml^{-1}) were prepared by spiking the matrix after evaporation and sample extraction. The LOD and LOQ were calculated. The LOD was defined as 3 × standard deviation of the blank, and the LOQ was defined as 10 × the standard deviation of the blank. The mean LOD and LOQ from each sample was calculated, with n = 10 independent of detector used.

2.6.2. Recovery

The recovery of the analyte (all *trans* vitamin K_2MK-7) was studied with the application of the standard addition method. The recovery was studied at three different levels of enrichment (10 μg, 20 μg and 30 μg per ml of a final solution, which is equal to the average mass of a pill). The recovery was studied independently for each supplement and detector used (DAD, CAD and QTOF). Global recovery was calculated for each level of fortification (the average of all the studied dietary supplements, n = 10), and also for each detector.

2.6.3. Matrix effect (MF)

MF was calculated by 100% − {[peak area of K_2MK-7 in the presence of the matrix (post extracted sample)/mean peak area of K_2MK-7 in the absence of the matrix (all *trans* K_2MK-7 in methanol: THF solution)] × 100%}. The mean MF was calculated from n = 10 independent of detector used.

2.6.4. Precision and accuracy

Precision and accuracy were evaluated by intra-day and inter-day parameters. Intra-day precision was calculated as % CV from the mean amount of quality control sample (all-trans K_2MK-7 United States Pharmacopeia (USP) Reference Standard, n = 10 and concentration 20 μg ml^{-1}) by a single person using the same equipment, under the same conditions, and in a short period of time (one day). Inter-day precision was calculated as % CV from the mean amount of the quality control sample (all trans K_2MK-7 United States Pharmacopeia (USP) Reference Standard, n = 10 and concentration 20 μg ml^{-1}) by three independent people using the same equipment, under the same conditions, and over a long period of time (3 days). The accuracy of this analytic method was assessed as the percentage relative error (100 × [found − added]/added]. The analytical standard was added after the whole procedure of sample preparation and 20 μg of all trans K_2MK-7 per sample was always added (the final concentration was 20 μg ml^{-1}). The accuracy was measured for one randomly selected dietary supplement, n = 10. Precision and accuracy were determined according to Yilmaz, Kadioglu, Meral, and Onganer (2012).

3. Results

To mark possible *cis/trans* isomers of menaquinone-7 in the dietary supplements, a stereo-selective chromatography column filled with chemically modified silica containing approximately 20% basic cholesterol was used. Geometric isomers of vitamin K_2MK-7 were marked with the application of a DAD and charged aerosol detectors (CAD). Identification of the *cis/trans* of isomers of vitamin K_2MK-7 was conducted by using a high-resolution quadrupole analyser coupled with a time of flight analyser (QTOF).

Fig. 2 presents examples of the chromatograms of the analysed dietary supplements, an analytical standard of all *trans* vitamin K_2MK-7, and the extract obtained from dried natto, in accordance with Schurgers and Vermeer (2000). In certain samples (in particular, of the Swiss producer and Polish producer-1), apart from all *trans* vitamin K_2MK-7, the presence of different chemical compounds was ascertained as well (they are identified with a question mark). An attempt was made to identify unknown substances by using a high-resolution mass spectrometer combined with a high-efficiency liquid chromatograph (HRMS–QTOF). Fig. 1 in the Supplementary materials shows the fragmentary spectra of the supplementary materials of chromatographically separated substances. The chemical compound known as C4 (Fig. 1 Supplementary materials) is all *trans* vitamin K_2MK-7, with an *m/z* = 667.00, which is a pseudo-molecular ion formed as the result of the adduction of NH_4^+ to 649.00 $(M+H+NH_3)^+$.

The retention time of the chemical compound known as C4 and its fragmentary spectrum are identical to those of the analytical standard of vitamin K_2MK-7 (Figs. 2 and 3). The remaining recorded spectra of unknown substances C1, C2, C3, C5, and C6 (Fig. 1 Supplementary

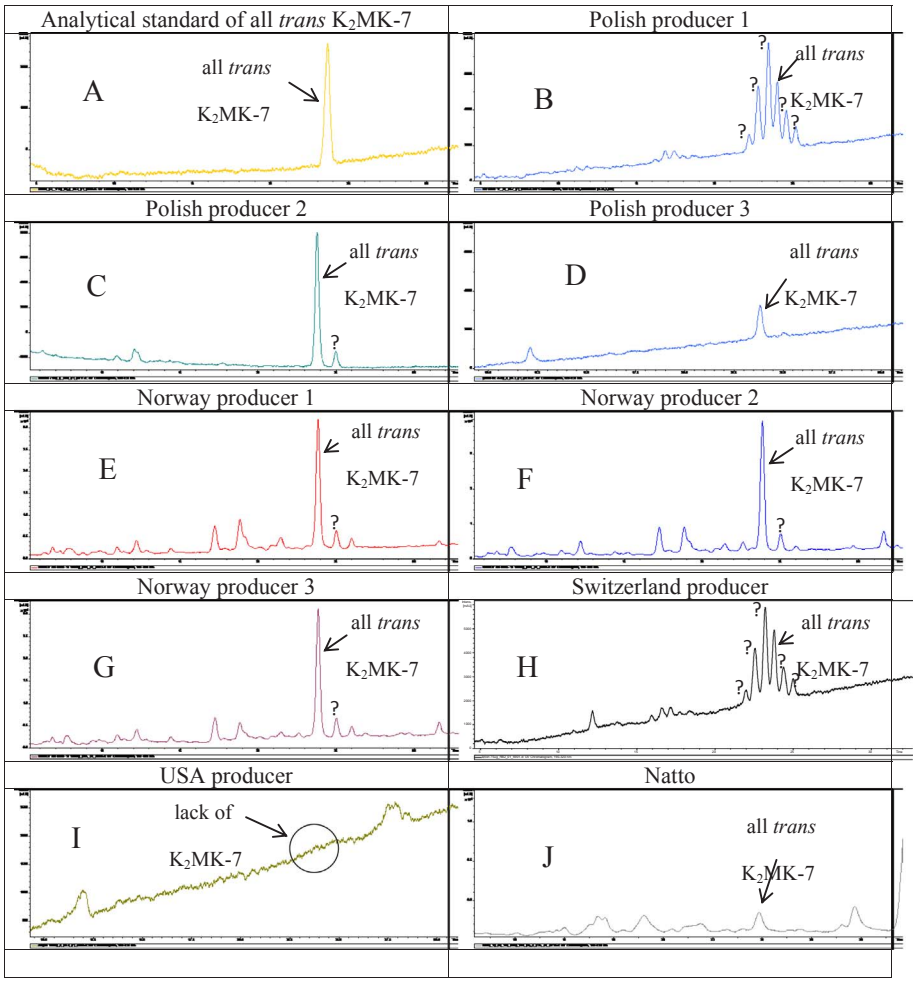

Fig. 2. Example chromatograms from CAD detection of an analytical standard vitamin of all *trans* K_2MK-7, various dietary supplements from various producers from various canaries and chromatograms of dry natto extract. ? – unknown substance.

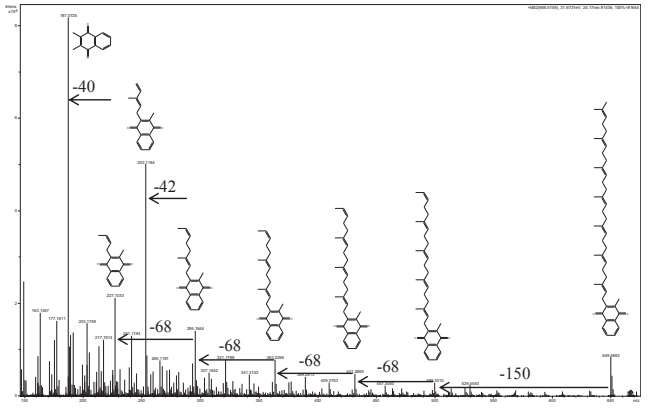

Fig. 3. Spectrum of all *trans* vitamin K_2MK-7.

materials) do not statistically differ from the spectrum of all *trans* vitamin K_2MK-7. Moreover, these substances undergo ionization in precisely the same way as all *trans* vitamin K_2MK-7 $(M+H+NH_3)^+$, which results in the formation of an adduct of m/z = 667.00 and a pseudomolecular ion of a chemical compound with a theoretical mass of 649.00 g mol^{-1}. As a result of the fragmentation of ion m/z = 667.00, there is decrease in the amount of ammonium radical, which causes the formation (in each case) of a fragment of m/z = 649.5 and is a pseudomolecular ion with the molecular formula $C_{46}H_{64}O_2$. For all the studied substances visible in Fig. 2 and Fig. 1 (Supplementary materials), the mass measurement error is smaller than 1.5 ppm and the isotope profiles (mSigma) have low values from adjusting the molecular formula amount to 99.9999%. Larger values of mSigma (an isotope profile not so well adjusted) are observed in the case of chemical compounds C1 and C6, which result from the relatively low concentrations of these substances, causing the heaviest isotopes to appear at the limit of detection of the mass spectrometer.

From this research, it is possible to determine that the identified unknown substances (C1, C2, C3, C5, and C6 Fig. 1 Supplementary materials) are likely to be the *cis/trans* isomers of vitamin K_2MK-7, which, for the first time, were identified in food. Unfortunately, in the case of the use of HRMS, it is not possible to determine where in the isoprene chain of menaquinone-7 the *cis* configuration exists.

Table 1 presents the validation parameters of the developed method of marking vitamin K_2MK-7 in dietary supplements. The developed method was applied to marking vitamin K_2MK-7 in dietary supplements available on the market in the form of a hard pill. Table 2 includes the results for the content of the *cis/trans* isomers of vitamin K_2MK-7 in the studied samples.

In Table 1, LOD, LOQ, and linear values are expressed in μg ml^{-1}; however, a more practical unit should be μg in the pill. However, because the masses of pills are very different (Table 2), it was decided to express all units in μg ml^{-1} because in accordance with the described procedure of preparing a sample, one pill of the studied supplement is prepared in 1 ml of the final solution (the mixture of methanol:THF, 7:3 v/v). On the basis of the results in Table 1, a major feature of the developed method is a sufficient sensitivity in relation to marking vitamin K_2MK-7 in dietary supplements. The sensitivity of the method (LOD and LOQ) depends on the detector. The highest sensitivity (the lowest LOD

Table 1
Validation parameters of all *trans* K_2MK-7 in dietary supplements.

	DAD detector	CAD detector	QTOF detector (SIM, *m/z* = 666,5 ± 0,5 *m/z*)
LOD [ng ml^{-1}]	378.7	678.2	206.5
% CV	8.2	5.5	10.4
LOQ [ng ml^{-1}]	1262.4	2260.8	690.0
% CV	0.6	0.1	8.1
Recovery			
Spiked level 1: 10 μg ml^{-1} [%]	89.1	90.1	91.3
SD	1.2	0.2	4.6
Spiked level 2: 20 μg ml^{-1} [%]	90.2	90.0	92.6
SD	1.6	0.6	5.9
Spiked level 3: 30 μg ml^{-1} [%]	96.5	95.8	93.7
SD	0.6	0.5	3.5
Matrix effect (ME) [%]	< 5	< 5	< 5
SD	–	–	2.2
Linear (range in μg ml^{-1} and R^2)	1.3–250 μg ml^{-1} R^2 = 0.998	2.3–250 μg ml^{-1} R^2 = 0.999	Very low, for example: 2–4 μg ml^{-1} R^2 = 0.991, but for 2–20 μg ml^{-1} R^2 = 0.870
Precision and accuracy			
Intra-day precision in % CV	0.5	0.6	7.7
Intra-day accuracy	2.5	2.8	5.2
Inter-day precision in % CV	3.3	3.9	9.1
Inter-day accuracy	−1.9	−2.1	−4.3

and LOQ) was obtained by using the mass spectrometer (QTOF). However, in the case of QTOF, the linear character of the method is very low, which virtually disqualifies this detector in terms of quantitative marking. A QTOF is the perfect tool for the identification or confirmation of marked substances.

For marking vitamin K_2MK-7 in dietary supplements, a DAD or CAD are perfect. The LOD and LOQ for these detectors are sufficient to mark the *cis/trans* form of vitamin K_2MK-7. Moreover, a major feature of these detectors is the very wide range of linearity from LOQ to 250 μg ml^{-1} (virtually μg pill^{-1}). The retrieval in the method statistically does not depend on the detector, which results from a small influence of the matrix (ME < 5%) and is within the range 89–96.5%. A major feature of the developed method is its being precise, which, in the case of DADs and CADs, amounts to an intra-day precision of 0.5–0.6% and an accuracy of 2.5–5.2%, whereas the inter-day precision amounts to 3.3–3.9% and the accuracy from −1.9 to −4.3% (Table 1). All the studied validation parameters were determined on the basis of the isomer of all *trans* K_2MK-7. Unfortunately, there are no forms of *cis/trans* vitamin K_2MK-7 available for sale. However, the application of a charged aerosol detector (CAD) for quantitative marking essentially eliminates the problem of the lack of standards for *cis/trans* isomers because the signal of this detector does not depend on the chemical structure of the molecules but only on their concentration. For that reason, the developed method of marking all *trans* K_2MK-7 with a CAD was used for marking the different form of vitamin K_2MK-7 in the dietary supplements of this vitamin available on the pharmaceutical market.

Based on this research (Table 2) it can be concluded that the composition of dietary supplements in terms of the content of vitamin K_2MK-7 is very varied. In the dietary supplement produced in USA, despite label claim, vitamin K_2MK-7 was not detected. The remaining supplements contained, without exception, *trans* K_2MK-7 in the quantity between 5.5 μg pill^{-1} and 248.1 μg pill^{-1}. All the studied supplements, with the exception of the Polish product (of the third producer), contained a varied content of *cis/trans* isomers K_2MK-7; however, the smallest share of them was ascertained in the case of the supplements produced in Poland and Norway. In the set of the studied samples, it is only the dietary supplement of vitamin K_2MK-7 from the Norwegian producer that contained the declared content of all the *trans* vitamin K_2MK-7. In the remaining supplements, the vitamin was either not ascertained, or its content was several times lower.

4. Discussion

Marking *cis/trans* isomers of vitamin K_2MK-7 was possible by means of the application of a stereo-selective chromatography column that was composed of chemically modified silica containing approximately 20% cholesteryl. This type of column was used by Nannapaneni, Jalalpure, Muppavarapu, and Sirigiri (2017) to separate *cis* and *trans* isomers of vitamin K_1 in biological samples. On the basis of the obtained mass spectra (Fig. 1 Supplementary materials), the separated substances, shown in Fig. 2, are *cis/trans* isomers of vitamin K_2MK-7. When ionized, all these chemical compounds produce a pseudo-molecular ion with a theoretical *m/z* = 667.00 and are adducts of NH_4^+ with a theoretical mass of 649.00. The mechanism of ionization can be expressed in the form $(M+H+NH_3)^+$. The formation of the NH_4^+ adduct was connected with the fact that in the mobile phases used for chromatographic separation, ammonium acetate was applied as a phase

Table 2
All trans K_2MK-7 and *cis/trans* isomers contents in dietary supplements obtained from local markets, n = 10.

Sample	Average weight of pill [mg]	SD	Declared amount of K_2MK-7 vitamin by producer [μg pill^{-1}]	All *trans* K_2MK-7 [μg pill^{-1}]	SD	Sum of *cis/trans* isomers of vitamin K_2MK-7 [μg pill^{-1}]	SD
Polish producer 1	350.2	11.0	100 (from natto)	22.6	0.9	81.5	1.1
Polish producer 2	322.9	2.7	75 (no data)	49.1	3.7	3.2	0.5
Polish producer 3	252.5	2.2	75 (no data)	5.5	0.1	< LOQ	–
Norway producer 1	564.5	19.1	100 (from natto)	139.8	1.9	15.7	0.3
Norway producer 2	677.5	42.9	200 (from natto)	248.1	3.4	30.5	0.2
Norway producer 3	623.3	31.5	100 (from natto)	133.2	1.1	16.4	0.2
Switzerland producer	389.1	3.9	100 (from natto)	11.9	1.1	31.2	1.0
US producer	620.4	30.9	100 (from natto)	< LOD	–	< LOD	–

modifier. The formation of such an adduct is typical for these phases (Fouquet, Humbel, & Charles, 2011; Huang & Siegel, 1999). Adducts of NH_4^+ are specific as fragments of these ions $(M+H)^+$ form, as well as neutral NH_3. Ions $(M+H)^+$ undergo fragmentation, leading to a typical spectrum of menaquinones-7 (Fig. 3 and Fig. 1 Supplementary materials). The use of HRMS (QTOF) makes it impossible to ascertain where the *cis* bond is located in the isoprenoid chain. Both *cis* and *trans* isomers undergo fragmentation in an identical way (Carlone & Anet, 1983; Huang et al., 2012). According to quality, dietary supplementation and biological functions, identification of the isomer of all *trans* K_2MK-7 and *cis/trans* isomers of K_2MK-7 is sufficient. To determine where the *cis* bonds are found in an isoprenoid chain of vitamin K_2MK-7, it is necessary to conduct fractionation of the identified substances that are shown in Fig. 2 and determine their chemical structure by NMR.

Studying the *cis/trans* isomers of vitamin K_2MK-7 is very important from a biological point of view. Only the *trans* forms of vitamin K_1 and K_2 have biological activity. The *cis* or *cis/trans* forms (in the case of menaquinones) do not have biological activity, or it is at a very low level (Beulens et al., 2013; Huang et al., 2012; Pucaj, Rasmussen, Møller, & Preston, 2011; Yamaguchi, 2014). This was shown for vitamin *cis* K1 (Lowenthal & Rivera, 1979) and mixtures of *cis/trans* isomers K_2MK-6 (Weber & Wiss, 1959). There are no biological data concerning the activity, including the toxicity, of the *cis/trans* form of vitamin K_2MK-7. However, on the basis of the biological function of the all *trans* isoprene units, it is possible to ascertain that *cis* isomers or *cis/trans* isomers constitute chemical pollution, which has no positive biological role, as does all *trans* vitamin K_2MK-7. Geometric isomerization of isoprenoid units in vitamin K_2MK-7 is possible in a few cases. *Cis* isomers are formed during the course of the chemical synthesis of this vitamin (Baj et al., 2016; Daines et al., 2003; Sato et al., 1973; Snyder & Rapoport, 1974) and also during the course of incorrect technological processes, the objective of which is to obtain different preparations of vitamin K_2MK-7, mainly by the impact of light (in particular, UV radiation), which causes geometrical isomerization of the isoprenoid units in menaquinone-7 (Huang et al., 2012). Afterwards, oxidization catalysed by a high temperature, or the impact of radicals, causes the formation of epoxides and *cis* isomers at different locations in the isoprenoid chain of vitamin K_2MK-7 (Yamada, Aoki, & Tahara, 1982). Such a process may take place while generating microcapsule preparations of vitamin K_2MK-7, which is a stock raw material for hard pills and capsules. It can be presumed that the geometrical isomerization of all *trans* vitamin K_2MK-7 occurs as the result of the autoxidation processes in the course of storing dietary supplements, which are in contact with oxygen in the atmosphere, light and elevated temperature. Unfortunately, there are no relevant data in the literature. However, we found different *cis/trans* isomers of vitamin K_2MK-7. Moreover, in certain supplements, the sum of the *cis/trans* isomers was significantly larger than the content of the pure form of *trans* K_2MK-7 (Fig. 2, Table 2). The explanation for the occurrence of a large quantity of *cis/trans* isomers of K_2MK-7, in particular in supplements from the first Polish producer and Swiss producer, is that they are likely the result of using synthetic vitamin K_2MK-7. The producers of these supplements declare that the vitamins contained in them were from natural sources (extract from natto). However, natto does not contain the *cis/trans* form of vitamin K_2MK-7, it only contains *trans* K_2MK-7, as shown by the chromatogram of the extract of natto obtained from dried natto in Fig. 2 (J), which matches the data in the literature (Kamao et al., 2007; Sato et al., 2001; Schurgers & Vermeer, 2000; Walther et al., 2013). In turn, during chemical synthesis of vitamin K_2MK-7 *cis/trans* isomers in relation to the pure form, all *trans* isomers are formed at a ratio of 1:3 or 1:2 in/in or not at all, depending on the method of synthesis (Baj et al., 2016; Daines et al., 2003; Sato et al., 1973; Snyder & Rapoport, 1974). In the analysed samples, large quantities of *cis/trans* isomers were found, indicating the use of synthetic vitamin K_2MK-7 as well as a likely adverse impact of the technological process generating vitamin K_2MK-7 in pills. These factors might have contributed to the occurrence of the large quantity of *cis/trans* isomers of vitamin K_2MK-7 in the studied samples. In the remaining supplements, no more than 5% *cis/trans* isomers were found. Moreover, only one geometric isomer of this vitamin was found (Fig. 2). The vitamins in these supplements were obtained either from natto or were synthesized by using a method similar to the process described by Baj et al. (2016). The presence of a small quantity of isomeric *cis/trans* was most likely the result of the technological process used to prepare vitamin K_2MK-7, which is the basis for obtaining a hard pill. Most likely, it was from a microcapsule preparation, during which the geometrical isomerization of all *trans* K_2MK-7 occurred.

Apart from the occurrence of *cis/trans* isomers of menaquinone-7, a serious problem with the declared content of vitamin K_2MK-7 was found. Only in supplements produced in Norway was the declared content of vitamin K_2MK-7 the same as the actual value. Moreover, a larger content of all *trans* vitamin K_2MK-7 was found in the Norwegian supplements, which was likely caused by the distant expiration date of these supplements. During production, the producers of supplements assume a certain surplus of vitamin so that by the expiration date its content is the same as the declared value. In all the remaining studied supplements, the content of vitamin K_2MK-7 was below the declared value, and in the case of the American supplement, it was not found at all. The results of this study allow us to formulate a number of conclusions. Producers of dietary supplements take advantage of the lack of legal regulations regarding official control of the quality of dietary supplements. In accordance with European law and with laws in different countries, dietary supplements are treated as food, which means they only have to be safe. There are no effective regulations or solutions that would force producers to thoroughly control the quality of supplements as is the case for producing medicinal products, which leads the actual content of vitamin in the final product to be different from that declared by the producer. This difference may result from the dishonesty of producers or errors in the supplement production process (errors in recipes) as well as from oxidation or disintegration of the vitamin during production, transport or storage of supplements. The high content of *cis/trans* isomers of vitamin K_2MK-7 in reference to all *trans* K_2MK-7 results in the need for determining the biological activity as well as the toxicity of the *cis/trans* forms of K_2MK-7. Moreover, results for supplements of vitamin K_2MK-7 in the form of hard pills were presented. This vitamin is, however, the most frequently sold in the form of heterogeneous capsules, in which it is dissolved in different oils. It is known that vitamin K_2MK-7 is susceptible to oxidation; therefore, there is a question regarding the quality of vitamin K_2MK-7 supplements.

5. Conclusion

A novel method that produces all *trans* vitamin K_2MK-7 and *cis/trans* isomers in dietary supplements sold in the form of hard pills was developed. The method is based on chromatography with detection (DAD, CAD, and also QTOF) and made it possible to separate and mark 5 different *cis/trans* isomers of vitamin K_2MK-7, as well as the isomer of all *trans* K_2MK-7. The developed and validated method was used to study the content of different isomeric forms of vitamin K_2MK-7 in different commercially available dietary supplements produced in different countries. This study demonstrated that there were large variations of the content of vitamin K_2MK-7 in the studied dietary supplements; usually, its concentration was below the declared content. In one of the supplements, it was not found at all. Moreover, large quantities of the *cis/trans* isomers of vitamin K_2MK-7 were identified, which likely did not have vitamin K_2 activity. Alternatively, vitamin K_2 activity might have been significantly reduced. Different contents of vitamin K_2MK-7 in the studied dietary supplements and the occurrence of large quantities of *cis/trans* isomers may indicate dishonesty of producers who claim that they use natural extracts of natto, which do not contain *cis* isomers of this vitamin. It can be presumed, that, in reality,

producers use cheap preparations of vitamin K_2 obtained by means of chemical synthesis and use production methods in which *cis/trans* isomers or the sold product are chemically transformed (e.g., oxidized) during storage, causing the transformation of all *trans* vitamin K_2MK-7 into *cis/trans* isomers. For the first time, dietary supplements with vitamin K_2MK-7 were studied. It was determined that there is a serious problem with their quality, which is caused by a number of factors.

Acknowledgements

This research is purely scientific. The aim of the research was not to compare products between themselves (for example - in relating range of the safety and of the quality), but merely to determine the actual state of the chemical form of vitamin K_2MK-7 in dietary supplements and the names of the products mentioned are only intended to enable the reproduction of the method and the results to other researchers. Moreover this work was co-financially supported by Polish Ministry of Science and Higher Education statutory grant number: 1.34/2017.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.10.001.

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