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Fermentation of Menaquinone-7: The Influence of Environmental Factors and Storage Conditions on the Isomer Profile

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Abstract: Menaquinone-7 (MK-7) provides significant health gains due to its excellent pharmacokinetic properties. However, MK-7 occurs at low concentrations in mainstream foods, heightening the demand for nutritional supplements. MK-7 exists as geometric isomers, and only all-*trans* MK-7 is bioactive. Exposure to certain environments impacts the isomer profile. Knowledge of these factors and their influence on the isomer composition is important, as the efficacy of fermented MK-7 end products is solely determined by the all-*trans* isomer. This investigation aimed to evaluate the short- and long-term effect of atmospheric oxygen, common temperatures, and light on the isomer profile. From the short-term study, it was ascertained that MK-7 is moderately heat-stable but extremely light-sensitive. The stability of all-*trans* MK-7 was then examined during 8 weeks of storage at a low temperature with minimal oxygen exposure in the absence of light. Negligible change in the all-*trans* MK-7 concentration occurred, suggesting it is reasonably stable during prolonged storage in this environment. These findings will aid the development of optimal storage conditions to preserve bioactive MK-7 in fermented nutritional supplements, the large-scale availability and consumption of which will help compensate for the dietary deficit of this essential vitamin and provide consumers with better health outcomes.

Keywords: menaquinone-7 isomer profile; bioactivity; fermentation; environmental factors; storage conditions

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1. Introduction

The vitamin K family consists of a set of fat-soluble vitamins, namely vitamin K1 (phylloquinone), vitamin K2 (menaquinones), and vitamin K3 (menadione). The various K vitamers are structurally similar, as they all contain a 2-methyl-1,4-naphthoquinone group [1]. However, they differ in the nature of an isoprenoid side chain at the 3-position, the length and degree of unsaturation of which confers unique properties to each kind of vitamin K [2]. Phylloquinone (PK) and menaquinones (MK) are the natural forms of the vitamin and play an essential role in human health and nutrition [3]. PK has one unsaturated and three saturated isoprenoid units in its side chain. It is ubiquitous within the chloroplasts of photosynthetic plants and algae, where it functions as an electron carrier during photosynthesis [2,4]. Therefore, PK can be consumed from a selection of everyday foods, including leafy greens, vegetable oils, and products resulting from such plant oils, and is the most abundant type of dietary vitamin K [4,5]. Conversely, MK are a series of compounds with isoprenoid chains of various lengths and degrees of unsaturation. The structure of the side chain can be represented by the format MK-*n*, where *n* is generally between four and thirteen and signifies the number of unsaturated isoprenoid residues in the chain [2]. MK are typically of microbial origin and are present in small quantities in specific animal, dairy, and fermented goods [2,5–8].

It is well-known that all vitamin K subtypes perform essential functions in the coagulation cascade and haemostasis. However, recent research has uncovered numerous other

roles and health gains of vitamin K. The intake of vitamin K2, in particular, has been related to the maintenance of bone and cardiovascular health, the suppression of neurological conditions, the prevention of cancer, aiding the functional recovery of the liver, reducing the likelihood of many health disorders, and decreasing the morbidity and mortality linked to coronavirus disease 2019 (COVID-19) [2,9–17].

MK-7 is the most notable vitamin K2 isoform due to its superior physicochemical characteristics and long plasma half-life (72 h), which enhance its extrahepatic bioavailability and therapeutic value [17–19]. Despite the significant health gains associated with MK-7, obtaining sufficient levels of the vitamin from regular food products is challenging for most consumers, as it is present in insufficient concentrations in limited foods. Natto, a Japanese fermented soybean containing nearly 800–1000 µg of MK-7 per 100 g of natto, is the richest source of MK-7 [6,8,20]. However, owing to its strong flavour and pungent aroma, most individuals perceive natto as unappetising and, hence, it tends to be a niche product that is not universally consumed by all populations. The MK-7 concentration in other dietary sources that appeal to mainstream consumers is inadequate. Consequently, meeting the daily intake requirements without the aid of nutritional supplements is not achievable for most populations, as it would require the consumption of unfeasibly large amounts of MK-7-containing foods [6]. This has increased the demand and created a lucrative market for MK-7 dietary supplements and enriched foods to complement natural sources, and the availability of such products has become progressively widespread [21].

It must be acknowledged that MK-7 demonstrates *cis*-*trans* isomerism, a feature that is common to most biomolecules. The all-*trans* isomer is the naturally occurring and bioactive form of the vitamin, whereas the various *cis* isomers are biologically ineffectual [7,22,23]. The bioactivity of MK-7 is related to its shape and structure, which depend on the arrangement of unsaturated bonds in the side chain. All-*trans* MK-7 has a straight isoprenoid chain (Figure 1), as all double bonds have the *trans* organisation [24]. In contrast, one or more unsaturated bonds in the *cis* conformation deform the linear molecular structure (Figure 1), and different numbers and combinations of *cis* double bonds can give rise to several *cis* MK-7 isomers [24]. The shape of MK-7 molecules determines their capacity to engage with subcellular components, and the non-linear configuration of the *cis* isomers impairs their ability to perform their biological role [25]. The *cis* isomers of vitamin K only sustain 1% of the biological significance of the all-*trans* form [26–28]. More recently, it has been established that *cis* MK-7 isomers have considerably diminished carboxylative potential and compromised bioactivity compared to all-*trans* MK-7 [25]. It is anticipated that the presence of *cis* MK-7 does not diminish the activity of the all-*trans* isomer. When the geometric isomers of the vitamin co-exist in a formulation, it is unlikely for interaction with the *cis* isomer to change the structure and bond arrangement of all-*trans* MK-7. As a result, the shape and, thus, the biological function of the all-*trans* isomer is expected to be unaffected. However, the remedial value of the preparation is only determined by the quantity of the biologically important isomer. Therefore, while the presence of *cis* MK-7 does not explicitly impact the activity of the all-*trans* isomer, its presence in the formulation is essentially an impurity, which decreases the biological function and therapeutic efficacy of all-*trans* MK-7 end products. In this regard, the isomer profile of MK-7 functional foods and dietary supplements is noteworthy, as their effectiveness is fundamentally governed by the proportion of the all-*trans* isomer.

MK-7 can be synthesised via chemical methods or microbial fermentation. The isomer composition of the MK-7 product is determined by various factors, primarily the manufacturing process and the techniques used to purify the post-reaction mixture [7,23]. Although chemical approaches are likely to be cost-effective, fermentation is more favourable from the perspective of both consumers and the environment. This is predominantly due to the recent consumer market trend promoting natural alternatives over synthetic formulations and the call for sustainable production methodologies. Fermentation is not only a natural approach for the synthesis of MK-7, but it is also more eco-friendly for the industrial

production of the vitamin. Hence, microbial fermentation can fulfil both consumer demand and environmental sustainability goals [29].

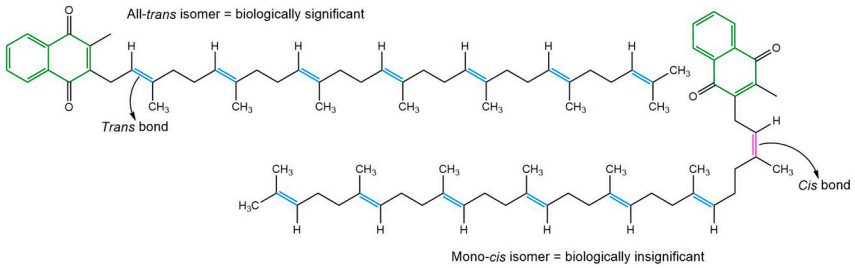

Figure 1. Chemical structure and bond organisation of MK-7 isomers.

The MK-7 isomer composition achieved from fermentation has not been widely examined, and it is generally believed that MK-7 resulting from fermentation-based synthesis exclusively occurs in the all-*trans* conformation. However, it has been proposed that exposure to particular factors may induce the transformation of the all-*trans* isomer to *cis* MK-7, and our previous studies were the first to ascertain the existence of *cis* MK-7 in fermented samples [30,31]. The occurrence of a *cis* isomer in samples obtained from fermentation implies that although the bacterium produces the naturally occurring all-*trans* isomer intracellularly, secretion into the fermentation broth exposes all-*trans* MK-7 to the extracellular milieu, which can promote its isomerisation to the *cis* form. It is important to appreciate that the exact structural identity (the number and location of *cis* double bonds in the isoprenoid side chain) of the *cis* MK-7 isomer produced from fermentation under the investigated conditions cannot be established in the absence of nuclear magnetic resonance (NMR) spectroscopy methods. It has been suggested that all-*trans* MK-7 is most likely to isomerise to the mono-*cis* isomer under certain conditions [32]. Therefore, it is anticipated that the *cis* MK-7 observed is a mono-*cis* isomer, which contains a single *cis* double bond in its isoprenoid chain. Nevertheless, complete structure determination is not essential when considering the bioactivity of fermented MK-7 end products, as all *cis* isomers, irrespective of the number and location of *cis* bonds in their isoprenoid side chain, have significantly reduced biological efficacy compared to the all-*trans* isomer.

Although our prior investigations have focused on optimising the extracellular environment, specifically the fermentation media [30] and key fermentation parameters [31], to increase the synthesis of the all-*trans* isomer and reduce the production of *cis* MK-7 during fermentation, it is crucial to guarantee that the quantity of the bioactive isomer is maintained in the final product. Hence, not only is it necessary to achieve a high concentration of all-*trans* MK-7 from fermentation, but its quantity must also be preserved in dietary supplements and functional foods to develop effective fermented MK-7 consumer end products. It has been postulated that exposure to light (especially ultraviolet (UV) light), atmospheric oxygen, and elevated temperatures may encourage the geometric isomerisation of isoprenoid residues in the side chain of MK-7 and result in the formation of *cis* isomers [7,23,25,33,34]. However, this aspect has not been explicitly explored, particularly in the context of MK-7 isomers produced from fermentation. The effect of typical environmental and storage conditions is worthy of consideration from the perspective of fermented MK-7 dietary supplements and fortified or functional foods, as these consumer end products are likely to be subjected to such factors during their manufacture, use, and shelf life, which will influence their effectiveness and therapeutic value.

Therefore, the objective of this study was to assess the effect of various storage conditions on the isomer profile of fermented MK-7 from the perspective of MK-7 end products. Accordingly, factors representing possible conditions and environments that MK-7 dietary supplements and fortified or functional foods may be subjected to during their production, consumption, and general shelf life were selected and examined. These include exposure to atmospheric oxygen, different temperatures, and light. All factors were initially investigated over a short interval, and the conditions that resulted in the least isomerisation

and/or degradation of all-*trans* MK-7 were further evaluated to explore the stability of the all-*trans* isomer in an ideal storage environment over an extended timeframe. The outcomes of this study will offer valuable insights for the development of optimum storage conditions to preserve the quantity of the all-*trans* isomer in fermented MK-7 end products. This will likely be an important progression in improving the accessibility of bioactive fermented MK-7 nutritional supplements and functional foods, as the *cis* isomers of the vitamin are effectively contaminants that have little therapeutic value. The widespread availability and consumption of such products by diverse populations will help boost the dietary intake of MK-7 and offer more significant health benefits to consumers.

2. Materials and Methods

2.1. Chemicals and Materials

The all-*trans* MK-7 reference standard (98.1% purity) was acquired from ChromaDex (Los Angeles, CA, USA). Glucose was obtained from Ajax Finechem Pty Ltd. (Taren Point, NSW, Australia). Yeast extract and tryptone were supplied by Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Soy peptone, methanol, 2-propanol, and *n*-hexane were obtained from Merck Millipore (Burlington, MA, USA). NaCl was acquired from a local supplier, and $CaCl_2$ was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Nutrient agar plates were procured from Fort Richard Laboratories (Auckland, New Zealand). All media components were microbiology grade, and all solvents were analytical grade.

2.2. Microorganism and Inoculum Preparation

Bacillus subtilis natto was selected for the fermentation experiments since it results in a high MK-7 yield and is considered the most suitable strain for industrial MK-7 production. Furthermore, there are no safety issues accompanying the *B. subtilis natto* strain, and it is generally recognised as safe (GRAS). Consequently, it is ideal for synthesising fermented MK-7 end products expected for human consumption. The procedure outlined by Berenjian et al. [20] was used to prepare the *B. subtilis natto* strain. The microbial cells were grown in an aqueous culture medium comprising yeast extract, tryptone, and NaCl and streaked on nutrient agar plates, which were incubated for 48 h at 37 °C. After incubation, the cells were removed from the plates and submerged in a sterile NaCl solution. The mixture was then put in a water bath for 30 min at 80 °C to inactivate the vegetative cells and stimulate the production of spores prior to centrifuging (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min to remove the cell debris. The resulting bacterial spore suspension functioned as the inoculum for the fermentation studies.

2.3. Fermentation Procedure

MK-7 was synthesised from fermentation using the optimal media and conditions determined from our previous investigations [30,31] to enable maximal all-*trans* and minimal *cis* MK-7 isomer production. The media, containing 1% (*w*/*v*) glucose, 2% (*w*/*v*) yeast extract, 2% (*w*/*v*) soy peptone, 2% (*w*/*v*) tryptone, and 0.1% (*w*/*v*) $CaCl_2$ [30], was prepared in bulk to maintain consistency and sterilised at 121 °C for 20 min by autoclaving (TOMY SX-700E, Tokyo, Japan). Afterwards, the samples were inoculated with 2% (*v*/*v*) of the *B. subtilis natto* spore suspension and fermented in individual McCartney bottles under aerobic conditions at 40 °C and 200 rpm for 7 days [31].

2.4. MK-7 Extraction

Following fermentation, MK-7 was extracted from the samples with 2-propanol and *n*-hexane, which were mixed in a ratio of 1:2 (*v*/*v*), and the liquid-to-organic ratio was 1:4 (*v*/*v*) [20]. The solution was vortexed for 2 min, and phase separation was achieved by centrifugation (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min. Due to the fat-soluble nature of MK-7, it favourably

dissolves in non-polar solvents, such as *n*-hexane. Thus, the hexane layer was isolated from the mixture and evaporated in another set of McCartney bottles under a vacuum to obtain the extracted MK-7.

2.5. Exposure Studies

The extracted MK-7 samples (contained in transparent McCartney bottles) were then exposed to various environmental and storage conditions to explore the short- and long-term impact of these factors on the MK-7 isomer composition. Different temperature (low (4 °C), ambient (20 °C), and high (100 °C)), light (no light/dark, ambient light, and UV light), and oxygen (exposed to atmospheric oxygen and not exposed to atmospheric oxygen) conditions were selected to simulate likely storage environments for fermented MK-7 consumer end products, such as MK-7-enriched fortified or functional foods and dietary supplements. Possible conditions that fermented MK-7 could be exposed to during the manufacture of these products were also considered.

The effect of short-term exposure to the different temperature, light, and oxygen conditions on the isomer profile of fermented MK-7 was initially assessed. As a part of this process, samples were subjected to the factors outlined in Table 1 for 0, 3, 6, and 9 days to investigate the variation in the isomer composition over a brief timeframe for all conditions.

Table 1. Environmental and storage conditions for the short-term exposure study.

Sample	Conditions
1	Low temperature (4 °C) and exposed to atmospheric oxygen = stored in the fridge with the lid off
2	Low temperature (4 °C) and not exposed to atmospheric oxygen = stored in the fridge with the lid on (purged with nitrogen)
3	High temperature (100 °C) and exposed to atmospheric oxygen = stored in the oven with the lid off
4	High temperature (100 °C) and not exposed to atmospheric oxygen = stored in the oven with the lid on (purged with nitrogen)
5	No light and exposed to atmospheric oxygen = stored in the dark with the lid off at ambient temperature (by default)
6	No light and not exposed to atmospheric oxygen = stored in the dark with the lid on (purged with nitrogen) at ambient temperature (by default)
7	Ambient light and exposed to atmospheric oxygen = stored in ambient light (lamp) with the lid off at ambient temperature (by default)
8	Ambient light and not exposed to atmospheric oxygen = stored in ambient light (lamp) with the lid on (purged with nitrogen) at ambient temperature (by default)
9	UV light and exposed to atmospheric oxygen = stored in UV light (lamp) with the lid off at ambient temperature (by default)
10	UV light and not exposed to atmospheric oxygen = stored in UV light (lamp) with the lid on (purged with nitrogen) at ambient temperature (by default)

The optimum storage conditions, which resulted in the least deterioration of all-*trans* MK-7, determined from the short-term investigation, were further analysed in a monitoring study to explore the stability of the all-*trans* isomer and variation in the isomer profile over an extended period. Accordingly, samples were prepared in triplicates and stored at a low temperature (4 °C) with minimal oxygen exposure in the absence of light for 8 weeks. The MK-7 isomer composition was analysed after 0, 1, 2, 3, 4, 5, 6, 7, and 8 weeks of storage.

2.6. MK-7 Analysis

At the conclusion of the exposure period, the MK-7 isomer composition of all samples was analysed, as discussed in our earlier study [30]. The all-*trans* and *cis* MK-7 concentrations were determined using a Dionex high-performance liquid chromatography (HPLC) instrument (Thermo Fisher Scientific, Waltham, MA, USA) composed of four P680 pumps, an ASI-100 automated sample injector, a TCC-100 thermostatted column compartment,

and a UVD340U photodiode array UV detector. A packed column (COSMOSIL Cholester, 100 mm × 2 mm × 2.5 µm; Nacalai Tesque Inc., Kyoto, Japan) was used to separate the compounds at 40 °C. Pure methanol constituted the mobile phase, and the compounds were eluted isocratically at a flow rate of 0.2 mL/min. The run-time, analytical wavelength, autosampler temperature, and injection volume were 30 min, 248 nm, 10 °C, and 10 µL, respectively. The Chromeleon 7 program (Thermo Fisher Scientific, Waltham, MA, USA) was used for data collection, and a relative retention time (RRT) of 1.12 was used to ascertain the *cis* isomer.

Liquid chromatography–mass spectrometry (LC–MS) methods were employed to verify the identity and corroborate the retention times of all-*trans* and *cis* MK-7, using the approach described in our previous investigation [30]. The LC–MS platform comprised a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC) system and a QExactive mass spectrometer with a HESI II source (Thermo Fisher Scientific, Waltham, MA, USA). The Thermo XCalibur 4.3 package (Thermo Fisher Scientific, Waltham, MA, USA) was used to operate the equipment, and data were obtained using the Chromeleon 7.3 application (Thermo Fisher Scientific, Waltham, MA, USA). The conditions summarised above were implemented for liquid chromatography; however, the injection volume was modified to 5 µL, and the run-time was increased to 37 min to suit the LC–MS system. Data were collected in the positive ionisation mode with a resolution of 70,000, a MS1 scan range of 150–1000 m/z, a maximum injection time of 200 ms, and an AGC target of 3×10^6. The Thermo FreeStyle 1.6 software (Thermo Fisher Scientific, Waltham, MA, USA) was utilised to evaluate the mass spectrometry (MS) data.

The MK-7 concentration of the samples was determined using a calibration curve (linear between 0.1 mg/L and 50 mg/L ($R^2 = 0.99$)), which was created with reference to the peak area corresponding to known concentrations of the analytical standard.

2.7. Statistical Methods

Statistical significance was determined by analysis of variance (ANOVA), and a two-sample *t*-test was used to compare the mean values of different groups. The data were reported as the mean ± standard deviation (SD) of three replicates, and significance was accepted at $p < 0.05$.

3. Results and Discussion

The various factors explored were selected to represent the likely storage environments for fermented MK-7 supplements and fortified or functional foods, together with potential conditions to which fermented MK-7 may be exposed during the manufacture of such products. For example, fermented MK-7-enriched dairy goods or supplements requiring cold temperature storage will probably be stored in the fridge at a low temperature (4 °C). In comparison, most other supplements and MK-7-enriched foods are likely to be stored and consumed at ambient temperature (20 °C). The packaging material and design for MK-7 products can influence the amount of light that MK-7 supplements and functional foods are exposed to, as dark, opaque, and transparent materials all permit the passage of variable amounts of light. Additionally, exposure to atmospheric oxygen is inevitable in the case of all MK-7 end products. High temperature (100 °C) conditions and exposure to UV light are likely to represent conditions that fermented MK-7 may be exposed to during the production, transportation, and storage of certain MK-7-enriched supplements and fortified or functional foods. All variables were first considered over a short timeframe. The optimal conditions that promoted minimal degradation and/or isomerisation of bioactive MK-7 were further explored to evaluate the stability of all-*trans* MK-7 over a longer period.

3.1. Effect of Environmental Factors and Storage Conditions on the MK-7 Isomer Composition

3.1.1. Light

The MK-7 isomer profile resulting from short-term storage in the dark at room temperature is outlined in Figure 2. Approximately 61% and 39% of the all-*trans* isomer and

87% and 67% of the *cis* isomer remained in the presence and absence of oxygen after 9 days of exposure. In addition, there is also no statistically significant difference in the all-*trans* and *cis* MK-7 isomer concentrations for the dark samples between days 3, 6, and 9 when assessing the presence and absence of oxygen independently ($p = 0.652$ and $p = 0.115$ for all-*trans* MK-7 in the presence and absence of oxygen and $p = 0.785$ and $p = 0.797$ for *cis* MK-7 in the presence and absence of oxygen). These findings indicate that no considerable reduction in the all-*trans* and *cis* MK-7 concentration occurs during short-term storage in the dark at ambient temperature, both with and without exposure to atmospheric oxygen.

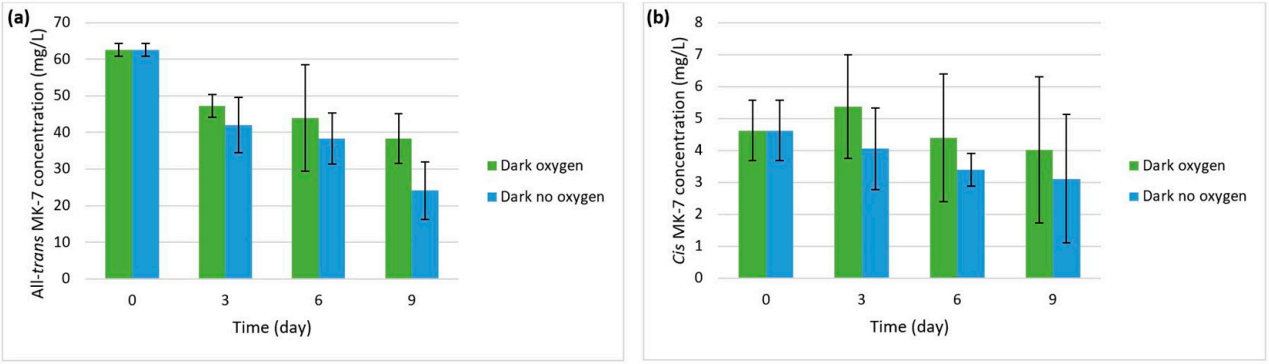

Figure 2. MK-7 isomer composition following short-term storage in the dark at ambient temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

Figure 3 illustrates the effect of ambient light at room temperature on the all-*trans* and *cis* MK-7 concentration, and it is evident that exposure to ambient light has a detrimental impact on the isomer concentration. Over 99% of all-*trans* and 100% of *cis* MK-7 was degraded to undetectable levels within 3 days of exposure to ambient light with and without oxygen, implying that contact with oxygen does not have a noticeable effect on MK-7 stability in the presence of ambient light. The influence of UV light exposure on the MK-7 isomer concentration is displayed in Figure 4. It is apparent that MK-7 is very unstable in UV light, as both all-*trans* and *cis* MK-7 were not detected over the entire exposure period, regardless of the presence or absence of atmospheric oxygen.

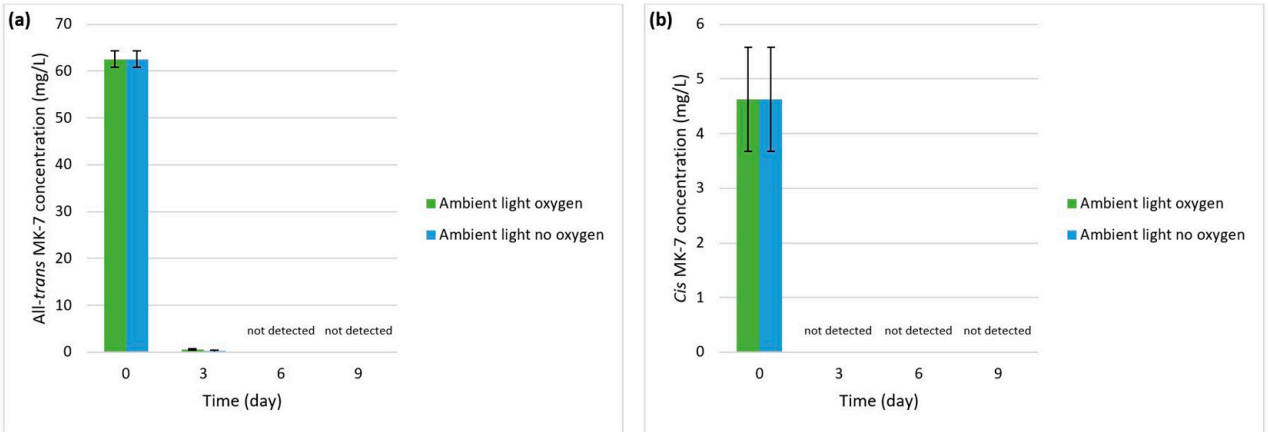

Figure 3. MK-7 isomer profile resulting from short-term exposure to ambient light at room temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

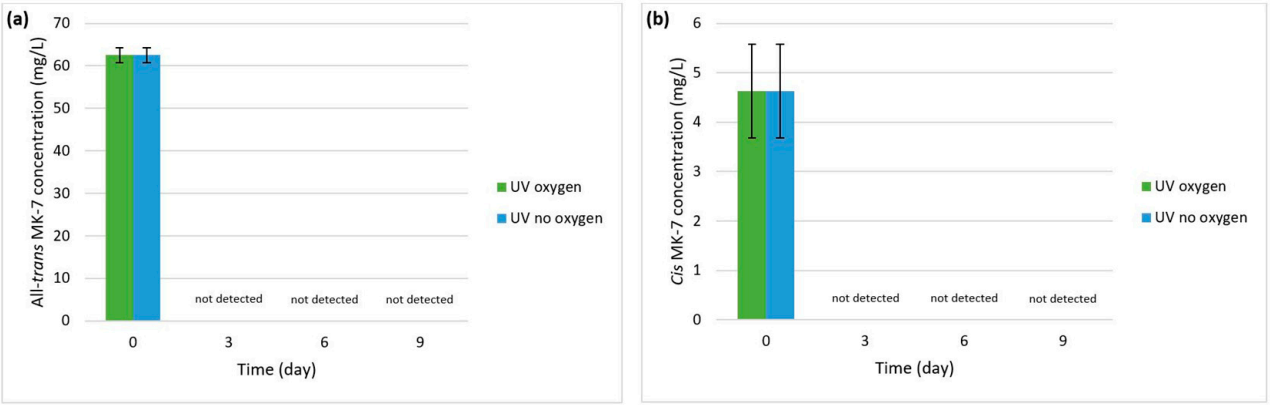

Figure 4. MK-7 isomer composition obtained from short-term exposure to UV light at ambient temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

Fat-soluble vitamins, including vitamin K, tend to be light-sensitive and are degraded by exposure to various forms of light, such as ambient light, daylight, and UV light [35–38]. It has been suggested that light exposure may promote photoisomerisation, which refers to the conversion of one isomer of a molecule to another by light. Transformation of all-*trans* MK-7 to one or more *cis* forms of the vitamin may potentially occur due to light; however, there are no specific reports in the literature [7,23,34]. Geometric isomerisation of all-*trans* MK-7 as a result of exposure to ambient or UV light was not observed in the current study. Instead, the concentration of all-*trans* and *cis* MK-7 was quickly reduced to undetectable levels in the presence of both types of light. It is also worth noting that the degree of photosensitivity of all-*trans* MK-7 might vary depending on the intensity and wavelength of light. Additionally, only particular intensities or wavelengths of light may induce the isomerisation of all-*trans* MK-7. Therefore, it could be that the light sources used in this investigation were not of the appropriate intensity or wavelength to promote this effect.

While it has been established that K vitamins are destroyed by light, limited research has been conducted to explore the stability of MK in the presence of different light sources. Moreover, studies explicitly considering MK-7 and its isomers in this context are absent. Ferland and Sadowski [39] assessed the PK content of several vegetable oils and evaluated the effect of light (daylight and fluorescent light) exposure on the stability of vitamin K1 in rapeseed and safflower oils over 22 days. It was determined that after only 2 days of exposure, the PK content of rapeseed and safflower oils was reduced by 46% and 59%, respectively, in the presence of fluorescent light and by 87% and 94%, respectively, when exposed to daylight. The effect of the type of storage container was also examined for rapeseed oil. It was ascertained that after 36 h of daylight and fluorescent light exposure, the PK content decreased by 93% and 44% for oil stored in clear bottles, respectively. In contrast, storage in amber bottles did not have a significant impact. Despite consideration of different types of light and vitamin K compounds, the results of this research are similar to the present study and demonstrate that vitamin K forms are highly vulnerable to light.

Collectively, these observations illustrate the destructive effect of various light sources on MK-7 and emphasise the importance of using dark or amber bottles and opaque packaging materials for the storage of fermented all-*trans* MK-7 dietary supplements and fortified or functional foods to preserve the quantity of the vitamin over its shelf life. UV exposure must also be avoided and is unsuitable for the manufacture of all-*trans* MK-7-containing supplements, milk, and other products that may require UV treatment as a means of processing or sterilisation. Strategies such as encapsulation, especially for dietary supplements, can also be implemented to further protect the all-*trans* isomer from exposure to light. Furthermore, it would be worthwhile to increase the awareness of consumers and state that exposure to light should be avoided on the product packaging to ensure the optimal performance of fermented all-*trans* MK-7 nutraceuticals.

3.1.2. Temperature

The impact of low (4 °C) and high (100 °C) temperatures on the MK-7 isomer composition was investigated in the presence and absence of oxygen. The results are depicted in Figures 5 and 6 for storage in the fridge (4 °C) and oven (100 °C), respectively. Around 68% and 61% of all-*trans* MK-7 and 79% and 61% of *cis* MK-7 remained in the presence and absence of oxygen at the end of the exposure period under low temperature conditions. There is also no statistically significant difference in the all-*trans* and *cis* MK-7 concentration for the fridge samples between days 3, 6, and 9 when considering the presence and absence of oxygen separately ($p = 0.997$ and $p = 0.944$ for all-*trans* MK-7 in the presence and absence of oxygen and $p = 0.749$ and $p = 0.098$ for *cis* MK-7 in the presence and absence of oxygen). This indicates that there is no notable decrease in the all-*trans* and *cis* isomer concentration over short-term exposure to low temperature conditions, both with and without contact with atmospheric oxygen. In comparison, approximately 17% and 33% of the all-*trans* isomer and 43% and 43% of *cis* MK-7 remained in the presence and absence of oxygen after 9 days of storage at a high temperature. Furthermore, there is no statistically significant difference in the all-*trans* and *cis* isomer concentrations for the oven samples between days 3, 6, and 9 when individually examining the effect of atmospheric oxygen ($p = 0.062$ and $p = 0.488$ for all-*trans* MK-7 in the presence and absence of oxygen and $p = 0.830$ and $p = 0.689$ for *cis* MK-7 in the presence and absence of oxygen). However, there is a statistically significant difference in the all-*trans* isomer concentration for the oven samples in the presence of oxygen between days 3 and 9 ($p = 0.002$). These observations suggest that although there is no appreciable difference in the isomer concentrations with and without contact with atmospheric oxygen between all three days of exposure holistically, there is a substantial decrease in the all-*trans* MK-7 concentration in the presence of oxygen between days 3 and 9. Thus, oxygen seems to accelerate the decomposition of the biologically active isomer at high temperatures. Although the effect of heat treatment and oxygen exposure has not been previously investigated for MK-7 isomers, it has been observed that during heating, oxygen increases the rate of degradation of all-*trans*-β-carotene, a precursor of vitamin A, which, similar to MK-7, is a lipid-soluble vitamin [40].

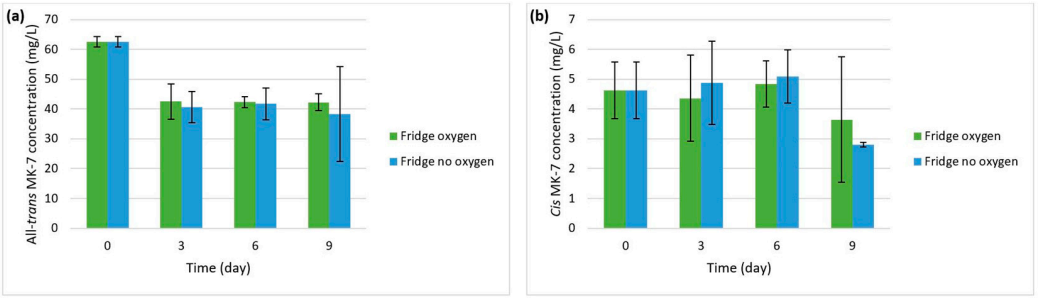

Figure 5. MK-7 isomer profile arising from short-term storage in the fridge at a low temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

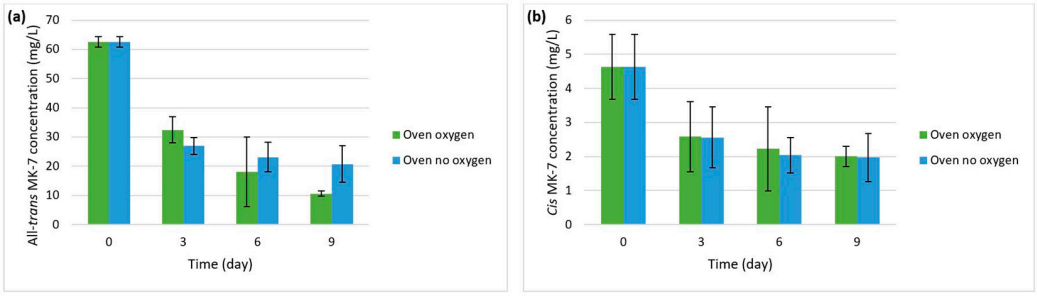

Figure 6. MK-7 isomer composition occurring from short-term storage in the oven at a high temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

The stability of vitamin K compounds is only slightly affected by heat exposure; hence, they are regarded as fairly heat-stable [35,36,41]. While there are no prior studies assessing the influence of heat on the stability of MK-7 isomers specifically, Ferland and Sadowski [39] have investigated the thermal stability of vitamin K1 in different vegetable oils at temperatures between 185 and 190 °C over 20 and 40 min. A slight loss of PK was observed, and approximately 7% and 11% of the original vitamin K was lost over 20 and 40 min of exposure, respectively. The findings of this research are largely comparable with the present study. Despite the differences in the investigated K vitamers, temperatures, and exposure times between the two studies, they both demonstrate a moderate loss of vitamin K upon heating, suggesting that MK-7 is relatively stable when exposed to reasonably high temperatures over a short period.

These observations imply that thermal sterilisation and manufacturing processes involving high temperatures, such as milling and drying for the synthesis of MK-7 dietary supplements and extrusion cooking for the production of cereals and other foods fortified with MK-7, may not reduce the MK-7 content significantly. Although such technological processes could require greater temperatures than that investigated in the current study (100 °C), the exposure times are expected to be much shorter (over a few minutes or hours rather than for 3, 6, or 9 days), which will likely mitigate the detrimental effect of higher temperatures. Additionally, encapsulation methods could be used to protect all-*trans* MK-7 in processes involving extreme temperatures and/or long handling times.

It is also evident that storage of MK-7 at ambient temperature is acceptable. This is demonstrated by the relatively high MK-7 content remaining after 9 days for the samples that were stored in the dark at room temperature (Figure 2). Moreover, there is no statistically significant difference in the all-*trans* and *cis* MK-7 concentrations between the dark and fridge conditions in the presence and absence of oxygen following short-term exposure ($p = 0.498$ for all-*trans* MK-7 and $p = 0.832$ for *cis* MK-7 from the overall ANOVA analysis of the dark and fridge groups). These outcomes also indicate that the negligible MK-7 isomer concentrations observed for both the ambient and UV light samples were due to exposure to the different light conditions and did not result from storage at room temperature.

The amount of light exposure was similar between the samples kept in the dark, fridge, and oven, as closure of the fridge and oven doors also eliminated light exposure in the low and high temperature conditions. This allows the effect of temperature to be meaningfully assessed between these groups. The ANOVA results indicate that there is a statistically significant difference in the all-*trans* isomer concentration between the dark, fridge, and oven conditions in the presence and absence of oxygen over the entire exposure period ($p = 2.201 \times 10^{-4}$). However, there is no statistically significant difference in the *cis* MK-7 concentration between these three groups in the presence and absence of oxygen over the investigated timeframe ($p = 0.149$). This suggests that while exposure to high temperatures negatively impacts the concentration of the bioactive isomer, it does not considerably affect the concentration of the biologically insignificant isomer.

3.1.3. Atmospheric Oxygen

Vitamin K is slowly affected by exposure to atmospheric oxygen [35,41]. It has been proposed that contact with atmospheric oxygen during the storage of MK-7 dietary supplements may lead to autoxidation processes, which adversely impact the concentration of the all-*trans* isomer and promote its isomerisation to *cis* MK-7 [7,23,34]. Nevertheless, this phenomenon has not been explicitly assessed, particularly for MK-7 isomers produced from fermentation.

The experimental observations from the present investigation suggest that contact with atmospheric oxygen does not have a substantial negative effect on the stability of MK-7 over a short period. The statistical analysis for the dark, fridge, and oven conditions (Table 2) indicates that there is no statistically significant difference ($p > 0.05$) in the all-*trans* and *cis* MK-7 concentrations between the samples that were and were not in contact with atmospheric oxygen for each day of the exposure period. For the ambient and UV light

conditions, all-*trans* MK-7 was only noted in the presence and absence of oxygen for the ambient light samples on day 3 of exposure, and there was no statistically significant difference in the all-*trans* isomer concentration between these two groups (p = 0.109). Furthermore, since all-*trans* and *cis* MK-7 were essentially undetectable following short-term exposure to ambient and UV light, it is evident that both isomers are rapidly degraded due to the different light exposures, irrespective of contact with atmospheric oxygen.

Table 2. Comparison of the MK-7 isomer concentrations between the oxygen and no oxygen samples on each day of the exposure period for the dark, oven, and fridge conditions using a two-sample *t*-test.

DARK		**FRIDGE**		**OVEN**	
All-*trans* MK-7 concentration		**All-*trans* MK-7 concentration**		**All-*trans* MK-7 concentration**	
Groups compared	*p*-value	Groups compared	*p*-value	Groups compared	*p*-value
Day 3 oxygen and no oxygen	0.416	Day 3 oxygen and no oxygen	0.754	Day 3 oxygen and no oxygen	0.212
Day 6 oxygen and no oxygen	0.653	Day 6 oxygen and no oxygen	0.893	Day 6 oxygen and no oxygen	0.610
Day 9 oxygen and no oxygen	0.125	Day 9 oxygen and no oxygen	0.748	Day 9 oxygen and no oxygen	0.085
***Cis* MK-7 concentration**		***Cis* MK-7 concentration**		***Cis* MK-7 concentration**	
Groups compared	*p*-value	Groups compared	*p*-value	Groups compared	*p*-value
Day 3 oxygen and no oxygen	0.416	Day 3 oxygen and no oxygen	0.736	Day 3 oxygen and no oxygen	0.984
Day 6 oxygen and no oxygen	0.454	Day 6 oxygen and no oxygen	0.772	Day 6 oxygen and no oxygen	0.852
Day 9 oxygen and no oxygen	0.699	Day 9 oxygen and no oxygen	0.603	Day 9 oxygen and no oxygen	0.960

Therefore, oxygen exposure is unlikely to significantly influence the all-*trans* MK-7 concentration of products that are consumed quickly and have a short shelf life, such as fortified or functional dairy products containing bioactive MK-7. However, it may adversely affect the all-*trans* MK-7 content of dietary supplements and products that are consumed over a longer period and have an extended shelf life. Encapsulation may be an effective technique to minimise the oxygen exposure of MK-7 contained in dietary supplements. Including labels on products to lessen their contact with oxygen (by closing the bottle lid or securing the packaging material) is also advisable for MK-7-enriched goods with a long shelf life.

3.1.4. Geometric Isomerisation of All-*trans* MK-7

It is interesting to notice that although it has been proposed that vitamin K, particularly all-*trans* MK-7, is susceptible to isomerisation upon exposure to various conditions, such as light, atmospheric oxygen, and elevated temperatures [7,23,34], conversion of the all-*trans* isomer to the *cis* form was not observed in the current study (only degradation of the vitamin and reduction in the concentration of both isomers was noted). A decrease in the concentration of the biologically effective isomer and a concurrent increase in the concentration of the *cis* isomer over time would denote the geometric isomerisation of all-*trans* MK-7. The *cis* MK-7 isomer concentration fluctuated considerably over the different days of exposure. While a slight increase in the concentration of *cis* MK-7 was observed for the fridge and dark storage conditions, there is no statistically significant difference in the *cis* isomer concentration in the presence and absence of oxygen between days 0 and 9 for the fridge and dark samples (p = 0.850). Moreover, a general downward trend in the *cis* isomer concentration can be noted over the entire exposure period for not only the fridge and dark samples but for all investigated storage conditions. This indicates a gradual decline, rather than an increase, in the *cis* isomer concentration over the 9-day storage period. Hence, it can be concluded that geometric isomerisation of all-*trans* MK-7 did not occur during the short-term exposure study. A potential explanation for the lack of isomerisation observed in this investigation could be that the conditions explored were reasonably mild and may not be sufficient to stimulate the isomerisation of all-*trans*

MK-7 over the timeframe explored. However, it may be possible for isomerisation to occur following longer periods of exposure to the same conditions. Alternatively, different conditions and/or harsher and more extreme environments may be required to promote the geometric isomerisation of the all-*trans* isomer.

3.2. Stability of All-trans MK-7 in an Optimal Storage Environment

A monitoring study was conducted to assess the stability of all-*trans* MK-7 and variation in the isomer composition over an extended timeframe during storage at a low temperature (4 °C) with minimal oxygen exposure in the absence of light.

In the short-term exposure study, the smallest decline in the concentration of the bioactive isomer was observed for the samples stored in the fridge at a low temperature and in the dark at ambient conditions. As previously outlined, the results obtained for the dark and fridge samples were comparable over a short span (no statistically significant difference in the MK-7 isomer concentration existed between these two groups). However, it is recognised that heat has a negative impact on the stability of MK-7 over a longer interval. Therefore, it was decided to consider storage at low rather than ambient temperature conditions over an extended period. The samples were also stored in dark/opaque bottles to further eliminate any light exposure, as it was determined from the short-term study that light has a detrimental effect on the stability of MK-7. In addition, no statistically significant difference in the MK-7 isomer concentration was noted between the samples that were and were not in contact with atmospheric oxygen during the short-term exposure investigation. Although oxygen is known to slowly impact the stability of MK-7, the effect of which is only likely to be observed after a prolonged period, the samples for the long-term study were just stored with the lid on (to decrease oxygen exposure) and not purged with nitrogen. This was done to simulate the potential environmental conditions that bioactive fermented MK-7 consumer end products will likely be subjected to during their manufacture, consumption, and overall shelf life, as, in reality, it would not be feasible to avoid exposure to atmospheric oxygen completely.

The samples were kept in an optimum storage environment for an extended timeframe. Figure 7 illustrates the variation in the all-*trans* and *cis* isomer concentrations over the long-term storage investigation. There was no appreciable change in the concentration of both isomers during 8 weeks of storage at a low temperature in the dark with minimal oxygen exposure. This implies that all-*trans* MK-7 is reasonably stable in this environment and is not susceptible to geometric isomerisation under optimal conditions.

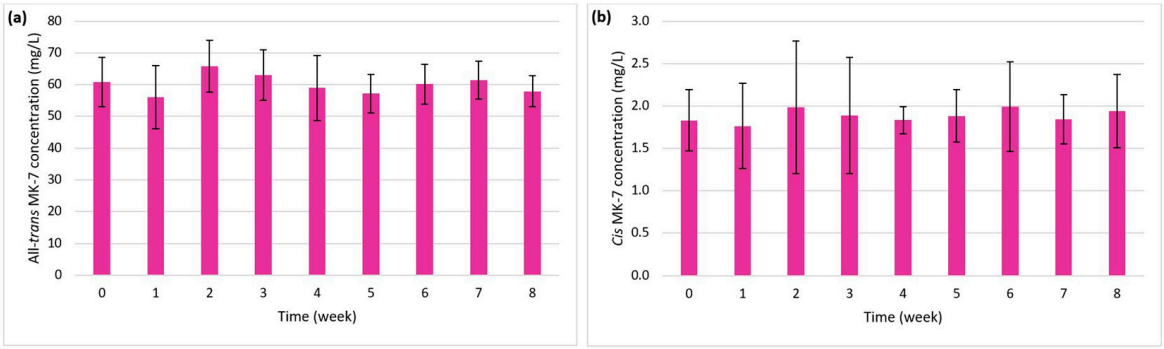

Figure 7. Variation in the isomer concentration over 8 weeks of storage at a low temperature with minimal oxygen exposure in the absence of light for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

These observations are supported by the ANOVA assessment, which indicates no statistically significant difference in the all-*trans* and *cis* isomer concentrations between the different weeks of exposure (p = 0.951 for all-*trans* MK-7 and p = 1.00 for *cis* MK-7). Additionally, a comparison of the MK-7 isomer concentration for every week of storage (weeks 1–8) with the control (week 0) via a t-test revealed that both the all-*trans* and *cis*

MK-7 concentration for each week of storage did not differ significantly from that of the control ($p > 0.05$).

Overall, the results of the long-term monitoring study demonstrate that low temperature conditions, reduced oxygen exposure, and the absence of light constitute the ideal storage environment for fermented MK-7. These conditions prevent the deterioration and preserve the concentration of the bioactive isomer, thereby retaining the therapeutic value of fermented MK-7 products.

3.3. Study Limitations

The findings of this investigation offer key insights into the effect of typical environmental and storage conditions that fermented MK-7 supplements and fortified or functional foods are likely to be subjected to on the MK-7 isomer profile and have shed light on the stability of the all-*trans* isomer over an extended period in an optimal storage environment. The ideal conditions to preserve the concentration of fermented all-*trans* MK-7 constituted the absence of light, low temperatures, and minimal oxygen exposure. Therefore, it is proposed that, where appropriate, fermented MK-7 products are packaged in dark/opaque bottles or materials and stored with the lid on or packaging tightly secured in the fridge at low temperature conditions (around 4 °C) to ensure that they retain their biological efficacy.

Although the impact of these factors on the isomer composition has been considered from the perspective of fermented MK-7 consumer end products, they have been examined in isolation. Thus, the experimental observations are restricted, and the conclusions drawn may differ slightly when the fermented MK-7 is actually formulated into supplements and fortified or functional foods.

It has been established that MK-7 is liable to degradation during storage, and the rate at which this occurs is accelerated in certain environments. Whilst exposure to specific storage conditions has been investigated in this study, it only covers a subset of the many factors that may be encountered during the manufacture and overall shelf life of a particular product. In addition, different preparations of bioactive fermented MK-7 may be exposed to unique storage environments depending on the characteristics of the final product (tablets, capsules, or fortified/functional foods).

For instance, fermented all-*trans* MK-7 formulated into tablets or capsules is likely to be exposed to various excipient compounds and active ingredients (in the case of multi-nutrient supplements), such as magnesium oxide (MgO), calcium carbonate ($CaCO_3$), calcium citrate ($Ca_3(C_6H_5O_7)_2$), cellulose, gelatine, and other vitamins and minerals. Certain compounds, including MgO, may also promote alkalinisation, and since MK-7 is vulnerable to alkaline conditions, such additives can create an unfavourable milieu that may enhance its deterioration. Hence, the inclusion of additional compounds and ingredients and their different combinations can create and expose the vitamin to different environments, which can affect the isomer profile and stability of all-*trans* MK-7 in different preparations. In contrast, fermented bioactive MK-7-enriched fortified or functional foods will likely be subjected to a different set of environmental factors specific to the selected food matrix and its storage requirements. For example, fresh dairy products require refrigeration at low temperatures and have a relatively short shelf life compared to cereals and other dry goods, commonly stored at ambient conditions over a longer period. Therefore, the isomer composition and stability of all-*trans* MK-7 will likely vary with the nature of the end product. Consequently, future research efforts need to be directed towards exploring and comprehensively understanding the effect of different environmental factors and storage conditions on the isomer composition and stability of all-*trans* MK-7 in context rather than independently of the desired application.

While prior studies have examined the stability of commercially available MK-7 dietary supplements and similar preparations, there have been no attempts thus far to explore the stability and isomer profile of fermented MK-7 in different types of formulated products. Furthermore, since the therapeutic benefits of fermented MK-7 nutritional supplements and fortified or functional foods solely result from the quantity of all-*trans* MK-7, it is essential

to ensure that they contain the bioactive isomer almost exclusively or in the most significant proportion following their manufacture, during their consumption, and throughout their overall shelf life. Therefore, in subsequent investigations, it would be advantageous to consider formulating fermented all-*trans* MK-7 into various consumer end products, such as tablets, capsules, and fortified or functional foods, to develop a deeper understanding of the effect of different production processes, preparations, and foods matrices on the MK-7 isomer profile.

In future work, it would also be valuable to carry out shelf life or degradation studies to explore both the short- and long-term stability of fermented all-*trans* MK-7 when formulated in various dietary supplement preparations and fortified or functional foods. This will allow the impact of a range of environmental and storage conditions, including product-specific features and those relating to the proposed packaging materials and design, on the quantity of bioactive MK-7 and the isomer composition resulting from different end uses to be elucidated. Factors often governed by commercial pressures also contribute to the overall shelf life of a product, and these include the time it takes for it to reach the consumer, the range of temperatures and climates that it is likely to be subjected to between production and consumption, and the rate at which it is expected to be consumed. Thus, such aspects also need to be considered in future research when assessing the stability of fermented all-*trans* MK-7 over its shelf life after it has been formulated into a diverse range of consumer products. Additionally, it is vital to ensure that a therapeutically significant concentration of the vitamin remains in the product at the end of its shelf life after taking into account the impact of the many factors that contribute to its holistic storage environment.

Essentially, the outcomes of appropriate shelf life and degradation studies examining the stability of fermented all-*trans* MK-7 in different product formulations will aid the estimation of a realistic shelf life and initial content (overage) of the vitamin in various end products in the future. This will also inform decisions regarding the product packaging and its recommended storage conditions to preserve the quantity of the bioactive isomer in specific applications.

4. Conclusions

This investigation presents unique insights into the impact of various environmental factors and storage conditions that fermented MK-7 consumer end products, such as dietary supplements and fortified or functional foods, may be subjected to during their production, consumption, and overall shelf life. The selected parameters included exposure to atmospheric oxygen, different temperature conditions, and light. These factors were first considered over a short interval to determine the optimal storage conditions. Essentially, there appeared to be no discernible differences in the degradation profiles of all-*trans* and *cis* MK-7 under the studied conditions, as, despite minor dissimilarities (possibly due to experimental variation), the overall trends in the reduction of both isomers with exposure to the various factors were comparable. Storage in the absence of light at a low temperature with minimal oxygen exposure preserved the quantity of the all-*trans* isomer to the greatest extent and was the ideal storage environment for fermented MK-7. The stability of the biologically significant MK-7 isomer under the optimal conditions was then evaluated over an extended period, and negligible change in the concentration of all-*trans* MK-7 occurred after 8 weeks of storage. This implies that the all-*trans* isomer is reasonably stable and not prone to substantial degradation during long-term storage in this environment. The findings of this study are significant, as they will facilitate the development of suitable storage conditions to maintain the concentration of the all-*trans* isomer in fermented MK-7 end products. The results will also aid the estimation of suitable overage levels of the vitamin in different products to account for its deterioration during storage, which will ensure that the amount of the biologically important isomer remaining at the end of a product's shelf life is not below the required or stated quantity. Collectively, this will be a significant advancement in improving the availability of bioactive fermented MK-7 nutritional supplements and fortified or functional foods, as the *cis* isomers

have considerably compromised biological function and therapeutic value. The broad consumption of such efficacious products by a range of populations will boost the dietary intake of MK-7 and help decrease the risk and progression of several age-related disorders and diseases of global relevance.

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