

# A Novel Fast HPLC-UV Method for Menaquinone-7 Determination in Fermentation Broth

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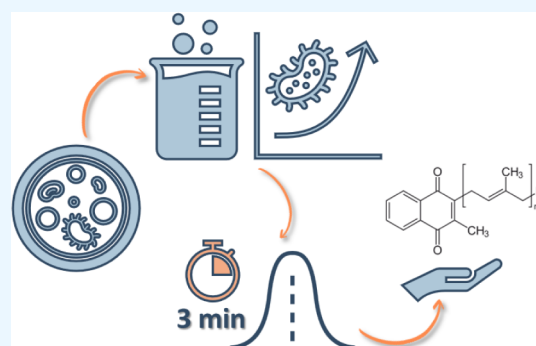


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**ABSTRACT:** Menaquinone-7 (MK-7) plays a key role in bone and cardiovascular health, and it is often included in multivitamin supplements and functional foods. The aim of this study is the development and validation, according to ICH guidelines, of a fast, highly sensitive, and reliable reverse-phase HPLC-UV quantification of vitamin K<sub>2</sub> as MK-7 in a common biological matrix sample, such as fermentation broth medium from *Bacillus subtilis*, with the advantage of single-step extraction and short analysis time. MK-7 was successfully isolated from the fermentation broth and its interfering residues by thermo-acidic extraction using 5% H<sub>2</sub>SO<sub>4</sub> and ethanol, without derivatization, and was retained using a C8 column. The isocratic elution and UV detection at 268 nm within a 3-min run time allowed quantification over a wide accuracy range of 0.10–18.00 µg/mL, with an LOQ value of 0.10 µg/mL and an LOD value of 0.03 µg/mL. The method was found to be precise, accurate, and reliable, with RSD% lower than 5% and recoveries varying between 96.0% and 108.9%. The proposed method unlocks the opportunity to be suitable for routine analysis in the screening of bacterial strains producing MK-7, as well as to be successfully employed in manufacturing processes in the nutraceutical and food industries where complex matrices are involved.



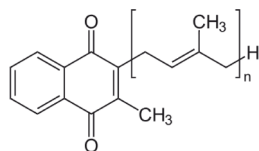
## INTRODUCTION

Vitamin K<sub>2</sub> or menaquinone (MK) is part of a family of naturally occurring fat-soluble vitamins. It is mainly present in fermented foods<sup>1</sup> (e.g., natto,<sup>2</sup> fresh fermented dairy products, cheese<sup>3</sup>) and animal products. The vitamin K<sub>2</sub> family exists in various forms, sharing a common core structure of a methylated naphthoquinone ring scaffold (2-methylnaphthalene-1,4-dione) and differing in the isoprenoid side chain length, which ranges from 4 to 13 isoprenyl units, as shown in Figure 1.

Menaquinones play a key role in bone and cardiovascular health. They are directly linked to calcium homeostasis by preventing bone calcium dissolution and mobilization, and they contribute to bone formation by promoting calcium deposition. Menaquinones also regulate osteoblast function,

and they can effectively stabilize mobile calcium, reduce artery calcium levels, inhibit calcium deposition in blood vessel walls, and prevent the occurrence of cardiovascular diseases. In particular, there is a growing interest in menaquinone-7 (MK-7) because of its involvement in bone formation, as well as its higher bioavailability<sup>4</sup> and longer half-life<sup>5</sup> in blood, due to its more hydrophobic nature compared to other homologues of the menaquinone family. Therefore, MK-7 can be considered the obvious choice to be included in multivitamin supplements and functional foods<sup>6</sup> for the prevention of cardiovascular diseases and calcification of blood vessels.

Highly sensitive, reliable, and fast determination of vitamin K<sub>2</sub> as MK-7 in complex matrices remains a crucial issue for several reasons, considering its important role in the human organism.<sup>7</sup> In particular, routine analysis is a significant challenge since MK-7 is mainly produced by a metabolic pathway, which involves *Bacillus subtilis* natto.<sup>8–10</sup> Indeed,



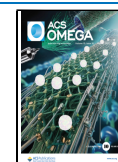
**Figure 1.** General structure of vitamin K<sub>2</sub>, i.e., MK-*n* where *n* stands for the number of isoprenoid side chain residues ranging from 4 to 13.

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*Bacillus subtilis*, originating from *natto*, is the most employed microorganism for the industrial production of dietary MK-7 supplements.<sup>11</sup> The bacterium growth during fermentation can be optimized by selecting appropriate fermentation process parameters<sup>12–14</sup> such as temperature, pH, oxygen levels, and nutrient availability, to maximize MK-7 production. In this context, an agile, fast, and efficient analytical method is needed to screen for menaquinone-producing strains. The bacterial cultures resulting from fermentation processes, unlike synthetic processes, may be complex in composition and contain different classes of molecules.<sup>15</sup> Several consecutive steps of preliminary cleanup and extraction are often needed<sup>16,17</sup> because of the presence of lipids, such as esters of fatty aromatic acids, or interfering substances coming from bacterial cell debris and fermentation broth-related byproducts. The major consequences are the difficulty of having an accurate extraction of the desired metabolite from the broth and the contamination of the chromatographic profile by coeluting peaks. Currently, the quantitative determination of vitamin K<sub>2</sub> as MK-7 in fermentation broth of bacterial cultures is mostly performed using chromatographic techniques. The most commonly reported chromatographic technique for separating, identifying, and quantifying vitamin K<sub>2</sub> homologues in biomatrices is reverse-phase HPLC. The stationary phases most frequently employed for separation are C18 and C30 phases, combined with mobile phase mixtures typically containing polar and nonpolar solvents, such as methanol and water as polar solvents, or isopropanol, acetonitrile, and *n*-hexane<sup>18</sup> as nonpolar solvents, often separating analytes by gradient elution.<sup>19</sup> Chromatographic runs developed with combinations of C18 or C30 phases and the aforementioned solvent mixtures often result in time-consuming analytical methods for fast screening.<sup>20</sup> Some elutions also involve acidic mobile phase pH to optimize peak shape; therefore, acetic acid or orthophosphoric acid are commonly added to the mobile phase for this purpose. Moreover, a major problem in MK-7 quantification is its low solubility in polar solvents. Indeed, the analysis is frequently carried out after evaporation of an MK-7 extract in a nonpolar solvent, such as a mixture of isopropanol and *n*-hexane,<sup>21,22</sup> followed by resolubilization in polar solvents like methanol or a mixture of methanol and dichloromethane. This procedure, however, introduces errors in the accuracy of MK-7 quantitation in fermentation broths.<sup>23</sup> On the other hand, MK-7 is often difficult to dissolve in the polar mobile phases typically used for reverse-phase HPLC due to its low polarity; this behavior could result in lower quantitation of the actual MK-7 concentration present in the samples.

LC-MS and LC-MS/MS<sup>24–27</sup> analytical methods for MK-7 have been developed and have significantly advanced over the past decade, offering the advantages of high sensitivity and accuracy; however, many laboratories are not equipped with this instrumentation, as it is very expensive in terms of cost, maintenance, and sample prepurification.

HPLC analysis with fluorescence detection is reported because of its greater sensitivity compared to UV detection, but it involves derivatization of the molecule before injection and the need for an internal standard,<sup>28</sup> thus increasing the risk of experimental errors due to several manipulation steps, or post-column derivatization, thereby increasing run time.

Voltammetric determination of menaquinone produced by *Bacillus subtilis* cultures is indicated and characterized by fast voltammogram recording, albeit with the inability to

distinguish between the homologues of the vitamin K<sub>2</sub> family.<sup>29</sup>

In this study, a novel, fast method for the determination of vitamin K<sub>2</sub> as MK-7 in biological matrix samples, such as fermentation broth medium, was developed and validated, offering the advantages of accurate extraction followed by rapid C8 reverse-phase HPLC-UV quantification at low cost and within a short time.

## EXPERIMENTAL SECTION

**Reagents and Materials.** Methanol (MeOH), ethanol (EtOH), isopropyl alcohol (IPA), and tetrahydrofuran (THF), all HPLC grade, were purchased from VWR (part of Avantor, USA). Sulfuric acid 98% (H<sub>2</sub>SO<sub>4</sub>) was purchased from Carlo Erba (Italy). A Milli-Q water system (Millipore, USA) was used to obtain ultrapure water (18 MΩ cm<sup>-1</sup>). The vitamin K<sub>2</sub> MK-7 reference standard was obtained from Gnosis by Lesaffre (France).

**Chromatographic Analysis.** An HPLC instrument (Agilent Technologies, USA), model 1260, equipped with a VialSampler (G7129A), a QuatPump (G7111B), an MCT (G7116A), and a DAD-HS (G7117C), was used. System control and data analysis were carried out with OpenLab CDS 2.4 software (Agilent, USA). A Kinetex reverse-phase C8 column (2.6 μm, 100 mm × 4.6 mm, Phenomenex, USA) was achieved. An isocratic elution was performed at a flow rate of 1 mL/min at 35 °C. A mobile phase consisting of MeOH:EtOH: water (80:19.5:0.5, v/v/v) was used. The chromatographic run time was 3 min, with a retention time (RT) of 2.18 min for MK-7. The sample injection volume was 5 μL, and the autosampler temperature was set at 12 °C. MK-7 was detected at a wavelength of 268 nm.

**Preparation of Standard Solutions.** A stock standard solution of MK-7 (400 μg/mL) was prepared by dissolving the MK-7 reference standard in a mixture of THF:EtOH (2:98, v/v) as the diluent. Working standard solutions were prepared by diluting the stock standard solution in EtOH to concentrations of 0.10, 0.15, 0.37, 0.75, 1.50, 7.50, 12.00, 13.50, 15.00, and 18.00 μg/mL. Stock and working standard solutions were stored at –20 °C and protected from light.

**Extraction of MK-7 from Fermentation Broth.** MK-7 was extracted from the fermentation broth using a thermo-acidic extraction procedure. 400 μL of fermentation broth, 200 μL of H<sub>2</sub>SO<sub>4</sub> 5%, and 5 mL of EtOH were sequentially transferred into a 15 mL centrifuge tube. After brief mixing, the centrifuge tube was placed in an ultrasonic bath at 70 °C for 15 min, with shaking every 5 min, to facilitate the extraction of MK-7 from the cells. The resulting mixture was centrifuged at 7800 rpm for 5 min at room temperature. The supernatant was filtered through a 0.45 μm RC filter and transferred to an amber glass vial before injection into the chromatographic system. The samples were protected from light during their preparation.

**Validation Method.** The proposed analytical method was validated following ICH guideline Q2(R2).<sup>30</sup> The parameters selected for validation were selectivity, limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, range, precision, and solution stability.

**Selectivity.** Selectivity is defined as the ability of a method to discriminate a specific analyte in a mixture without interference from other components. The selectivity of the proposed chromatographic method was evaluated by analyzing blank (EtOH), diluent (200 μL of H<sub>2</sub>SO<sub>4</sub> + 5 mL of EtOH),

matrix solution (cell culture medium), reference solution (MK-7 solution at target level), and test solution (fermentation broth sample). The blank, diluent, and matrix solutions should not exhibit any peak or shoulder peak at the retention time of MK-7. The retention time of the MK-7 peak in reference and test solutions should be consistent. The MK-7 peak should be well-resolved from any other peak.

**Limit of Detection (LOD).** The limit of detection is defined as the lowest concentration of the analyte in a sample that can be detected but not necessarily quantified. The LOD was determined based on a signal-to-noise ratio (S/N) between 3 and 10. To evaluate the LOD, serial dilutions of the MK-7 stock standard solution were prepared, and each dilution was injected six times.

**Limit of Quantification (LOQ).** The limit of quantification is defined as the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The LOQ was determined based on a S/N greater than or equal to 10. To evaluate the LOQ, serial dilutions of the MK-7 stock standard solution were prepared, and each dilution was injected six times. The mean and relative standard deviation (% RSD) of the peak area response were calculated. The % RSD should be less than 15%, and the mean % recovery value should be between 70% and 130%.

**Linearity.** The linearity of the proposed method was evaluated at ten concentrations of the working standard solutions (0.10, 0.15, 0.37, 0.75, 1.50, 7.50, 12.00, 13.50, 15.00, and 18.00  $\mu\text{g/mL}$ ), corresponding to 0.7%, 1.0%, 2.5%, 5.0%, 10.0%, 50.0%, 80.0%, 90.0%, 100%, and 120% of the target concentration, respectively. Three injections at each concentration level were analyzed. The linearity graph of the concentration versus peak response was plotted. Regression analysis was used to determine the slope, y-intercept, and correlation coefficient ( $R^2$ ). The correlation coefficient should be greater than or equal to 0.998.

**Accuracy.** Accuracy is defined as the closeness of agreement between the result obtained using the analytical method and the true value of the analyte in the sample. Accuracy was determined through recovery studies for MK-7 from the matrix solution. The matrix solution was spiked with an MK-7 stock standard solution at seven different concentration levels (0.10, 0.75, 3.00, 7.50, 10.50, 15.00, and 18.00  $\mu\text{g/mL}$ ), corresponding to 0.7%, 5.0%, 20.0%, 50.0%, 70.0%, 100%, and 120% of the target concentration, respectively. At each concentration level, three samples were prepared and analyzed. The percentage recovery was calculated as follows: Recovery (%) = (amount detected/amount expected)  $\times$  100. Recovery should be between 85% and 115%.

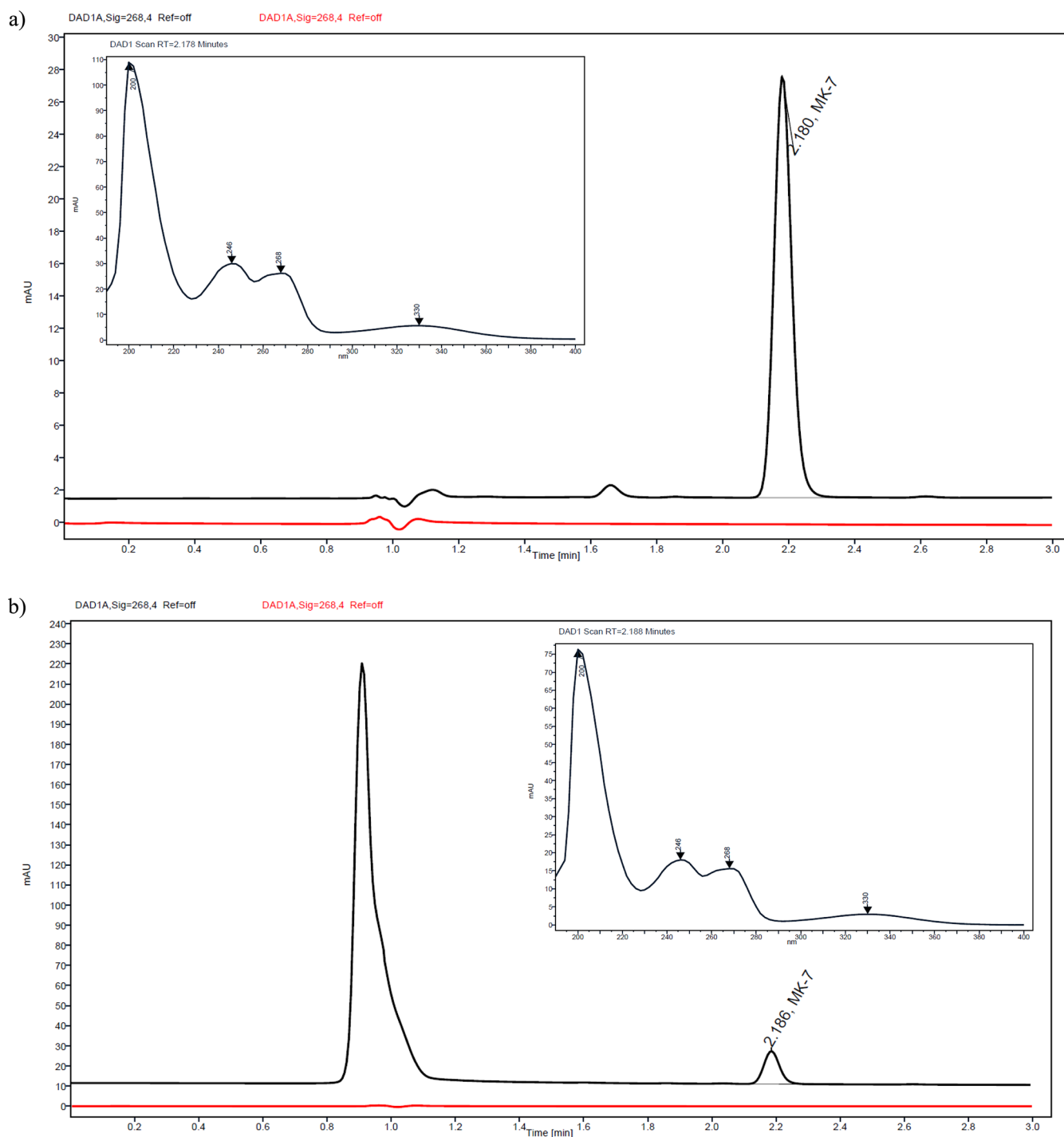
**Precision.** Precision is defined as the measure of the degree of scattering among a series of measurements obtained from the same homogeneous sample. The precision of the analytical method was evaluated at two levels: repeatability and intermediate precision. Repeatability (intraday precision) was determined by performing six independent measurements of the test solution on the same day. The % RSD ( $n = 6$ ) should be less than 5%. Intermediate precision (interday precision) was assessed by repeating the repeatability test on a different day, with a different analyst and chromatographic equipment. To enhance the reliability of the proposed chromatographic method, the MK-7 reference standard batch, laboratory consumables, solvents, and reagents were also included as variables to evaluate intermediate precision. The % RSD ( $n = 12$ ) should be less than 5%.

**Solution Stability.** The stability of the solutions is defined as the ability of a solution to maintain its physical and chemical properties over a specified period of time under defined storage conditions. The stability of the MK-7 reference solution and test solution was evaluated at time intervals of 0, 24, and 48 h, stored in the autosampler at 12  $^{\circ}\text{C}$  in amber glass vials. For the MK-7 reference solution, a single preparation was injected three times at each time point. The stability of the reference solution was determined by assessing the percentage difference (diff. %) in the average peak area relative to the measurement at  $t = 0$  h. For the test solution, three independent preparations were generated, each one injected as a singleton at each time point. The MK-7 concentration was calculated using a fixed calibration curve injected at  $t = 0$  h. The stability of the test solution was assessed by evaluating the diff. % in the concentration of each preparation relative to the corresponding concentration at  $t = 0$  h.

**Statistical Analysis.** The statistical analysis was performed using Minitab version 19 (Minitab, LLC, State College, PA). Least squares regression was applied to determine the optimal correlation between the predictor variable (concentration of MK-7) and the response variable (peak area). The statistical significance of the linear correlation was evaluated by using analysis of variance (ANOVA). The  $p$ -value was derived from the  $F$ -test, which compares the variance of the model to the residual error, providing the strength and reliability of the observed correlation. The data were reported as mean  $\pm$  standard deviation, and a  $p \leq 0.05$  was considered to be statistically significant.

## RESULTS AND DISCUSSION

**Sample Preparation Optimization.** The sample preparation for the extraction of MK-7 from fermentation broths required careful optimization to ensure high accuracy and reliability. As a starting point, a multistep liquid–liquid extraction approach was employed, utilizing a combination of organic solvents commonly used for extracting fat-soluble vitamins. This procedure involved the use of a phyloquinone internal standard and an extraction mixture of  $n$ -hexane, isopropanol, and water in a 50:30:20 (v/v/v) ratio, followed by evaporation to dryness under a nitrogen stream and a final reconstitution step in ethanol. However, this approach required a large volume of solvents (20–25 mL) and repeated extractions. Additionally, recovery tests conducted on spiked broths with an MK-7 stock standard solution yielded unsatisfactory accuracy results, with 80% recovery. To develop a more accurate, efficient, and rapid sample preparation, ethanol was selected as the extraction solvent with the addition of  $\text{H}_2\text{SO}_4$  (5%) for cell lysis. A subsequent step of sonication at 70  $^{\circ}\text{C}$  for 15 min improved the extraction of MK-7 from the bacterial cells. The selected solvent was compatible with the mobile phase due to its similar polarity, ensuring efficient separation and optimal interaction between the sample and the chromatographic system. This approach also improved the peak shape and resolution, enabling accurate quantitative measurement of MK-7 during the fermentation process. Based on these results, the extraction of MK-7 was also tested through enzymatic hydrolysis using lysozyme, followed by ethanol extraction. The results obtained were highly comparable to those achieved through thermo-acidic hydrolysis, which was preferred over enzymatic hydrolysis due to its shorter duration, fewer steps, and enhanced reproducibility in MK-7 extraction from fermentation broths.



**Figure 2.** HPLC-UV chromatogram of MK-7 working standard solution (a) at a concentration of 0.75  $\mu\text{g/mL}$  and UV trace extracted at RT = 2.18 min (black); HPLC-UV chromatogram of MK-7 test solution (b) extracted from fermentation broth and UV trace extracted at RT = 2.18 min (black). In both chromatograms (a and b) HPLC-UV chromatogram of blank solution is shown as an overlay (red). The x-axis of chromatograms is time (min) and y-axis is UV response at  $\lambda = 268$  nm.

**HPLC Method Optimization.** Preliminary experiments were performed according to the USP monograph for menaquinone-7, with the purpose of developing a faster HPLC-UV assay method. The chemical and physical properties of the column stationary phase are known to influence the resolution, shape, and retention time of the chromatographic peak. Even though the C18 column chemistry used in the USP assay method provided the best chromatographic separation for nonpolar compounds, the reverse-phase Kinetex C8

column was tested for the analysis of MK-7. This column, which has a core-shell-based particle with a bonded silica phase, helps to obtain a chromatogram ideal for the fast analysis of hydrophobic compounds such as MK-7, with good separation and the best compromise among sensitivity, selectivity, and run time. The elution time could be influenced by the column temperature. At room temperature (25  $^{\circ}\text{C}$ ), the retention time value increased despite the run time of the USP method, while at higher temperatures, the retention time value



**Table 1. List of Regression Parameters: Retention Time, Calibration Range,  $R^2$ , LOD, and LOQ**

Component	Retention time (min)	Calibration range ( $\mu\text{g/mL}$ )	Regression equation <sup>a</sup>	$R^2$	LOD ( $\mu\text{g/mL}$ )	LOQ ( $\mu\text{g/mL}$ )
MK-7	2.18	0.10–18.00	$y = 6.067x + 0.199$	0.9995	0.03	0.10

<sup>a</sup> $y$  = Peak area;  $x$  = Concentration of MK-7.

decreased; therefore, column temperature was accordingly set at 35 °C for optimum separation, and the run time was reduced to 3 min. All chromatograms of MK-7 were obtained by using an isocratic elution of the mobile phase. Different mobile phase compositions containing ethanol and Milli-Q water (97:3, v/v), or ethanol, methanol, THF, and Milli-Q water in the ratio of 15:80:10:1 (v/v/v/v), were tested according to the USP procedure.<sup>31</sup> Nevertheless, good separation in a reduced run time was achieved using a mixture of methanol, ethanol, and Milli-Q water in the ratio of 80:19.5:0.5 (v/v/v). The wavelength of 268 nm was chosen because it produced the best signal. Figure 2a,b shows the chromatographic separation of MK-7 (RT = 2.18 min) performed on the C8 column over a run time of 3 min.

**Validation of the HPLC Method.** Fermentation broth is a complex biological matrix that contains a variety of components, including cells and cellular debris from the microbial culture, nutrients such as carbon and nitrogen sources, byproducts of microbial metabolism, and impurities that may coelute with MK-7, interfering with its detection. The application of an HPLC-UV analytical method for the determination of MK-7 in the fermentation broth is a crucial aspect of the fermentation process. By employing robust sample preparation techniques and optimized chromatographic conditions, this method allows for precise and reliable quantification of MK-7, even in a complex fermentation matrix. The proposed method for the analysis of MK-7 was validated following the official ICH guidelines in terms of accuracy, linearity, specificity, precision, LOQ, LOD, and solution stability. A summary of the validation results is presented in Tables 1 and 2.

**Selectivity.** The HPLC method must discriminate MK-7 without interference from the other components. Chromatograms of blank, diluent, matrix solution, reference solution, and test solution were compared to ensure there were no interfering peaks at the RT of MK-7. As observed in the

overlaid chromatograms shown in Figure 3, the RT of the MK-7 peak in the reference solution is consistent with the RT of the MK-7 peak in the test solution, and the peak appears well resolved from any others. Chromatograms of blank, diluent, and matrix solution demonstrate that there is no interference from other compounds, thus confirming the selectivity of the method.

**LOD and LOQ.** LOD and LOQ were calculated based on the S/N approach, where the peak-to-peak noise around the analyte retention time was measured. The noise value was calculated based on the peak height of the blank around the retention time of the analyte using OpenLab CDS 2.4 software. As shown in Table 1, the LOD value obtained is 0.03  $\mu\text{g/mL}$  (average S/N = 8.78), and the LOQ value obtained is 0.10  $\mu\text{g/mL}$  (average S/N = 45.3). The LOQ value complied with the acceptance criteria; indeed, the % RSD for peak area ( $n = 6$ ) is 2.7%, and the mean recovery percentage is 85%. These results underline the relevance of the developed method's high sensitivity for MK-7 determination. As a matter of course, this approach highlights the method's capability to empirically determine the lowest concentration value of MK-7 that could be produced in the early stages of a fermentation process, thereby ensuring the reliability of direct measurements when monitoring the process itself. Nevertheless, some analytical methods report competitive LOD and LOQ values, making it necessary to perform an additional calculation of these validation parameters based on the linear regression approach as well.<sup>32</sup> This was intended to account for real-world measurement variations and enable meaningful cross-method comparability of results. Specifically, the LOD derived from the regression curve is 0.17  $\mu\text{g/mL}$ , and the LOQ is 0.50  $\mu\text{g/mL}$ . It is evident that the recalculated data are slightly higher than the former ones calculated based on the S/N approach because they are the outcome of a statistical extrapolation, yet they maintain the advantage of being comparable.

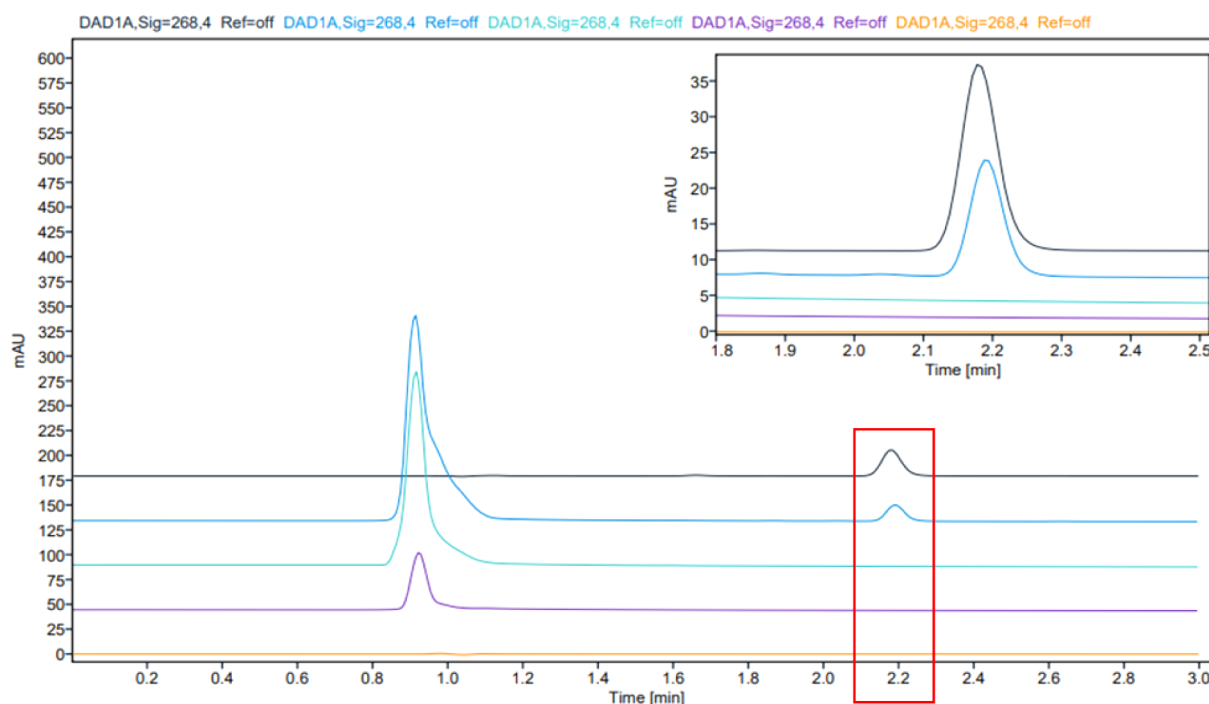
**Linearity and Range.** The linearity was evaluated at ten concentrations of the working standard solutions, ranging from 0.10 to 18.00  $\mu\text{g/mL}$ . These values were chosen to achieve reasonable coverage of the range of concentrations of MK-7 in fermentation broths, taking into consideration the dilution of the extract before HPLC-UV analysis. The mean peak areas ( $n = 3$ ) were plotted against the corresponding concentrations in  $\mu\text{g/mL}$ . The calibration curve, with 95% confidence bands obtained from unweighted least-squares linear regression analysis, is shown in Figure 4. The results showed good correlation ( $R^2 = 0.9995$ ). Hence, it was concluded that the method is linear in the range between 0.10  $\mu\text{g/mL}$  and 18.00  $\mu\text{g/mL}$ .

**Accuracy.** Since the fermentation broth is a challenging matrix, the method's accuracy is critical to ensure that MK-7 is accurately quantified. The accuracy of the proposed method was determined using a matrix solution spiked with different amounts of MK-7 stock standard solution. The calculated percentage recovery values are presented in Tables 2. The recovery of MK-7 from the samples varied between 96.0% and 108.9%. The extraction method and analytical procedure

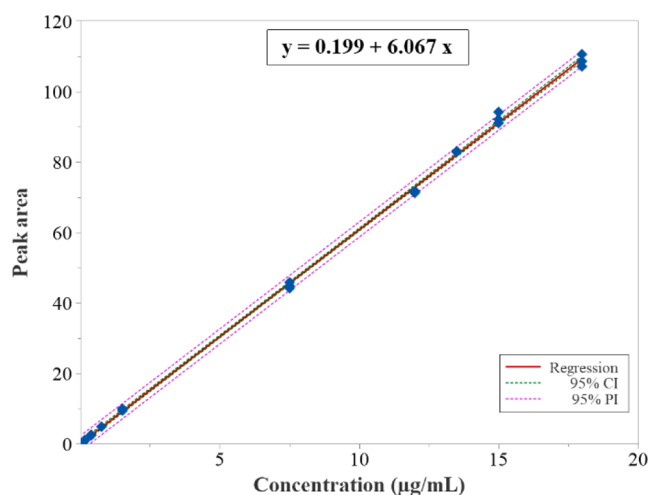
**Table 2. Chromatographic Method Validation Parameters**

Accuracy				
Theoretical (% of target level)	Expected concentration ( $\mu\text{g/mL}$ )	Recovery RE % <sup>a</sup>		
		Inj.1	Inj.2	Inj.3
0.7	0.10	96.0	99.2	105.0
5	0.75	99.3	100.8	99.2
20	3.00	108.9	102.8	103.5
50	7.50	101.2	101.4	100.9
70	10.50	103.0	100.5	103.2
100	15.00	101.8	101.7	101.2
120	18.00	100.6	101.8	101.4
Precision				
Repeatability (mean $\pm$ SD, $\mu\text{g/mL}$ ) <sup>b</sup>	% RSD	Intermediate precision (mean $\pm$ SD, $\mu\text{g/mL}$ ) <sup>c</sup>		% RSD
8.69 $\pm$ 0.08	0.97	8.34 $\pm$ 0.38		4.61

<sup>a</sup>Average of three determinations ( $n = 3$ ). <sup>b</sup>Average of six determinations ( $n = 6$ ). <sup>c</sup>Average of 12 determinations ( $n = 12$ ).



**Figure 3.** HPLC-UV overlaid chromatograms at  $\lambda = 268$  nm of blank (orange), diluent (violet), matrix solution (green), test solution (blue), and reference solution (black). The magnification square shows the baseline separation of the peak of the MK-7 at the retention time 2.18 min.



**Figure 4.** Calibration curve of ten levels of the MK-7 working standard from 0.10 to 18.00  $\mu\text{g/mL}$ .

developed were accurate for the determination of MK-7 in fermentation broths within the range of 0.10 to 18.00  $\mu\text{g/mL}$ .

**Precision.** The precision of the chromatographic method was estimated by measuring repeatability (intraday assay precision,  $n = 6$ ), giving the % RSD value as 0.97%, and intermediate precision (interday variation,  $n = 12$ ), giving the % RSD value as 4.61%, thus satisfying the acceptance criteria. These results provide evidence of the good precision of the proposed analytical method.

**Solution Stability.** As shown in Table 3, no changes were observed in the diff. % of the average peak area of the MK-7 reference solution compared to  $t = 0$  h. Furthermore, as shown in Table 4, no changes were observed in the diff. % of MK-7 concentration in the test solution relative to  $t = 0$  h. The negligible percentage differences observed support the assess-

**Table 3.** Stability of Reference Solution over Time

Time point	$t = 0$ h	$t = 24$ h	$t = 48$ h
Reference Solution Average Area	98.27	97.44	97.94
Diff. % vs $t = 0$ h	-	-0.8	-0.3

ment of the stability of both reference and test solutions over the 48-h period.

**Determination of MK-7 in Fermentation Broths.** The validated method in this study was successfully used to monitor MK-7 production during fermentation. By analyzing fermentation broths at specific time points, the method allowed for the evaluation of how different strains contribute to MK-7 production and how factors such as nutrient availability and pH influence microbial metabolism. Three strains were compared across three separate batches, with samples taken at six time intervals. This enabled a comprehensive evaluation of strain-specific differences in MK-7 yield, as shown in Table 5. The results provide timely insights into production kinetics, from low to high concentrations of MK-7, facilitating the screening and comparison of different strains and the consequent identification of the microorganism with the highest MK-7 production potential. Furthermore, the method demonstrated high reliability in detecting MK-7 in complex fermentation matrices, whose composition can vary significantly depending on the strain. The fast, reliable, and highly accurate nature of this analytical method is particularly relevant as an essential tool for strain selection and optimization in the fermentation process.

**Opportunity and Challenges of the Proposed Method.** The aim of this study was the development and validation of an agile and rapid HPLC-UV method for the analysis of MK-7. The procedure was refined by employing robust yet practical sample preparation and optimized chromatographic conditions, thus allowing accurate and reliable quantification of MK-

**Table 4. Stability of Test Solution over Time**

Sample	$t = 0\text{h}$		$t = 24\text{h}$		$t = 48\text{h}$	
	MK7 concentration [ $\mu\text{g/mL}$ ]		MK7 concentration [ $\mu\text{g/mL}$ ]		MK7 concentration [ $\mu\text{g/mL}$ ]	
Test Solution 1	123.290		122.029		123.032	
Test Solution 2	121.616		120.551		120.454	
Test Solution 3	120.308		121.022		119.874	
			Diff. % vs $t = 0\text{ h}$		Diff. % vs $t = 0\text{ h}$	
			−1.0		−0.2	
			−0.9		−1.0	
			0.6		−0.4	

**Table 5. MK-7 Assay in Fermentation Broths**

Strain of <i>Bacillus subtilis</i> natto	Batch	Concentration MK-7 ( $\mu\text{g/mL}$ )					
		Time points (hours)					
		24	30	48	54	72	78
Strain A	1	35.4	53.4	92.0	96.9	131.4	139.1
	2	32.6	37.7	81.0	93.8	130.2	136.0
	3	23.4	34.9	95.4	114.0	149.6	155.0
Strain B	1	43.8	62.1	106.4	130.9	153.6	174.2
	2	29.0	35.4	77.0	91.0	113.0	121.0
	3	31.4	34.9	82.6	140.6	137.6	150.3
Strain C	1	44.3	71.8	130.2	147.7	173.9	188.9
	2	46.7	76.1	137.9	153.6	174.5	175.0
	3	38.8	47.2	138.2	148.3	176.4	189.7

7, despite the copresence of interfering compounds coming from the complexity of a biological matrix. Indeed, the developed chromatographic method was applied to the analysis and quantitative determination of MK-7 in fermentation broths of bacterial culture samples, where MK-7 levels are significantly low, making it important to eliminate interfering compounds using sample cleanup techniques. MK-7 molecules are typically extracted with mixtures of a polar solvent and a nonpolar solvent. The present procedure combines thermo-acidic hydrolysis with 5%  $\text{H}_2\text{SO}_4$  and extraction with ethanol, followed by direct injection into reversed-phase HPLC-UV. This method reduces the number of steps involved in sample preparation, leading to accurate quantification of MK-7. Furthermore, the present method requires a run time of 3 min, which is significantly shorter than other methods reporting similar chromatographic and detection conditions or comparable sensitivity. For instance, Rishipal et al. reported a retention time of less than 3 min for MK-7 analysis using HPLC-UV detection; however, their method showed higher LOD and LOQ values and a run time of 10 min.<sup>33</sup> Furthermore, both Ranmadugala et al.<sup>18</sup> and Çömez and Koroğlu.<sup>28</sup> calculated lower LOD and LOQ values compared to the proposed analytical method, demonstrating their high sensitivity. However, their analytical methods involve multistep extraction procedures, the use of enzymatic hydrolysis or internal standards, nonpolar and volatile solvents for sample preparation, and mobile phases, sometimes with the addition of salts. In this regard, the mobile phase used in the present method is highly applicable in terms of preparation, being a mixture of methanol, ethanol, and Milli-Q water (80:19.5:0.5, v/v/v), thus not requiring the use of salts or acids to improve the chromatographic profile<sup>34</sup> and retention time of the analyte. Moreover, the isocratic elution of the MK-7 peak at  $\text{RT} = 2.18\text{ min}$  enables fast analysis of the samples. The proposed validated method proves to be a competitive compromise between high sensitivity and wide applicability, as it can be used to monitor the impact of different bacterial strains on MK-7 production during the fermentation process,<sup>35</sup>

as well as being useful for manufacturing purposes in the nutraceutical and food industries.

## CONCLUSIONS

In this study, a fast analytical method for the quantitative determination of vitamin  $\text{K}_2$  as MK-7 in biological matrix samples, such as fermentation broth from *Bacillus subtilis* has been proposed. The MK-7 molecule has been successfully isolated from the fermentation broth by thermo-acidic extraction without derivatization, and it has been retained and separated from interfering residues using a C8 column with UV detection at 268 nm. The HPLC-UV method has been carefully optimized and validated within a brief run time of 3 min and a wide accuracy range, with an LOQ value equal to  $0.10\text{ }\mu\text{g/mL}$ , thus guaranteeing high sensitivity while employing common instrumentation and materials.

The single-step extraction with limited manipulation of the fermentation broth sample, the short-time analysis and evaluation of the analyte, the low cost, and the affordable materials of the analytical procedure make the current method suitable for application in the screening of bacterial strains and their impact on MK-7 production in a fermentation process. Moreover, it could also be employed in the nutraceutical and food industries for manufacturing purposes, thanks to the simplicity of the overall procedure and the enhanced selectivity, which makes it adaptable for different complex matrices.

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### Author Contributions

The manuscript was written through contributions from all the authors. A. C. and M. d.N. conceived and proposed the idea; A.C., A.G., and M. L. designed all the experiments; A.G. and G.R. performed the experiments; A.G. analyzed the data; A.C., A.G., and M. L. wrote the manuscript; all the authors helped



revise and edit the manuscript. All the authors have given approval to the final version of the manuscript.

## Notes

The authors declare the following competing financial interest(s): A. Celiento, A. Giuliani, M. Laini, M. Longoni, G. Rizzi and M. dei Nobili are employees of Gnosis SpA, a pharmaceutical company producing vitamin K2.

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