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Development of isotope dilution-liquid chromatography tandem mass spectrometry for the accurate determination of vitamin K_1 in spinach and kimchi cabbage

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Abstract

In the human body, vitamin K_1 is important for bone and cardiovascular health and blood coagulation. To assess the correlation between vitamin K_1 intake and health outcomes, the accurate determination of the amounts of vitamin K_1 in green leafy vegetables, which are its major source in the diet, is needed. In this study, an accurate method for quantifying naturally occurring *trans*- and *cis*-vitamin K_1 in spinach and kimchi cabbage was developed on the basis of isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LC/MS/MS). A C30 column was employed for proper separation of *trans*- and *cis*-vitamin K_1 isomers, and vitamin K_1-d_7 was used as an internal standard. The developed method was validated by measuring gravimetrically fortified samples, and its performance parameters were evaluated. The measured results agreed with the gravimetric results with a difference of less than 3%. The repeatability and reproducibility of the vitamin K_1 analysis were less than 2% relative standard deviation, indicating that the method had a higher-order metrological quality as a reference method.

Keywords *Trans*- and *cis*-vitamin K_1, Spinach, Kimchi cabbage, Isotope dilution-liquid chromatography/tandem mass spectrometry, Higher-order reference method

Introduction

Vitamin K has a distinct 2-methyl-1,4-naphthoquinone ring structure and is classified as either vitamin K_1 (phylloquinone) or vitamin K_2 (menaquinone) according to the structures of their side chains. Vitamin K_1 is a naturally occurring form of vitamin K in plants and the major dietary form of vitamin K in most diets; vitamin K_2 has a more restricted distribution in meat, liver, and certain fermented foods (Booth 2012). In plants, vitamin K_1 is found in large quantities in photosynthetic tissues of dark green leafy vegetables since it functions as an electron acceptor during photosynthesis (Gross et al. 2006). In humans, vitamin K functions as a coenzyme during the post-translational conversion of specific glutamyl residues in certain proteins. Therefore, vitamin K deficiency may cause failure in the regulation of vitamin K-dependent physiological processes (Stenflo et al. 1974). Vitamin K was originally noted for its role in blood clotting. In addition, studies have shown that supplementation with vitamin K_1 may be important for maintaining bone and cardiovascular health in elderly people (Feskanich et al. 1999; Shea et al. 2009).

The United States Institute of Medicine reported that the adequate intake level of vitamin K is 120 μg/day for men and 90 μg/day for women (Trumbo et al. 2001). In Japan, the recommended average daily intake level

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of vitamin K has been set at 75 μg/day for males and 65 μg/day for females (Kamao et al. 2007). However, recent studies indicate that the current guideline, which is based on the very low requirements to maintain normal blood coagulation, might not be sufficient for those functions and suggest a reconsideration of the vitamin K requirement that is based upon bone and cardiovascular health (Binkley et al. 2002; Vermeer et al. 2004). To set dietary vitamin K requirements for health, an accurate assessment of usual population intakes in relation to measurable health outcomes should be performed. A comprehensive evaluation of vitamin K_1 contents in most commonly consumed foods showed that only a small number of food items contribute substantially to dietary vitamin K_1 intake. It was found that green leafy vegetables contribute more than one-half of total vitamin K_1 intake (Booth et al. 1996; Kamao et al. 2007; Kim et al. 2013; Thane et al. 2002; Yan et al. 2004). A few green vegetables, such as collards, spinach, and salad greens, contain more than 300 μg of vitamin K_1/100 g, while other green vegetables contain smaller amounts. In Korean diet, among various green vegetables, spinach and kimchi cabbage are the main sources of vitamin K_1, providing 19% and 17% to total intake, respectively (Kim et al. 2013).

Vitamin K_1 is routinely measured using high-performance liquid chromatography (HPLC)–fluorescence detection (FLD), as its detection sensitivity is greatly enhanced by derivatization (AOAC 2012; Booth et al. 1994; CEN 2003; Woollard et al. 2002). However, the multistep sample preparation and chemical derivatization resulted in insufficient accuracy and precision required for reliable measurements (Kamao et al. 2007). The problems have been overcome with the use of liquid chromatography/mass spectrometry (LC/MS). In particular, the stable isotope dilution method with LC/MS detection is considered the most accurate method. As it uses isotopically labeled vitamin K_1 as an internal standard, the method corrects for losses of target compounds during sample clean-up and compensates for the variation in ionization efficiency due to matrix interferences (Trufelli et al. 2011). Studies have reported liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods for the determination of the content of vitamin K_1 in vegetables (Campillo et al. 2019; Huang et al. 2016; Jäpelt and Jakobsen 2016; Jensen et al. 2021). However, the methods determine the total vitamin K_1 content without separating *trans* and *cis* isomers. The accuracies and precisions of those methods were not sufficient as a method with higher-order metrological quality; the relative standard deviations (RSDs) of the multiple analyses were approximately 6–10%, and the method recovery was in the range of 85% to 120%. In this respect, this laboratory, the National Metrology Institute (NMI) of Korea, developed isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LC/MS/MS) for the accurate determination of individual vitamin K_1 isomers. In our previous article, we reported an ID-LC/MS/MS method for the accurate measurement of *trans*- and *cis*-vitamin K_1 in infant formula (Lee et al. 2017). In the current study, application of the method was expanded to leafy vegetables, i.e., spinach and kimchi cabbage. As vitamin K_1 in infant formula is mostly from fortification with synthetic vitamin K_1, sample preparation procedures adopted in the previous method had to be altered for the proper extraction and clean-up of naturally occurring vitamin K_1 in raw food materials. In this study, vitamin K_1 was extracted from the plant matrix by liquid–liquid extraction and the remaining lipophilic pigments were eliminated by solid-phase extraction (SPE). To better evaluate the nutritional value of foods, we separated and quantified two vitamin K_1 isomers individually, considering their unique biological activities (Bus and Szterk 2021). It may enhance method accuracy by using each standard that is chemically identical to the corresponding analyte present in the sample. Matrix effects that may cause measurement bias were carefully examined and controlled. The performance of the method, including its accuracy, repeatability, reproducibility, and measurement uncertainty, was evaluated to test if it provides higher-order metrological quality to be used for the assignment of certified values in green leafy vegetable reference materials that are under development in this laboratory.

Materials and methods

Chemicals and reagents

Vitamin K_1 was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and was used as a primary reference material. The purity of the vitamin K_1 standard was determined using a mass-balance method including LC/UV analysis for structurally related impurities, thermogravimetric analysis for non-volatile impurities, Karl-Fisher titration for water content, and headspace GC/MS for residual solvents (Lee and Kim 2014). ^{1}H nuclear magnetic resonance (NMR) was used to measure the ratio of *trans*- and *cis*-vitamin K_1 contents in the primary material as described in our previous publication (Lee et al. 2017). Combining the results of the mass-balance analysis and the NMR analysis, the *trans*- and *cis*-vitamin K_1 contents were assigned to be 86.28 ± 0.57% and 13.17 ± 0.25%, respectively. An internal standard, vitamin K_1-d_7 (5,6,7,8-d_4, 2-methyl-d_3), was purchased from Cambridge Isotope Laboratories (Andover, MA, USA); the ratio of *trans*- and *cis*-vitamin K_1-d_7 was measured by LC/UV analysis and determined to be 69.13:30.87.

HPLC grade methanol, isopropanol, n-hexane, and diethyl ether were obtained from Burdick Jackson (Muskegon, MI, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used were of analytical grade. Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of standard solutions

Four replicates of standard solutions containing 10 mg/kg vitamin K_1 in methanol were independently prepared gravimetrically. An internal standard solution containing 10 mg/kg vitamin K_1-d_7 in methanol was prepared in the same manner. For each of the four standard solutions, two isotope ratio standard solutions with a 1:1 isotope ratio were prepared by gravimetrically spiking the internal standard solution. A total of eight isotope ratio standard mixtures were cross-checked by the LC/MS/MS to test consistency among the standard solutions, and one isotope ratio standard solution was selected for calibration of the sample analysis.

Sample preparation and extraction

Freeze-dried and pulverized spinach and kimchi cabbage flours, which were produced in this laboratory as candidate reference materials for the analysis of organic nutrients, were used as homogeneous samples during the development and validation of this method. A 0.1 g portion of sample (dried flour form, 0.1 g is equivalent to 2 g of raw spinach or raw kimchi cabbage) was weighed into a 30 mL amber bottle. Using a gas tight syringe, an appropriate amount of the internal standard solution was gravimetrically spiked to match an approximate 1:1 isotope ratio. Then, 9 mL of isopropanol, 6 mL of n-hexane, and 4 mL of water were added. The solution was sonicated for 5 min, vortexed for 5 min, and then sonicated for another 5 min. The sample was centrifuged at 1800 × *g* for 10 min, and the upper organic layer was collected and evaporated to dryness under a stream of nitrogen at 35 °C. The dried residue was reconstituted with 2 mL of n-hexane.

Normal phase solid-phase extraction (SPE) was used to remove remaining lipophilic pigments from the extract. Briefly, the n-hexane extract was loaded onto a silica column (Sep-Pak Vac Silica, 3 cc; Waters, Milford, USA) that was preconditioned by washing with 8 mL of hexane/diethyl ether (93:3, v/v) and 8 mL of n-hexane. After loading the sample extract, the SPE cartridge was washed with 8 mL of n-hexane, and then the vitamin K_1-containing fraction was eluted with 8 mL of hexane/diethyl ether (93:3, v/v). The eluent was collected and evaporated to dryness under nitrogen gas. It was reconstituted with 1 mL of methanol for the analysis. All procedures were performed in subdued lighting.

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis

The LC/MS/MS system consisted of a Waters Xevo TQS triple quadrupole mass spectrometer connected to an Acquity UPLC system (Manchester, UK) with an atmospheric pressure chemical ionization (APCI) interface. The corona discharge current was set to 3.0 μA, and the cone voltage was 30 V. The flow rate of the desolvation gas was 1000 L/h, and the desolvation temperature was 600 °C. The mass spectrometer was operated in the positive ion mode. The columns used were an analytical C30 column (150 × 4.6 mm, particle size 3 μm; YMC Co., Komatsu, Japan) and a guard column (23 × 4.0 mm; YMC Co.). The injection volume was 10 μL. The isocratic mobile phase was methanol/water (96:4, v/v) with 0.1% formic acid, and the flow rate was set to 1.0 mL/min. The selected reaction monitoring (SRM) channels were *m/z* 452 → 187 for vitamin K_1 and *m/z* 459 → 194 for vitamin K_1-d_7. The collision energy was 20 eV, and the dwell time for each SRM channel was 0.5 s. HPLC-MS/MS conditions are summarized in Additional file 1: Table S1.

Post-column infusion

To investigate the matrix effect, we obtained the matrix effect profiles for the vegetable samples. A sample extract after the sample preparation processes (without spiking the internal standard) was subjected to the LC/MS/MS run as described in "LC/MS/MS Analysis." The LC eluent was infused with a standard solution containing 500 μg/kg vitamin K_1 and vitamin K_1-d_7 at a constant flow of 30 μL/min via a T-connector installed in front of the ionization interface. A syringe pump (Harvard, South Natick, MA) was used to achieve continuous post-column infusion. MS was performed in SRM mode to obtain the matrix effect profiles of the analyte and its isotopic analog. The SRM chromatogram obtained in this way represents the matrix effect profile of the corresponding sample extract.

Method validation

The amounts of *trans*- and *cis*-vitamin K_1 in the sample were separately determined by comparing the isotope ratios of *trans*-vitamin K_1/*trans*-vitamin K_1-d_7 and *cis*-vitamin K_1/*cis*-vitamin K_1-d_7 to those of the isotope ratio standard as described in a previous publication (Lee et al. 2017). The ID-LC/MS/MS method was evaluated if it can provide higher-order metrological quality as a reference method. The performance parameters of the method, including accuracy, repeatability, reproducibility, limits of detection and quantification, and

measurement uncertainty, were evaluated, following the IUPAC guidelines (Thompson et al. 2002).

The vegetable candidate reference materials (freeze-dried kimchi cabbage and spinach flours) under production in this laboratory were used as well-homogenized samples for the course of the method validation. The repeatability was tested by measuring multiple subsamples from the two candidate reference materials within a day, and the reproducibility was evaluated by conducting the same repeatability test on different days at one-month intervals. The accuracy of the method was evaluated by measuring gravimetrically fortified samples. As vegetable samples without vitamin K_1 are not available, we used the same vegetable reference materials as the blank sample after measuring the pre-existing (blank) levels of the two vitamin K_1 isomers. The samples were spiked with known amounts of vitamin K_1 such that the fortified levels were approximately 0.5-, 1.0- and 3.0-fold higher than the pre-existing *trans*-vitamin K_1 levels in blank samples. Subsequently, blank and fortified samples were analyzed using the proposed ID-LC/MS/MS method. The measured values were determined by the difference in the pre-existing blank levels and the measured results of the fortified sample and compared with the gravimetrically prepared values.

Results and discussion

Separation of vitamin K_1 isomers

At the beginning of this study, we used the same LC separation conditions used in the ID-LC/MS/MS method developed for an infant formula sample (Lee et al. 2017), which used a C30 stationary phase with an isocratic mobile phase (3% water and 97% methanol with 0.1% formic acid). However, in the vegetable samples, there were several unidentified peaks between *cis*- and *trans*-vitamin K_1, which were observed in oils and margarines (Woollard et al. 2002), and other peaks after *cis*-vitamin K_1. To better separate those peaks, the water content in the isocratic mobile phase was slightly increased to 4%. With the new separation conditions, the peaks from the sample matrix were observed near *cis*-vitamin K_1, but were separated from the analytes even though their baselines partly overlapped (Fig. 1). The mass spectrum of the unknown peak between *cis*- and *trans*-vitamin K_1 also showed a dominant peak at *m/z* 452, indicating that the unknown compound may have the same molecular weight with the vitamin K_1 isomers. We obtained MS/MS spectra of *trans*- and *cis*-vitamin K_1 and unknown compounds in order to find alternative SRM channels that could distinguish those peaks. The MS/MS fragmentation spectra of *m/z* 452 ions of *trans*- and *cis*-vitamin K_1 and the unknown were quite similar (Fig. 2). The major fragment ion was at *m/z* 187, and a series of other fragments with differences of 14 or 16 were found. This result indicated that the unknown compound was a structurally similar isomer that could not be distinguished by selecting different SRM channels.

Investigation of the matrix effect on the isotope ratio measurement

In the isotope ratio measurement using the developed method, the deuterium-labeled internal standards (vitamin K_1-d_7) eluted slightly earlier than their native analytes (vitamin K_1) (Fig. 1c). A potential bias in the measurement of their ratio could be caused if their elution times were in a region in which the ionization efficiency underwent a significant change due to matrix effects (Kim et al. 2015). APCI is less susceptible to matrix effects than electrospray ionization (ESI), and our previous study analyzing vitamin K_1 in infant formula showed similar results (Lee et al. 2017). Therefore, we used APCI as the ionization method in this study. Additionally, to systematically investigate the matrix effects in the two vegetable samples, we displayed matrix effect profiles of vitamin K_1 and vitamin K_1–d_7 using the post-column infusion system as described in the "LC/MS/MS analysis" section (Fig. 3). The matrix effect profiles were overlaid on top of their SRM chromatograms for normal LC/MS/MS operation. The profile data indicated that there was no significant change in ionization efficiency observed from 17 to 21 min, where the analytes eluted. This observation indicated that *trans*- and *cis*-vitamin K_1 and their respective internal standards experienced similar matrix effects, and thus, possible biases in the measurement of the ratio were minimized (Fig. 3). Additionally, the measurement was confirmed to be unbiased by testing it on fortified samples during the following validation process.

Method validation

The developed ID-LC/MS/MS method was evaluated if it could be used as a reference method with higher-order metrological quality. The accuracy of the method was examined by measuring gravimetrically fortified samples. The performance of the method was also evaluated by testing key parameters, such as repeatability, reproducibility, detection limit, and uncertainty level.

Accuracy

As vegetable samples without vitamin K_1 are not available, the same freeze-dried vegetable flour reference materials were used as blank samples after measuring the pre-existing (blank) levels of the two vitamin K_1 isomers. To investigate the accuracy of the method,

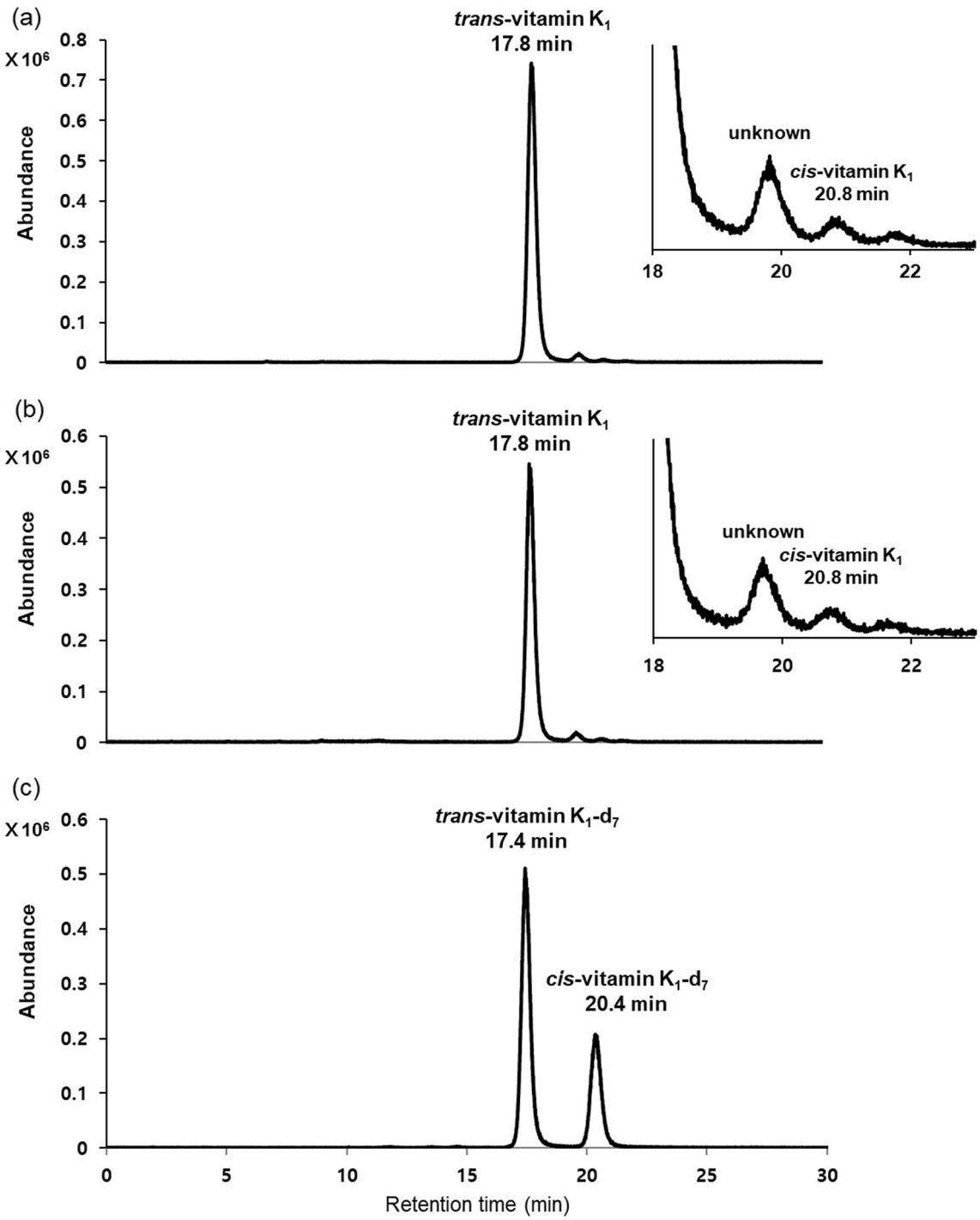

Fig. 1 SRM chromatograms of **a** vitamin K_1 in spinach, **b** vitamin K_1 in kimchi cabbage, and **c** vitamin K_1-d_7. The SRM transitions were *m/z* 452 → 187 for vitamin K_1 and *m/z* 459 → 194 for vitamin K_1-d_7

we gravimetrically fortified three different levels (low, medium, and high) by spiking the vitamin K_1 standard solution into the vegetable candidate reference materials. We measured the levels of the two isomers in the blank samples and fortified samples using the developed ID-LC/MS/MS method. The measured values for the fortified samples were calculated by subtracting the pre-existing blank levels from the measured results of the fortified samples. The results are summarized in Table 1. The measured values were

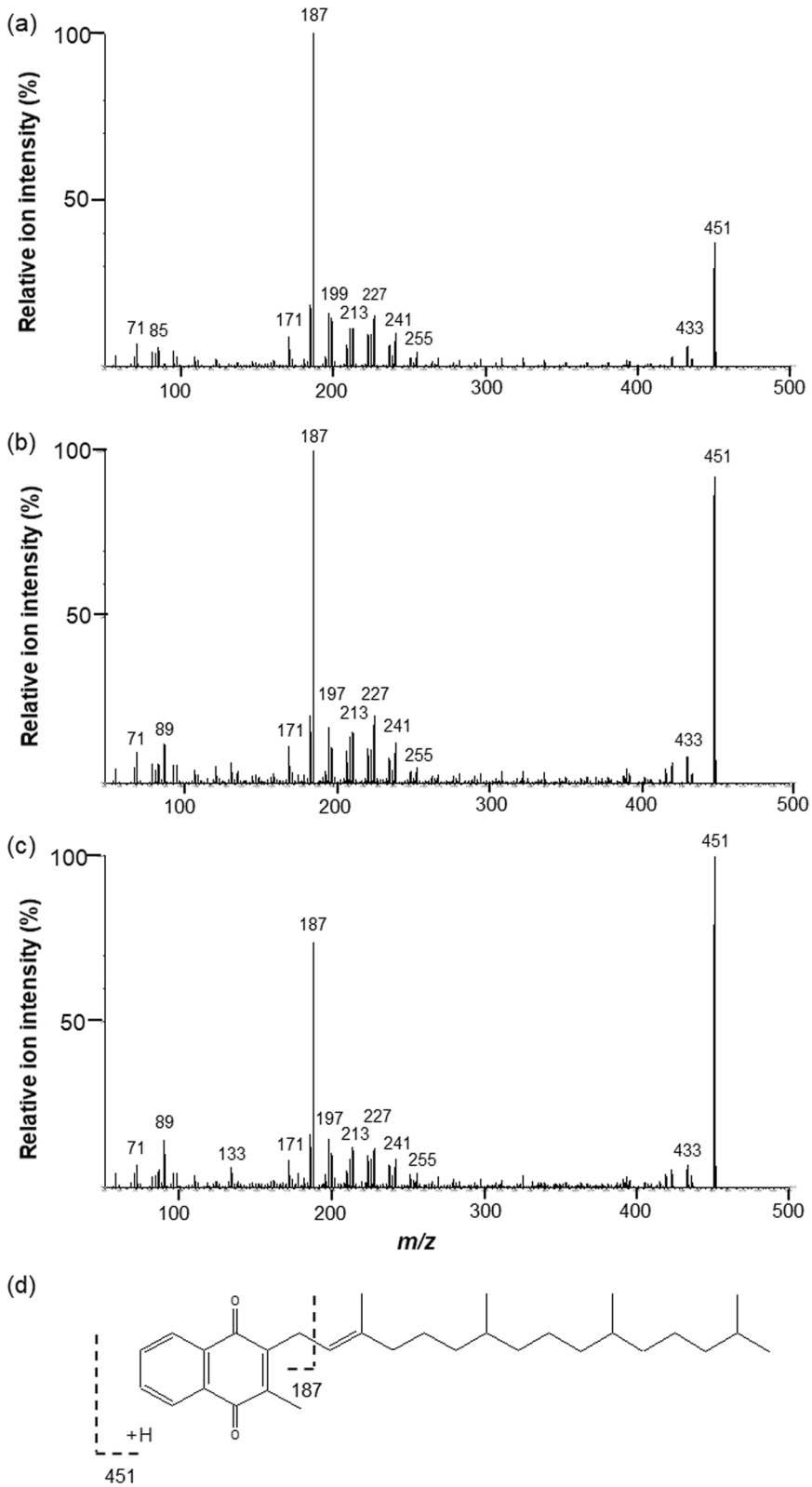

Fig. 2 Collisionally induced dissociation (CID) mass spectra of the molecular ions, $[M+H]^+$ of **a** *trans*-vitamin K_1, **b** the unknown, and **c** *cis*-vitamin K_1 in positive APCI mode. A schematic illustration of the fragmentation pathways of the precursor ions is shown in figure **d**. The collision energy was 20 eV, and cone voltage was 60 V

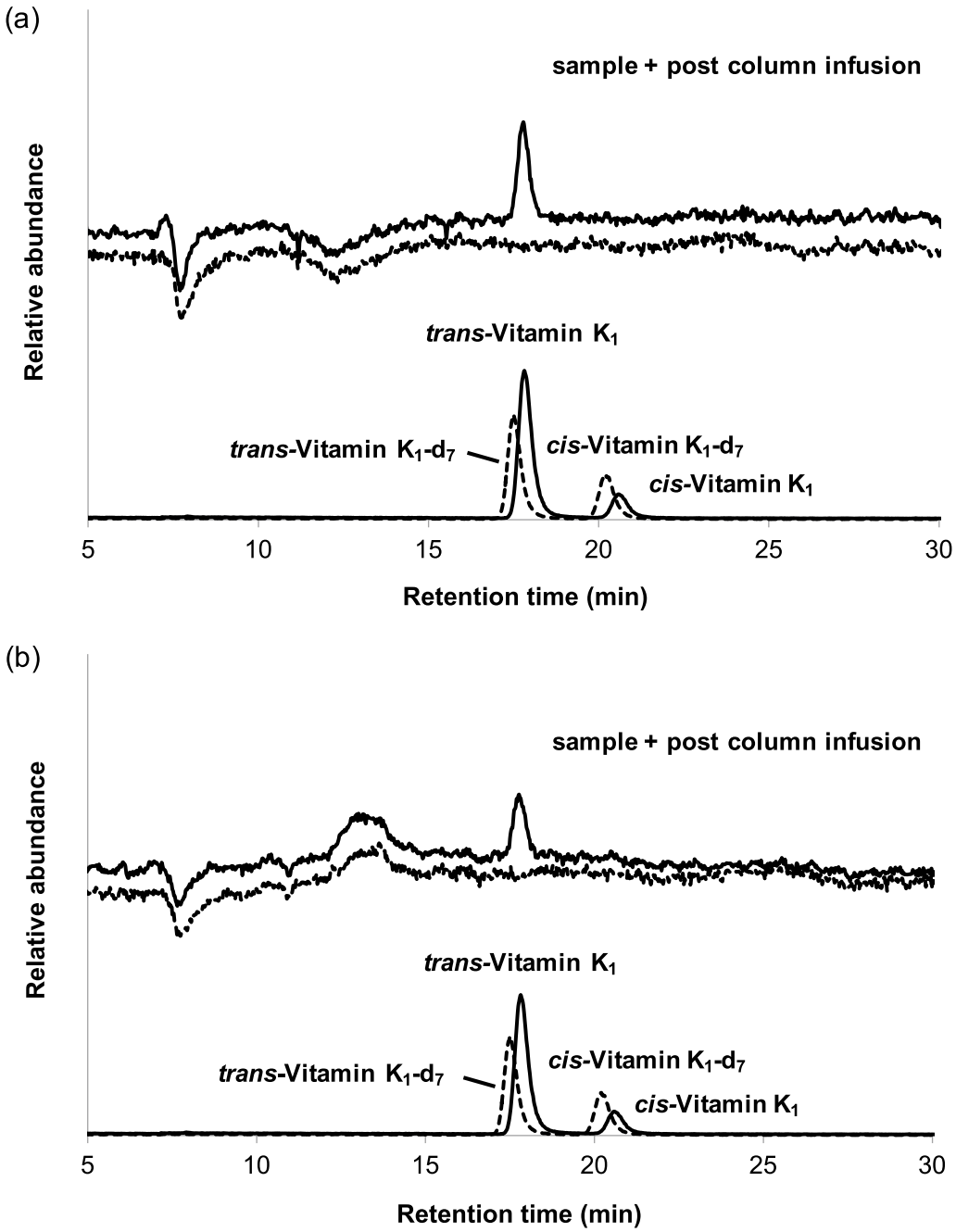

Fig. 3 Matrix effect profiles of vitamin K_1 (analyte) and vitamin K_1-d_7 (isotope analog) for the developed method. The profiles were obtained by recording the SRM chromatograms from the LC runs of **a** spinach and **b** kimchi cabbage extract with the post-column infusion of an isotope ratio standard solution. A YMC C30 column (150 × 4.6 mm, particle size 3 µm) was used for isocratic elution with methanol/water (96/4, v/v) containing 0.1% formic acid. The solid lines represent vitamin K_1, and the dotted lines indicate vitamin K_1-d_7

in good agreement with the gravimetrically fortified values within their uncertainties, indicating the validity of the analytical method. The differences between the two values ranged from − 2.5 to 2.0% and − 0.2 to 2.7% for spinach and kimchi cabbage, respectively.

Repeatability and reproducibility

By measuring multiple subsamples of the candidate reference materials within a day, the repeatability was tested and variations among the results from the multiple subsamples could be evaluated. The reproducibility

Table 1 Measurement results of gravimetrically fortified (A) spinach and (B) kimchi cabbage samples by the ID-LC/MS/MS method

Analytes	Gravimetric value (mg/kg)[a]	Measured value (mg/kg)[a,b]	Difference (%)
(A)			
trans-vitamin K_1	12.764 ± 0.049	13.02 ± 0.69 (5.2%)[c]	2.0
	26.86 ± 0.10	27.0 ± 1.1 (4.2%)	0.7
	73.01 ± 0.27	71.2 ± 3.6 (5.1%)	− 2.5
cis-vitamin K_1	1.955 ± 0.004	1.96 ± 0.18 (9.3%)	0.1
	4.114 ± 0.008	4.14 ± 0.37 (8.8%)	0.7
	11.184 ± 0.020	11.1 ± 1.1 (10.2%)	− 1.0
(B)			
trans-vitamin K_1	7.191 ± 0.030	7.34 ± 0.38 (5.1%)	2.0
	15.298 ± 0.058	15.68 ± 0.57 (3.6%)	2.5
	45.64 ± 0.17	46.0 ± 2.7 (4.9%)	0.7
cis-vitamin K_1	1.102 ± 0.003	1.13 ± 0.18 (15.8%)	2.7
	2.343 ± 0.005	2.34 ± 0.38 (16.1%)	− 0.2
	6.990 ± 0.013	7.1 ± 1.1 (15.7%)	1.6

[a] The number after "±" is the expanded uncertainty of the proceeding value with the 95% confidence level

[b] Measured values were calculated by subtracting the levels of the blank spinach or kimchi cabbage samples from the measurement results of spiked samples

[c] The percentage value provided in parentheses indicates the relative extended uncertainty compared to the corresponding mean value

Table 2 Repeatability and reproducibility of the developed ID-LC/MS/MS method. (A) Spinach and (B) kimchi cabbage

(A)

	Subsample No	ID-LC/MS/MS Results (mg/kg)	
		***trans*-vitamin K_1**	***cis*-vitamin K_1**
Period 1	#1	24.26 ± 0.65[d]	0.217 ± 0.014
	#2	23.80 ± 0.64	0.237 ± 0.012
	#3	24.59 ± 0.61	0.241 ± 0.018
	#4	24.14 ± 0.59	0.220 ± 0.009
	Average	24.2 ± 1.0	0.229 ± 0.004
	Standard deviation[a]	0.33 (1.4%)	0.012 (5.3%)
	Average of total vitamin K_1[b]	24.43	
	Standard deviation combined	0.33 (1.4%)	
	Expanded uncertainty combined	1.1 (4.3%)	
Period 2	#1	24.12 ± 0.94	0.218 ± 0.023
	#2	24.57 ± 0.97	0.235 ± 0.043
	#3	24.51 ± 0.97	0.233 ± 0.024
	#4	24.56 ± 0.96	0.234 ± 0.056
	Average	24.4 ± 1.3	0.230 ± 0.008
	Standard deviation	0.22 (0.88%)	0.008 (3.6%)
	Average of total vitamin K_1	24.67	
	Standard deviation combined 1.69%	0.22 (0.90%)	
	Expanded uncertainty combined	1.29 (5.3%)	
Period 3	#1	24.22 ± 0.91	0.250 ± 0.020
	#2	24.13 ± 0.89	0.242 ± 0.019
	#3	23.91 ± 0.86	0.247 ± 0.020
	#4	23.97 ± 0.88	0.247 ± 0.020
	Average	24.1 ± 1.0	0.243 ± 0.024
	Standard deviation	0.14 (0.60%)	0.008 (3.09%)

Table 2 (continued)

(A)

	Subsample No	ID-LC/MS/MS Results (mg/kg)	
		***trans*-vitamin K_1**	***cis*-vitamin K_1**
	Average of total vitamin K_1	24.30	
	Standard deviation combined	0.15 (0.61%)	
	Expanded uncertainty combined	1.0 (4.1%)	
Average		24.23	0.23
Standard deviation among period[c]		0.19 (0.80%)	0.008 (3.3%)

(B)

	Subsample No	ID-LC/MS/MS Results (mg/kg)	
		***trans*-vitamin K_1**	***cis*-vitamin K_1**
Period 1	#1	13.39 ± 0.32	0.148 ± 0.013
	#2	13.630 ± 0.34	0.149 ± 0.006
	#3	13.81 ± 0.34	0.161 ± 0.014
	#4	13.31 ± 0.33	0.139 ± 0.008
	Average	13.53 ± 0.47	0.149 ± 0.008
	Standard deviation	0.23 (1.7%)	0.009 (6.0%)
	Average of total vitamin K_1	13.68	
	Standard deviation combined	0.23 (1.7%)	
	Expanded uncertainty combined	0.68 (5.0%)	
Period 2	#1	13.68 ± 0.54	0.173 ± 0.009
	#2	13.58 ± 0.54	0.162 ± 0.013
	#3	13.61 ± 0.54	0.153 ± 0.011
	#4	13.56 ± 0.54	0.151 ± 0.011
	Average	13.61 ± 0.75	0.160 ± 0.010
	Standard deviation	0.005 (0.40%)	0.010 (6.2%)
	Average of total vitamin K_1	13.77	
	Standard deviation combined	0.06 (0.46%)	
	Expanded uncertainty combined	0.75 (5.5%)	
Period 3	#1	13.29 ± 0.47	0.151 ± 0.024
	#2	13.35 ± 0.49	0.131 ± 0.014
	#3	13.43 ± 0.50	0.137 ± 0.011
	#4	13.28 ± 0.58	0.138 ± 0.014
	Average	13.34 ± 0.54	0.138 ± 0.014
	Standard deviation	0.067 (0.51%)	0.009 (6.59%)
	Average of total vitamin K_1	13.48	
	Standard deviation combined	0.07 (0.49%)	
	Expanded uncertainty combined	0.62 (4.6%)	
Average		13.49	0.149
Standard deviation among period		0.16 (1.2%)	0.011 (7.4%)

[a] The value provided in parentheses indicates the relative standard deviation compared to the mean value

[b] Total vitamin K_1 is the sum of *trans*- and *cis*-vitamin K_1

[c] Standard deviation of the results among three time periods, indicating the method's reproducibility

[d] The number after "±" is the expanded uncertainty of the proceeding value with the 95% confidence level

Table 3 Uncertainty sources in the determination of *trans*- and *cis*-vitamin K_1 by the developed ID-LC/MS/MS method. The samples are spinach (A) and kimchi cabbage (B)

Uncertainty components	Sources (evaluation method)	Typical value (%)	
		***trans*-vitamin K_1**	***cis*-vitamin K_1**
(A)			
Standard solution	Purity of the reference material (from the KRISS purity analysis)	0.2	0.1
	Gravimetric preparation (from the cross-check of the independent sets of calibration solutions)	2.0	2.1
Isotope ratio standard	Gravimetric mixing (from the cross-check of multiple isotope ratio standards from each individual standard solution)	2.1	2.1
Peak area ratio of the standard and isotope-labeled standard from the LC/MS/MS measurements of the isotope ratio standard	Repeatability of multiple measurements	1.1	1.4
Mass of the sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01	< 0.01
Mass of the isotope-labeled standard solution spiked into the sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01	< 0.01
Peak area ratio of the standard and isotope-labeled standard from the LC/MS/MS measurements of the sample extracts	Repeatability of multiple measurements	0.4–1.5	0.5–2.6
(B)			
Standard solution	Purity of the reference material (from the KRISS purity analysis)	0.2	0.1
	Gravimetric preparation (from the cross-check of the independent sets of calibration solutions)	2.0	2.1
Isotope ratio standard	Gravimetric mixing (from the cross-check of multiple isotope ratio standards from each individual standard solution)	2.1	2.1
Peak area ratio of the standard and isotope-labeled standard from the LC/MS/MS measurements of the isotope ratio standard	Repeatability of multiple measurements	1.1	1.4
Mass of the sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01	< 0.01
Mass of the isotope-labeled standard solution spiked into the sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01	< 0.01
Peak area ratio of the standard and isotope-labeled standard from the LC/MS/MS measurements of the sample extracts	Repeatability of multiple measurements	0.5–1.3	0.8–2.5

was evaluated by conducting a repeatability test on different days. The measurement results of the three different time periods are summarized in Table 2. In most cases, the RSDs of the multiple subsamples within a time point were less than 2% for *trans*-vitamin K_1, and the RSD of the means at the three different time points were approximately 1%; these values were superior to those obtained from previous studies: 6.3 ~ 7.4% (Booth et al. 1994), 1.1 ~ 8.8% (Huang et al. 2016), 3.4 ~ 5.2% (Jäpelt and Jakobsen 2016), 8.8 ~ 9.2% (Campillo et al. 2019), and 5.5 ~ 5.9% (Jensen et al. 2021). This indicated that the overall process of the method showed improved repeatability and reproducibility, which resulted in increased confidence in the validity of the results. In the case of *cis*-vitamin K_1, its level in the materials was much lower compared to *trans*-vitamin K_1, and its RSDs were slightly higher than *trans*-vitamin K_1, ranging from 3 to 7%. We note that the higher RSDs for *cis*-vitamin K_1 are attributed to the partial overlapping of its peak with the structural isomer peaks as shown in Fig. 1.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were estimated based on the signal-to-noise ratios obtained in the SRM chromatograms of spinach and kimchi cabbage flour. The LODs (level of the analyte in the sample, which gives a signal-to-noise ratio of 3:1) were 0.031 mg/kg for *trans*-vitamin K_1 and 0.026 mg/kg for *cis*-vitamin K_1. LOQs (level of the analyte in the sample, which gives a signal-to-noise ratio of 10:1) of *trans*- and *cis*-vitamin K_1 were both 0.1 mg/kg.

Uncertainty evaluation

We evaluated the measurement uncertainty using an established protocol maintained in our laboratory at the NMI of Korea. The detailed protocol has been described in our previous studies (Choi et al. 2003). Uncertainty sources of the results are itemized in Table 3. The uncertainty components are as follows: the area ratio of analytes and isotope-labeled standards from the LC/MS/MS measurements of the isotope ratio standard (1.1% for *trans*- and 1.4% for *cis*-vitamin K_1) and sample extracts (0.4 ~ 1.5% for *trans*- and 0.5 ~ 2.6% for *cis*-vitamin K_1), the gravimetric preparation of the standard solution (2.0% for *trans*- and 2.1% for *cis*-vitamin K_1), and the gravimetric mixing of the isotope ratio standard solution (2.1% for both *trans*- and *cis*-vitamin K_1). For measuring dried vegetable flours, the combined RSD was less than 2.0%, and the combined relative expanded uncertainty (at the 95% confidence level) was approximately 5.0% (Table 2), which were similar levels to the measurement uncertainties in Table 1.

The measurement uncertainties from the method are much lower than those from widely used analytical methods. In Table 2, the RSDs of multiple measurements (less than 2.0%) were much less than those obtained from the HPLC-FLD method (6.3–7.4%) (Booth et al. 1994) and previously published ID-MS/MS methods (3.4–9.2%) (Campillo et al. 2019; Huang et al. 2016; Jäpelt and Jakobsen 2016; Jensen et al. 2021). The comparison of the method's performance in our study with that of other HPLC-based methods is presented in Additional file 1: Table S2. The overall measurement uncertainties of this method were much less than one-third of the expected relative standard deviations of testing laboratories, which was estimated by the Horwitz equation (9–11% for the 10–50 mg/kg range), indicating that the developed method has adequate metrological quality, which is required for a higher-order reference method (ISO 2015).

Conclusions

We established an isotope dilution-liquid chromatography/tandem mass spectrometric (ID-LC/MS/MS) method as a candidate reference method for the accurate determination of *trans*- and *cis*-vitamin K_1 in two leafy vegetables (spinach and kimchi cabbage). The chromatographic conditions were optimized to ensure the proper separation of two vitamin K_1 isomers without bias caused by matrix interference. Method accuracy was validated by ensuring only a small difference between the measured values and gravimetrically fortified amounts. In addition, a small measurement uncertainty of the developed method was observed. These results indicated that the developed method has a higher level of accuracy and precision compared to other methods. Therefore, it could be used as a standard method for other measurements.

Abbreviations

APCI	Atmospheric pressure chemical ionization
ESI	Electrospray ionization
GC/MS	Gas chromatography/mass spectrometry
HPLC-FLD	High-performance liquid chromatography-fluorescence detection
ID-LC/MS/MS	Isotope dilution-liquid chromatography/tandem mass spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LC/UV	Liquid chromatography/ultraviolet
LOD	Limit of detection
LOQ	Limit of quantification
NMI	National Metrology Institute
NMR	Nuclear magnetic resonance
RSD	Relative standard deviation
SPE	Solid-phase extraction
SRM	Selected reaction monitoring

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-023-00399-w.

Additional file 1: Table S1. HPLC-MS/MS conditions for the analysis of trans- and cis-vitamin K1. **Table S2.** Comparison with other HPLC-based methods proposed for vitamin K1 analysis in foods.

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Author contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by HL, JL and HL. HL and BK wrote the main manuscript text. The final manuscript has been read and approved by all authors.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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