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DETERMINATION OF VITAMIN K CONCENTRATION IN CANADIAN CHEESE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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**DETERMINATION OF VITAMIN K CONCENTRATION IN CANADIAN CHEESE
USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY**

by

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We accept this thesis as conforming to the required standards:

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ABSTRACT

Vitamin K refers to a family of fat soluble vitamins which includes phyloquinone (vitamin K₁) and menaquinones (vitamin K₂). All forms of vitamin K act as cofactors for γ -glutamyl carboxylase, an enzyme which adds Gla residues to certain proteins and are required for the protein to bind calcium. Differences in metabolism of phyloquinone and menaquinones result in phyloquinone being primarily used to help activate blood clotting factors while menaquinones help activate other proteins such as osteocalcin and Matrix-Gla protein. These two proteins have been shown to help fight osteoporosis and heart disease. Increased vitamin K intake has been shown to fight these diseases, but little information is available on the amount of vitamin K in food. To gain more information on the amount of vitamin K in Canadian foods, liquid chromatography-mass spectrometry was used to measure phyloquinone, menaquinone-4 (MK-4), and menaquinone-7 (MK-7) in Canadian cheese obtained from grass-fed cows and conventional supermarket cheese. Samples were homogenized in water, melted in a boiling water bath with HCl and then extracted with hexanes before filtration and injection into the LC-MS. A calibration curve from 0.1 to 1 $\mu\text{g/g}$ was obtained for phyloquinone and MK-4, but was unsuccessful for MK-7. Gouda cheese made from grass-fed cows during the early spring and summer contained very high amounts of phyloquinone and MK-4, as did Maasdammer cheese from grass-fed cows. Vitamin K was not detected in conventional supermarket cheddar cheese and only MK-4 was detected in Gouda from the spring. The extraction procedure likely had a low recovery as the peak area of the internal standards was 10-20x larger in the standards than in the samples. A LC-MS method was successfully used to detect low concentrations of phyloquinone and MK-4, but further optimization of the extraction procedure is required for analysis of a larger number of samples.

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INTRODUCTION

Vitamin K refers to a group of fat soluble vitamins originally discovered in the 1930s by Hendrik Dam who was awarded the 1943 Nobel Prize in Physiology or Medicine for his discovery. Dam shared the Nobel Prize with Edward A. Doisy who characterized vitamin K and was able to isolate two forms of vitamin K (Cranenburg et al. 2007, Nobelprize.org). The first form of vitamin K was called vitamin K₁ or phylloquinone and is found in green leafy vegetables. The second form of vitamin K, vitamin K₂ which is found in animal fat and some fermented foods, consists of a group of homologues called menaquinones that vary in the number of isoprene units in their side chain (Figure 1). Menaquinones are referred to as menaquinone-n (MK-n) where “n” indicates the number of isoprene units in the side chain (Cranenburg et al. 2007).

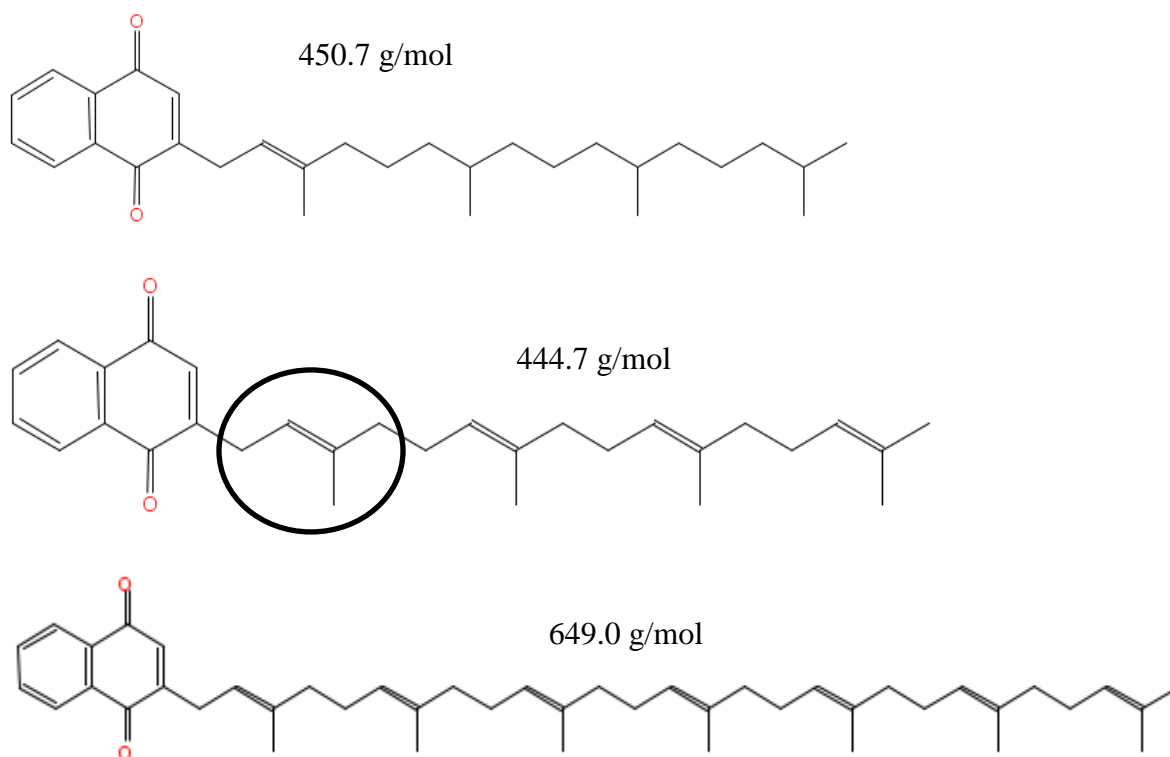


Figure 1. Structure and molecular mass of phylloquinone (top), MK-4 (middle), and MK-7 (bottom). All molecules are structurally similar. Menaquinones contain differing lengths of isoprene units (circled) and are named according to the number of isoprene repeats.

The functions of K vitamins are very similar. Both phylloquinone and all menaquinone homologues act as cofactors for γ -Glutamyl carboxylase enzymes. These enzymes are responsible for the carboxylation and conversion of glutamic acid to γ -carboxyglutamate (Gla) residues in certain proteins. Gla residues are able to bind calcium ions with high affinity and are essential for the proper functioning of several proteins (Beulens et al. 2009). The first protein found to contain Gla was prothrombin, which phylloquinone helps activate. Menaquinones help activate a variety of proteins including osteocalcin, which transports calcium to the bones preventing osteoporosis (Aaseth et al. 2012), and Matrix-Gla Protein (MGP), which removes calcium plaques from arteries (Adams and Pepping 2005). Removing calcium plaques can significantly help reduce the risk of heart disease and studies have shown that a high menaquinone intake can decrease the risk of coronary heart disease (Beulens et al. 2009; Gast et al. 2008). Due to differences in metabolism of phylloquinone and menaquinones, only menaquinones are thought to reduce arterial calcification (Beulens et al. 2009). Increased dietary consumption of menaquinones could help fight osteoporosis as well as decrease arterial calcification, but first more information is needed regarding the amount of menaquinones in food products.

Menaquinones are mostly found in animal fat and in some fermented foods. There are relatively few studies which have measured menaquinone levels in foods, and none that have looked at foods in Canada. The most comprehensive studies have determined the amount of menaquinones in European dairy, fish, and meat products (Schurgers and Vermeer 2000; Koivu-Tikkanen et al. 2000; Ostermeyer and Schmidt 2001; Manoury et al. 2013). These studies found a large range of menaquinone concentrations from trace amounts to just over 1 $\mu\text{g/g}$ in some cheeses. Studies on American food have found similarly low concentrations and broad ranges of menaquinone concentration (Elder et al. 2006; Ferreira et al. 2006). Due to the large ranges of concentrations found in previous studies it is difficult to speculate on the amount of vitamin K in Canadian meat and dairy products. It is therefore important to determine the vitamin K content of Canadian food products, starting with cheese as it was one of the best menaquinone sources reported in Europe (Schurgers and Vermeer 2000; Koivu-Tikkanen et al. 2000; Ostermeyer and Schmidt 2001; Manoury et al. 2013). Cheese is also a commonly consumed food, with the average Canadian consuming 12.32 kg of cheese

in 2013 (Canadian Dairy Information Centre). Although there is no official recommended daily intake for menaquinones, it has been suggested that total vitamin K (phylloquinone and menaquinone) intake should be between 200 and 500 µg/day (Vermeer et al. 2005), which is much higher than current government recommendations of 90 µg/day for adult females and 120 µg/day for adult males (Health Canada). Based on the low concentrations of vitamin K found in most studies, it is unlikely most people are receiving enough vitamin K.

One factor that has not been previously investigated, but could provide higher vitamin K concentrations in animal products is the animals' diet. For example, cows like all animals, produce menaquinones by converting phylloquinone from grass into MK-4 while their gut bacteria produce longer chain menaquinones such as MK-7 (Okano et al. 2008). Since phylloquinone comes from chloroplasts in plants, it follows that grass-fed cattle would produce food products with more vitamin K than cattle fed a high grain diet.

The goal of this study was to use liquid chromatography-mass spectrometry, a method commonly used to measure fat soluble vitamins due to its ability to separate and identify analytes, to determine the phylloquinone, MK-4, and MK-7 concentrations in Canadian conventional and grass-fed cheese. This will provide insight into the amount of K vitamins in the Canadian diet and whether cows with a higher phylloquinone intake, via grass diet, produce food products with higher vitamin K concentrations.

MATERIALS AND METHODS

Materials and Reagents

Cheese samples were purchased from local supermarket and health food stores in Kamloops, BC (Superstore, Nature's Fare). All cheese samples contained 33% fat. Phylloquinone, MK-4, and δ-tocopherol were purchased from Supelco (Bellefonte, PA) and MK-7 was purchased from Chromadex (Irvine, CA). All chromatography solvents were of LC-MS grade.

Methanol was purchased from Caledon Laboratory Chemicals (Georgetown, ON), tetrahydrofuran was purchased from Honeywell Burdick & Jackson (Mississauga, ON), and water and hexanes were purchased from Fisher Scientific (Ottawa, ON).

Sample Preparation

Fat soluble vitamins were extracted from cheese samples using an extraction procedure modified from Manoury et al. (2013). Approximately 2 g of cheese was weighed into a centrifuge tube to which 10 mL of water was added. The sample was homogenized for 30 s at 13,500 rpm, 400 μ L of internal standard (20 μ g/g δ -tocopherol) and 5 mL of 1M HCl were added, and the mixture was placed in a boiling water bath for half an hour. The mixture was then cooled in an ice bath and 10 mL of 2-propanol and 2 mL of hexanes were added. The sample was shaken and centrifuged at 3,700 x g for 10 min. Following centrifugation, the top organic layer was filtered twice through a 0.45 μ m nylon filter. The filtered solution was transferred to an amber LC-MS vial and 5 μ L was injected into the LC-MS. All samples were extracted once and then run in triplicate on the LC-MS.

Standard Solutions

Standard solutions of phyloquinone, MK-4, and MK-7 were prepared by dissolving the vitamins in methanol to make stock solutions. The stock solutions were diluted to make working solutions of 2 μ g/g which were used to produce the calibration curve. The standard solution for MK-4 was run on the LC-MS in duplicate with ten concentrations ranging from 0.1 to 1 μ g/g. Phyloquinone and MK-7 standard solutions were run in duplicate with five concentrations from 0.1 to 1 μ g/g. All standards contained 20 μ g/g δ -tocopherol as internal standard.

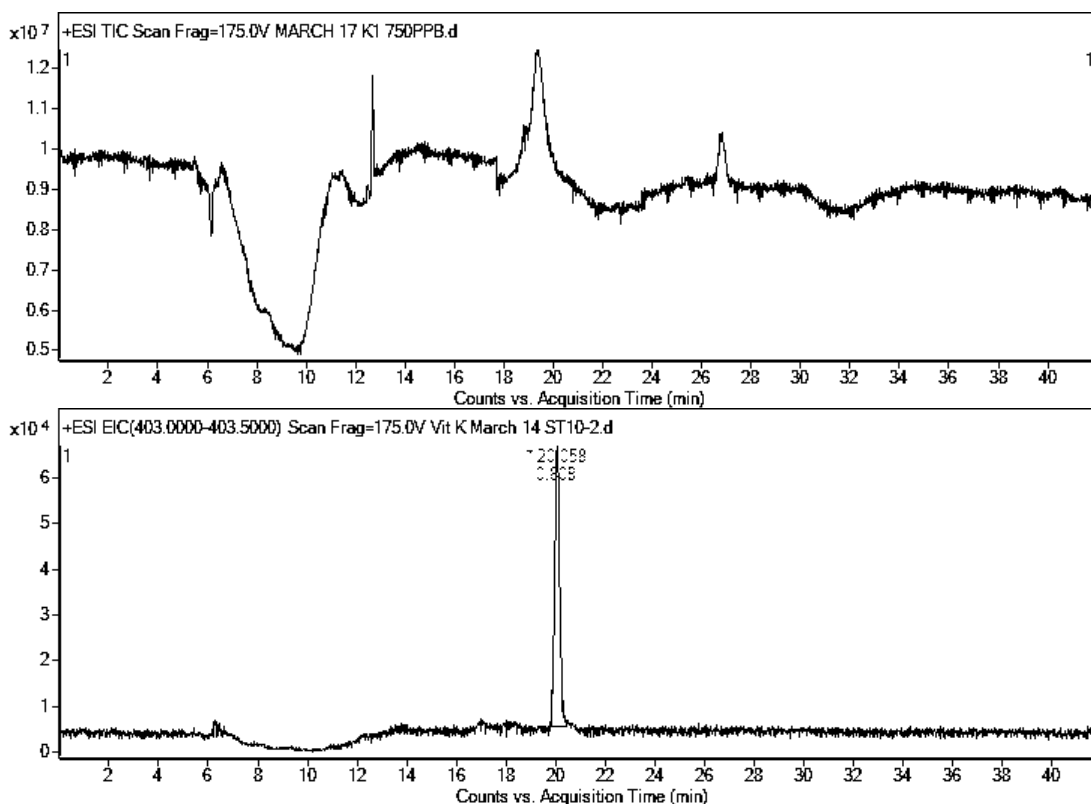
Instrument Parameters

The LC-MS system consisted of a 1200 Series HPLC system (Agilent, Mississauga, ON) paired with a 6530 Accurate-Mass quadrupole-time-of-flight mass spectrometer (Agilent, Mississauga, ON) with an electrospray ionization source. The column (4.6 mm x 250 mm) was packed with 3 μ m zorbax C₁₈ (Agilent, Mississauga, ON). The column temp was 45° C with a 0.5 mL/min flow rate. A 42 min run time was used and the gradient started at 0% B, linearly increasing to 75% B by 2 min, then isocratic at 75%B until 40 min, and linearly dropping back to 0% B by 42 min. The mobile phase A consisted of methanol/water (90:10, v/v) and mobile phase B was methanol/tetrahydrofuran (70:30, v/v). To each liter of mobile phase, 0.5 mL of formic acid was added. Detection by the MS was performed in positive mode with a gas temp of 300° C, sheath gas of 350° C, drying gas flow rate of 8 L/min,

sheath gas flow of 10 L/min, and nebulizer at 8 psig. The VCap was 3000 V, the fragmentor voltage was 175 V, skimmer 65 V, and nozzle 1000 V.

RESULTS

Phylloquinone, MK-4, and MK-7 were not readily detectable in the total ion chromatogram for any of the samples or standards so analysis of the LC-MS data primarily used the extracted ion chromatograms (Figure 2). Extracted ion chromatograms showed very clear peaks for all K vitamins and the internal standard. The peak order consisted of phylloquinone eluting first (~6 min), then δ -tocopherol, MK-4, and MK-7 eluted last (data not shown as peaks were too small in concentrations analyzed). There was a significant peak at approximately 12 min which was present in all standards, but was not attributed to any one vitamin. A blank using methanol also contained the peak at ~12 min.



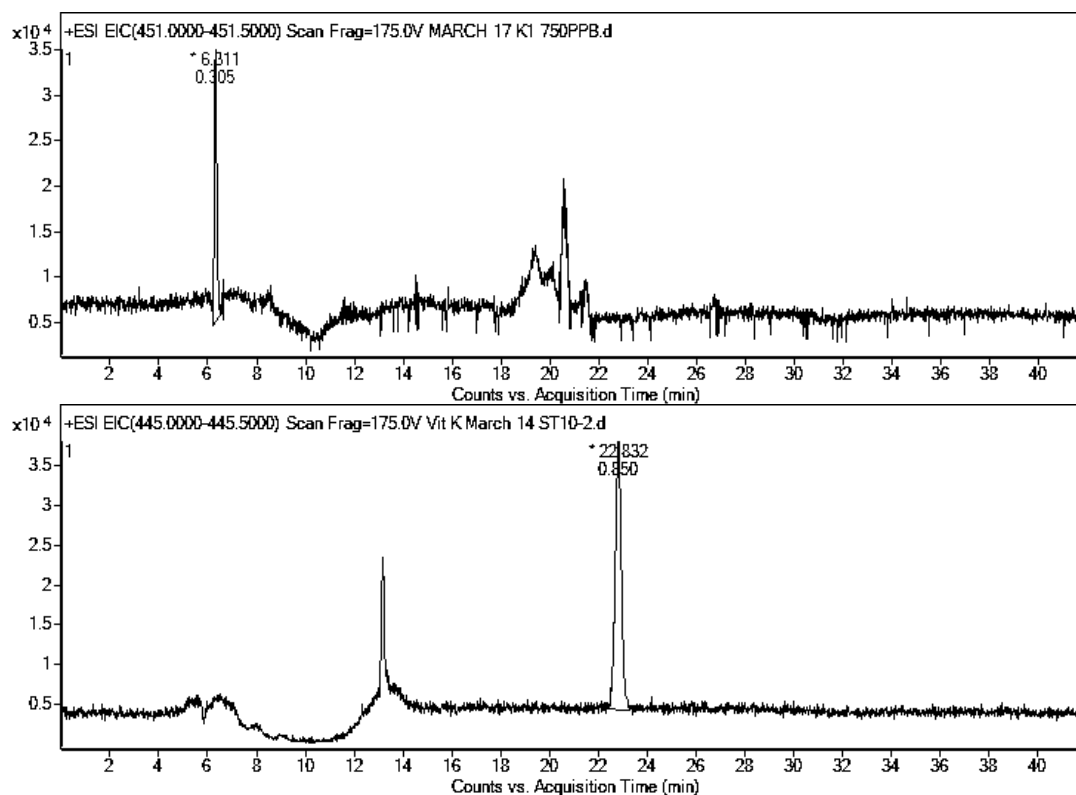


Figure 2. LC-MS chromatograms of vitamin K standards. The total ion chromatogram (top) of a 0.75 µg/g phylloquinone standard did not provide much useful data. Extracted ion chromatograms were used to measure the peak area of each vitamin. The internal standard (second from top) eluted at ~20 min, phylloquinone (third from top) eluted at ~6 min, and MK-4 (bottom) eluted at ~23 min. All peaks were baseline resolved.

Calibration curves were prepared for each of the three K vitamins using the ratio of the peak area of the vitamin to the peak area of the internal standard (δ -tocopherol). Calibration curves for phylloquinone, MK-4, and MK-7 were obtained by running standards of each vitamin separately. Calibration used standards ranging from 0.1 to 1 µg/g for both phylloquinone and MK-4. Calibration of MK-4 used 10 concentrations and calibration with phylloquinone used 5 standard concentrations. An internal standard, δ -tocopherol, was used in all standards and samples. Both standard and sample concentrations were measured using the peak area ratio of the vitamin over peak area of the internal standard. The calibration of both phylloquinone and MK-4 showed a linear instrument response to concentration with a R^2 value of 0.9881 for MK-4 and 0.9703 for phylloquinone (Figure 3).

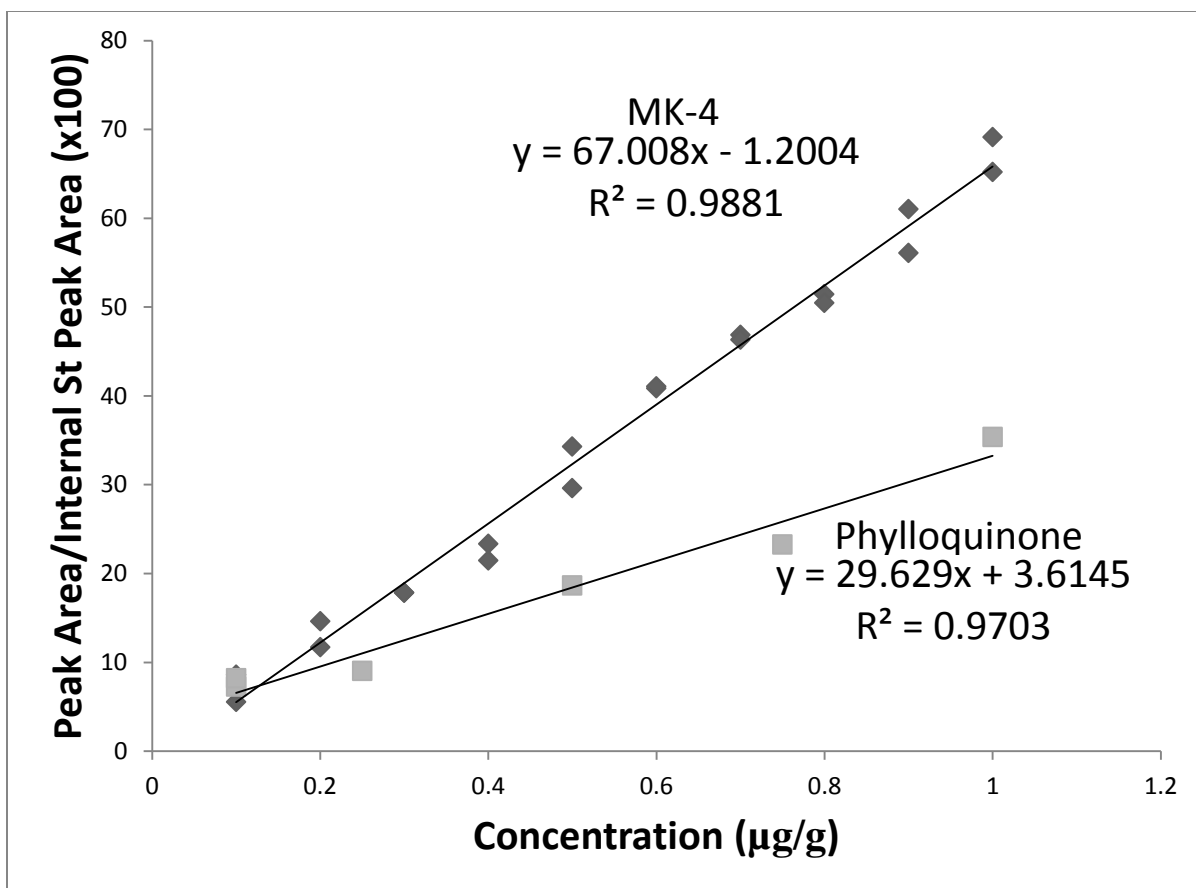


Figure 3. Calibration curve obtained for phylloquinone (squares) and MK-4 (diamonds) using LC-MS. δ -tocopherol was used as an internal standard and instrument response was measured using the peak area ratio of analyte to internal standard. LC-MS had a linear response to both vitamins between 0.1 and 1 $\mu\text{g/g}$.

Cheese samples from grass-fed cattle and conventional supermarket cheese were investigated. Gouda investigated. Gouda cheese made from milk collected in the early spring, spring, and summer were looked at to see if the time of year affects the vitamin K concentrations. The summer samples contained close to twice as much phylloquinone and MK-4 as the early spring sample (sample (

Table 1). Only one replicate of the spring sample contained any vitamin K and it did not contain any phylloquinone. The Maasdammer cheese had lower concentrations of MK-4 than the early spring and summer Gouda cheeses, and had a phylloquinone concentration between that of the early spring and summer Gouda cheeses. No phylloquinone or MK-4 was detected in the cheddar cheese.

Table 1. Concentration of phyloquinone and MK-4 (mean \pm SD of three replicates of one extract) measured in four cheese samples from grass-fed cattle and conventional supermarket cheese sample. Gouda samples were made from milk collected in the early spring, spring, and summer.

Sample	Phylloquinone ($\mu\text{g/g}$)	MK-4 ($\mu\text{g/g}$)
Gouda – grass-fed, early spring	2.61 \pm 0.39	2.98 \pm 2.53
Gouda – grass-fed, spring	n.d	1.20*
Gouda – grass-fed, summer	6.37 \pm 0.48	4.97 \pm 1.05
Maasdammer – grass-fed	4.76*	1.57*
Cheddar	n.d	n.d

*Vitamin K was only detected in one of the three replicates

n.d: not detected

The relative levels of phyloquinone to MK-4 changed between the cheese samples. The early spring and summer Gouda had approximately equal amounts of phyloquinone and MK-4, while the Maasdammer had significantly more phyloquinone than MK-4.

DISCUSSION

Cheese is one of the best sources of vitamin K, yet even in cheese it is difficult to measure the vitamin K concentration due to the low concentration and complex matrix. The total ion chromatograms of all standards and samples were not useful due to the low concentrations, but the ability to extract ion chromatograms allowed for the measurement of 100 ng/g concentrations of vitamin K in the standards. Lower concentration standards were also able to be detected although the detection limit was not determined. LC-MS proved to be an invaluable tool in this study allowing calibration curves from 0.1 to 1 $\mu\text{g/g}$ to be successfully prepared for phyloquinone and MK-4. A calibration curve for MK-7 was attempted, but the peak for MK-7 did not respond in a concentration dependant manner. This was likely due to being below or very close to the detection limit as a similar problem arose for phyloquinone and MK-4 when lower concentration standards were run.

All standards and samples contained an unknown peak at ~12 min with very high intensity. This peak was well separated from the three vitamins analyzed, but could become a problem if trying to analyze other menaquinones or other fat soluble vitamins. The contamination peak was present when running methanol blanks and when bypassing the sampling system

altogether. This would suggest the contamination is from the solvents. New solvents were used to try eliminate the peak, but were unsuccessful. One other possibility is algae growth in the solvent filters/lines. Removal of the contaminant peak is desirable as it may allow for analysis of more vitamins and potentially increase the detection limit.

The concentration of both phyloquinone and MK-4 were extremely high in all samples in which vitamin K was detected. The concentrations of MK-4 in both the early spring Gouda and summer Gouda were close to 3-5 times higher than the highest concentration (1.18 $\mu\text{g/g}$) reported by Manoury et al. (2013) for menaquinones 6-10 combined. The vitamin K concentrations were above the range of the calibration curve, limiting the confidence of the concentrations. We expect the linear range of the calibration curve would extend well above 1 $\mu\text{g/g}$.

Comparing the Gouda cheese from different seasons appears to show that there is more vitamin K in cheese from the summer. Based on the results, summer cheese had the highest vitamin K levels followed by early spring, then spring. The higher vitamin K levels in the summer Gouda may indicate variation based on the time of year. Grass-fed cows spend the most time grazing in the summer, so summer cheese would be expected to have higher vitamin K concentrations as was observed. In the early spring and spring, cows would still be eating hay and grass harvested the previous year which would not provide as much phyloquinone. It is important to note that only one sample of each cheese was used and while a trend may be observed, it is not statistically significant until more samples are measured.

The relative amount of phyloquinone to menaquinone varied between samples. This was most noticeable between the Gouda and Maasdammer cheeses. The Maasdammer cheese had a much higher ratio of phyloquinone to MK-4 than any Gouda cheese. This could be due to differences in the manufacturing process, such as different bacterial strains, as previous studies have shown some bacterial strains are better than others at converting phyloquinone to MK-4 (Manoury et al. 2013). As both the early spring and summer Gouda cheeses contained similar amounts of phyloquinone and MK-4, the presence of only MK-4 in the spring cheese is highly suspect and could indicate problems in the extraction procedure.

The peak areas of the internal standard was much smaller (~10-20x) in the samples than standards (data not shown). This means there was a significant loss of vitamins during the extraction procedure and indicates the extraction procedure needs further optimization. In particular, solid phase extraction (SPE) could be useful if used following filtration of the organic layer. This would remove fatty acids and produce a cleaner chromatogram and it would be easier to detect K vitamins. Also, loss of the internal standard may have been greater than that of the vitamins in the sample as δ -tocopherol is easily oxidized. Using sonication as some studies have done (Gomis et al. 2000) instead of acid and a hot water bath may help reduce loss of δ -tocopherol if this is the case. Sonication may also provide better results for measuring more fat soluble vitamins.

This study successfully demonstrated a LC-MS method for the measurement of phyloquinone and MK-4. The LC-MS was able to detect standard concentrations as low or lower than 0.1 $\mu\text{g/g}$ and a calibration curve from 0.1 to 1 $\mu\text{g/g}$ was obtained for both phyloquinone and MK-4. The measurement of vitamin K in five cheeses showed very high levels of vitamin K in Gouda and Maasdammer cheeses from grass-fed cows, but not in conventional supermarket cheddar. Measurement of vitamin K in cheese samples was problematic and further optimization of the extraction procedure is required before any larger scale analysis can be performed.

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