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Quantitative measurement of vitamin K_2 (menaquinones) in various fermented dairy products using a reliable high-performance liquid chromatography method

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ABSTRACT

We evaluated menaquinone contents in a large set of 62 fermented dairy products samples by using a new liquid chromatography method for accurate quantification of lipo-soluble vitamin K_2, including distribution of individual menaquinones. The method used a simple and rapid purification step to remove matrix components in various fermented dairy products 3 times faster than a reference preparation step. Moreover, the chromatography elution time was significantly shortened and resolution and efficiency were optimized. We observed wide diversity of vitamin K_2 contents in the set of fermented dairy products, from undetectable to 1,100 ng/g of product, and a remarkable diversity of menaquinone forms among products. These observations relate to the main microorganism species currently in the different fermented product technologies. The major form in this large set of fermented dairy products was menaquinone (MK)-9, and contents of MK-9 and MK-8 forms were correlated, that of MK-9 being around 4 times that of MK-8, suggesting that microorganisms able to produce MK-9 also produce MK-8. This was not the case for the other menaquinones, which were produced independently of each other. Finally, no obvious link was established between MK-9 content and fat content or pH of the fermented dairy products.
Key words: vitamin K_2, menaquinone, fermented milk, cheese

INTRODUCTION

Vitamin K_2 (menaquinone) is a natural form of vitamin K that occurs in food. Vitamin K_2 is mainly present in fermented food (e.g., natto, fresh fermented dairy product, cheese), whereas the other form of vitamin K, vitamin K_1 (phylloquinone), is abundant in leafy green vegetables (e.g., cabbage, spinach, lettuce). It is generally presumed that vitamin K_2 is produced by microorganisms. Vitamin K_2 refers to a series of naphthoquinones with a variable side chain that is usually between 4 and 10 isoprene units long. The compounds in the series are referred to as menaquinone (**MK**)-n, where n denotes the number of isoprene units.

Vitamin K_2 exhibits significant health benefits, particularly for cardiovascular (Beulens et al., 2009) and bone health (Schurgers et al., 2007). The daily intake dose recommended by European Food Safety Authority (EFSA, 1990) is 75 μg for adults and 12 μg for children from 6 mo to 4 yr. Some studies suggest that the bioavailability of vitamin K_2 is related to the length of the side chain, with medium-length menaquinone (e.g., MK-7) being more bioavailable than short-chain menaquinone (e.g., MK-4; Schurgers and Vermeer, 2000). Therefore, we assumed that not only the total amount of vitamin K_2 but also the distribution of vitamin K_2 forms could be of interest in supporting or enhancing health benefits.

Although the presence of menaquinones in food is well known and demonstrations of its health benefit are increasing, publications on reliable and accurate quantification methods (and efficient quantitative extraction methods) from food matrices are scarce.

The dosage of phylloquinone has been studied and quantified in different foodstuffs (Booth et al., 1993). A European standard method (ISO, 2004; NF EN 14148) exists to determine vitamin K_1 by HPLC, but no official method exists for the measurement of vitamin K_2. We propose an optimized technique of dosage and quantification of vitamin K_2 adapted to fermented dairy products. Using this method, we quantified total vitamin K_2 and evaluated the distribution of side-chain forms MK-6 to MK-10 in fermented dairy products obtained by different dairy technologies (e.g., thermophilic cheeses, mesophilic cheeses, fresh fermented milks).

MATERIALS AND METHODS

Fermented Dairy Samples

Sixty-two fermented dairy samples were purchased in late 2010 from several retail stores in France, Germany,

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Table 1. Fermented dairy samples in this study

Sample category and ID	Country (production country, if different)	Shelf life date (month/day/year)	Analysis date (month/day/year)	DM (g/100 g)	Fat (g/100 g)	pH
Blue cheese						
BlueC1	France	11/09/2010	11/09/2010	66.8	41.0	5.57
BlueC2	France	11/08/2010	11/08/2010	51.7	29.0	6.44
BlueC3	France	12/13/2010	11/30/2010	54.3	35.0	7.19
BlueC4	England	Unknown	12/15/2010	65.7	39.5	6.47
Caerphilly						
HardC-Caer	England	11/30/2010	11/30/2010	58.2	32.8	4.57
Cheddar						
HardC-Ched1	England	12/28/2010	12/03/2010	65.4	35.0	4.81
HardC-Ched2	England	12/02/2010	12/02/2010	62.3	33.0	5.08
HardC-Ched3	England	12/08/2010	12/03/2010	64.1	35.0	5.01
HardC-Ched4	England	11/22/2010	11/22/2010	61.1	32.8	5.01
HardC-Ched5	England	01/08/2011	12/03/2010	67.1	36.0	5.20
HardC-Ched6	England	12/31/2010	12/03/2010	66.0	36.0	5.22
Cheshire						
HardC-Ches	England	12/10/2010	12/03/2010	58.3	31.5	4.36
Comté						
HardC-Co1	France	11/12/2010	10/29/2010	65.2	34.5	5.57
HardC-Co2	France	12/13/2010	10/29/2010	65.2	34.5	5.68
Emmental						
HardC-Em1	France	12/06/2010	10/29/2010	61.3	30.5	5.59
HardC-Em2	France	12/28/2010	10/29/2010	61.0	29.5	5.52
Leicester						
HardC-Leic	England	12/01/2010	12/01/2010	63.0	33.0	4.97
Mesophilic fermented milk						
MFM1	France	10/31/2010	10/29/2010	16.3	9.5	4.34
MFM2	France	11/14/2010	10/29/2010	20.7	8.5	4.72
MFM3	France	11/11/2010	10/29/2010	17.2	2.8	4.51
MFM4	France	12/09/2010	11/30/2010	18.0	3.5	4.52
MFM5	France	12/05/2010	11/30/2010	24.0	10.0	4.57
MFM6	France	12/06/2010	11/30/2010	22.8	0.3	4.33
MFM7	France	05/01/2011	11/30/2010	18.6	0.3	3.84
MFM8	France	12/03/2010	11/30/2010	16.9	6.0	4.29
MFM9	Germany	12/12/2010	12/10/2010	25.0	10.0	4.37
MFM10	Germany	01/05/2011	12/15/2010	11.2	<0.3	4.09
MFM11	Germany	12/09/2010	12/09/2010	19.2	4.0	4.28
MFM12	Poland	11/26/2010	11/26/2010	9.3	0.5	4.40
MFM13	Poland	12/06/2010	12/06/2010	26.5	ND[1]	4.36
MFM14	Poland	12/10/2010	12/10/2010	11.9	1.5	4.52
MFM15	Poland	12/05/2010	12/03/2010	11.3	1.3	4.24
MFM16	Poland	12/11/2010	12/10/2010	25.1	4.0	4.34
MFM17	Poland	12/05/2010	12/03/2010	29.2	8.0	4.39
MFM18	Poland	12/06/2010	12/06/2010	21.9	6.5	5.05
MFM19	Poland	12/09/2010	12/09/2010	20.6	7.0	4.50
MFM20	Poland	12/16/2010	12/15/2010	26.8	18.0	4.36
MFM21	Poland	12/16/2010	12/15/2010	24.4	0.5	4.41
Mozzarella cheese						
Mozz	France (Germany)	11/28/2010	11/26/2010	43.7	23.5	5.79
Semihard cheese						
SemiHC1	France (the Netherlands)	01/24/2011	10/29/2010	57.8	29.5	5.04
SemiHC2	France (the Netherlands)	Unknown	10/29/2010	60.9	29.5	5.25
SemiHC3	France	11/30/2010	10/29/2010	55.7	27.5	5.41
SemiHC4	France (the Netherlands)	01/14/2011	10/29/2010	57.1	24.0	5.20
SemiHC5	France	11/16/2010	10/29/2010	51.3	26.0	5.05
SemiHC6	France (the Netherlands)	12/27/2010	10/29/2010	58.5	27.0	5.43
SemiHC7	Denmark	Unknown	12/15/2010	58.8	29.0	5.96
SemiHC8	Denmark	Unknown	12/15/2010	56.4	29.0	6.11
SemiHC9	Denmark	12/07/2010	12/07/2010	53.7	25.1	6.41
SemiHC10	Poland	01/31/2011	12/15/2010	56.8	27.0	5.31
SemiHC11	Poland	11/30/2010	11/30/2010	59.3	27.8	6.12
Soft cheese						
SoftC1	France	11/22/2010	11/09/2010	51.2	25.0	6.62
SoftC2	France	11/16/2010	11/09/2010	54.4	27.0	6.60
SoftC3	France	12/12/2010	11/30/2010	65.6	34.0	5.45

Continued

Table 1 (Continued). Fermented dairy samples in this study

Sample category and ID	Country (production country, if different)	Shelf life date (month/day/year)	Analysis date (month/day/year)	DM (g/100 g)	Fat (g/100 g)	pH
SoftC4	France	12/10/2010	11/30/2010	51.9	25.5	5.95
SoftC5	Germany	12/08/2010	12/08/2010	45.2	14.5	7.32
SoftC6	Germany	12/03/2010	12/03/2010	52.4	33.0	7.37
SoftC7	Germany	12/20/2010	12/15/2010	59.7	35.5	6.69
Thermophilic fermented milk						
TFM1	France	11/08/2010	10/29/2010	12.2	3.0	4.39
TFM2	France	10/28/2010	10/28/2010	12.6	0.5	4.15
TFM3	France	11/12/2010	10/29/2010	16.0	<0.3	4.02
TFM4	France	11/06/2010	10/29/2010	15.0	3.0	4.30
TFM5	France	11/23/2010	11/23/2010	9.4	2.0	4.59

[1]ND = not determined.

Denmark, England, and Poland. Table 1 shows the different types of dairy products used in the study. The samples were stored at 4°C in the dark and analyzed before their sell-by date and at the age at which they are typically consumed.

The products were all dairy foods fermented with lactic acid bacteria (e.g., *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Lactococcus*, and *Leuconostoc* spp.) and some also contained *Propionibacterium* and *Corynebacterium* spp., yeasts, and molds. The dairy products were grouped in categories based on their cheese technologies: 4 blue cheeses (**BlueC**), 13 hard cheeses (**HardC**), including 1 Caerphilly cheese (**HardC-Caer**), 6 Cheddar cheeses (**HardC-Ched**), 1 Cheshire cheese (**HardC-Ches**), 2 Comté cheeses (**HardC-Co**), 2 Emmental cheeses (**HardC-Em**) and 1 Leicester cheese (**HardC-Lei**), 1 Mozzarella cheese (**Mozz**), 21 mesophilic fermented milks (**MFM**), 11 semihard cheeses (**SemiHC**), 7 soft cheeses (**SoftC**), and 5 thermophilic fermented milks (**TFM**).

Analyses of DM and Fat Contents and pH

The DM content was determined following an adaptation of the ISO 5534 standard method (ISO, 2004; ISO 5534), with desiccation done for a constant time. The Heiss method (AFNOR, 2002; AFNOR NF V04-287) was used to determine the fat content of the fermented dairy products, and pH was measured by using a penetration probe introduced into the samples. All evaluations were performed once.

Chemicals and Reagents

All solvents used for vitamin K_2 analysis (extraction and chromatography) were of HPLC grade, including propan-2-ol, hexane, ethanol, and methanol. Reagents (hydrochloric acid, sodium acetate, sodium hydrogenophosphate, potassium dihydrogenophosphate and zinc chloride) were purchased from VWR (Fontenay-sous-Bois, France). Acetic acid was purchased from Fluka (Saint-Quentin-Fallavier, France). Zinc powder was supplied by Aldrich (Saint-Quentin-Fallavier, France). Phylloquinone (vitamin K_1) used as internal standard and *Candida rugosa* lipase (type VII, 1,449 U/mg) were purchased from Sigma (Saint-Quentin-Fallavier, France). Menaquinone-9 was purchased from Seebio Biotechnology Inc. (Shanghai, China). Ultrapure water was produced by a Purelab option Q system from Elga LabWater (Antony, France).

The stock standard solutions of phylloquinone and MK-9 (10 mg/mL) were prepared in ethanol and stored in the dark at 4°C. Working solutions were prepared from the stock solutions by appropriate dilution in ethanol (5 μg/mL for phylloquinone solution and 10 μg/mL for MK-9 solution) and shielded from light at 4°C.

Extraction of Vitamin K from Fermented Dairy Products

The method used to extract vitamin K_2 was adapted from Koivu-Tikkanen (2000). Two grams of each cheese or 10 g of thermophilic fermented milk was weighed in 50-mL amber polytetrafluoroethylene (PTFE) tubes with 10 mL of ultrapure water. Samples were mixed with an Ultraturrax mixer at 13,500 rpm for about 30 s. Then, 400 μL of internal standard (solution of vitamin K_1 at 5 μg/mL in ethanol) and 5 mL of 1 *N* HCl were added to this mixture (instead of the 10 mL of 37% HCl used by Koivu-Tikkanen, 2000). The sample was heated in a water bath at 100°C for 30 min (instead of the 10 min used by Koivu-Tikkanen, 2000) and then cooled in an ice bath. A heat treatment of 60 min was also tested and gave similar results to the treatment for 30 min, showing no destruction of vitamin K_2 even with the longer heating step. Ten milliliters of propan-2-ol and 2 mL of hexane were added to extract menaqui-

Table 2. Comparison of vitamin K_2 concentrations measured in milk samples with different fat levels (spiked with 200 ng/mL of menaquinone-9) and a full-fat soft cheese and treated with either lipase (reference method in the literature) or acid (proposed new method)

Item	Lipase treatment	Acid treatment
0% Fat (n = 5)		
Mean ± SD (ng/mL)	200.5 ± 2.8	187.6 ± 2.8
RSD[1] (%)	1.4	1.5
5% Fat (n = 5)		
Mean ± SD (ng/mL)	222.8 ± 6.9	198.5 ± 4.0
RSD (%)	3.1	2.0
15% Fat (n = 5)		
Mean ± SD (ng/mL)	229.8 ± 3.0	195.5 ± 4.7
RSD (%)	1.3	2.4
30% Fat (n = 5)		
Mean ± SD (ng/mL)	222.5 ± 15.6	195.8 ± 8.8
RSD (%)	7.0	4.5
Full-fat soft cheese (n = 5)		
Mean ± SD (ng/g)	665.6 ± 15.3	840.7 ± 30.6
RSD (%)	2.3	3.6

[1]RSD = relative standard deviation.

nones. Samples were shaken and centrifuged at 3,700 × *g* for 10 min at room temperature. After filtration through a 0.45-μm nylon filter, the organic layer was placed in an amber vial and injected into the liquid chromatograph. The extraction was performed 4 times independently. All steps of the extraction were carried out under reduced light to prevent deterioration of the vitamin K.

In this study, 2 preparation steps were tested to obtain a fast and reproducible extraction method for various fermented dairy products. Most of the published studies about vitamin K extraction use a lipase treatment; for example, Pérez-Ruiz et al. (2007) and Indyk and Woollard (1997, 2000). We decided to test whether an acid treatment in the extraction step would avoid the time-consuming and unreliable lipase treatment.

Milk samples containing different levels of fat (0, 5, 15, and 30%) and free from vitamin K_2 were spiked with 200 ng/mL of MK-9 and extracted 4 times. Following extraction with an acid treatment or a lipase treatment, milk samples were analyzed by HPLC. For the HCl test, 10 mL of milk was added to 10 mL of ultrapure water and 5 mL of 1 *N* HCl and heated for 30 min at 100°C. For the lipase test, 0.5 g of *Candida rugosa* lipase and 10 mL of PBS (pH 7.70) were added to 10 mL of milk sample and incubated for 90 min at 45°C. Following pretreatment with acid or lipase, vitamin K_2 was extracted.

A French full-fat soft cheese (same brand as the SoftC3 cheese, with 63.4 g/100 g of DM, 33.5 g/100 g of fat, and pH 5.67) was analyzed following the 2 preparations step outlined here to compare the acid treatment with the lipase treatment for a complex fermented dairy product.

Analytical HPLC of Menaquinones

Chromatography analysis was performed by using an Agilent Technologies HPLC 1100 series system (Palo Alto, CA) in reverse phase with fluorescence detection. The HPLC system comprised a model G1379A online degasser, a model G1379A quaternary pump, a model G1367A WPALS autosampler set at 10 μL, a model G1316A COLCOM temperature-controlled column oven set at 55°C, and a model G1321A FLD fluorescence detector. The menaquinones were separated on a Gemini column (3 μm C18, 100 × 4.6 mm, Phenomenex, Le Pecq, France). They were eluted in isocratic mode with a mobile phase composed of an 83:17 (vol/vol) mixture of methanol and ethanol added with

Table 3. Resolution (R) between peak 2 and peak marked with * (undefined compound) for both columns[1]

Column[2]	RT_2 (peak 2; min)	RT_* (peak *; min)	ω_2 (peak 2; min)	ω_* (peak *; min)	Resolution
Capcell Pak column	22.71	24.21	1.82	1.63	0.870
Gemini column	12.35	14.03	1.47	0.77	1.500

[1]RT_2 = retention time for peak 2; RT_* = retention time for peak *; ω_2 = peak width at base for peak 2; ω_* = peak width at base for peak *.

[2]Capcell Pak column was from Shiseido Co. Ltd. (Tokyo, Japan); Gemini column was from Phenomenex (Le Pecq, France).

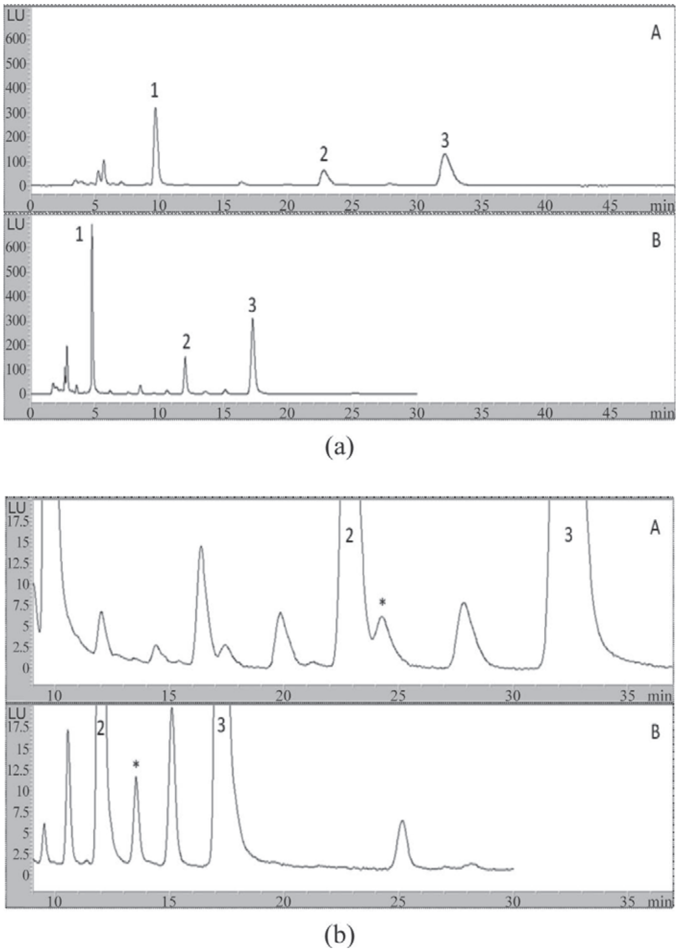

Figure 1. Chromatograms of a microorganism extract eluted on a Capcell Pak column (A; Shiseido Co. Ltd., Tokyo, Japan) and a Gemini column (B; Phenomenex, Le Pecq, France). Panel (a) shows the chromatogram of the full elution time, whereas panel (b) shows the portion of the full elution time between 9 and 37 min). Peak 1 = vitamin K_1, peak 2 = menaquinone (MK)-8, peak 3 = MK-9, peak * = undefined compound.

5 mmol/L of sodium acetate, 10 mmol/L of $ZnCl_2$, and 5 mmol/L of acetic acid. The flow rate of the mobile phase was set at 1.4 mL/min. Vitamin K compounds do not exhibit natural fluorescence so they need to be reduced in hydroquinones that are fluorescent to improve the sensitivity of fluorescent detection. This step was carried out after separation by chemical reduction into a postcolumn (50 × 4 mm) filled with zinc powder (Haroon et al., 1987). The menaquinones were detected with a fluorescence detector set to an excitation wavelength of 220 nm and an emission wavelength of 436 nm.

Different forms of vitamin K_2 (MK-6 to MK-10) were identified by retention time and quantified by internal calibration with vitamin K_1 used as internal standard. A 5-level calibration was obtained by injection of standards containing vitamin K_1 (2,000 ng/mL) and MK-9 (20 to 5,000 ng/mL) before each sample analysis series and by using peak areas. Linear regression was performed to quantify the menaquinones. Because no standard was commercially available, quantification of MK-6 to MK-10 was achieved by using MK-9 response factor. Concentrations were then corrected by the molecular mass ratio of MK-6, MK-7, MK-8, and MK-10 to MK-9.

Several publications deal with the use of a Capcell Pak column C18 manufactured by Shiseido Co. Ltd. (Tokyo, Japan); for example, Hojo et al. (2007) and Kamao et al. (2005, 2007). To reduce elution time and obtain better resolution, we tested a column with lower particle size and length. We compared the Gemini column (3 µm, C18, 150 × 4.6 mm, Phenomenex) with the Capcell Pak column (5 µm SG-C18, 250 × 4.6mm, Shiseido Co. Ltd.). A solvent flow rate of 1.4 mL/min was chosen for the Gemini column, whereas a flow rate of 1 mL/min was used with the Capcell Pak column. A menaquinone extract was injected with both chromatography methods onto both columns.

Resolution (R) between 2 peaks a and b was calculated using the formula

$$R = 2\times \frac{RT_a - RT_b}{\omega_a + \omega_b},$$

where RT_a and RT_b are the retention times of the first and the second peaks, respectively, and ω_a and ω_b are the peak width at base for the first and second peaks, respectively. Efficiency (N) and height equivalent to a theoretical plate ($HETP$) for both columns were calculated as follows:

Table 4. Efficiency and height equivalent to a theoretical plate (HETP) for both columns

Column[2]	RT (peak 3; min)	ω (peak 3; min)	Length (mm)	Efficiency	HETP (mm)
Capcell Pak column	32.11	4.30	250	892.2	0.280
Gemini column	17.19	2.10	150	1,072.1	0.140

[1]RT = retention time for peak 3; ω = peak width at base for peak 3.

[2]Capcell Pak column was from Shiseido Co. Ltd. (Tokyo, Japan); Gemini column was from Phenomenex (Le Pecq, France).

Table 5. Concentration (ng/g of product; means ± SD) of menaquinones (MK-6 to MK-10) and total amount of vitamin K_2 measured in the 62 fermented dairy products

Cheese sample category and ID	MK-6	MK-7	MK-8	MK-9	MK-10	Total K_2
Blue cheese						
BlueC1	14.4 ± 0.8	24.6 ± 1.2	59.8 ± 2.0	189.0 ± 5.0	30.0 ± 1.2	317.7 ± 8.1
BlueC2	35.4 ± 6.4	ND[1]	67.6 ± 5.2	230.4 ± 8.6	27.8 ± 3.5	361.3 ± 10.7
BlueC3	ND	ND	ND	52.3 ± 1.8	ND	52.3 ± 1.8
BlueC4	96.7 ± 12.0	223.3 ± 19.0	103.1 ± 6.7	301.7 ± 17.1	ND	724.8 ± 54.5
Caerphilly						
HardC-Caer	15.8 ± 1.3	ND	78.9 ± 2.2	324.3 ± 8.0	ND	418.9 ± 7.8
Cheddar						
HardC-Ched1	8.7 ± 5.9	ND	37.9 ± 1.6	155.5 ± 3.9	ND	202.2 ± 3.6
HardC-Ched2	16.0 ± 2.9	ND	10.5 ± 2.2	24.8 ± 2.7	ND	51.3 ± 3.2
HardC-Ched3	27.5 ± 2.3	21.7 ± 0.9	61.8 ± 0.8	253.3 ± 1.5	64.9 ± 2.6	429.2 ± 3.9
HardC-Ched4	24.5 ± 0.7	ND	15.2 ± 0.8	ND	49.4 ± 1.5	89.1 ± 2.1
HardC-Ched5	29.9 ± 3.6	23.1 ± 0.3	44.1 ± 3.6	143.4 ± 6.4	52.9 ± 2.2	293.4 ± 4.4
HardC-Ched6	26.4 ± 1.1	17.2 ± 1.7	19.4 ± 0.5	69.5 ± 5.2	39.5 ± 5.6	172.0 ± 9.9
Cheshire						
HardC-Ches	15.7 ± 0.7	ND	57.9 ± 1.7	241.7 ± 4.0	ND	315.4 ± 3.2
Comté						
HardC-Co1	ND	ND	ND	ND	ND	ND
HardC-Co2	ND	ND	ND	ND	ND	ND
Emmental						
HardC-Em1	ND	ND	ND	ND	35.0 ± 3.1	35.0 ± 3.1
HardC-Em2	ND	ND	ND	ND	45.1 ± 6.1	45.1 ± 6.1
Leicester						
HardC-Leic	20.0 ± 1.1	21.5 ± 1.3	47.6 ± 2.4	162.4 ± 2.8	43.8 ± 2.2	295.4 ± 3.5
Mesophilic fermented milk						
MFM1	4.1 ± 0.3	3.8 ± 0.2	21.2 ± 0.3	85.6 ± 0.9	5.9 ± 0.5	120.6 ± 2.0
MFM2	3.9 ± 0.3	3.8 ± 0.8	20.3 ± 1.1	56.4 ± 3.0	ND	84.4 ± 4.4
MFM3	1.3 ± 0.3	1.2 ± 0.2	7.2 ± 0.8	29.1 ± 1.4	ND	38.8 ± 2.2
MFM4	1.9 ± 0.2	2.8 ± 0.1	25.9 ± 0.8	144.7 ± 4.6	6.0 ± 0.4	181.2 ± 5.6
MFM5	4.9 ± 0.3	6.1 ± 0.2	37.9 ± 1.7	145.5 ± 5.6	6.1 ± 0.5	200.5 ± 7.5
MFM6	ND	ND	9.6 ± 1.0	21.1 ± 1.8	ND	30.7 ± 2.8
MFM7	ND	ND	ND	10.2 ± 0.4	ND	10.2 ± 0.4
MFM8	ND	ND	7.0 ± 0.6	60.4 ± 4.9	ND	67.4 ± 5.4
MFM9	6.0 ± 0.2	6.3 ± 0.3	31.1 ± 1.7	88.4 ± 3.2	5.5 ± 1.0	137.3 ± 1.6
MFM10	ND	ND	ND	1.9 ± 0.2	ND	1.9 ± 0.2
MFM11	2.1 ± 0.4	4.1 ± 0.3	42.3 ± 2.8	198.5 ± 8.8	6.5 ± 0.5	253.6 ± 12.3
MFM12	ND	4.3 ± 0.4	17.4 ± 0.3	98.1 ± 1.5	9.9 ± 0.8	129.7 ± 1.9
MFM13	4.8 ± 0.6	7.7 ± 0.2	25.3 ± 1.5	104.5 ± 4.9	5.7 ± 0.9	147.9 ± 7.4
MFM14	ND	ND	7.1 ± 0.8	31.1 ± 0.7	ND	38.2 ± 1.4
MFM15	ND	ND	4.4 ± 0.5	19.3 ± 1.7	ND	23.7 ± 2.0
MFM16	4.3 ± 0.8	9.3 ± 0.5	82.3 ± 2.7	414.2 ± 13.0	16.1 ± 1.0	526.2 ± 16.3
MFM17	7.9 ± 0.8	10.9 ± 0.4	89.3 ± 3.5	394.8 ± 5.1	16.2 ± 1.1	519.2 ± 9.8
MFM18	0.5 ± 1.0	ND	8.4 ± 1.0	17.0 ± 1.9	ND	26.0 ± 3.1
MFM19	2.6 ± 0.4	3.2 ± 0.7	18.0 ± 2.8	37.1 ± 4.1	ND	60.9 ± 5.0
MFM20	11.9 ± 0.6	3.6 ± 0.1	16.5 ± 1.3	82.1 ± 3.0	ND	114.1 ± 4.8
MFM21	2.2 ± 0.4	3.4 ± 0.3	20.7 ± 1.1	76.1 ± 2.2	ND	102.4 ± 3.3
Mozzarella cheese						
Mozz	ND	ND	ND	ND	ND	ND
Semi-hard cheese						
SemiHC1	29.0 ± 3.6	ND	33.9 ± 4.5	168.8 ± 23.3	ND	231.6 ± 31.1
SemiHC2	34.5 ± 6.5	ND	73.1 ± 6.5	321.1 ± 24.2	ND	428.7 ± 36.2
SemiHC3	21.0 ± 0.8	20.6 ± 1.2	51.1 ± 9.0	100.4 ± 24.5	30.2 ± 1.3	223.3 ± 32.1
SemiHC4	14.5 ± 1.0	5.5 ± 6.3	37.3 ± 1.8	169.4 ± 16.8	26.0 ± 2.4	252.8 ± 16.2
SemiHC5	15.3 ± 0.2	9.1 ± 6.1	39.2 ± 3.8	217.7 ± 11.9	27.0 ± 2.0	308.3 ± 15.8
SemiHC6	15.9 ± 0.6	14.1 ± 1.7	61.3 ± 4.7	176.6 ± 12.4	ND	267.9 ± 16.7
SemiHC7	16.1 ± 2.0	13.5 ± 3.4	35.8 ± 1.4	115.3 ± 3.6	13.7 ± 3.1	194.3 ± 10.2
SemiHC8	19.8 ± 0.7	6.2 ± 1.0	25.0 ± 0.6	185.1 ± 2.1	ND	236.1 ± 2.7
SemiHC9	16.3 ± 0.7	7.1 ± 0.8	45.0 ± 4.5	182.9 ± 18.2	ND	251.3 ± 23.3
SemiHC10	9.8 ± 2.1	ND	27.8 ± 1.5	124.5 ± 4.1	ND	162.1 ± 5.2
SemiHC11	15.8 ± 1.0	ND	56.4 ± 1.7	166.3 ± 1.6	138.0 ± 9.6	376.6 ± 9.3
Soft cheese						
SoftC1	18.5 ± 2.4	ND	89.2 ± 17.9	176.1 ± 19.1	ND	283.8 ± 37.1
SoftC2	13.7 ± 1.1	13.4 ± 2.0	103.7 ± 9.0	201.7 ± 13.5	ND	332.6 ± 19.4
SoftC3	25.9 ± 4.0	17.1 ± 1.2	139.9 ± 12.9	939.7 ± 15.5	56.5 ± 3.1	1,179.1 ± 22.7

Continued

Table 5 (Continued). Concentration (ng/g of product; means ± SD) of menaquinones (MK-6 to MK-10) and total amount of vitamin K_2 measured in the 62 fermented dairy products

Cheese sample category and ID	MK-6	MK-7	MK-8	MK-9	MK-10	Total K_2
SoftC4	12.7 ± 4.2	16.1 ± 1.3	78.9 ± 3.7	355.5 ± 12.6	ND	463.2 ± 18.4
SoftC5	3.7 ± 0.6	3.2 ± 0.9	23.4 ± 3.3	76.4 ± 7.8	1.8 ± 1.4	108.4 ± 13.2
SoftC6	26.1 ± 2.6	16.3 ± 4.9	21.3 ± 1.2	65.8 ± 3.9	ND	129.6 ± 7.9
SoftC7	16.9 ± 1.7	4.6 ± 1.6	33.7 ± 1.3	126.0 ± 5.3	ND	181.2 ± 6.3
Thermophilic fermented milk						
TFM1	ND	ND	ND	ND	ND	ND
TFM2	ND	ND	ND	ND	ND	ND
TFM3	ND	ND	ND	ND	ND	ND
TFM4	ND	ND	ND	ND	ND	ND
TFM5	ND	ND	ND	ND	ND	ND

[1]ND = not detected.

$$N = 16\left(\frac{RT}{\omega}\right)^2,$$

$$HETP = \frac{L}{N},$$

where RT is the retention time of the peak (min), ω is the peak width at base (min), and L is the length of the column (mm).

RESULTS AND DISCUSSION

Method Optimization

The goals of this optimization were to obtain a fast and reproducible preparation step for fermented dairy products and high resolution of chromatography. For that purpose, we replaced the lipase treatment commonly used as a preparation step by a chemical step. We also tested a second column for chromatography analysis.

Acid Versus Lipase Treatment. As observed in Table 2, samples treated with acid (and particularly milk samples with higher fat levels) gave results closest to the added amount of 200 ng/mL compared with samples treated with lipase. The level of total vitamin K_2 in soft cheese was higher with the acid treatment than with the lipase treatment (Table 2). This could be explained by the fact that the action of the lipase might have been incomplete. Based on these experiments and results, we decided to implement the acid treatment

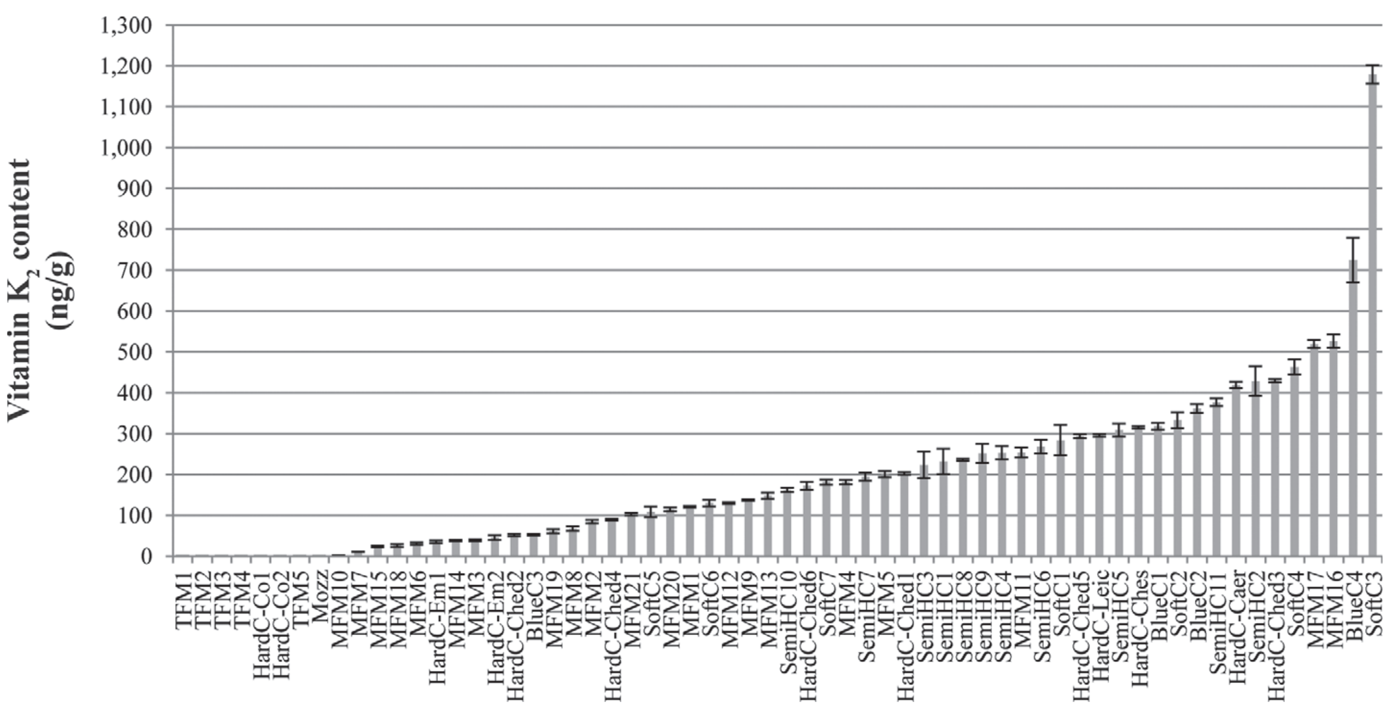

Figure 2. Total amount of vitamin K_2 for the 62 fermented dairy products. See Table 1 for sample ID codes.

for the extraction step for all types of fermented dairy products.

Chromatography Parameters. Because of the dimensions of the Gemini column, peaks had a more Gaussian shape, as observed in Figure 1; fewer co-elutions can be seen in Figure 1. The calculated resolution for 2 selected peaks was almost twice as high with the Gemini column compared with the Capcell Pak column (Table 3). Resolution with the Gemini column was even better than the recognized low resolution limit of 1.3; this was not the case for the Capcell Pak column.

Efficiency was calculated for both chromatography columns to compare peak dispersion (Table 4) and was clearly higher with the Gemini column. The height equivalent to a theoretical plate (HETP) was half as much with the Gemini column, as demonstrated by higher and thinner peaks. The use of the Gemini column reduced the chromatography analysis time and resolved much more efficiently, giving Gaussian-shaped peaks for all menaquinones. Results presented in this publication are based on this optimized method.

Concentration of Menaquinones in Fermented Dairy Products

The concentrations of menaquinones and the total amount of vitamin K_2 measured in the 62 fermented dairy products (ng/g of product) are given in Table 5. The total amount of vitamin K_2 showed a wide range of values among the fermented dairy products under study, from undetectable vitamin K_2 values (for TFM1) to values up to 1,100 ng/g of product (for SoftC3), as illustrated in Figure 2. The daily intake of cheese is estimated at around 30 g, which could thus provide up to 33 μg of vitamin K_2 or 44% of the daily dose recommended for adults by the European Food Safety Authority. The literature reports vitamin K_2 content generally ranges from undetectable concentration to around 400 ng/g in fermented dairy products (Koivu-Tikkanen, 2000). However, other authors noticed values up to 800 ng/g in some cheeses (Schurgers and Vermeer, 2000; Schurgers, 2002).

Eight fermented dairy products did not contain detectable amounts of vitamin K_2: 5 TFM (TFM1 to TFM5), 2 Comté cheeses (HardC-Co1 and HardC-Co2), and 1 Mozzarella cheese (Mozz). Similarities in cheese process explain this absence of vitamin K_2. For these types of products, usually only thermophilic species (*Streptococcus thermophilus*, *Lactobacillus delbrueckii*, *Bifidobacterium*) are used as lactic acid bacteria starter (Fox et al., 2000), and those thermophilic species are not known to be vitamin K_2 producers (Collins and Jones, 1981; Morishita et al., 1999). This is also the case for a yogurt-type product also made with pure thermophilic species (Hirauchi et al., 1989; Koivu-Tikkanen et al., 2000).

All other fermented dairy products evaluated in this study contained some vitamin K_2; 60% of the products had >100 ng/g, a high value for animal products (Koivu-Tikkanen et al., 2000). All of those products contained mesophilic lactic acid bacteria species as starters and especially *Lactococcus* spp., which are reported to be the source of vitamin K_2 (Collins and Jones, 1981; Morishita et al., 1999).

The contents of vitamin K_2 for semihard cheeses were homogeneous, with vitamin K_2 concentrations never less than 100 ng/g. This is in agreement with previous studies; for example, approximately 400 ng/g of vitamin K_2 has been reported for an Edam-type cheese (Koivu-Tikkanen et al., 2000).

Two products had very high concentrations of vitamin K_2: a soft cheese (SoftC3, up to 1,100 ng/g) and a blue cheese (BlueC4, around 700 ng/g). Further investigations are needed to better understand these high contents of vitamin K_2. This level of vitamin K_2 in cheeses has never been reported in the literature because soft cheeses and blue cheeses have never been studied in respect to their vitamin K_2 content. This high level is mostly due to the lactic acid bacteria used in making these cheeses, in particular *Leuconostoc* species (Morishita et al., 1999) and the yeasts or molds used as ripening flora. Most of the soft and blue cheeses analyzed in this study had high contents of vitamin K_2.

The literature typically reports the total amount of vitamin K_2, and information about contents of individual menaquinones in fermented dairy products is limited. Figure 3 illustrates the distribution of individual menaquinones from MK-6 to MK-10 that were quantified in 54 fermented dairy products. The 8 fermented dairy products that did not contain any vitamin K_2 were excluded from this analysis.

Clearly, the major form quantified in fermented dairy products was MK-9; at least 83% of the fermented dairy products studied contained >50% of MK-9. The second major form was MK-8. This finding is in accordance with the literature, which indicates that MK-9 and

Table 6. Pearson correlation coefficient (r) and significance of r performed using the Student *t*-test[1]

	MK-9	MK-8	pH	Fat content
MK-9	1.00	0.88***	0.08	0.24
MK-8	0.88***	1.00	0.23	0.33
pH	0.08	0.23	1.00	0.60
Fat content	0.24	0.33	0.60	1.00

[1]MK = menaquinone.

***$P < 0.001$.

MK-8 are dominant in various cheese types (Koivu-Tikkanen, 2000). However, our results showed that MK-9 was much more significant in fermented dairy products.

Figure 3 shows that 2 Emmental cheeses (HardC-Em1 and HardC-Em2) contained only MK-10. A report from the literature reported that Emmental-type cheeses did not have any menaquinones (Koivu-Tikkanen et al., 2000). The HardC-Em1 and HardC-Em2 cheeses were the 2 products in this series of 54 fermented dairy products that mostly contained thermophilic lactic acid bacteria (*Streptococcus thermophilus*), but they also

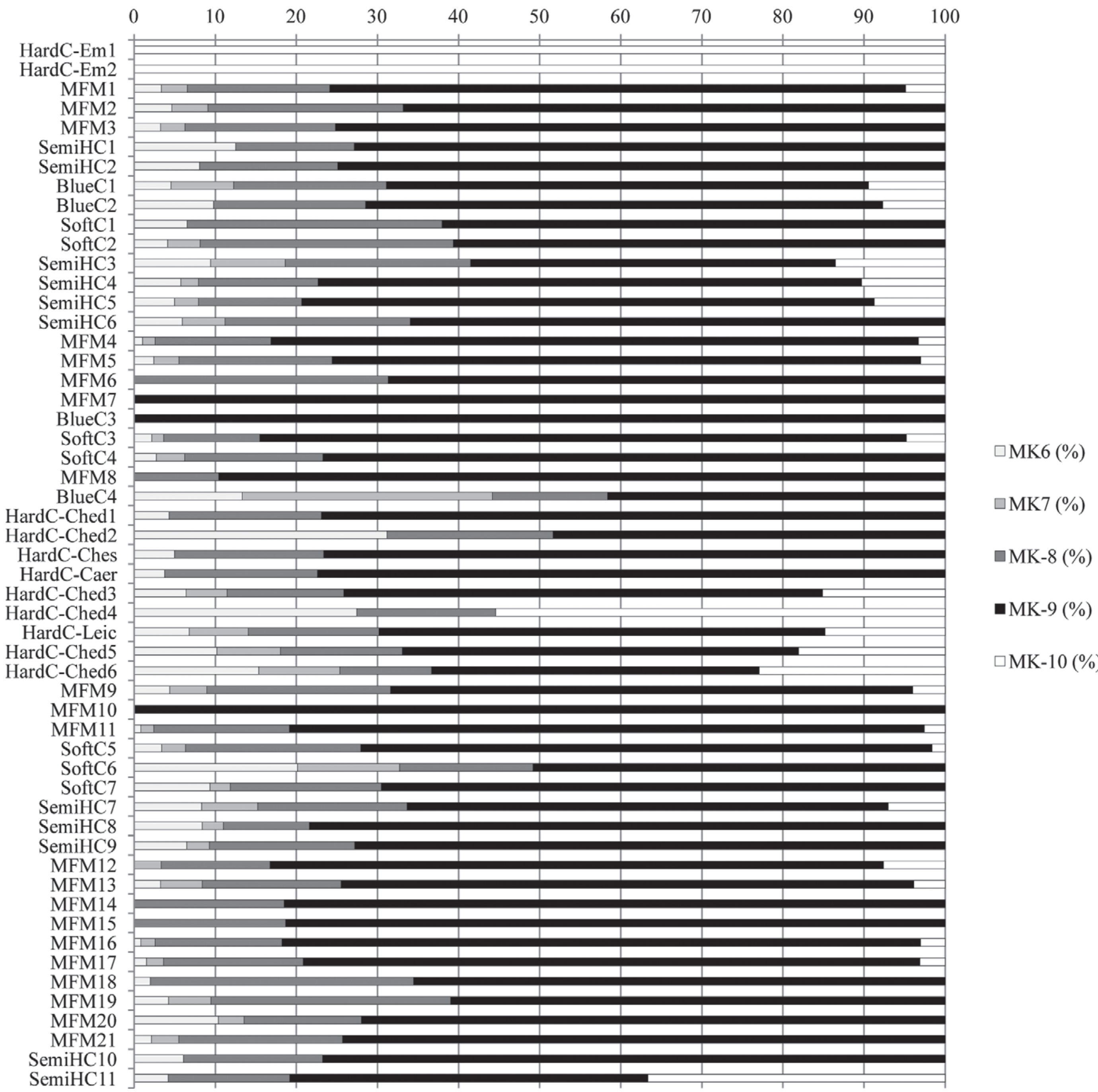

Figure 3. Distribution of the different forms of menaquinones (MK) for the 54 fermented dairy products (8 samples are not shown because they did not have detectable contents of vitamin K_2). See Table 1 for sample ID codes.

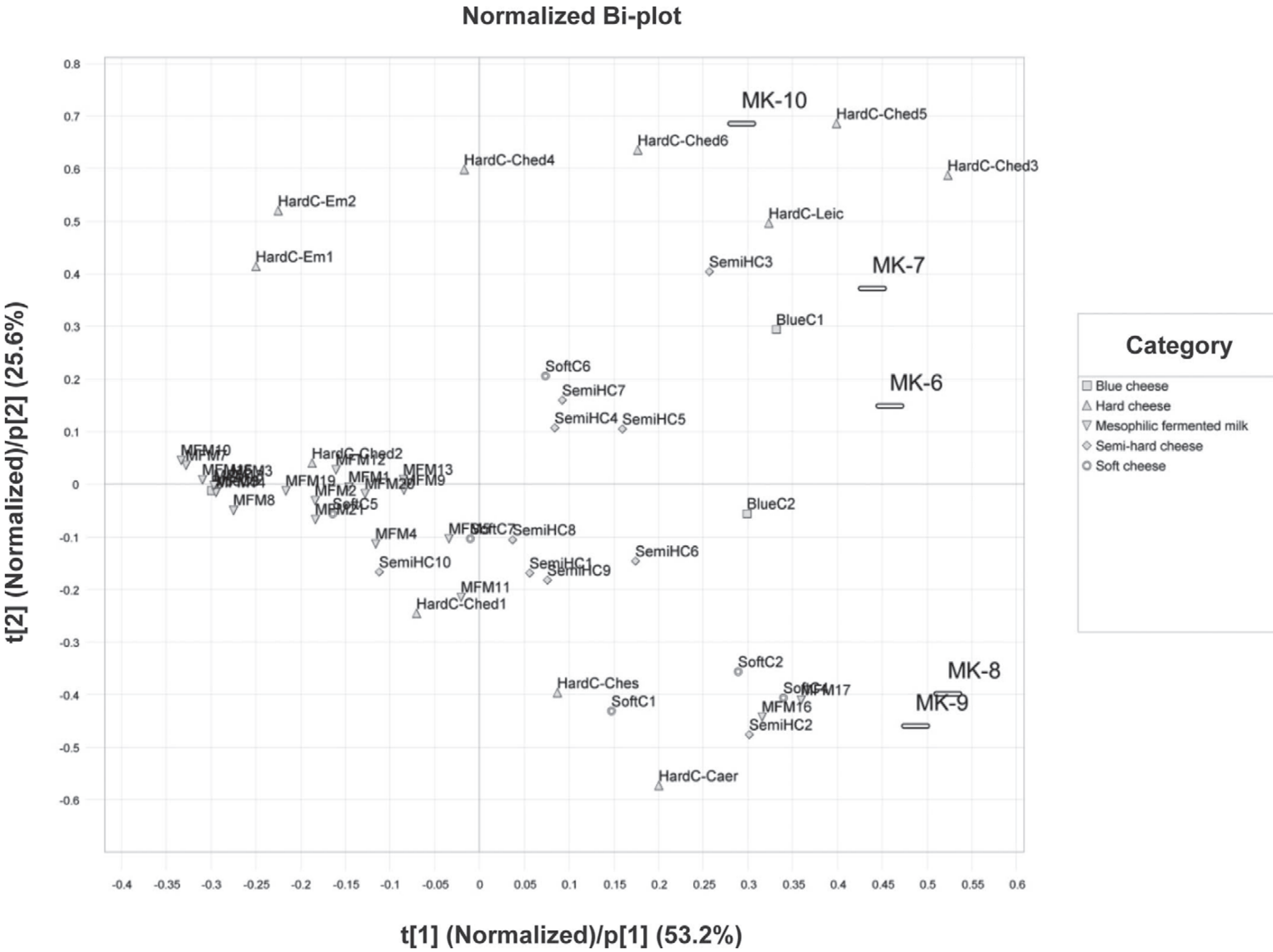

Figure 4. Principal component analysis (PCA) of the 52 fermented dairy products (8 samples are not shown because they did not have detectable contents of vitamin K_2, and 2 outlier samples, BlueC4 and SoftC3, did not undergo this PCA), distributed by menaquinones (MK) concentrations. t[1] and t[2] = samples in axis 1 and axis 2, respectively; p[1] and p[2] = MK concentration in axis 1 and axis 2, respectively. See Table 1 for sample ID codes.

contained specific species as propionibacteria and probably some lactobacilli. Unfortunately, none of those species is known to be a producer of MK-10.

Among the hard cheeses, the Cheddar cheese HardC-Ched4 was clearly differentiated by having no MK-9, whereas most of the other fermented dairy products contained some; it also possessed a high proportion of MK-10 compared with other fermented products. This was a surprising finding, and further research is needed to explain this observation. Cheddar cheese HardC-Ched2 also had a high proportion of MK-6, which was generally a minor form for most of the fermented dairy products studied here.

Among blue cheeses, the blue cheese BlueC3 had an unusual profile, containing only MK-9. Moreover, blue cheese BlueC4, which had one of the highest amounts of total vitamin K_2, had a high percentage of MK-7, which was otherwise not common in this type of cheese. Among the semi-hard cheeses, SemiHC11 was clearly distinguished from the others by its higher proportion of MK-10.

Data analysis was performed using principal component analysis (**PCA**) on fermented dairy samples with 5 menaquinone forms (MK-6 to MK-10). The biplot of principal component (PC) 1 compared with PC2 is depicted in Figure 4; PC1 explained 53.2% of the variation, whereas PC2 explained 25.6% of the variation. The 2 cheeses that were previously identified as outliers, BlueC4 and SoftC3 (with differentiating high values of menaquinones), did not undergo PCA.

Principal component analysis enabled us to visualize the position of the 52 fermented dairy products based on menaquinone content and highlighted some correlations between the different menaquinone forms. Trends toward grouping were apparent: some fermented dairy products were placed in the west quadrant of the plot, indicating a negative correlation with most forms of vitamin K_2. Most of the products located in that quadrant were mesophilic fermented milks. Two exceptions were MFM16 and MFM17, which had high MK-8 and MK-9 concentrations and so were located in the east region of the map. Those 2 products were Tvarog, from Poland, and were mainly distinguished by their *Leuconostoc* content.

Mainly hard cheese (Emmental, Cheddar, and Leicester cheeses) technology was represented in the north region of the map, representative of cheeses having a high content of MK-10. Nevertheless, 2 distinguishing products in this category, HardC-Ches and HardC-Caer, were located in the south region of the PCA map, with higher MK-8 and MK-9 levels. The technology used for these 2 hard cheeses is similar; Caerphilly is often compared with a Cheshire, although its format is smaller and moisture content is higher (Fox et al., 2004). Both cheeses are known to have lower pH than Cheddar cheeses (confirmed in this study: pH around 4.3 compared with 5 for Cheddar cheeses), with higher amounts of lactic acid bacteria, particularly *Lactococcus* species.

As observed in the PCA map, concentrations of MK-8 and MK-9 were correlated ($r = 0.88$; $P < 0.001$), indicating a linear correlation between those 2 forms of menaquinone. For most fermented dairy products, the level of MK-9 was 4 times higher than that of MK-8. This suggested that microorganisms (most probably lactococci) able to convey MK-9 in those fermented dairy products also produced MK-8. No obvious correlation could be established for MK-6, MK-7, or MK-10. Further investigations would be necessary to better understand the origin of those menaquinones, including microbial enumeration of the different fermented dairy products.

Because the MK-9 form was predominant, we studied how the composition of the fermented dairy products (and especially fat and pH) could affect MK-9 content; results are given in Table 6. The level of MK-9 was clearly not dependent on the fat level content of the fermented dairy products, meaning that low-fat dairy products could contain as much MK-9 as high-fat products. Moreover, we found no link between pH and MK-9 content in those fermented milks.

CONCLUSIONS

An HPLC method for quantitative measurement of menaquinones in fermented dairy products was developed with 2 main objectives: to reduce the analytical time and obtain better resolution for chromatographic peaks. The initial lipase treatment was replaced by an acid treatment. This modification made the sample preparation step 3 times faster, elution time was reduced by using a different column, and resolution and efficiency were optimized. Menaquinone-9 was the major form quantified in a variety of fermented dairy products. Levels of MK-9 and MK-8 were correlated, although the levels of MK-9 were approximately 4 times those of MK-8. This suggests that the lactococci that produced MK-9 in these fermented dairy products also produced MK-8 in a relatively constant ratio. We found no link between fat level or pH of the fermented dairy products and MK-9 content.

REFERENCES

AFNOR. 2002. AFNOR NF V04-287: Fromages—Détermination de la teneur en matière grasse—Méthode de Heiss. Agence Française de Normalisation, La Plaine Saint-Denis Cedex, France.

Beulens, J. W., M. L. Bots, F. Atsma, E. L. Bartelink, M. Prokop, J. M. Geleijnse, C. M. Witterman, D. E. Grobbee, and Y. T. Van Der Schouw. 2009. High dietary menaquinone intake is associated with reduced coronary calcification. Atherosclerosis 203:489–493.

Booth, S. L., J. A. Sadowski, J. J. Weihrauch, and G. Ferland. 1993. Vitamin K_1 (phylloquinone) content of food: A provisional table. J. Food Compost. Anal. 6:109–120.

Collins, M. D., and D. Jones. 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. Microbiol. Rev. 45:316–354.

EFSA (European Food Safety Authority). 1990. Directive 90/496/EEC on Nutrition Labelling for Foodstuffs. European Food Safety Authority, Parma, Italy.

Fox, P., P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee. 2000. Fundamentals of Cheese Science. Aspen Publishers Inc., Gaithersburg, MD.

Fox, P., P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee. 2004. Cheese: Chemistry, Physics and Microbiology. Major Cheese Groups. Vol. 2. 3rd . Elsevier Academic Press, London, UK.

Haroon, Y., D. S. Bacon, and J. A. Sadowski. 1987. Chemical reduction system for the detection of phylloquinone (vitamin K_1) and menaquinones (vitamin K_2). J. Chromatogr. A 384:383–389.

Hirauchi, K., T. Sakano, S. Notsumoto, T. Nagaoka, A. Morimoto, K. Fujimoto, S. Masuda, and Y. Suzuki. 1989. Measurement of K vitamins in foods by high-performance liquid chromatography with fluorometric detection. Vitamins (Japan) 63:147–151.

Hojo, K., R. Watanabe, T. Mori, and N. Taketomo. 2007. Quantitative measurement of tetrahydromenaquinone-9 in cheese fermented by propionibacteria. J. Dairy Sci. 90:4078–4083.

Indyk, H. E., and D. C. Woollard. 1997. Vitamin K in milk and infant formulas: Determination and distribution of phylloquinone and menaquinone-4. Analyst 122:465–469.

Indyk, H. E., and D. C. Woollard. 2000. Determination of vitamin K in milk and infant formulas by liquid chromatography: Collaborative study. J. AOAC Int. 83:121–130.

ISO. 2004. ISO 5534: Cheese and processed cheese: Determination of the total solids content (Reference method). International Organization for Standardization (ISO), Geneva, Switzerland.

ISO. 2004. NF EN 14148: Foodstuffs: Determination of vitamin K1 by HPLC. International Organization for Standardization (ISO), Geneva, Switzerland.

Kamao, M., Y. Suhara, N. Tsugawa, and T. Okano. 2005. Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 816:41–48.

Kamao, M., N. Tsugawa, Y. Suhara, A. Wada, T. Mori, K. Murata, R. Nishino, T. Ukita, K. Uenishi, K. Tanaka, and T. Okano. 2007. Quantification of fat-soluble vitamins in human breast milk by liquid chromatography–tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 859:192–200.

Koivu-Tikkanen, T. J. 2000. Determination of phylloquinone and menaquinones in foods by HPLC. PhD Thesis. EKT-series 1216. Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland.

Koivu-Tikkanen, T. J., V. Ollilainen, and V. I. Piironen. 2000. Determination of phylloquinone and menaquinones in animal products with fluorescence detection after postcolumn reduction with metallic zinc. J. Agric. Food Chem. 48:6325–6331.

Morishita, T., N. Tamura, T. Makino, and S. Kudo. 1999. Production of menaquinones by lactic acid bacteria. J. Dairy Sci. 82:1897–1903.

Pérez-Ruiz, T., C. Martinez-Lozano, M. D. Garcia, and J. Martin. 2007. High-performance liquid chromatography-photochemical reduction in aerobic conditions for determination of K vitamins using fluorescence detection. J. Chromatogr. A 1141:67–72.

Schurgers, L. J. 2002. Studies on the role of vitamin K_1 and K_2 in bone metabolism and cardiovascular disease. Structural differences determine different metabolic pathways. PhD Thesis. University of Maastricht, Maastricht, the Netherlands.

Schurgers, L. J., M. H. J. Knapen, and C. Vermeer. 2007. Vitamin K_2 improves bone strength in postmenopausal women. Int. Congr. Ser. 1297:179–187.

Schurgers, L. J., and C. Vermeer. 2000. Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. Haemostasis 30:298–307.