FOOD COMPOSITION AND ADDITIVES

Liquid Chromatographic Determination of Folate Monoglutamates in Fish, Meat, Egg, and Dairy Products Consumed in Finland

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A liquid chromatographic (LC) method with fluorescence and UV detection was used to determine the folate contents of fish, meat, fish and meat products, chicken, eggs, and milk consumed in Finland. 5-Methyltetrahydrofolate, tetrahydrofolate, 5-formyltetrahydrofolate, 10-formylfolic acid, and folic acid from 24 commodities obtained from supermarkets, retail stores, and different outlets in the Helsinki area were analyzed. Pooled samples were extracted at pH 6.0 in the presence of antioxidants and deconjugated with hog kidney deconjugase. Very low levels of folates were detected in meat and meat products. Fresh fish, fish sticks, and chicken meat contained reasonable amounts (3–13 μg/100 g) of tetrahydrofolate and 5-methyltetrahydrofolate. Egg yolk contained high concentrations of 5-methyltetrahydrofolate (140–150 μg/100 g); 10-formylfolic acid was also detected (14–17 μg/100 g). Between-species differences in folate monoglutamate distributions were observed. The highest levels of tetrahydrofolate, >5 μg/100 g, were found in chicken meat and fillets of rainbow trout, whitefish, and baltic herring. Tetrahydrofolate was most abundant in fresh fish. LC was well suited for analyzing folate compositions of meat, fish, and other foods of animal origin. Recovery of added folates ranged from 49 to 96%.

The folate content of meat is low. Pork and lamb contain only a few micrograms of folate, and most raw beef cuts contain <10 μg folate/100 g. Most fish also contain <15 μg folate/100 g, but extensive variation exists even in recently reported values (e.g., from 3.4 to 26 μg/100 g for salmon). The folate contents of chicken meat are in the same range as those of fish (1–3). The folate content of pasteurized milk is about 5 μg/100 g, and the major folate is 5-methyltetrahydrofolate (4, 5). Folates are well concentrated in egg yolk, but egg white is a poor folate source (2, 6). These mostly low levels of folate in foods of animal origin are important as far as folate intake from diet is concerned because large amounts of foods of animal origin, particularly liquid dairy products, are consumed. The distribution of folates in these foods is not well known, and up-to-date food composition data based on modern analytical techniques are needed to estimate the daily folate intake from the diet. Also, the influence of folate distribution on folate stability or bioavailability is still unclear.

Folate bioavailability from natural sources may be limited or impaired when compared with folic acid (supplemental or through fortification) (7–9). Thus, it is important to obtain more information of the folate forms in foods. Folate fortification of food is not practiced in many countries, and staple foods usually are good folate sources. Some data on the distribution of folate monoglutamates after deconjugation in foods of animal origin are available (4, 5, 10–14). Selhub and coworkers, (15, 16) reported the distribution of folate polyglutamates in some foods. As more bioavailability studies are performed with natural foods, data on folate composition and polyglutamate chain lengths should also be available.

The aim of this work was to study the folate content and distribution in foods of animal origin. We studied the staple meat and fish products consumed in Finland, including both raw and processed products. Some dairy products and eggs also were analyzed. Foods were obtained as they would be by consumers to take into account storage and handling losses of analytes or possible interconversions in fresh products. This study is part of a larger survey to determine folate contents of foods consumed in Finland (14, 17, 18).

Experimental

Reagents

(a) *Standards.*—Tetrahydrofolic acid trihydrochloride, 5-methyltetrahydrofolic acid (calcium salt), 5-formyltetrahydrofolic acid (calcium salt), folic acid, pteroyltri-γ-L-glutamic acid ($PteGlu_3$), and 10-formylfolic acid were obtained from Dr. Schirck's Laboratories, Jona, Switzerland. Standards were dissolved as described by van den Berg et al. (19), and purities were calculated from molar extinction coefficients at pH 7 (20). Standard solutions were stored at −18°C in 0.01M acetate buffer (pH 4.9) with 1% ascorbate.

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(**b**) *Extraction buffer.*—0.075M phosphate buffer (pH 6.0), 1.0% ascorbate, and 0.1% mercaptoethanol.

(**c**) *Solvents for liquid chromatography (LC).*—LC grade acetonitrile (Rathburn, Walkerburn, Scotland) and 0.03M phosphate buffer (pH 2.2).

(**d**) *LC mobile phase.*—Acetonitrile–phosphate buffer (pH 2.2) gradient (9% acetonitrile increased to 24% within 8 min after 4 min lag phase, acetonitrile decreased to 9% at 14 min for equilibration); 0.8 mL/min.

(**e**) *Enzymes.*—Hog kidney deconjugase was prepared according to the method of Gregory et al. (21). Fifty microliters of that preparation yielded almost complete deconjugation of 17 μg $PteGlu_3$ (in excess compared with samples) to folic acid within 40 min.

Apparatus

(**a**) *Liquid chromatograph.*—Varian Vista 5500 equipped with UV-200 detector (Varian, Surrey, UK); scanning fluorescence detector, Model 470 (Waters, Milford, MA); Millennium 2010 chromatography data acquisition system (Waters).

(**b**) *LC columns.*—Hypersil ODS 150 × 4.6 mm id, packed with 3 μm silica particles (Shandon, Cheshire, UK); Spherisorb ODS column (Phase Separations Ltd, Clwyd, UK) packed with 5 μm silica particles (250 × 4.6 mm id) mounted in front of a Hypersil column in case peak purities were checked; Novapak C_{18} Guard-Pak guard columns (Waters).

Samples

Samples were collected in the Helsinki area from May 1995 to July 1996. A composite sample was prepared usually from 10 subsamples obtained from stores and supermarkets (10 in total) belonging to 4 of the main retail chains in Finland, according to their market share.

Samples were collected in a manner that brand effects were included either subjectively by sampling the most popular brands the most or objectively by using sales estimates given by wholesale food chains or food manufacturers. Each commodity was collected once within a day, providing one composite sample, except for rainbow trout and eggs, which were sampled twice during the study. The first composite sample of eggs was collected to represent regular eggs, and the second pool of eggs consisted of specialty eggs (e.g., from hens kept on litter). Vendace (*Coregonus albula*) was sampled from marketplaces, retail shops, and fish distributors.

The goal was to obtain a representative sample that will provide average compositional data for each food. About 17% of the Finnish population lives in the sampling area. Food distribution is centralized in Finland, and no geographical variations in vitamin, mineral, or proximate content of foods in Finland have been found previously (Ollilainen et al., 1996, unpublished results, University of Helsinki, Finland).

Extraction, Deconjugation, and Purification of Samples

Procedures for sample preparation, extraction, deconjugation, and purification were reported elsewhere (17). Fresh fish was prepared for frozen storage in extraction buffer on the day of purchase. Other samples were prepared within 2 days of purchase. The storage time in buffer at –20°C before analysis was kept to a minimum, being at most 3 weeks. Five grams of solid samples was weighed in duplicate for extraction at pH 6.0. Milk and buttermilk (10 g) were stored in 5 mL 0.15M phosphate buffer containing 2% sodium ascorbate and 0.2% mercaptoethanol. Because of its low pH and strong buffering capacity, buttermilk was also extracted with 0.15M phosphate (1% ascorbate, 0.1% mercaptoethanol). Deconjugation was performed at pH 4.9 with hog kidney deconjugase for 2 h at 37°C. Usually, 3 mL sample extract was used with 0.8–1.0 mL hog kidney deconjugase. For samples with very low folate content, 4 or 5 mL was applied. Deconjugated sample extracts were purified with strong-anion-exchange cartridges prior to LC analysis.

Table 1. Recoveries of folate monoglutamates added to food samples[a]

Folate	Amount added, μg/100 g	Recovery, %
	Beef steak[b]	
H_4	6	64 ± 4.8
5-CH_3	6	76 ± 2.1
5-HCO	28	90 ± 27.6
10-HCO-FA	22	70 ± 3
FA	22	66 ± 11.0
	Egg yolk[b]	
H_4	1.2	61 ± 3.0
5-CH_3	168	67 ± 5.8
5-HCO	42	82 ± 10.6
10-HCO-FA	18	49 ± 5.8
FA	44	49 ± 3.1
	Milk powder[c]	
H_4	—	ND
5-CH_3	18	88
5-HCO	—	ND
10-HCO-FA	—	ND
FA	220	77
	Lyophilized pig liver[c]	
H_4	—	ND
5-CH_3	200	96
5-HCO	—	ND
10-HCO-FA	—	ND
FA	—	ND

[a] Key: H_4 = tetrahydrofolate, 5-CH_3 = 5-methyltetrahydrofolate, 5-HCO = 5-formyltetrahydrofolate, 10-HCO-FA = 10-formylfolic acid, FA = folic acid, ND = not determined.
[b] Recoveries are means of 3 determinations ± standard deviations.
[c] Recoveries are means of 2 determinations.

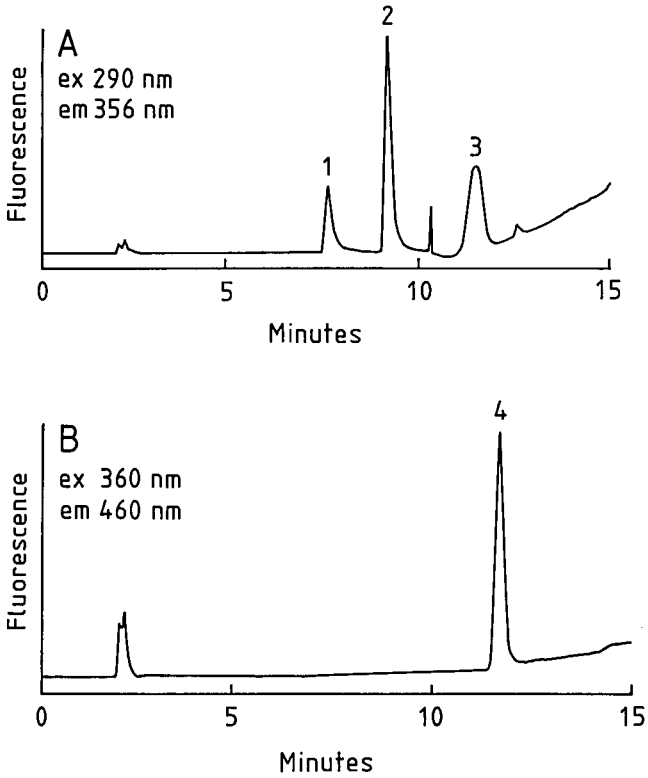

Figure 1. Liquid chromatograms of reduced folate monoglutamate standards: (A) peak 1, tetrahydrofolate (1 ng), peak 2, 5-methyltetrahydrofolate (3 ng), and peak 3, 5-formyltetrahydrofolate (4 ng). Fluorescence detection wavelengths: excitation, 290 nm; emission 356 nm. (B) peak 4, 10-formylfolic acid (2 ng). Fluorescence detection wavelengths: excitation, 360 nm; emission, 460 nm. Detector gain change at 10.4 min.

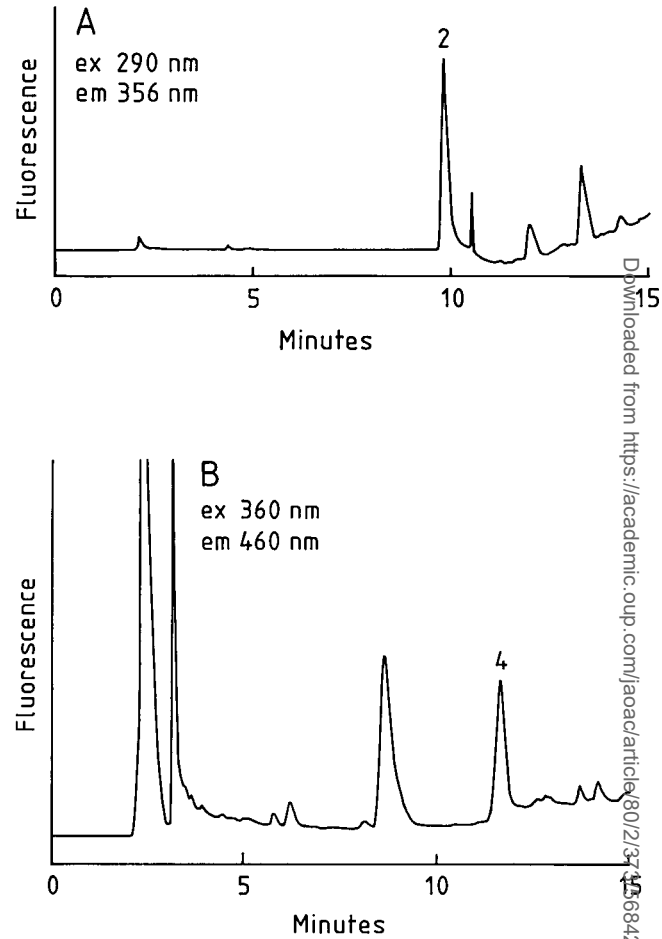

Figure 2. Liquid chromatograms of folate monoglutamates in egg yolk: (A) peak 2, 5-methyltetrahydrofolate (1.7 ng), (B) peak 4, 10-formylfolic acid (1.8 ng).

Liquid Chromatography

Samples and standards were analyzed in a liquid chromatograph equipped with a fluorescence detector. Excitation wavelength was 290 nm and emission wavelength was 356 nm (reduced folate monoglutamates). An excitation wavelength of 360 nm and emission wavelength of 460 nm were used for 10-formylfolic acid. Folic acid was determined with a UV detector at 290 nm. The capacity factors (k′) of tetrahydrofolate, 5-methyl-tetrahydrofolate, 10-formylfolic acid, 5-formyltetrahydrofolate, and folic acid were 3.7, 4.6, 5.6, 5.9, and 6.3, respectively. Ten to 100 μL of sample extract after anion-exchange purification was injected into the column in duplicate.

Moisture and Fat

Moisture content was determined in duplicate from the overnight loss of weight at 102°C (22). Fat content was determined in triplicate by petroleum ether extraction (22).

Recovery and Assay Studies

Beef steak and egg yolk were used in recovery studies with 5 folate derivatives. In addition, milk powder and lyophilized pigs' liver were used for recovery studies with 5-methyltetrahydrofolate and folic acid (milk). Standards were added prior to homogenization, and recoveries were calculated according to AOAC methods (22).

A control sample (milk powder or lyophilized pigs' liver), a sample extract with $PteGlu_3$ to control deconjugation efficiency, and an enzyme blank were assayed with each set of analysis.

Folate monoglutamates were determined by their retention times and spiking. UV detection was used to verify peaks on the basis of their response ratios with fluorescence detection, particularly for 5-formyltetrahydrofolate. Because of the low amounts of folate in the samples, only a few more peaks could be quantitated with UV detection (5-methyltetrahydrofolate in egg yolk and 5-formyltetrahydrofolate in Edam cheese). Quantitation was based on external calibration plots of the peak heights of the standards vs their concentration fitted by the least-square regression equation. The amount of each folate is presented in its free acid form. Standards (untreated) were injected from the same buffer as the samples.

Results and Discussion

Mean recoveries ranged from 49 to 96% (Table 1). Egg yolk was a difficult matrix, as reflected by the low recoveries. Addi-

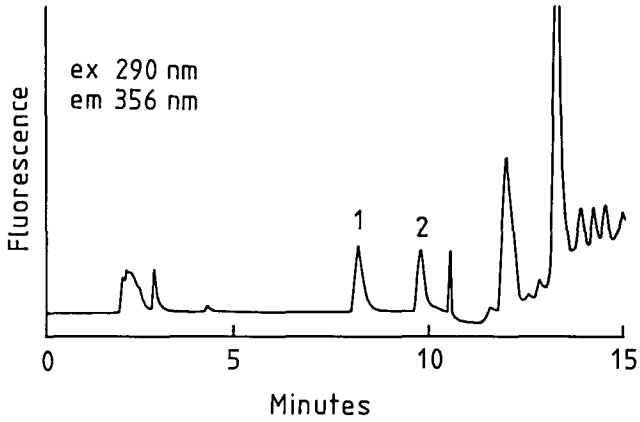

Figure 3. Liquid chromatogram of folate monoglutamates in raw vendace: peak 1, tetrahydrofolate (0.6 ng) and peak 2, 5-methyltetrahydrofolate (0.4 ng).

tion of very low levels of tetrahydrofolate might have influenced recoveries. In these samples, folic acid was detected only in trace amounts, and therefore, its poor recovery did not influence the folate content of these foods. Unfortunately, only folate monoglutamates could be used for recovery studies, whereas most food folates are expected to exist as polyglutamates. None of the results were corrected for recoveries.

Method performance was tested in intercomparison studies with vitamin-enriched milk powder and pigs' liver (23). Our results were slightly lower for milk powder (95 μg) and pigs' liver (1222 μg) than the mean results obtained with the microbiological method (*Lactobacillus rhamnosus* at pH 6.2): 124 and 1499 μg/100 g, respectively. Method repeatability, expressed as the relative coefficient of variation of the results for control milk sample during 1.5 years, was 12% for the sum of 3 folates (103 ± 12.3 μg/100 g) and 11% for 5-methyltetrahydrofolate (28 ± 3.1 μg/100 g).

Representative chromatograms of folates in standards, egg yolk, and vendace samples are shown in Figures 1–3. Separation was achieved in 15 min (one column), but for peak verification, separation with 2 columns was sometimes used (e.g., 5-formyltetrahydrofolate in chicken meat, Edam cheese, and butter milk and 10-formylfolic acid in egg yolk).

Folate contents of 5 species of raw fish, some fish products, and frozen shrimps are shown in Table 2. Folate levels in meat and meat products and in eggs and few milk products are presented in Tables 3 and 4. Results are means of 2 determinations for one pooled sample.

The most important folates in these foods were 5-methyltetrahydrofolate and tetrahydrofolate. 10-Formylfolic acid was detected in egg yolk. Other samples (e.g., herring and smoked whitefish) showed small peaks with similar retention values, but their identities were not ascertained with additional measures. 5-Formyltetrahydrofolate was present in a few samples, but because of its low fluorescence intensity, its detection in small quantities (<5 μg/100 g) was difficult. Method detection limits were presented previously (18).

In fresh fish fillet, 5-methyltetrahydrofolate ranged from 1.4 μg (pike) to 6.1 μg (vendace) per 100 g and tetrahydrofolate ranged from 2.8 μg (pike) to 8.9 μg (vendace). Smoked

Table 2. Folate monoglutamates in fresh fish and fish products[a]

Item	Folate, μg/100 g fresh weight					Dry matter, %	Description of pooled sample
	H_4	5-CH_3	5-HCO	10-HCO-FA	FA		
Pike (*Esox lucius*)	2.8[b]	1.4	2	—	—	19.8	8 fillets, skinned
Baltic herring (*Clupea harengus* spp. *membras*)	5.5	5.4	msk (—)	msk(7)	—	22.1	Fillets from 10 stores; average fillet,, 19 g
Rainbow trout (*Salmo gairdneri*)	5.6	4.1	msk (2)	0.3	—	32.7	9 fillets, 340–674 g each, skinned
Rainbow trout	8.5	4.8	msk	ND	—	ND	Few fillets
Vendace (*Coregonus albula*)	8.9	6.1	msk	—	—	24.9	10 subsamples; 57 fish in pool; average size, 27 ± 10.6 g; heads and gut removed in the laboratory
Whitefish (*Coregonus* sp.)	6.5	2.7	<2	—	—	26.0	6 fillets, skinned
Whitefish, smoked	2.2	2.7	—	msk (8)	—	27.2	Obtained as 10 whole fish; size,, 710 ± 178 g; flesh only
Fish sticks, cod	3.1	5.1	—	<0.5	—	39.1	11 samples, 3 brands, frozen, about 60% fish
Herring, pickled	0.3	0.2	msk (<2)	—	<2	46.0	9 samples in glass containers, 7 brands, seasoned, drained
Tuna fish, canned in water	0.7	1.1	msk (<5)	—	<1	26.6	10 samples, 6 brands, from Thailand, drained for 15 s; tuna was 81% of total weight
Brine drained from canned tuna fish	0.5	0.7	—	—	<1	8.9	*See* above
Shrimps, frozen	2.0	13	msk (<8)	<1	msk (<5)	15.9	10 samples, 5 brands

[a] Key: H_4 = tetrahydrofolate, 5-CH_3 = 5-methyltetrahydrofolate, 5-HCO = 5-formyltetrahydrofolate, 10-HCO-FA = 10-formylfolic acid, FA = folic acid, — = below detection limit, ND = not determined, msk = masked by impurities (highest possible level given in parenthesis).

[b] Mean of duplicate determinations for one pooled sample.

Table 3. Folate monoglutamates in meat and meat products[a]

Item	Folate, µg/100 g fresh weight				Dry matter, %	Fat, %	Description of pooled sample
	H_4	5-CH_3	5-HCO	FA			
Beef meat, steak	0.7[b]	0.5	msk	—	26.8	ND	10 samples
Pork meat, shoulder	0.8	0.2	—	—	28.0	6.6	10 samples, meat only
Minced meat, beef and pork	1.2	0.3	—	—	35.0	14.5	10 samples, about 1:1 ratio
Chicken meat	7.6	1.5	6 (msk)	<2	23.6	ND	11 legs, deboned and skinned
Ham, cooked, smoked	0.5	0.1	2–3 (msk)	—	26.0	3.3	10 samples, chunks or sliced
Sausage, cooked, cutlet type	1.1	0.7	—	—	34.7	14.7	9 samples, packed, sliced
Sausage, cooked, lightly smoked	1.6	0.6	—	—	36.8	14.2	10 samples, 8 brands, 'lenkki'

[a] Key: H_4 = tetrahydrofolate, 5-CH_3 = 5-methyltetrahydrofolate, 5-HCO = 5-formyltetrahydrofolate, FA = folic acid, — = below detection limit, ND = not determined, msk = masked by impurities.
[b] Mean of duplicate determinations for one pooled sample.

whitefish and fish sticks contained >5 µg total folates. Considering its high lability to oxidation, tetrahydrofolate was well retained in processed fish. Brine drained from canned tuna fish contained almost as much folates as the canned tuna itself.

All meat and meat products, excluding chicken, contained <5 µg folates per 100 g. Milk contained mainly 5-methyltetrahydrofolate; buttermilk and cheese contained more 5-formyltetrahydrofolate than other folates. Here the 5-formyltetrahydrofolate was quantitated with UV detection because of traces of fluorescing impurities.

Laukkanen et al. (11, 12) studied several liquid milk products and cheeses for their vitamin content. Their folate contents, determined also by LC for liquid milk products, are very similar to ours, except for Edam cheese. Their result for Edam cheese was as high as 34.8 µg/100 g by microbiological method. Our LC result (8 µg) was impaired by impurities or other matrix effects (e.g., extraction efficiency). For liquid milk products, Laukkanen et al. (11) found good correlation between LC and microbiological results.

Egg yolk contains mainly 5-methyltetrahydrofolate in conjugated form (16). We also found mostly 5-methyltetrahydrofolate (87%) in egg yolk, but the level we detected was almost twice that reported earlier (16). Different feeding practices could explain the differences, because folates in egg yolks become saturated with increasing dietary folate (6). The 2 pools of egg yolk we analyzed produced very similar values.

Very limited data exist on folate composition of fish or fish products. Müller (13) reported folate derivatives in 2 fish species and considered further analysis necessary. Other data are mostly on total folates determined by microbiological assay (1, 24, 25), but the folate levels are about the same as reported here. However, our analysis indicates that tetrahydrofolate is one of the important folates in fish.

Folate levels were very low in meat and meat products. But folate contents can be very high in liver, liver foods, and kidneys (14, 25). Our results for 5-methyltetrahydrofolate and tetrahydrofolate in beef and pork are very much in line with those of Müller (13), but not for 5-formyltetrahydrofolate. Our results indicate that tetrahydrofolate is the main folate in chicken meat, particularly in the dark leg meat. Data on total folates of meat reported here are at the lower range of previously published data (1–3), perhaps because of low recoveries (Table 1) or the lack of reference compounds or suitable methods for other unknown folates. Our data, however, provide information

Table 4. Folate monoglutamates in egg yolk, egg white, milk, buttermilk, and cheese[a]

Item	Folate, µg/100 g fresh weight					Dry matter, %	Description of pooled sample
	H_4	5-CH_3	5-HCO	10-HCO-FA	FA		
Egg yolk I	<0.5[b]	148	—	17	5	48.8	40 eggs from 10 cartons; average egg size, 60 g
Egg yolk II	<0.1	139	msk	14	7	48.1	18 eggs from 6 cartons; average egg size, 65 g
Egg white I	0.3	0.4	—	—	6	11.4	*See* above
Egg white II	0.1	0.2	—	—	—	11.5	*See* above
Milk, 1.5% fat	0.3	4.1	—	—	—	10.1	10 samples, semiskimmed, pasteurized
Buttermilk, 2.5% fat	0.6	3.7	5	1	—	10.5	10 samples, cultured with *Lactobacillus acidofilus* and *Bifidobacterium* starters
Buttermilk, nonfat	2.0	4.0	9	ND	—	ND	10 samples
Yoghurt, plain	1.9	1.2	3	ND	—	ND	10 samples
Cheese, Edam type	1.2	2.2	4/msk	1	msk	58.1	9 samples, 24% fat

[a] Key: H_4 = tetrahydrofolate, 5-CH_3 = 5-methyltetrahydrofolate, 5-HCO = 5-formyltetrahydrofolate, 10-HCO-FA = 10-formylfolic acid, FA = folic acid, — = below detection limit, ND = not determined, msk = masked by impurities.
[b] Mean of duplicate determinations for one pooled sample.

for further evaluation of these foods as folate sources, their bioavailability, and their stability during storage or processing.

10-Formylfolic acid is not expected to be naturally present in food. It is an oxidation product of 10-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate, and 5,10-methylenetetrahydrofolate (26, 27). Its presence in egg yolk has not been reported previously, possibly because no one has looked for it. Although it is an oxidation product, 10-formylfolic acid is included in microbiological folate assays (*Lactobacillus casei*) and in chick bioassays, because it exhibits the similar biological activity as folic acid (28).

Composite samples were used in this study; results therefore do not indicate possible within-species variation. When 2 separate samples of eggs and rainbow trout were analyzed, the variation after compositing was not high. Our unpublished results indicate only a small variation (4–22% relative coefficient of variation) in the folate content of fish or chicken meat sampled at the same time from several outlets.

On the basis of average food consumption (29, 30), the best folate sources from the studied foods (of animal origin) were eggs, milk, and cultured milk, including yoghurt. With the folates from liver and other edible offals, the average daily intake of folates from the foods of animal origin would be around 100 μg. Correcting recoveries by the mean recovery of 5-methyltetrahydrofolate (82%) or tetrahydrofolate (63%) will moderately increase the average daily intake. On the other hand, a more careful estimation of daily folate intake that includes cooking losses, for example, would possibly decrease the average intake.

Conclusion

Tetrahydrofolate and 5-methyltetrahydrofolate were the most important folates in the Finnish foods studied. Although the microbiological method for folate analysis is well established and is well suited for determining total folate in foods, significant additional data on folates in foods can be obtained accurately with LC and fluorescence detection.

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