Determination of Folates in Vegetables and their Retention During Boiling

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Abstract

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HPLC method for 5-methyltetrahydrofolate (5-MTHF) determination in vegetables was optimised for the folate release from the food matrix. Enzymatic hydrolyses using the subsequent addition of α-amylase, protease, and conjugase from hog kidney, or their combinations, were tested. The highest release values were obtained with the application of enzymes α-amylase and conjugase, amounting to 112.4–127.0% of the values obtained in the processing with sole conjugase. The simultaneous addition of both enzymes and the incubation at pH 4.9 did not suppress the release of folates. Spinach, Chinese cabbage, lettuce, cauliflower, and broccoli contained more than 50 µg of 5-MTHF/100g, whereas less than 25 µg/100 g was found in potatoes, carrot, white cabbage, green and yellow pepper. Individual vegetables differed in the folate retention during their boiling under constant conditions. The highest retention was found in Brussels sprouts, cauliflower, and broccoli. After 8 min boiling more than 75% of the initial amount of 5-MTHF remained in these vegetables. Lower values of 5-MTHF retention, between 37% and 52% of their initial content, were found in spinach, savoy cabbage, and carrot.

Keywords: vegetables; folate; determination; retention

The essentiality of folates for humans has been known for many years. Folates play a key role in DNA biosynthesis and in the methylation cycle. Deficiency of folates may influence the ability to synthetise DNA and result in anemia. A high level of plasma homocysteine, which is considered as a risk factor in cardiovascular diseases, corresponds with a low folate intake. It has been proved that a reduced folate status in pregnant women leads to an increased risk of neural tube defects in infants (Scott *et al.* 2000).

Folate levels in vegetables are relatively high. Considering their consumption, vegetables represent a rich source of folates in the human diet. Folates contained in raw vegetables, as analysed by HPLC method, were found to range between 27 and 187 µg/100 g by Müller (1993), and between 9 and 114 µg/100 g by Vahteristo *et al.* (1997), with 5-methyltetrahydrofolate as the predominant derivative. The total folate content up to 425 µg/100 g was established by microbiological assay of Australian vegetables (Iwatani *et al.* 2003). The comparison of the data contained in various national food tables and summarised by Witthöft *et al.* (1999) also reveals many differences in the folate contents of respective vegetables. The probable reason for this is not only the variation in variety, season, climate, and postharvest handling, but primarily the differences in analytical procedures.

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Microbiological and HPLC methods are most often used to determine total folates. This determination generally consists in sample homogenisation, extraction, enzymatic hydrolysis, and the microbiological or HPLC folate assay. Conjugase is usually used for the hydrolysis of polyglutamyl folates. Due to the fact that folates might be bound to polysaccharides or proteins, α-amylase and protease are sometimes recommended for enhancing their release from the food matrix. The experimental conditions chosen should respect the low folate stability and will always be a compromise between the maximal release of folates and the stability of monoglutamylfolate released.

Some authors found that the trienzyme method led to a better folate release and thus to higher folate concentrations than the application of sole conjugase (Tamura 1998; Aiso & Tamura 1998; Johnston *et al.* 2002a; Yon & Hyun 2003). However, Iwatani *et al.* (2003) and Pandrangi and LaBorde (2004) recorded higher release values after the application of a single enzyme or two enzymes in the analyses of broccoli and spinach.

The reactivity and the solubility of folates are the reasons for expecting substantial folate losses during cooking. Leaching and oxidative degradation represent the main causes of the decrease of folate values in this case and may thus influence the folate intake. Extensive folate losses in the course of spinach and broccoli boiling were found by McKillop *et al.* (2002). Steaming, in contrast, resulted in a minimal decrease of the folate content. In a laboratory-simulated water and steam blanching of spinach, 83% and 42%, respectively, of folates were lost (De Souza & Eitenmiller 1986). The effect of the treatment was found to be strongly plant species dependent (Puuponen-Pimia *et al.* 2003).

The aim of the present study was to evaluate the analytical procedure of folate determination in vegetables with respect to the conditions of enzymatic hydrolysis. This method was applied to the determination of folates contained in their main vegetable sources. The retention of folates in the course of vegetables boiling was determined.

MATERIAL AND METHODS

Vegetables were bought at local retail stores between September 2005 and May 2006. The samples were purchased one day before conducting the experiments and stored overnight at 4–8°C. The standard of 5-methyltetrahydrofolate (5-MTHF) was obtained from Schircks Laboratories, Switzerland. Conjugase (EC 3.4.19.9: γ-glutamyl hydrolase) from hog kidney prepared according Gregory *et al.* (1984) was used for enzymatic hydrolyses. The efficacy of the isolated enzyme was tested using pteroyltri-γ-L-glutamic acid (Vahteristo *et al.* 1996). Pteroyltri-γ-L-glutamic acid, α-amylase (EC 3.2.1.1: 1,4-α-D-glucan glucanohydrolase), and protease (EC 3.4.24.31: actinase E) were purchased from Sigma, the certified material CRM 485 – lyophilised mixed vegetable – from Fluka.

Optimisation of enzymatic hydrolysis. The basic procedure for the determination of 5-methyltetrahydrofolate (5-MTHF), developed for its assay in milk products (Holasová *et al.* 2004), included homogenisation of the sample using the homogeniser IKA A11 and the extraction into phosphate buffer, pH 6 (100°C, 10 min) in the presence of antioxidants. After cooling and pH adjustment to 4.9, conjugase from hog kidney was added, followed by incubation at 37°C for 3 hours. The enzymes were inactivated by boiling, the samples were then cooled, centrifuged, purified by SPE on a SAX column, and analysed by RP HPLC with fluorimetric detection. To find optimal conditions for enzymatic hydrolysis, selected vegetable matrices (lettuce, spinach, cabbage, Chinese cabbage, and broccoli) were subjected to the following procedures in order to evaluate the influence of individual enzymes or their combinations on the folate release:

(a) 5 ml extract (pH 6) + 1 ml α-amylase (20 mg per ml); incubation 37°C/3 h; adjustment to pH 4.9; 5 ml sample + 2 ml conjugase from hog kidney; incubation 37°C/2 h (A + HK);
(b) 5 ml extract (pH 6) + 2 ml protease (2 mg/ml); incubation 37°C/3 h; adjustment to pH 4.9; 5 ml sample + 2 ml conjugase; incubation 37°C per 2 h (P + HK);
(c) 5 ml extract (pH 6) + 1 ml α-amylase; incubation 37°C/3 h + 2 ml protease; incubation 37°C/3 h; adjustment to pH 4.9; 5 ml sample + 2 ml conjugase; incubation 37°C/2 h (A + P + HK);
(d) 5 ml extract (pH 6) + 1–3 ml H_2O; adjustment to pH 4.9; 5 ml sample + 2 ml conjugase; incubation 37°C/2 h (HK).

All samples were analysed in duplicates.

With the aim to shorten the time of incubation and to minimise the possible losses of folates, the influence of pH on α-amylase activity and the

simultaneous application of α-amylase and conjugase were tested in the following experiments using a sample of lettuce:

(e) 5 ml extract (pH 6) + 1 ml α-amylase (20 mg per ml); incubation 37°C/3 h; adjustment to pH 4.9; 5 ml sample + 2 ml conjugase from hog kidney; incubation 37°C/2 h;
(f) 5 ml extract (pH 4.9) + 1 ml α-amylase (20 mg per ml); incubation 37°C/3 h, 5 ml sample + 2 ml conjugase from hog kidney; incubation 37°C/2 h;
(g) 5 ml extract (pH 4.9) + 1 ml α-amylase + 1 ml conjugase; incubation 37°C/3 h.

In all experiments, blank samples and 5-MTHF external standard were included. Two independent samples of lettuce were analysed in duplicates.

Determination of 5-MTHF in selected vegetables. To determine 5-MTHF in selected vegetables (potatoes, broccoli, onion tops, cauliflower, carrot, pepper, curled parsley, Brussels sprouts, lettuce, spinach, and cabbage), the previously applied procedure (Holasová *et al.* 2004), modified for the conditions of enzymatic hydrolysis, was used. The modification included the simultaneous addition of α-amylase and conjugase from hog kidney to the extract and the incubation at pH 4.9 and 37°C for 3 h [procedure (g)]. The samples of each vegetable kind were analysed in duplicates. The method chosen was validated by repeatability (RSD = 7.91%, n = 5), the recovery of the added standard (94.1–108.4%, average 99.6%), and by the analyses of the certified material CRM 485 – lyophilised mixed vegetable (101.9% of certified value found).

Boiling of selected vegetables. The retention of 5-MTHF during 2–12 min boiling of broccoli, cauliflower, spinach, savoy cabbage, Brussels sprouts, and carrot was followed. Approximately 30 g samples of vegetables (1 floret of broccoli or cauliflower, 2–3 heads of Brussels sprouts, slices of carrot 1 cm thick, several leaves of spinach or savoy cabbage) were weighed and subsequently put into boiling water. The vegetable/water weight ratio was 1/3. The treatment lasted 2, 4, 8, and 12 min respectively; the exposure was assumed to start after the temperature inside the sample had reached 90°C. The treated samples were immediately plunged into iced water for 2 min, then dried and weighed. The boiled vegetables as well as the raw material were stored in vacuumed polyethylene bags at –40°C until analysed. A modified method, which involved the simultaneous application of α-amylase and conjugase from hog kidney to the extract and incubation at pH 4.9 and 37°C for 3 h [procedure (g)], was applied in the 5-MTHF determination. All kinds of vegetables were sampled and boiled in duplicates and the final 5-MTHF concentrations were calculated from two replicate determinations. The retention was expressed as the actual retention and computed as follows: % TR = (5-MTHF content per 100 g of boiled vegetables × g of vegetables after boiling)/(5-MTHF content per 100 g of raw vegetables × g of vegetables before boiling) × 100.

RESULTS AND DISCUSSION

Optimisation of enzymatic hydrolysis

Table 1 shows the 5-MTHF contents in lettuce, spinach, cabbage, Chinese cabbage and broccoli, as found by the application of enzymatic hydrolysis.

Table 1. Effect of α-amylase (A), protease (P), conjugase (HK) and their combinations on 5-MTHF content in vegetables

Vegetable	Content* of 5-MTHF using hydrolysis methods (a) to (d)							
	(a) A + HK		(b) P + HK		(c) A + P + HK		(d) HK	
	(μg/100 g)	(%)	(μg/100 g)	(%)	(μg/100 g)	(%)	(μg/100 g)	(%)
Lettuce	78.8	122.4	72.5	113	81.3	126.0	64.6	100
Spinach	98.3	112.4	82.3	94.0	85.2	97.4	87.5	100
Cabbage	12.3	123.0	9.9	99.0	9.1	91.0	10.0	100
Chinese cabbage	66.1	115.4	61.6	107.5	65.2	113.8	57.3	100
Broccoli	56.4	127.0	57.2	128.8	50.1	112.0	44.4	100

*Values are means of duplicates. Difference between duplicates was always less than 10%

Table 2. Effect of hydrolysis conditions on 5-MTHF content in lettuce using hydrolysis methods (e) to (g)

Conditions of hydrolysis	Incubation (h)	5-MTHF ± SD* (μg/100 g)
(e) pH 6.0, A + HK subsequently	3 + 2	74.7 ± 3.8
(f) pH 4.9, A + HK subsequently	3 + 2	72.6 ± 4.2
(g) pH 4.9, A + HK simultaneously	3	78.3 ± 4.8

*Values are means from two independent experiments

Sole conjugase (HK), or a combination of either conjugase and α-amylase (A + HK) or conjugase and protease (P + HK), or of all three enzymes, viz. conjugase, α-amylase, and protease (trienzyme method – A + P + HK), were applied.

The highest yields were obtained using the simultaneous application of α-amylase and conjugase (A + HK), amounting to 112.4–127.0% of the values obtained in the processing with sole conjugase (HK). With the exception of broccoli, the application of protease and conjugase (P + HK) did not increase the yield compared to the A + HK procedure. The trienzyme method (A + P + HK) led to higher yields only with lettuce, with all other vegetables it was less efficient than the combination of α-amylase and conjugase. In spinach and cabbage, the folate values were even lower than the yields of the single enzyme method. This corresponds to the results by Iwatani *et al.* (2003), who found the single enzyme treatment more effective than the trienzyme method in the analyses of spinach and Chinese broccoli. Shrestha *et al.* (2000) also found the single enzyme method more suitable than the trienzyme method. On the other hand, the effectiveness of the trienzyme method was established by several authors, mainly in complex food samples (Tamura *et al.* 1997) and in foods with high contents of protein and starch (De Souza & Eitenmiller 1990; Pfeiffer *et al.* 1997).

Whereas the incubation time in the dienzyme procedures under the conditions tested was 6 hrs,

Table 3. 5-MTHF content in selected vegetables

Vegetable	5-MTHF (μg/100 g)*	Total folates (μg/100 g) USDA (2005)	5-MTHF (μg/100 g) Vahteristo (1997)
Potatoes	15	16	21
Broccoli	56	63	98
Onion tops	50	14	
Cauliflower	89	57	80
Carrot	23	19	16
Red pepper	46	18	50
Green pepper	17	11	
Yellow pepper	23	26	
Curled parsley	104	152	
Brussels sprouts	50	61	88
Iceberg lettuce	73	38	44
Spinach leaves	87	194	
White cabbage	16	43	27
Chinese cabbage	72	79	50

*Results were obtained using hydrolysis method (g). Values are means of duplicates. Difference between duplicates was always less than 10%

Table 4. 5-MTHF content in raw vegetables used for boiling experiments

	Broccoli	Cauliflower	Spinach	Savoy cabbage	Brussels sprouts	Carrot
5-MTHF (μg/100 g)*	47.2	73.4	87.6	44.0	58.0	21.0

*Results were obtained using hydrolysis method (g). Values are means of duplicates. Difference between duplicates was always less than 10%

the trienzyme method under the conditions tested needed 8 h of incubation. This prolonged time might negatively influence the results. To minimise the losses of folates during incubation and to shorten the duration of analyses, the simultaneous application of α-amylase and conjugase was also tested. According to some authors (Vahteristo *et al.* 1997; Johnston *et al.* 2002b), α-amylase is often used at pH 6, whereas the optimal activity of hog kidney conjugase is at pH 4.9 (Gregory *et al.* 1984). The incubation of the lettuce extract with α-amylase at pH 6.0 and 4.9 [procedure (e) and (f)] did not reveal any differences in the folate readings (Table 2). Aiso and Tamura (1998) also failed to find any pH dependent differences in the folate values in spinach after α-amylase and protease testing. A simultaneous addition of α-amylase and conjugase [procedure (g)] and the incubation for 3 h at pH 4.9 resulted in a moderate increase of the folate readings (Table 2). The shorter incubation time might contribute to this increase.

Determination of 5-MTHF in selected vegetables

An optimised method was used for the folate determination in the selected vegetables that represent significant folate sources in the human diet either because of their high folate contents or their high consumption(Table 3). Spinach, Chinese cabbage, lettuce, cauliflower, and broccoli contain more than 50 μg of 5-MTHF in 100 g, their consumption in 2005 amounted to 0.7–2.5 kg/person/year. In potatoes, 15 μg/100 g was measured but their annual consumption was 72.5 kg/person. Less rich sources such as carrot or white cabbage contribute to the folate intake to lesser extents, 6.2 and 8.3 kg, respectively, being consumed per year (CZSO 2007). If compared with the published data, certain differences in the folate contents are found in several vegetables. The cause of this is obviously connected with the properties and

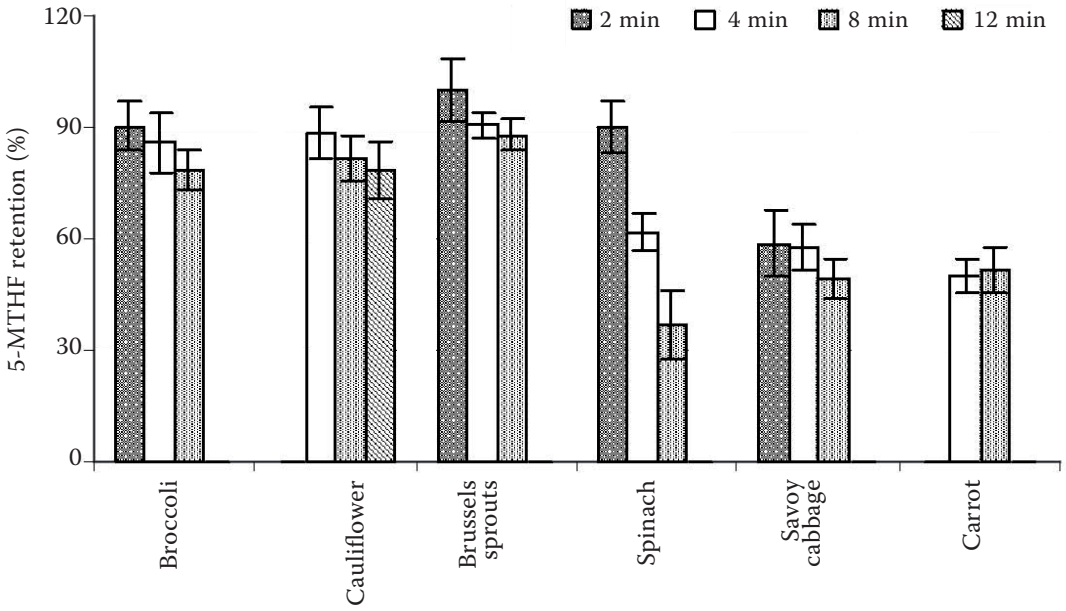

Figure 1. Retention of 5-MTHF during boiling of vegetables [Results were obtained using hydrolysis method (g). Values are means of two independent boilings with their standard error shown by vertical bars]

history of the analysed vegetables, like their variety, freshness, handling, climate, and agricultural conditions, as well as with the analytical method applied. The values published by Vahteristo *et al.* (1997) were obtained using HPLC method and refer to 5-MTHF, the USDA data represent total folates.

Retention of 5-MTHF during the boiling of selected vegetables

Broccoli, cauliflower, spinach, savoy cabbage, Brussels sprouts, and carrot were boiled in water and the retention of folates was determined in the samples of the respective vegetables boiled for 2, 4, or 8 minutes. In cauliflower, these intervals were prolonged to 4, 8, and 12 min corresponding better to its typical boiling time (Figure 1). Table 4 contains the values of 5-MTHF found in raw vegetables subsequently used in these experiments.

The folate retention during boiling under constant conditions differs between the individual vegetables. The highest retention values were found in Brussels sprouts, cauliflower, and broccoli. After 8 min boiling, more than 75% of the initial 5-MTHF content was retained. Lower values of retention, between 37% and 52% of the initial 5-MTHF content, were measured in spinach, savoy cabbage, and carrot. The differences in the folate retention between respective vegetables might be related to the vegetable properties such as the weight/surface ratio or the presence of endogenous antioxidants. A correlation between the folate losses caused by various ways of cooking and the kind of vegetable (peas, broccoli, and potatoes) was recently mentioned by Stea *et al.* (2006). Similar data were published by McKillop *et al.* (2002) for spinach, though a lower retention value was found in broccoli. The results of the present experiments with cauliflower are close to the data published by Melse-Boonstra *et al.* (2002) on the losses found after 8 min blanching. Generally, our results support the hypothesis that non-leafy vegetables retain more folates during their boiling than do leafy vegetables.

CONCLUSIONS

The determination of 5-MTHF in spinach, cabbage, Chinese cabbage, and broccoli after the folate release using α-amylase and conjugase proved a greater efficiency of this treatment in comparison with that using sole conjugase or trienzyme. A simultaneous addition of both enzymes with incubation at pH 4.9 does not suppress the folate release. Spinach, Chinese cabbage, lettuce, cauliflower, and broccoli contained more than 50 μg of 5-MTHF/100 g, whereas less than 25 μg/100 g were found in potatoes, carrot, white cabbage, green and yellow pepper. The folate retention during boiling under constant conditions differs between individual vegetables. The highest retention (more than 75%) was measured in Brussels sprouts, cauliflower and broccoli after 8 min boiling. The retention between 37 and 52% of the initial 5-MTHF content was found in spinach, savoy cabbage, and carrot.

References

Aiso K., Tamura T. (1998): Trienzyme treatment for food folate analysis: Optimal pH and incubation time for α-amylase and protease treatments. Journal of Nutritional Science and Vitaminology, **44**: 361–370.

CZSO (2007): www.czso.cz/csu/2006edicniplan.nsf/publ/3004-06-v_roce_2005

De Souza S.C., Eitenmiller R.R. (1986): Effect of processing and storage on the folate content of spinach and broccoli. Journal of Food Science, **51**: 626–628.

De Souza S.C., Eitenmiller R.R. (1990): Effects of different enzyme treatments on extraction of total folate from various foods prior to microbiological assay and radioassay. Journal of Micronutrient Analyses, **7**: 37–57.

Gregory J.F., Sartain D.B., Day B.P.F. (1984): Fluorometric determination of folacin in biological materials using high performance liquid chromatography. Journal of Nutrition, **114**: 341–353.

Holasová M., Fiedlerová V., Roubal P., Pecháčová M. (2004): Biosynthesis of folates by lactic acid bacteria and Propionibacteria in fermented milk. Czech Journal of Food Sciences, **22**: 175–181.

Iwatani Y., Arcot J., Shrestha A.K. (2003): Determination of folate content in some Australian vegetables. Journal of Food Composition and Analysis, **16**: 37–48.

Johnston K.E., DiRienzo D.B., Tamura T. (2002a): Folate content of dairy products measured by microbiological assay with trienzyme treatment. Journal of Food Science, **67**: 817–820.

Johnston K.E., Lofgren P.A., Tamura T. (2002b): Folate concentrations of fast foods measured by trienzyme extraction method. Food Research International, **35**: 565–569.

McKillop D.J., Pentieva K., Daly D., McPartlin J.M., Hughes J., Strain J.J., Scott J.M., McNulty H. (2002): The effect of different cooking methods on folate retention in various foods that are amongst the major contributors to folate intake in the UK diet. British Journal of Nutrition, **88**: 681–688.

Melse-Boonstra A., Verhoef P., Konings E.J.M., van Dusseldorp M., Matser A., Hollman P.C.H., Meyboom S., Kok F.J., West C.E. (2002): Influence of processing on total, monoglutamate and polyglutamate folate contents of leek, cauliflower and green beans. Journal of Agricultural and Food Chemistry, **50**: 3473–3478.

Müller H. (1993): Bestimmung der Folsäure-Gehalte von Gemüse und Obst mit Hilfe der Hochleistungsflüssigchromatographie. Zeitschrift für Lebensmittel-Untersuchung und -Forschung, **196**: 137–141.

Pandrangi S., LaBorde L.F. (2004): Optimization of microbial assay of folic acid and determination of folate content in spinach. International Journal of Food Science and Technology, **39**: 525–532.

Pfeiffer C.M., Rogers L.M., Gregory III J.F. (1997): Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. Journal of Agricultural and Food Chemistry, **45**: 407–413.

Puuponen-Pimia R., Hakkinen S.T., Aarni M., Suortti T., Lampi A.M., Eurola M., Piironen V., Nuutila A.M. (2003): Blanching and long-term freezing affect various bioactive compounds of vegetables in different ways. Journal of the Science of Food and Agriculture, **83**: 1389–1402.

Scott J., Rébeillé F., Fletcher J. (2000): Folic acid and folates: the feasibility for nutritional enhancement in plant foods. Journal of the Science of Food and Agriculture, **80**: 795–824.

Shrestha A.K., Arcot J., Peterson J. (2000): Folate assay of food by traditional and trienzyme treatments using cryoprotected *Lactobacillus casei.* Food Chemistry, **71**: 545–552.

Stea T.H., Johansson M., Jägerstad M., Frlich W. (2006): Retention of folates in cooked, stored and reheated peas, broccoli and potatoes for use in modern large-scale service systems. Food Chemistry, **101**: 1095–1107.

Tamura T., Mizuno Y., Johnston K.E., Jacob R.A. (1997): Food folate assay with protease, α-amylase and folate conjugase treatments. Journal of Agricultural and Food Chemistry, **45**: 135–139.

Tamura T. (1998): Determination of food folate. The Journal of Nutritional Biochemistry, **9**: 285–293.

U.S. Department of Agriculture, Agricultural Research Service (2005): USDA National Nutrient Database for Standard Reference, Release 18.Available at http://www.ars.usda.gov/ba/bhnrc/ndl

Vahteristo L.T., Ollilainen V., Koivistoinen P.E., Varo P. (1996): Improvements in the analysis of reduced folate monoglutamates and folic acid in food by high-performance liquid chromatography. Journal of Agricultural and Food Chemistry, **44**: 477–482.

Vahteristo L., Lehikoinen K., Ollilainen V., Varo P. (1997): Application of an HPLC assay for the determination of folate derivatives in some vegetables, fruits and berries consumed in Finland. Food Chemistry, **59**: 589–597.

Witthöft C.M., Forssén K., Johannesson L., Jägerstad M. (1999): Folates-food sources, analyses, retention and bioavailability. Scandinavian Journal of Nutrition, **43**: 138–146.

Yon M., Hyun T.H. (2003): Folate content of foods commonly consumed in Korea measured after trienzyme extraction. Nutrition Research, **23**: 735–746.

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