Journal of Chromatography, 449 (1988) 271–279
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 658

DETERMINATION OF NATIVE FOLATES IN MILK AND OTHER DAIRY PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

DOUGLAS L. HOLT*, RANDY L. WEHLING* and MICHAEL G. ZEECE
Department of Food Science and Technology, 134 Filley Hall, University of Nebraska, Lincoln, NE 68583-0919 (U.S.A.)
(Received May 16th, 1988)

SUMMARY

Folates were measured in dairy products by high-performance liquid chromatography without prior sample clean-up. Detection limits for individual folates range from 0.3 to 7.3 ng/g. The folates were extracted from the sample matrix by adjusting the pH to 4.5 with acetic acid, centrifuging to remove precipitated proteins, and treating with conjugase to remove multiple polyglutamate residues. Folates were separated from other sample components using a reversed-phase column with a methanol–phosphate buffer (pH 6.8), and ion-pairing with tetrabutylammonium ion. Fluorescence was found to be the most useful detection technique. Fluorescence detection of reduced forms of the vitamin was achieved by post-column pH adjustment of the eluent with phosphoric acid, while the parent folic acid molecule required chemical oxidation with hypochlorite in order to obtain a fluorescent response.

INTRODUCTION

Folic acid and several related compounds are important cofactors in human nutrition, where they play a role in one carbon transfers in the biosynthesis and degradation of proteins and nucleic acids[1–4]. Their activity in nucleic acid synthesis has led to the use of folate antagonists in anti-cancer therapy[5]. Folates are extremely labile when subjected to oxidation and heat, and it is thought that many food processing operations result in a significant destruction of the vitamers[6–10], although recent evidence has indicated that folates may be protected by other food components[8]. On the other hand, the instability of the vitamers may lead to underestimation of the levels naturally occurring in foodstuffs, due to the destruction of significant amounts during analysis[3,11]. The loss of folates during analysis is one of the major impediments to the measurement of these compounds by the nutritional chemist.

An additional complication in the analysis of folates is the extremely low levels

* Present address: Dole Packaged Foods, Technical Center, 2102 Commerce Drive, San Jose, CA 95131, U.S.A.

0021-9673/88/$03.50

present in most foods. Except for a few cases such as liver and yeast extract, the folate levels in most foods are less than 100 ng/g[3,5]. Thus, destruction of even limited amounts of folates during sample preparation may reduce the total folate level below detection limits.

Folates are usually measured by microbiological assay. This method, while highly sensitive (detection limits approach 10 ppt), is very tedious and can provide only limited information on the actual forms of folate present in a sample[12]. Additionally, microbiological assays are often subject to multiple interferences from the sample matrix. Several researchers have attempted to circumvent these problems by using a chromatographic separation prior to microbiological assay. The microbiological assay then serves as a sensitive detector for the chromatographic separation[13].

In recent years, high-performance liquid chromatography (HPLC) has been used to determine the levels of folates in several matrices including liver, blood, infant formula, and milk[14,15]. Gregory *et al.*[15] have described a procedure that uses sample clean-up on an open ion-exchange column, followed by HPLC separation and fluorescence detection. Certain folates required a post-column oxidation to form fluorescent derivatives. Other researchers have measured folates in plasma and serum by using a thin-layer electrochemical detector[16,17]. In this paper, we describe the application of these two detection techniques to the determination of folates at biological levels in dairy products, in combination with a reversed-phase column and ion-pairing to separate folates from interferences in the sample matrix. A method has been developed that eliminates the need for extensive sample clean-up prior to injection onto the HPLC column.

EXPERIMENTAL

Standards and samples

Individual folate standards were obtained from Sigma (St. Louis, MO, U.S.A.) or Dr. B. Schirks laboratory (Switzerland), and used without further purification. Purity of the standards was assessed by chromatographic separation with ultraviolet detection at 280 nm. Standard solutions (usually 1000 ng/ml) were prepared in 0.01 *M* sodium phosphate buffer (pH 4.5), containing 0.1% ascorbate and 0.01 *M* 2-mercaptoethanol (MCE). A 10 000 ng/ml solution (in 0.01 *M* phosphate buffer, pH 6.8, containing 0.1% ascorbate and 0.01 *M* MCE) was used to prepare spiked samples, which were allowed to stand for 1.0 h prior to extraction.

Raw milk was obtained from a mixed milking of approximately 50 cows at a local dairy and was analyzed on the day collected. Pasteurized milk and other processed dairy products were obtained from local retail outlets.

Extraction of folates from dairy products

Milk and other fluid dairy products were adjusted to pH 4.5 with glacial acetic acid, and then stirred for 1.0 h with a low speed magnetic stirrer. Samples of cottage cheese or yogurt were homogenized for 1 min in a Waring blender, then adjusted to pH 4.5. All samples were then centrifuged for 10 min at a minimum of 1000 *g*. The supernatant was decanted, and a solution of sodium phosphate buffer (pH 4.5) containing 10% ascorbate and 1 *M* MCE added, so that the final concentrations of ascorbate and MCE were 0.1% and 0.01 *M* respectively. The supernatant was then

treated with 0.1 ml of a conjugase preparation, previously isolated from hog kidneys[15], and incubated at 37°C in sealed tubes for 2 h. This conjugase treatment was determined to be sufficient to convert 10 μg/ml folic acid pentaglutamate to the monoglutamate within 30 min. After incubation, the samples were centrifuged at 10 000 *g* for 10 min, filtered through 0.45-μm filters, and stored on ice in the dark prior to injection. Standard solutions and spiked samples used for determining recoveries were treated in an identical manner.

Chromatography

Water for the liquid chromatographic mobile phase was distilled and deionized. HPLC grade methanol was used without further purification. Tetrabutylammonium dihydrogenphosphate was purchased as a 1.0 *M* solution (Aldrich, Milwaukee, WI, U.S.A.) and added to the mobile phase at 50 ml/l. The mobile phase was prepared by dissolving 0.560 g dipotassium hydrogenphosphate and 0.480 g potassium dihydrogenphosphate in a small amount of water in a 1-l flask. The pH of this aqueous buffer was carefully adjusted to 6.8 with phosphoric acid or potassium hydroxide, prior to dilution to 1.0 l with water or methanol. A phosphate buffer–methanol (50:50) solution, and a 100% aqueous phosphate buffer were maintained separately and mixed to form the mobile phase using the high pressure mixing system of the chromatographic hardware. All solvents were filtered through 0.45-μm filters prior to use.

The analytical column used was a Rainin (Woburn, MA, U.S.A.) C_{18} Microsorb Short-One™ (10 cm × 4.6 mm I.D., 3 μm spherical packing). A Brownlee (Santa Clara, CA, U.S.A.) cartridge system, equipped with a 1.5 cm × 3.2 mm I.D. guard column packed with a 7-μm widepore C_{18} packing material, was installed ahead of the analytical column. Flow-rate through the analytical column was 1.0 ml/min. The column was flushed with distilled water, followed by methanol, after each working day.

The chromatographic system consisted of two Beckman 110B solvent delivery pumps, a Beckman 420 system controller, and a Beckman Model 210A sample injection valve equipped with a 100-μl loop. Detection was either with a Bioanalytical Systems (West Lafayette, IN, U.S.A.) LC-4B amperometric detector fitted with a glassy carbon electrode, and operated at 900 mV *versus* an Ag/AgCl reference electrode, or with a Kratos (Ramsey, NJ, U.S.A.) 980 variable-wavelength fluorescence detector (excitation 238 nm, emission > 340 nm). Sensitivity was generally set at 10 nA f.s. for the amperometric detector. High voltage on the fluorescence detector photomultiplier tube was set at 10% over the autocalibration value (approximately 800 V), and the range setting was generally 0.01 μA. The time constant was set at 5 s. The entire chromatographic system was operated at ambient temperature. Chromatograms were recorded with a Spectra-Physics 4270A (San Jose, CA, U.S.A.) recording integrator.

Preceding the fluorescence detector, post-column pH adjustment of the eluent stream was achieved by pumping 0.25 ml/min of a 4.25% (v/v) aqueous phosphoric acid solution through a reaction tee, using a Milton Roy Mini-pump (Riviera Beach, FL, U.S.A.). The reaction tee has been previously described by Mauro *et al.*[18]. When required, a post-column chemical oxidant was substituted for the phosphoric acid solution. The oxidizing solution consisted of 0.005% sodium hypochlorite, 0.1

M sodium dihydrogenphosphate and 0.2 *M* sodium chloride in water (pH 3.0), and was added to the eluent stream at 0.25 ml/min, as suggested by Gregory *et al.*[15].

RESULTS AND DISCUSSION

Chromatography

The chromatographic system was a modification of that developed by Duch *et al.*[19]. Conditions were modified in order to optimize the separation of folates from sample interferences. Reversed-phase columns from several manufacturers were evaluated with respect to their suitability for this particular separation. A high efficiency column packed with a 3 μm C_{18} packing material (Rainin Short One) provided excellent separation of the various folate forms (Fig. 1), and a relatively long life (over 500 injections) with the mobile phase conditions used.

The separation of folate standards was only slightly affected by varying the pH of the mobile phase between 6.0 and 7.0. However, retention times of several components in milk were significantly changed by variations in mobile phase pH. The optimum separation of 5-methyltetrahydrofolate, the predominant form of folate in milk, from the sample matrix was obtained at pH 6.8 (Fig. 2). On the other hand, methanol concentration strongly influenced the retention of folates on the analytical column. Mobile phase methanol contents between 12 and 25% gave no change in the elution order of folates, but the retention times of 5-methyltetrahydrofolate, the most strongly retained vitamer, varied from 8 to 45 min depending on methanol concentration. The retention times and calculated efficiency factors (k') for various folates under selected isocratic conditions are presented in Table I. Gradient elution, where the methanol content was varied from 0 to 20% over 15 min, was successfully employed to separate

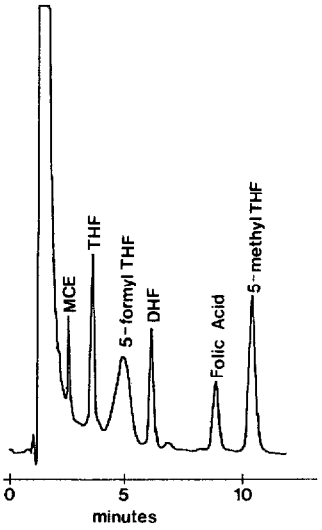

Fig. 1. Separation of several folates including tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-formyl THF), dihydrofolate (DHF), folic acid, and 5-methyltetrahydrofolate (5-methyl THF) under isocratic conditions using 20% methanol. Folates were detected by ultraviolet absorption at 280 nm.

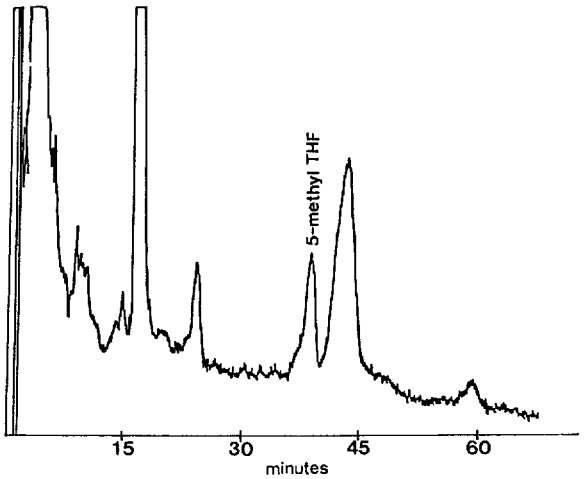

Fig. 2. Chromatogram of milk showing the naturally occurring 5-methyltetrahydrofolate (5-methyl THF). Fluorescence detection following post-column pH adjustment was used to quantitate the 5-methyl THF.

up to six different folates from interferences in the milk matrix. However, since only 5-methyltetrahydrofolate was found to occur naturally at significant levels in dairy products, isocratic conditions at 16% methanol can routinely be employed to quantitate this form.

Detection

As ultraviolet absorption detection was found to lack sufficient sensitivity to detect the low levels of folates naturally present in dairy foods, more sensitive detection techniques were investigated. Recent advances in electronics and detector cell design have led to an increased use of electrochemical detection (ED) with HPLC separations[20]. ED can be both highly selective and sensitive under ideal conditions. Examination of cyclic voltammograms of folates (Fig. 3) indicated that an oxidative cell potential of 900 mV would provide detection, and was compatible with the mobile

TABLE I

RETENTION TIMES (t_R) AND CAPACITY FACTORS (k') FOR VARIOUS FOLATES SEPARATED UNDER ISOCRATIC CONDITIONS USING DIFFERENT METHANOL CONCENTRATIONS

Folate	*14% Methanol*		*18% Methanol*		*20% Methanol*	
	t_R *(min)*	k'	t_R *(min)*	k'	t_R *(min)*	k'
Tetrahydrofolate	4.32	2.76	3.60	2.13	3.40	1.96
5-Formyltetrahydrofolate	16.87	13.67	10.80	8.39	7.90	5.87
Dihydrofolate	22.57	18.63	13.98	11.16	9.73	7.46
10-Formyltetrahydrofolate	30.75	25.74	16.05	12.96	11.20	8.74
Folic acid	32.36	27.14	16.32	13.19	11.32	8.84
5-Methyltetrahydrofolate	50.17	42.63	25.84	21.47	12.08	9.50

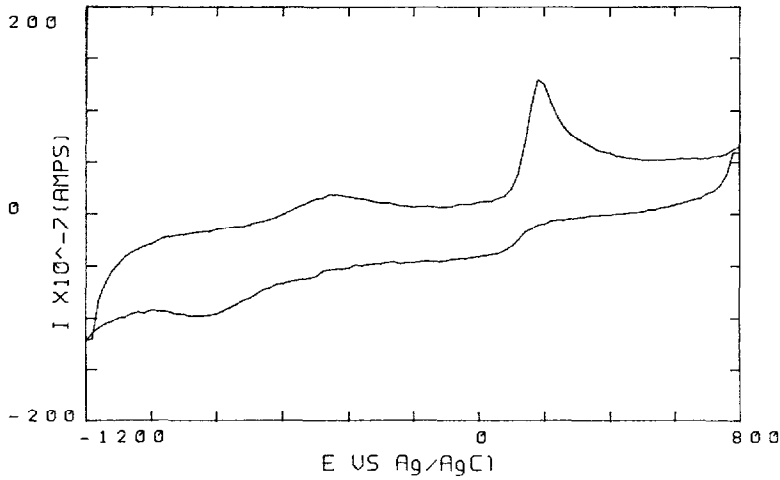

Fig. 3. Cyclic voltammogram of 2 m*M* 5-methyltetrahydrofolate dissolved in the methanol–phosphate buffer HPLC mobile phase. Potentials shown are measured *vs.* an Ag/AgCl reference electrode. The voltage was swept from +800 mV to −1200 mV and back at a scan rate of 250 mV/s.

phase used in this separation. Hydrodynamic voltammograms of several folates also indicated that ED would be a viable technique (Fig. 4). However, the instability of folates due to oxidation required that high levels of reducing agents, such as ascorbate and/or MCE, be included during the extraction steps[5,9,21]. These high levels (0.1% and 0.01 *M*, respectively) created significant interferences in the oxidative electrochemical detection of folates. Several attempts to remove these reducing agents with solid phase extraction techniques prior to injection were unsuccessful. No protocol could be devised that would eliminate ascorbate and MCE without loss of some early eluting folates. In addition, complete recovery of highly retained folates required the use of solvents that were detrimental to the stability of the vitamers, and to the

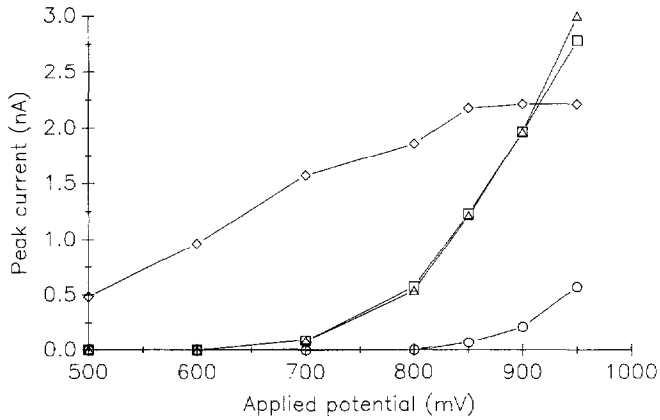

Fig. 4. Hydrodynamic voltammograms for 5-formyltetrahydrofolate (△), dihydrofolate (□), 5-methyltetrahydrofolate (◇), and folic acid (○). Potentials shown are measured *vs.* an Ag/AgCl reference electrode.

resolution of the HPLC separation. Attempts to use column switching techniques similar to those reported by Wegner *et al.*[17] were also unsuccessful. The slight changes in pressure and/or temperature induced during column switching resulted in severe baseline disturbances at the high detector sensitivity settings required to measure low levels of folates.

Gregory *et al.*[15] have reported the use of native fluorescence to detect extremely low levels of folates. Initial attempts to use this technique met with failure. However, it was discovered that the fluorescence of many folates is strongly dependent on pH. With a post-column adjustment of the mobile phase pH to <3, and use of an optimized excitation wavelength (238 nm), very satisfactory detection levels for the folates were achieved (Table II). The pH reduction also quenched the fluorescence of several interfering compounds from milk.

The parent folic acid molecule did not fluoresce after a simple pH adjustment. However, post-column chemical oxidation of folic acid with hypochlorite, as suggested by Gregory *et al.*[15], did produce a fluorescent compound that could be measured at slightly different wavelengths (365 nm excitation, >410 nm emission). Post-column oxidation destroyed the fluorescence of the other folates examined, resulting in the need for two injections of each sample in order to detect all possible folate forms. Folic acid was not expected to be present in biological samples[5,9], and was not found in any of the dairy products tested.

Sample preparation

In order to minimize potential losses of folates due to oxidation during extraction, every attempt was made to simplify sample preparation. Treatment with conjugase was necessary to remove interferences from polyglutamate forms of the individual vitamers. The polyglutamate forms of some folates can be separated under chromatographic conditions similar to those employed here, but resolution and detection of folylpolyglutamates is difficult[22].

Levels of ascorbate and MCE were optimized so that folate stability during extraction was maximized. It was discovered that high levels of ascorbate ($>0.5\%$)

TABLE II

DETECTION LIMITS, REPRODUCIBILITY OF INJECTION AND RECOVERY DATA FOR SEVERAL FOLATES

Folate	*Detection limit (ng/g)**	*Reproducibility, R.S.D. (%)*	*Recovery (%)*
Tetrahydrofolate	5.7	13.20**	73.4
5-Formyltetrahydrofolate	5.1	4.10***	99.4
Dihydrofolate	4.6	2.73§	79.6
10-Formyltetrahydrofolate	7.3	1.69§	82.3
Folic acid	1.7	6.43**	100.1
5-Methyltetrahydrofolate	0.3	3.46**	97.4

* Using 100 μl injection.
** Determined at 10 ng/ml.
*** Determined at 50 ng/ml.
§ Determined at 100 ng/ml.

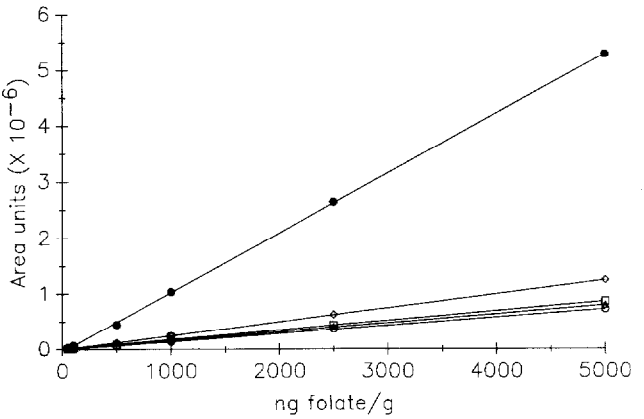

Fig. 5. Linearity of the fluorescence response for several folates including 5-methyltetrahydrofolate (●), tetrahydrofolate (◇), dihydrofolate (□), 10-formyltetrahydrofolate (△), and 5-formyltetrahydrofolate (○).

caused folic acid to precipitate in phosphate buffer. By reducing the ascorbate concentration to 0.1%, with the addition of 0.01 *M* MCE to the sample, most folates were adequately stabilized (Table II). Recoveries were determined by addition of standards to seven samples of pasteurized milk at levels of 100 ng/g sample. The low recovery of tetrahydrofolate (73.4%) is probably due to the extreme instability of this form of the vitamer. It is unlikely that the highly unstable tetrahydrofolate is present in dairy products at significant levels, and none was found in any of the samples analyzed.

The combination of ion-pairing separation and fluorescence detection provided selectivity sufficient to resolve the folates of interest from other sample components, making prior sample cleanup unnecessary. The elimination of all clean-up steps minimizes the chances for oxidation of the folates.

Statistical considerations

The reproduction of the chromatography, detection, and integration was evaluated by making a series of seven injections of a mixed folate standared (Table II). Relative standard deviations ranged from a low of 1.69% for 10-formyltetrahydrofolate to a high of 13.2% for tetrahydrofolate. The linearities of the fluorescence responses for several folates were determined by addition of a mixed standard to milk at levels equivalent to 50, 100, 500, 1000, 2500, and 5000 ng of individual folate/g sample, with each level in duplicate. Standards of 5-methyltetrahydrofolate were also included at levels of 1 and 10 ng/g. Correlation coefficients were greater than 0.98 in all cases (Fig. 5).

CONCLUSIONS

The method described above has been applied to the determination of folates in both raw and pasteurized bovine milk. In both cases, the only form of folate found in significant levels was 5-methyltetrahydrofolate. The average folate content of three samples of raw milk was 66.7 $\pm$ 3.3 ng/g, while seven samples of pasteurized milk

resulted in an average value of 49.6 ± 1.7 ng/g. These results agree well with literature values[3] obtained by microbiological assay using *Lactobacillus casei* as the test organism (55 ng/g and 50 ng/g for raw and pasteurized milk, respectively). The HPLC procedure has also been successfully applied to the determination of folates in other dairy products including cottage cheese, cultured buttermilk, and yogurt.

In summary, a reversed-phase separation with ion-pairing is coupled with fluorescence detection to measure six different folates at native levels in milk and dairy products. The response for each of the folates is linear over the range of interest. The method has an advantage over previous procedures, in that sample clean-up and pre-concentration steps on open columns are not required, thereby minimizing the potential for loss of the vitamers during sample preparation, and significantly reducing the analysis time.

ACKNOWLEDGEMENTS

This work was funded in part by the National Dairy Promotion and Research Board (U.S.A.), administered in cooperation with the National Dairy Council. This is Paper No. 8623, Journal Series, Nebraska Agricultural Station, Lincoln, NE 68583-0704, U.S.A.

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