Determination of folates in seaweeds by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method, with a combination of fluorescence and ultraviolet detectors, to determine the folate forms present in commercial macroalgae products form northwest Spain as part of nutritional studies in dehydrated and canned seaweeds is reported. The method includes extraction of folates from seaweed by heat treatment, deconjugation of folate polyglutamates by incubation with hog kidney conjugase and purification by solid-phase extraction (SPE) with strong anion-exchange (SAX) cartridges. Separation was achieved with a Tracer Extrasil ODS 5 μm 25 cm $\times$ 0.4 cm column using acetonitrile and potassium phosphate buffer (pH 2.2) as mobile phase. Good results were obtained with respect to repeatability (relative standard deviation (R.S.D.) $\leq$4.12%) and recovery ($\geq$90.80%). The amount of folate (as folic acid) in the six species ranged from 61.4 to 161.6 μg per 100 g dry mass. In all the seaweeds studied (*Himanthalia elongata, Laminaria ochroleuca, Palmaria* spp., *Undaria pinnatifida* and *Porphyra* spp. *and Saccorhiza polychides*) the single most abundant form is 5-CH$_3$–H$_4$-folate, except *Porphyra and Himanthalia*.
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Keywords: Food analysis; Seaweeds; Folates

1. Introduction

Seaweeds are used in many maritime countries as a source of food, for industrial applications and as a fertiliser. The major utilisation of these plants as food is in Asia. Japan and China have used them as staple item of diet for a very long time. In most western countries, food and animal consumption is restricted and there has not been pressure to develop seaweed cultivation techniques [1].

As a result of recent interests in simple living, the potentials of seaweed as a source of natural and healthy food became widely recognised and studies on the nutritional values of seaweeds have become more widespread [2]. In comparison with land vegetables, seaweeds are potentially good sources of polysaccharides, minerals, and certain vitamins [3].

Folate is the general term including folic acid (pteroylglutamate, PteGln) and poly-γ-glutamyl conjugates with the biological activity of folic acid. Folates present a diverse array of compounds that vary by oxidation state of the pteridine ring structure, one-carbon moieties carried by specific folate, and the number of conjugated glutamate residues on the folate [4]. These vitamins cofactors are essential for the synthesis of purines and pyrimidines and in the production of methionine from homocysteine [5]. They also play a role in neural tube defects.

Yeast, mushrooms, kidney, liver, and especially vegetables provide the richest vitamin sources for humans. Lesser amounts of folic acid are found in meats, cereals, fruits, and certain roots. Food storage and cooking markedly decrease concentrations levels of folic acid [6]. Because of the presence of multiple forms in food products and its instability, folate presents a difficult analytical problem.

The folate content in food and biological tissues is usually determined by a microbiological assay using *Lactobacillus casei* as test organism [7,8]. More specific high-performance liquid chromatography (HPLC) methods have been developed with UV and/or fluorescence detection to identify different forms of folates [9–25] and with electrochemical detection [26]. Recently, highly specific HPLC–mass spectrometry (HPLC–MS) methods have been reported for the analysis of folates in food [5,27].

In this work, we describe a reliable method for the simultaneous determination of folates and folic acid in edible

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seaweeds with a combination of fluorescence and ultraviolet. The method involved extraction of folates from seaweed by heat treatment followed by deconjugation of folate polyglutamates to their respective monoglutamate forms by incubation with hog kidney conjugase, the seaweed extracts were concentrated and purified with strong anion-exchange (SAX) solid-phase extraction (SPE) cartridges. Folates were analysed from seaweed by the method of Vahteristo et al. [13] with a modification.

2. Experimental

2.1. Chemicals and standard solutions

All chemicals were of analytical grade. Acetonitrile and ethanol were from Merck (Darmstadt, Germany). Potassium phosphate monobasic, sodium phosphate dibasic, potassium phosphate dibasic, sodium ascorbate, phosphoric acid and 2-mercapto ethanol were obtained from Sigma (Steinheim, Germany). Ascorbic acid, citric acid, and hydrochloric acid fuming 37% were from Merck (Darmstadt, Germany). Sodium acetate and sodium chloride were from Panreac (Barcelona, Spain). The folate standards [5-methyltetrahydrofolate (barium salt), tetrahydrofolic (THF) acid, 5-formyltetrahydrofolate (calcium salt) and folic acid] were obtained from Sigma (Steinheim, Germany). Pteroyltri-γ-glutamic acid (PteGlu$_3$) was from Dr. Schirck's Labs (Jona, Switzerland).

Standards solutions were prepared by dissolving 5-CH$_3$–H$_4$-folate, 5-HCO–H$_4$-folate and folic acid in 0.1 M phosphate buffer (pH 7.0) containing 1.0% (w/v) sodium ascorbate, H$_4$-folate was dissolved in 0.1M phosphate buffer (pH 7.0) containing 0.1% (v/v) 2-mercaptoethanol. Aliquots of these solutions were taken for preparing a solution with all folate standards and then dissolved in 0.01 M acetate containing sodium ascorbate 1% (w/v) at pH 4.9.

2.2. Equipment

The HPLC system (Hewlett-Packard, CA USA) consisted of an HP1100 quaternary pump, an HP1100 degassing device, a 20 μl injection loop (Rheodyne, Cotati, CA), a column heater SP8792, (San José, CA, USA), an HP1100 fluorescence detector and an HP1100 UV detector. The HPLC was controlled by a personal computer running HP Chem Station Software.

The separation was performed on a Tracer Extrasil ODS2 column 250 mm × 4.6 mm, 5 μm and a guard column TR-C160-1 ODS 15 mm × 4.0 mm, 5 μm (Teknokroma, Barcelona, Spain) at 30 °C.

2.3. Sampling

Dehydrated seaweed (*Himanthalia elongata, Laminaria ochroleuca, Palmaria* spp., *Undaria pinnatifida* and *Por-*

phyra spp.) were obtained from the factory Algamar (Redondela, Pontevedra, Spain). The samples were dried at 45 °C for 24 h, and then packed in polypropylene bags. Canned algae (*H. elongata* and *Saccorhiza polychides*) were obtained from a local canning factory named Conservas y Ahumados LOU (Ribeira, La Coruña, Spain). The algae are prepared canned and sterilised with water and salt. The canning process involves heating to 112 °C for 40 min in an autoclave. Both dehydrated and canned samples were collected on the Atlantic coastal region in Galicia (NW Spain).

Prior to analysis all samples were dried. In the canned algae, excess water was drained off and the algae were partially dried in an oven at 45 °C for 48 h and then 10 h at 50 °C in a vacuum oven. In the case of the dehydrated algae, the dry matter was determined by weighing before and after drying to constant mass in a vacuum oven at 50 °C.

2.4. Enzymes

The hog kidney enzyme (HK) was prepared from hog kidney acetone powder (Sigma–Aldrich, Steinheim, Germany) as previously described Kamen and Caston [28]. The activity of the HK was evaluated measuring the amount of folic acid produced from PteGlu$_3$. One millilitre of the HK preparation was added to 16 μg of PteGlu$_3$ in 1.5 ml of 0.1 M acetate buffer, 1% ascorbate (pH 4.9) as the reaction medium. The reaction was terminated after a 45 min of incubation period at 37 °C. The selection of pH 4.9 was based on previous studies [29] that showed that this pH is suitable for both effective extraction and deconjugation with hog kidney conjugase for HPLC analysis. The folic acid produced was analysed by HPLC.

The deconjugation efficiency was performed, as previously described [29], by mixing a 3 ml aliquot of each seaweed extract containing 57 nmol of PteGlu$_3$ with 1 ml of the HK conjugase preparation. The incubation were carried out in a water bath for 3 h at 37 °C.

2.5. Sample preparation

2.5.1. Sample extraction and incubation

Folates were extracted from seaweed by the method of Vahteristo et al. [13] with a slight modification. The samples (2 g) were weighed in a screw-cap centrifuge tube. A 30 ml volume of extraction buffer (75 mM K$_2$HPO$_4$ containing 52 mM ascorbic acid/ascorbate mixture and 0.1% (v/v) 2-mercaptoethanol, pH 6.0 adjusted with phosphoric acid) was added and immediately vortexed for 20 s. The homogenates were flushed with N$_2$, capped and placed in a water bath at 100 °C for 10 min. After cooling on ice, the mixtures were centrifuged at 11,000 × g for 20 min, followed by removal of supernatant. The residue was re-dissolved with 10 ml of extraction buffer and re-centrifuged for 10 min. The two supernatants were pooled and diluted

to 50 ml with the extraction buffer in a volumetric flask. A 3 ml aliquot of the sample extract was adjusted to pH 4.9 with acetic acid and mixed with 1 ml of hog kidney conjugase preparation. The mixture was flushed with nitrogen gas sealed and then incubated in a water bath for 120 min. In order to inactivate enzymes the extracts were kept 5 min in a boiling water bath, and finally cooled on ice.

2.5.2. Solid-phase extraction (SPE)

SPE was performed using 3 ml strong anion-exchange tubes (quaternary amine) (Supelco, Bellefonte, PA, USA). The sample extract was diluted to 6 ml with Milli-Q water and 15 μl of 2-mercaptoethanol was added before loading onto the column.

Prior to use, the extraction cartridge SPE (3 ml) was activated by washing successively with methanol (2 ml), and water Milli-Q (2 ml). After its activation, a 2 ml volume of 0.01 M phosphate buffer containing 0.1% 2-mercaptoethanol pH 7 was applied on the column. The sample extract was passed through with a flow-rate <1 ml/min. The column was washed twice with 0.01 M phosphate buffer (1.5 ml) and the folates were eluted with 3 ml of 0.1 M sodium acetate containing 10% (w/v) sodium chloride and 1% (w/v) ascorbic acid. Prior to HPLC analysis, all samples were filtered through a 0.5 μm Millipore filter (Bedford, MA, USA) and then injected into the chromatograph.

2.6. Chromatographic conditions

Operating conditions were as follows: column temperature 30 °C; flow-rate, 0.8 ml/min; injection volume 20 μl. The mobile phase used was a gradient of acetonitrile and 30 mM potassium phosphate buffer (pH 2.2). Within the first 4 min, the mobile phase was isocratically with 10% acetonitrile and 90% phosphate buffer, after which the acetonitrile was raised 15% within 8 min and back to the original composition after 3 min.

The absorbance of all eluted folate vitamers was monitored with an UV detector set at 290 nm for folic acid and with a fluorescence detector set at 290 nm excitation and a 356 nm emission wavelengths for H_4-folate, 5-CH_3–H_4-folate and 5-HCO–H_4-folate.

2.7. Quantification and identification

Quantification was performed on the basis of linear calibration plots of peak area against concentration. Calibration lines were constructed using a range of concentrations of the standard, selected such that concentration in the sample was at the middle of the range. Each line is based on five concentrations of standard.

Identification of the different compounds was made by comparison of their retention times with those of pure standards.

3. Results and discussion

3.1. Method optimisation

Several experiments were carried out in order to optimise the sample preparation and the chromatographic conditions. In the extraction procedure preliminary assays were performed in order to establish optimal sample amount: 1.00; 1.25; 2.00; and 2.50 g, were tried, the best results were obtained with 2.00 g which are in accordance with the amount of sample employed by Finglas et al. [30] to determine folates in lyophilised Brussel sprouts and by Vahteristo et al. [13] in white cabbage and spinach, respectively.

Optimal conditions for conjugase volume and deconjugation efficiency were investigated. To check the deconjugation different amounts of the substrate PteGlu$_3$ (23, 57 and 114 nmol) were added into the sample after heat extraction, these assays indicated that the best results was achieved with 57 nmol. The conversion of pteroyltriglutamic acid (PteGlu$_3$) into folic acid in the sample matrix was in all cases higher 90.87% as peak areas.

The effect of enzyme volume on the deconjugation in the seaweed extract was also evaluated, 0.5; 0.7; 0.8; 0.9; 1.0 and 1.2 ml of the HK preparation were added into the sample during the extraction procedure, it was found that the deconjugation was complete with 1 ml of the enzymatic solution.

As previous papers have reported [29,31] hog kidney conjugase is the most suitable enzyme to determine folates by HPLC once these methods are restricted to use of conjugases that yield a monoglutamyl folate as the terminal product.

In order to activate strong anion-exchange (SAX) cartridges preliminary assays were carried out with hexane and methanol as conditioning solvents, different elution volumes 1.0; 2.0; 2.5, 3.0; 3.5 and 4 ml were also assayed. The best results were achieved with methanol and using 3 ml to elute the sample. In developing the chromatographic method several conditions were assayed.

The mobile phase used is based on the phase described by Vahteristo et al. [13] for determination of folates in food. An increase of the proportion of phosphate buffer from 80 to 85% between 4 and 12 min was necessary to improve the resolution in seaweed sample.

The analysis was performed at room temperature and with the column thermostatted at 30 °C, different flow-rates were also tried, 0.8 and 1.0 ml/min. It was found that the best separation was achieved at 30 °C, with a flow-rate of 0.8 ml/min and when a C_{18} guard column was used.

3.2. Method validation

The method was calibrated using a series of folate standards of known concentrations. Parameters of linearity, intercept, slope, r^2, and range are presented in Table 1. Correlation coefficients were in all cases greater than 0.9988.

Table 1
Method validation parameters for determination of folates in seaweed samples

	5-CH_3–H_4-folate	5-HCO–H_4-folate	H_4-folate	Folic acid
Limit of detection (ng/ml)	1.2	2.1	1.7	0.9
Repeatability (R.S.D. %)	2.55	4.12	2.62	2.93
Recovery (%)	95.93	94.58	90.8	94.78
Parameters of calibration lines				
Intercept	−0.0069	0.0998	−0.5355	0.4088
Slope	107.41	44.519	470.49	63.605
Determination coefficient	0.9994	0.9994	0.9992	0.9988
Range (µg/ml)	0.01–0.12	0.02–0.22	0.01–0.17	0.02–4.00

$n = 6$.

Detection limits (defined as signal three times the height of the noise level) calculated in accordance with American Chemical Society [32] are shown in Table 1. Vahteristo et al. [13] have obtained similar results.

Repeatability, was estimated as relative standard deviation (R.S.D). for determination of six extracts (each one prepared separately from the same homogenised sample). The results are given in Table 1. The method has a satisfactory repeatability (never more than 4.12%), these results are low compared in previous papers [13,16,19].

Recoveries were estimated on the basis of determination after spiking six samples of *Palmaria* spp. with know amounts of standards (see Table 1). The lower recovery of tetrahydrofolate (90.80%) with regard to the others folates is probably due to the extreme instability of this form of vitamer [33].

3.3. Folates in seaweeds

Table 2 summarises the folate content of dehydrated and canned algae. Results are presented as µg of free folate forms and the sum of the vitamers calculated as µg folic acid per 100 g dry mass taking into account the differences in molecular mass of the different folate monoglutamates. The amount of folate in the samples ranged from 52.12 to 167.69 µg per 100 g dry mass.

Tetrahydrofolic acid, and 5-formyltetrahydrofolate were not detected in *S. polychides* and *Porphyra* spp.

The high folic acid content in seaweed analysed is probably as an oxidative product of tetrahydrofolic over the transport or washing with exposure to oxygen, previous of dried and canned processing.

The folates content of canned *H. elongata* was lower that dried samples, possibly because of the effects of processing. Fig. 1A–D show the chromatograms of a standard solution of folates and a seaweed sample with UV and fluorescence detection. H_4-folate, 5-CH_3–H_4-folate and 5-HCO–H_4-folate were quantified by fluorescence and folic acid by UV.

Comparing with USDA Nutrient Data base [34] the results achieved in this paper present lower levels. There are two possible ways to explain this fact. In one hand, the folates have been determined microbiologically, and microbiological values are frequently higher than concentrations determined by HPLC and this could be due to the presence of other compounds with folate activity that have not been identified [11,21]. In the other hand, during sterilisation and drying process the concentration of folates decrease considerably [6,14].

In *U. pinnatifida*, *Palmaria* spp., *L. ochroleuca* and *S. polychides* 5-CH_3–H_4-folate was the most predominant folate form as reported previously for fruits, berries, potato, carrot and white cabbage [35]. Mean folate levels determined in this study in algae, were similar to the amounts found in italian bread, and kiwi fruit [34]; lower than amounts found in frozen spinach and higher than in apple (*Malus domestica*) and banana (*Musa paradisiaca*) [35].

Table 2
Folate vitamers in seaweed samples (µg/100 g dry weight)[a]

	5-CH_3–H_4-folate	5-HCO–H_4-folate	H_4-folate	Folic acid	Sum (as folic acid)
Undaria pinnatifida[b]	66.54 ± 7.63	46.38 ± 2.62	12.54 ± 1.55	47.92 ± 3.17	149.61 ± 7.62
Himanthalia elongata[b]	30.14 ± 4.85	46.96 ± 11.64	10.82 ± 2.96	25.81 ± 1.73	99.42 ± 10.04
Laminaria ochroleuca[b]	83.65 ± 4.62	44.67 ± 5.24	13.20 ± 3.17	47.88 ± 11.97	161.59 ± 6.10
Porphyra spp.[b]	33.89 ± 4.41	nd	nd	36.25 ± 7.80	61.40 ± 9.28
Palmaria spp.[b]	88.67 ± 5.23	61.74 ± 5. 99	13.44 ± 2.16	26.71 ± 2.15	159.11 ± 6.18
Himanthalia elongata[c]	24.31 ± 0.83	32.34 ± 3.57	8.24 ± 0.83	17.59 ± 0.59	71.71 ± 5.67
Saccorhiza polychides[c]	43.00 ± 18.00	nd	9.52 ± 0.66	24.80 ± 1.55	66.5 ± 12.50

n.d.: not detectable.

[a] Each value represents the mean ± S.D. for six different samples. The value for each sample was the mid point of two replicate determinations of a single extract.
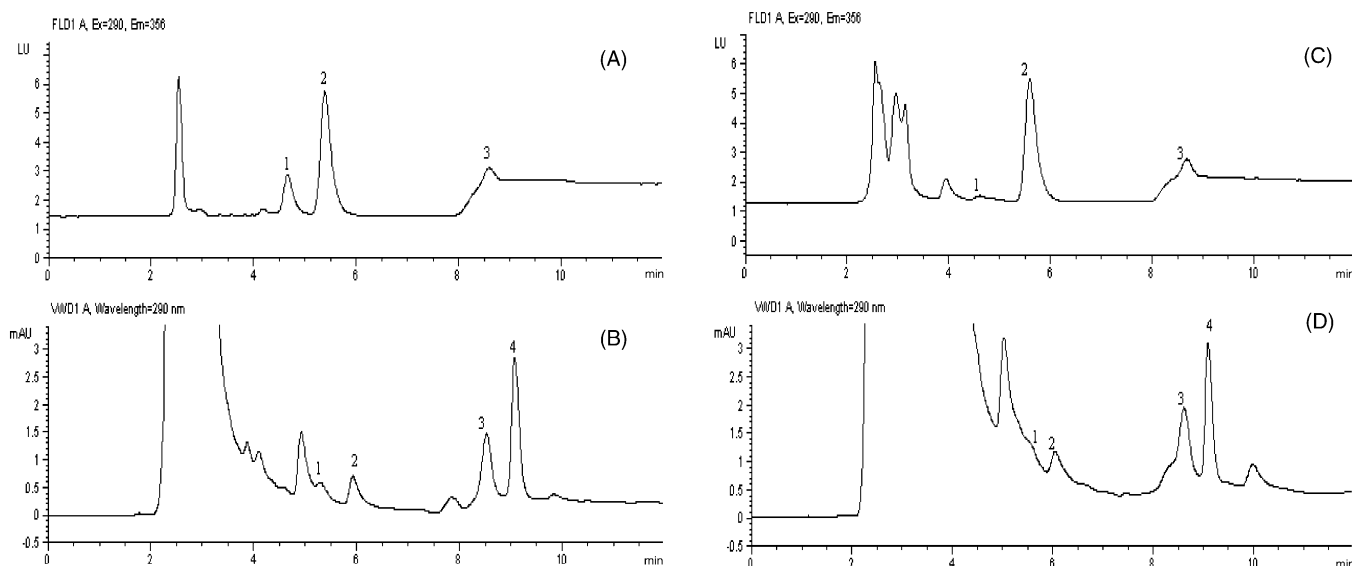
[b] Dehydrated samples.

[c] Canned samples.

Fig. 1. HPLC chromatograms of a standard solution: (A) with fluorescence detection λ_{em} 356 nm and λ_{ex} 290 nm (B) with UV detection at 290 nm and a dehydrated *Undaria pinnatifida* sample with (C) fluorescence and (D) UV detection. (1) H_4-folate; (2) 5-CH$_3$– H_4-folate; (3) 5-HCO– H_4-folate and (4) folic acid.

In conclusion, the method proposed is precise and may be considered suitable for the determination of folates by HPLC with UV and fluorescence detectors.

Acknowledgements

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