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Analysis of some folate monoglutamates by high-performance liquid chromatography–mass spectrometry. I

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Abstract

The use of high-performance liquid chromatography for the determination of folates is well documented. The methods used are based on reversed-phase chromatography with UV and/or fluorescence detection. In many instances it is difficult to reach the required detection limits and many of the methods lack specificity. High-performance liquid chromatography–mass spectrometry (LC–MS) has become a well established analytical tool in modern laboratories. It can offer superior specificity and often lower detection limits than conventional LC detection methods and thus is ideally suited to the analysis of folates. A system capable of separating the four main folates [folic acid (pteroylglutamic acid, PGA)], 5-methyltetrahydrofolic acid, terahydrofolic acid and 5- and/or 10-formyltetrahydrofolic acid [folinic acid (CHOTHF)] using LC–MS with negative ion electrospray has been developed. After optimisation, a system using a 12.5 cm×3 mm, 3 μm Hypersil ODS column and a mobile phase of 2.5 m*M* acetic acid–acetonitrile (88:12, v/v) was developed which was capable of separating the four folates in under 10 min without the use of a gradient system. Extraction of the folates is by heat treatment and sample clean-up is by solid-phase extraction (C_{18}). On column limits of confirmation are 0.6 ng for PGA and CHOTHF.

Keywords: Food analysis; Monoglutamates; Glutamates; Vitamins; Folic acid; Folinic acid

1. Introduction

The folates are a group of vitamins based on the parent compound folic acid [pteroylglutamic acid (PGA)]. The compounds differ in regard to the state of oxidation of the pteridine ring, the number of glutamate residues conjugated to the *para*-amino-benzoic acid moiety and the type of substitution at the 5- and/or 10-carbon position (Fig. 1) [1]. It has been found that the folates influence a number of biological processes in humans and are particularly important in the prevention of neural tube defects (associated with spina bifeda) in unborn children. This has led to the fortification of some foodstuffs with folic acid [2].

Only the reduced folates are found naturally in plants and animals. In humans, the predominant form of folate found in the blood is 5-methyltetrahydrofolic acid (5-MeTHF) whereas in the porcine species, it has been reported that tetrahydrofolic acid (H_4Folate) is the major circulating form. Several different forms have been found in foodstuffs, the most common being 5-MeTHF, 5- and/or 10-formyl

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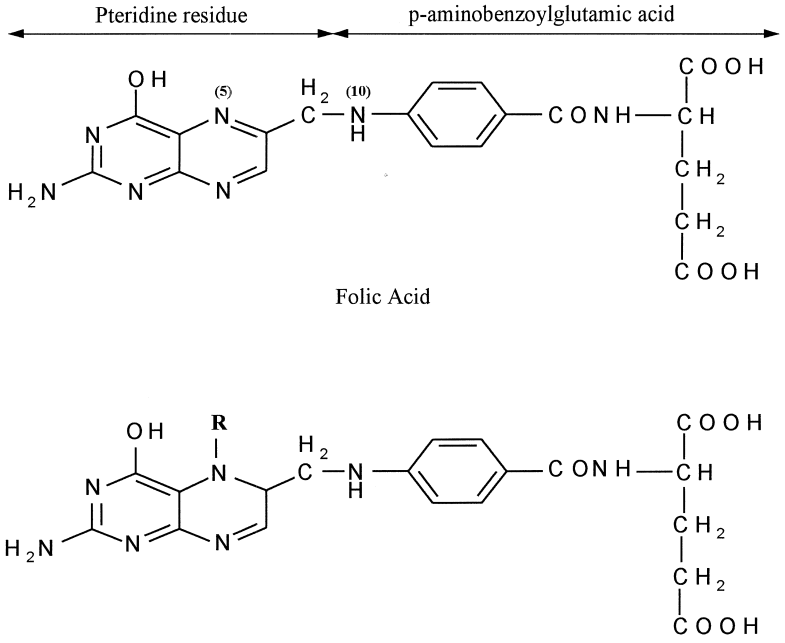


Substituant group (R)	Folate
H	H_4Folate
CHO	CHOTHF
CH_3	MeTHF

Fig. 1. Chemical Structure of folic acid and substituent groups of the principal folates.

tetrahydrofolic acid [folinic acid (CHOTHF)] and H_4Folate.

Traditionally a total folate content of food has been determined using a microbiological assay. When individual folate concentration is required separation of the various forms must be achieved. The different folate compounds exhibit small differences in their ionic character and are thus well suited to analysis by high-performance liquid chromatography (HPLC). A number of column liquid chromatography methods [1–9] have been established based on reversed-phase chromatography with UV and/or fluorescence detection. Dual detection is required because some folates e.g. PGA do not fluoresce naturally. In many instances it is difficult to reach the required detection limits and many of the methods lack specificity. Many of the folates are extremely prone to oxidation and require the presence of antioxidants such as ascorbic acid and/or 2-mercaptoethanol. The use of such compounds can cause interference at the detection stage of the analysis.

HPLC–mass spectrometry (LC–MS) has become an increasingly popular and available tool in modern laboratories. It can offer superior specificity and often lower detection limits than conventional LC detection methods and thus is ideally suited for the analysis of folates. At the time of writing there are no published LC–MS methods for the determination of folates in the literature. The purpose of this study was to produce an LC–MS method for both qualitative and quantitative determination of folates in foodstuffs.

This paper presents LC–MS conditions for the separation and identification of the four main folates.

The results from the examination of folinic and folic acid in a vitamin supplement and some foodstuffs using the described method are included.

2. Experimental

2.1. Reagents

2.1.1. Folate compounds

PGA was obtained from Fluka (Poole, UK). Tetrahydrofolic acid trihydrochloride dihydrate (H_4Folate), 5-methyltetrahydrofolic acid calcium salt (5-MeTHF) and 5-formyltetrahydrofolic acid calcium salt (CHOTHF) were obtained from Dr. Schirck' s Labs. (Jona, Switzerland).

Acetonitrile, methanol (HPLC grade) and ascorbic acid (SLR grade) were obtained from Fisher Scientific (Loughborough, UK). Ammonium acetate, ammonia solution sg 0.91 and glacial acetic acid (AristarR) were obtained from Merck (Lutterworth, UK). 2-Mercaptoethanol was obtained from Sigma (Poole, UK). High purity water with a conductivity of 18 MΩ cm^{-1} or better was also used.

2.1.2. Standard solutions

Stock standards (1 mg/ml) were prepared by dissolving 25 mg in 25 ml of 0.1 *M* ammonium acetate buffer containing 1% (w/v) ascorbic acid (adjusted to pH 7.2 with ammonia solution sp. gr. 0.91) and 0.1% (v/v) 2-mercaptoethanol (stock standard buffer). The solutions were degassed ultrasonically, flushed with nitrogen and stored in a freezer until required. Working standards were diluted in a solution containing 2% stock standard buffer (aq.)–mobile phase (50:50).

2.2. HPLC Instrumentation

Experimental work was carried out using a Waters (Watford, UK) LC system (616 pump, 717 plus autosampler and 600 S controller). Chromatographic separation was carried out on a Hewlett-Packard (Stockport, UK) Hypersil ODS (12.5 cm×3 mm, 3 μm) cartridge column using a mobile phase consisting of 2.5 m*M* acetic acid–acetonitrile (88:12) at a flow-rate of 0.5 ml/min. An injection volume of 50 μl was used.

2.3. Mass spectrometer instrumentation

A Finnigan SSQ7000 mass spectrometer fitted with an electrospray ionisation interface operated with a spray voltage of 3 kV was used throughout the study. The heated capillary was maintained at 300°C. Scanning work was carried out between 50–500 u with a scan cycle time of 0.5 s. For selected ion monitoring (SIM) work a scan window of 0.4 u and a scan cycle time of 0.5 s was used. Quantitation was carried out using the abundance (peak area) of the $[M-H]^-$ ion for each compound. Confirmation of identity was made if the ratios of three structurally significant ions (as shown in Figs. 3 and 4 in Section 3) from the sample matched the ion ratios for a standard of similar concentration to within ±20%.

2.4. Sample preparation

0.01–1 g of homogenous sample (depending on folate content) was weighed into a 15 ml polypropylene tube. Ten ml of extraction buffer [0.1 *M* ammonium acetate containing 1% (w/v) ascorbic acid (adjusted to pH 4 with glacial acetic acid) and 0.1% (v/v) 2-mecaptoethanol] was added to each tube. The folates were extracted by placing the samples in a boiling water bath for 1 h. The samples were shaken vigorously every 15 min. Following extraction the samples were cooled and centrifuged at 4000 rpm for 20 min.

2.5. Sample clean-up

Purification was carried out on C_{18} solid-phase extraction cartridges. Each cartridge was conditioned with 5 ml of acetonitrile followed by 5 ml of extraction buffer. An aliquot (5 ml) of sample was transferred to an extraction cartridge and this was allowed to flow through under gravity. The cartridge was rinsed with extraction buffer (5 ml) and the folates were subsequently eluted with 5 ml of mobile phase directly into an amber volumetric flask (10 ml). Samples were bulked to volume with 2% (v/v) of stock standard buffer. Aliquots of these solutions were taken for analysis by LC–MS.

3. Results and discussion

3.1. Optimisation of chromatography

The development of a completely new mobile phase system was necessary because existing LC methodologies utilise phosphate buffer mobile phase systems which are not compatible with the SSQ7000 mass spectrometer. No retention of the folate compounds was obtained using acetonitrile–water mobile phase systems. To obtain retention, acetic acid was added to the mobile phase to suppress the ionisation of the folate compounds thus allowing partitioning into the non-polar stationary phase. At a concentration of 0.1 *M* in the ratio acetic acid–acetonitrile (90:10) using a 10 cm×3 mm, 5 μm Hypersil cartridge the four folates could be separated within 15 min. Peak shape was further improved with a 12.5 cm×3 mm, 3 μm Hypersil column (Fig. 2.) The addition of weak acids to mobile phases is not recommended when operating in ESI negative ion mode because high spray currents leads to the generation of a corona discharge at the spray tip which results in a loss of sensitivity. For this reason the concentration of acetic acid in the mobile phase was reduced from 0.1 *M* to 2.5 m*M*. Under these conditions retention of the folates was still obtained and sensitivity was improved. No further work was carried out on H_4Folate and 5-MeTHF from this point because of their poor stability. It is intended to examine these folacins and other related compounds at a later date.

A decrease in peak area (the cause of which has not been established) was observed if a short analysis time was used. With a 5 min run time a 600 ng/ml standard of peak area 16 000 had decreased by 60% on the following injection. For this reason a total run time of 15 min was used between in-

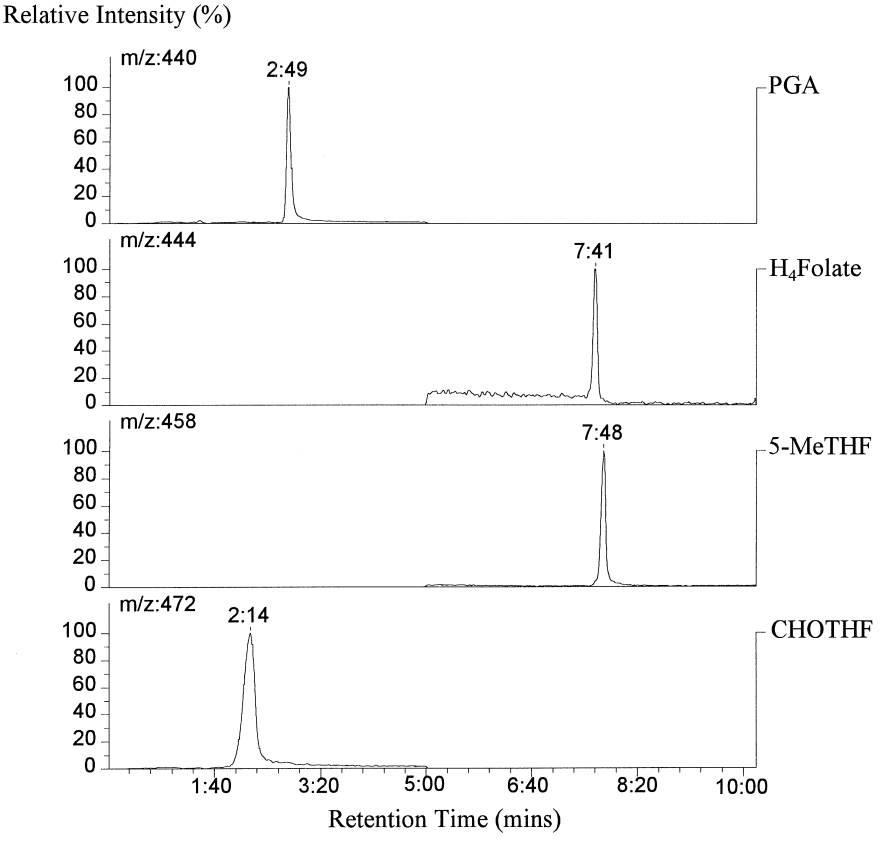


Fig. 2. ESI Mass chromatogram of $[M–H]^-$ ions of PGA, H_4Folate, 5-MeTHF and CHOTHF (250 ng/ml).

jections. Variations in retention time of standards and samples were also observed. Samples tended to elute up to 25% earlier than standards. The shift in retention time was stable (±0.04 min). This is not so much of a problem with mass spectrometric detection as the ratio of confirmation ions can be used for positive identification. In order to obtain linear calibration curves it was necessary to buffer the standard solutions. Buffering stabilises the ionisation of the folates in solution and enables linear calibration curves to be produced. A typical calibration curve consisting of 5 data points for PGA (between 50 and 600 ng/ml) is specified by the Eq. $y=38.408x-735.51$ ($r^2=0.9998$) where y denotes the peak area of the $[M-H]^-$ ion of PGA, x represents the concentration of PGA in ng/ml and r^2 is the correlation coefficient. Limits of detection based on the weakest of three ions with a signal-to-noise ratio of 3:1 were 12.5 ng/ml (0.6 ng on column) for both CHOTHF and PGA.

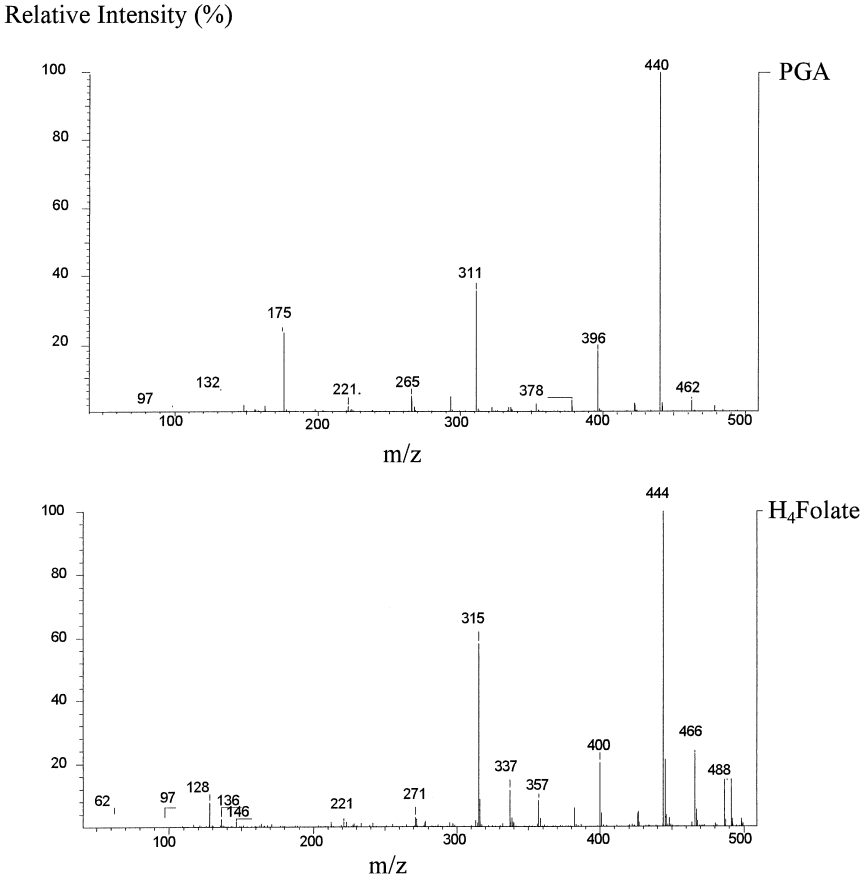
3.2. Optimisation of mass spectrometer

Direct injection (no column) of folate standards using both APCI (atmospheric pressure chemical ionisation) and ESI (electrospray ionisation) in both positive and negative ion modes identified ESI (negative ion mode) to be the most suitable. ESI was chosen because greater signal-to-noise ratios were obtained when compared to APCI. It is common to see only pseudomolecular ions, $[M-H]^-$ (and often adduct ions such as $[M-H+Na]^-$) with the ESI interface. For confirmation purposes it is desirable to monitor at least three structurally significant ions where possible. This can be achieved by collision induced dissociation (CID). The SSQ7000 mass spectrometer is equipped with a set of octapole rods located between the ionisation chamber and the quadrupole analyser. Fragmentation can be induced by applying a voltage to these rods. The resulting potential difference between the skimmer cone and rods cause the ions to accelerate and fragmentation is caused by collisions with gas molecules found between the source and analyzer regions of the mass spectrometer.

Individual folate standards were injected without application of a fragmentation voltage and little was seen apart from the $[M-H]^-$ ion for each compound. When a fragmentation voltage of around 30 V was applied other structurally significant fragmentation ions were observed. Figs. 3 and 4 show spectra obtained with collision induced dissociation for the four main folacins. Adduct ions at m/z's greater than the $[M-H]^-$ were also detected. Sensitivity was further improved by lowering the spray voltage from 4.5 kV to 3 kV and increasing the capillary temperature from 250°C to 300°C. Sheath gas and auxiliary gas were operated at 80 p.s.i. and 40 p.s.i., respectively (1 p.s.i.=6894.76 Pa).

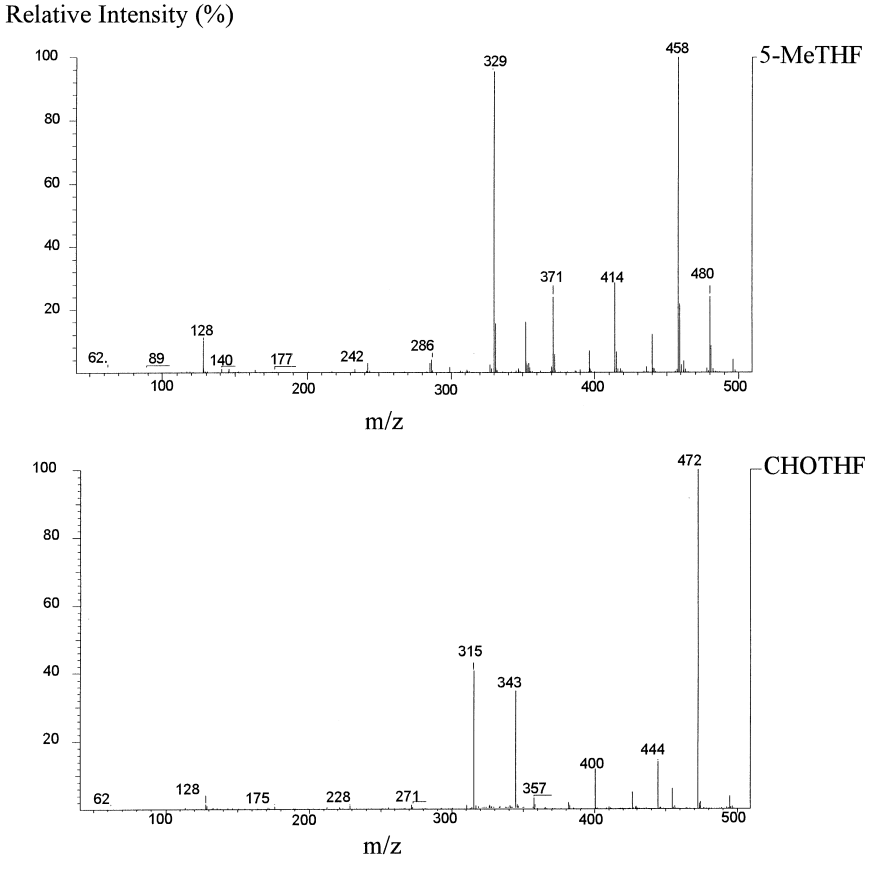
3.3. Results

A commercially available multivitamin tablet containing 200 μg folacin(PGA)/tablet and a breakfast cereal containing 200 μg/100 g of PGA, the same breakfast cereal spiked with 2 μg/g of PGA and CHOTHF, a concentrated beef and vegetable extract (833 μg/100 g PGA) and the same extract spiked with 8 μg/g of PGA and CHOTHF were taken through the described procedure. Typical examples of standard and sample chromatograms are shown in Fig. 5. Standards were also taken through the clean-up procedure in order to measure extraction efficiency, the results are shown in Table 1. The multivitamin was found to contain PGA at 217±18 μg/tablet, which is comparable with the declared amount. The breakfast cereal was found to contain significantly more PGA than expected. Recovery of PGA from spiked samples was good but no CHOTHF was recovered. A large peak at m/z 472 was observed in both spiked and un-spiked samples but the confirmation ion ratios did not compare with those obtained for the CHOTHF standard thus indicating that this was not CHOTHF. The beef and vegetable extract was also found to contain significantly more PGA than expected. CHOTHF and PGA were recovered from the spiked samples but quantitation of CHOTHF and PGA was not attempted because of decreasing peak areas for both CHOTHF and PGA during the run. Quantitation of CHOTHF was further complicated by an interfering peak. First appearances suggested that the samples extracts were unstable but a repeat analysis of an un-spiked extract the following day compared well with the results already obtained thus suggesting that this was not the case.



Folacin	Suggested Confirmation Ions
PGA	440 $[M-H]^-$
	396 $[M-H-CO_2]^-$
	311 $[M-HOOC-CH_2-CH-CH-COOH]^-$
H_4Folate	444 $[M-H]^-$
	400 $[M-H-CO_2]^-$
	315 $[M-HOOC-CH_2-CH-CH-COOH]^-$

Fig. 3. Fragmentation spectra of PGA and H_4 Folate.



Folacin	Suggested Confirmation Ions
5-MeTHF	458 $[M-H]^-$
	414 $[M-H-CO_2]^-$
	329 $[M\text{-}HOOC\text{-}CH_2\text{-}CH\text{-}CH\text{-}COOH]^-$
CHOTHF	472 $[M-H]^-$
	343 $[M\text{-}HOOC\text{-}CH_2\text{-}CH\text{-}CH\text{-}COOH]^-$
	315 $[M\text{-}HOOC\text{-}CH_2\text{-}CH\text{-}CH\text{-}COOH - C{=}O]^-$

Fig. 4. Fragmentation spectra of 5-MeTHF and CHOTHF.

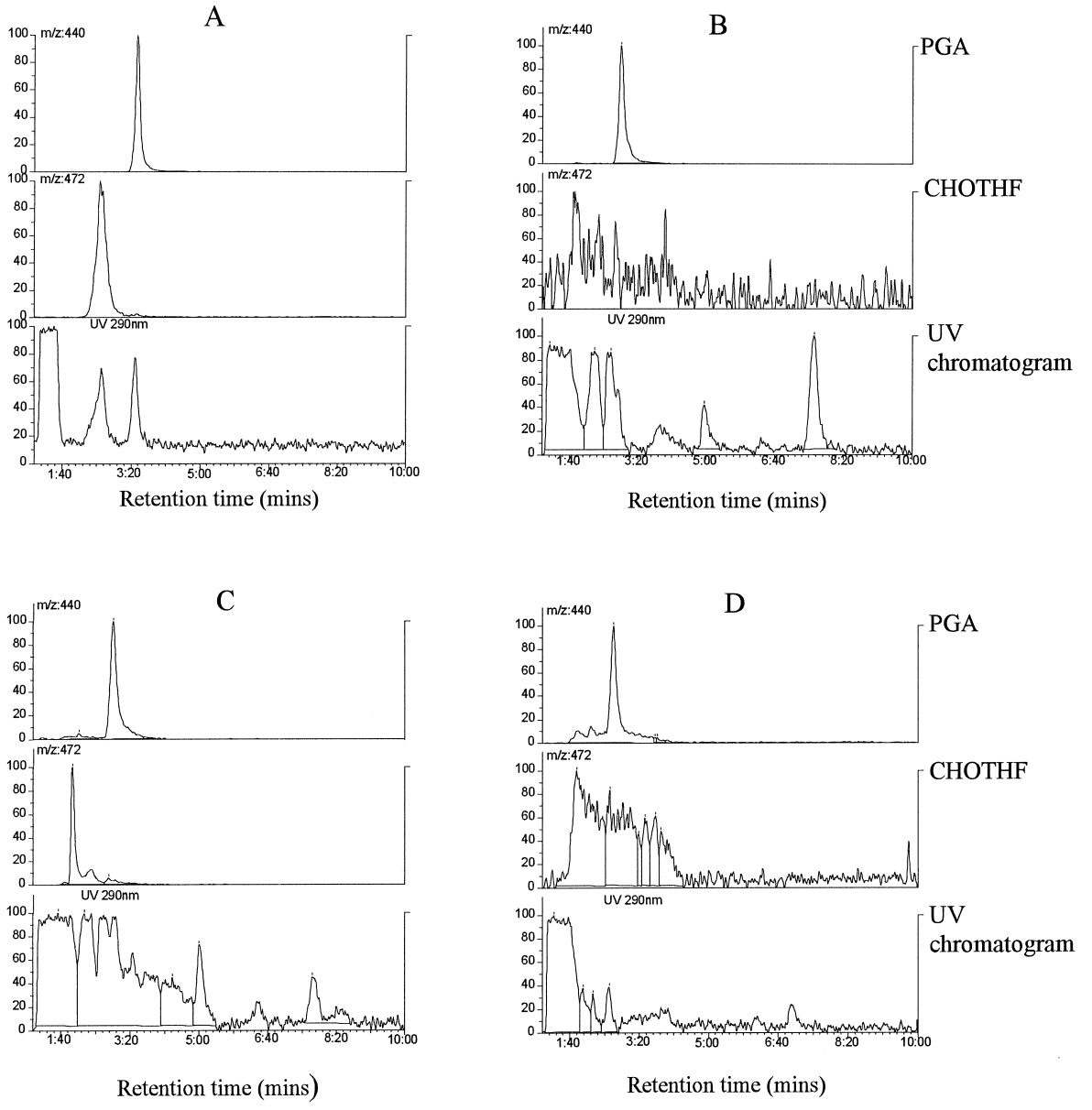


Fig. 5. UV and mass chromatograms of (A) PGA and CHOTHF (600 ng/ml), (B) multivitamin extract, (C) spiked ''Malt Bite'' breakfast cereal and (D) beef and vegetable extract.

4. Conclusion and recommendations

A LC–MS system, capable of separating the four main folates has been developed and suitable confirmation ions for each folacin have been identified. Application of the method to ''real'' samples has identified potential problems that appear to be matrix specific and these must be overcome before the method can be used routinely. The establishment of a LC–MS method for the key folates will enable

Table 1
Results of analysis of selected food matrices and a vitamin supplement by described procedure

	200 ng/ml standard solution (n=5)		Multi-vitamin (n=8) PGA	Breakfast cereal, day 1 (n=4) PGA	Spiked breakfast cereal, day 1 (n=4) PGA	Breakfast cereal, day 2 (n=4) PGA	Spiked breakfast cereal, day 2 (n=4) PGA	Beef and vegetable extract (n=4) PGA
	PGA	CHOTHF						
Mean recovery	91%	72%	217 μg/tablet	459 μg/100 g	79%	333 μg/100 g	94%	1770 μg/100 g
RSD (%)	13	8	8	7	23	6	15	5

further detailed investigation to be made into the problems associated with the stability and extraction of these demanding compounds.

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