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# Optimized high-performance liquid chromatographic procedure for the separation and quantification of the main folacins and some derivatives

## I. Chromatographic system

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ABSTRACT

An improved reversed-phase high-performance liquid chromatographic (HPLC) procedure for the rapid separation and sensitive fluorimetric quantification of the main folacins and some degradation products is presented. The system is the first to include two substances that can be used as internal standards. It allows the complete separation of all substances within 26 min. The fluorescence of reduced folates is measured directly after the column whereas other compounds are converted oxidatively to fluorescent products by post-column derivatization. Among several oxidants tested, potassium peroxodisulphate was found to be the most suitable.

## INTRODUCTION

The folacins or folates are a widespread group of vitamers of about 100–150 biologically active compounds differing in the redox state of the pteridine nucleus, the one-carbon substituents and the length of the γ-glutamyl side chain [1–3]. Although they have been known for more than 40 years, their analysis still represents a problem because of the extreme lability of many of the derivatives against light and oxygen and their low concentration in biological materials [4–6]. The simultaneous determination of all forms does not seem possible.

It is important to emphasize that the nutritional availability of different vitamers is varied. Especially the pteroylpolyglutamates representing the active coenzymes of $C_1$ metabolism must be hydrolysed before their intestinal absorption and thus are only partially utilized [7]. This is a factor which should be taken into account because several nutritional surveys have shown that there is a dietary deficiency of the folacins in some population groups [8,9]. From this point of view, it is important to differentiate between "free" folate, *i.e.*, the monoglutamate forms, and "total" folate, which encompasses all folates including the pteroylpolyglutamates.

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Within the last few years, a number of methods for the analysis of folacins have been developed, including microbiological, radiometric and chromatographic techniques. Microbiological tests are sensitive but are time consuming and do not allow differentiation between all forms of folates, especially between free and total folate [10,11]. Radiometric procedures have mainly been used to determine folates in blood, although they are not suitable for the analysis of complex materials because of the varying affinity of different forms of folates towards the binding proteins [12,13]. Conventional chromatographic procedures such as thin-layer [14,15] and column chromatography [16,17] need a lot of time and are often less reproducible and reliable.

These procedures are being replaced by high-performance liquid chromatography (HPLC), which is more rapid and in many instances yields a better resolution. Since 1976, numerous methods have been proposed [18–36], but all the systems lack some properties necessary for their application to biological materials or their application in metabolic studies using radioactive folacin compounds. Either they do not allow the separation of all important forms of folates [21,22,26,28] or they are not sensitive and specific enough for the direct determination of folacins [18,23]. For this reason, they often need prechromatographic steps to clean and concentrate the samples [29,33,35] or require microbiological quantification of the collected fractions [26,30,36].

Further, no successful attempt to use an internal standard has been described, although folacin analogues such as methotrexate and 3′,5′-dichlorofolic acid are suitable for this purpose. A system that allows the simultaneous determination of naturally occurring folates and methotrexate would also be useful for clinical surveys of patients receiving antitumor therapy with methotrexate.

This work was aimed at establishing a rapid technique for the separation and fluorimetric quantification of the main folacin monoglutamates and polyglutamates after enzymatic hydrolysis, some degradation products, methotrexate and dichlorofolic acid. The system was designed for the determination of folates in tissues and also for metabolism studies using radiolabelled folacin. The extraction techniques, the hydrolysis of the pteroylpolyglutamates and the application of the overall method will be presented in a forthcoming publication [44].

## EXPERIMENTAL

### *Folacin standards*

Pteroylglutamic acid (PteGlu), dihydrofolic acid (DHF), tetrahydrofolic acid (THF), sodium 5-methyltetrahydrofolate (5-$CH_3$-THF), calcium 5-formyltetrahydrofolate (5-CHO-THF), methotrexate (MTX), 3′,5′-dichlorofolic acid (DCF), *p*-aminobenzoylglutamic acid (pABG), pterin (Pt) and pterin-6-carboxylic acid (Pt-6-COOH) were obtained from Sigma (Deisenhofen, Germany). All substances were diluted in sodium ascorbate solution (1%), immediately flushed with nitrogen and kept at −80°C. Under these conditions, the derivatives were stable for at least 4 weeks, except for DHF, which had to be replaced weekly.

All other chemicals and solvents were obtained from Merck (Darmstadt, Germany) and were of the highest purity available. Water was purified with a Millipore Milli-Q system (Waters, Eschborn, Germany).

*Chromatography*

Several chromatographic systems were tested based on ion-pair reversed-phase HPLC and conventional reversed-phase HPLC. The best resolution of peaks was achieved using reversed-phase HPLC on a 3-μm ODS Hypersil (Shandon, Frankfurt, Germany) analytical column (250 × 4.6 mm I.D.) which had been filled by the upward slurry technique [37] using 2-propanol for preparing the slurry. The analytical column was connected with a C-135 B precolumn (Upchurch Scientific, Oak Harbour, WA, U.S.A.) containing dry-packed Shandon ODS Hypersil (10-μm).

The most suitable mobile phase was found to be a gradient of 5 m*M* $KH_2PO_4$ and acetonitrile (Table I), which was pumped at a flow-rate of 0.9 ml/min. The pH of the buffer was adjusted to 2.3 with $H_3PO_4$. The solvents were freshly prepared on the day of use, filtered through 0.45-μm filters (Schleicher & Schüll, Dassel, Germany) and degassed ultrasonically under vacuum.

TABLE I

GRADIENT COMPOSITION DURING THE HPLC ANALYSIS OF FOLACINS

| Component[a] | Time (min) | | | |
|---|---|---|---|---|
| | 0 | 15 | 18 | 21 |
| A (%) | 93 | 87 | 81 | 79 |
| B (%) | 7 | 13 | 19 | 21 |

[a] A = 5 m*M* potassium phosphate (pH 2.3); B = acetonitrile.

During the development of the separation system, we employed a Merck–Hitachi HPLC system consisting of an L-5000 gradient former, a 655A-11 solvent metering pump, a 655A-23 UV detector and a loop injector (Rheodyne, Model 7125) with a 250-μl syringe. Ultraviolet absorption was monitored at 295 nm and peaks were registered with a Waters D 740 integrator. All analyses were performed at room temperature.

*Fluorimetric detection of folacins*

THF, 5-$CH_3$-THF and 5-CHO-THF showing native fluorescence at excitation and emission wavelengths of 295 and 356 nm respectively, were determined with a Merck–Hitachi F-1000 fluorimeter which was placed in the eluent stream directly after the column. PteGlu, DHF, MTX and DCF were converted to fluorescent pterins using a post-column derivatization arrangement as described by Gregory *et al.* [33]. We tested several oxidant solutions [$Ce(SO_4)_2$, $KMnO_7$, $K_2Cr_2O_7$, $KClO_4$, $KIO_4$, $Ca(OCl)_2$, $K_2S_2O_8$, chloramine T (sodium N-chloro-4-toluenesulphonamide)] with standard redox potentials between 1 and 2 V in aqueous solutions of 0.1% and 1%. They were pumped into the eluent stream leaving the first detector through a T-junction either with a conventional HPLC pump or with a laboratory-constructed perfusor pump.

After a reaction zone consisting of a 10-m Teflon tube (0.8 mm I.D.), coiled three-dimensionally to minimize peak broadening [38] and kept at 60°C in a water-

bath, the fluorescence of the resulting pterins was monitored at excitation and emission wavelengths of 365 and 450 nm, respectively, with a second fluorescence detector (Merck-Hitachi F-1000). As observed by fluorescence spectroscopy of the different derivatives in the mobile phase, the chosen wavelengths corresponded to the maxima for the folacins and the products of the oxidative cleavage. The signal was recorded with a Merck D-2000 integrator. In all instances quantification was performed on the basis of peak area.

## RESULTS

### *Separation system*

Fig. 1 shows a typical chromatogram of natural folacins, the main degradation products, MTX and DCF. Under these conditions, all substances could be separated within 26 min and the next injection could be performed after 30 min. Without the internal standards MTX and DCF, separation was terminated after 18 min and the next sample could be injected after 22 min. The resolution was sufficient in all instances for quantification of the separated substances. The most critical derivatives were 5-CHO-THF and DHF, which could only be separated using a column filled with particles of 3-$\mu$m diameter. Using 5-$\mu$m particles of Shandon ODS Hypersil or 10-$\mu$m $\mu$Bondapak Phenyl (Waters), DHF and 5-CHO-THF could not be completely separated or could not be separated at all.

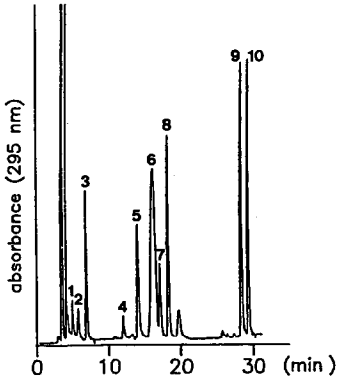


Fig. 1. Chromatographic separation of folacins and some of their degradation products. Conditions: column, Shandon Hypersil ODS (3 $\mu$m) (250 × 4.6 mm I.D.); mobile phase, gradient (Table I) of 5 m*M* potassium phosphate (pH 2.3) and acetonitrile at a flow-rate of 0.9 ml/min; ambient temperature; UV detection at 295 nm; 50–60 pmol of each compound were injected. Peaks: 1 = Pt; 2 = Pt-6-COOH; 3 = pABG; 4 = THF; 5 = 5-$CH_3$-THF; 6 = 5-CHO-THF; 7 = DHF; 8 = PteGlu; 9 = MTX; 10 = DCF.

### *Post-column derivatization*

Using a conventional HPLC pump to deliver the oxidant technical problems arose because the pump seals were rapidly destroyed. This led to an irregular output and caused high baseline noise, especially when reagents that fluoresce themselves, *e.g.*, $K_2Cr_2O_7$, were used. In contrast, a laboratory-constructed perfusor-like pump

guaranteed a continuous flow without pulsation and was not attacked by the chemicals.

Our experiments with $Ca(OCl)_2$, a reagent first used by Day and Gregory [32], were not successful. The peak areas of the derivatization products were even smaller than those obtained with UV detection. Further, MTX was hardly attacked and the reagent rapidly decomposed, which led to poorer derivatization within two or three analyses. Other reagents tested, *e.g.* $K_2Cr_2O_7$, $Ce(SO_4)_2$, $KClO_4$, $KIO_4$ $KMnO_4$ and chloramine T, were not suitable for derivatization. Either they did not improve the sensitivity or technical problems arose, *e.g.*, deposits of manganese dioxide in the tubes and on the walls of the detector cell. The best results were achieved with potassium peroxodisulphate ($K_2S_2O_8$), which was capable of oxidizing all substances of interest in a comparable manner. The detectable peak areas of the derivatization products depended very much on the flow-rate of the oxidant (Table II). It was clear that higher flow-rates resulted in smaller peak areas.

Fig. 2. shows a standard chromatogram of folacins using fluorimetric detection.

TABLE II

POST-COLUMN DERIVATIZATION OF FOLACINS: INFLUENCE OF FLOW-RATE OF THE DERIVATIZATION REAGENT (1% $K_2S_2O_8$) ON THE FLUORESCENCE OF THE RESULTING PRODUCTS

Values given represent the relative peak areas in comparison to a flow-rate of 0.39 ml/min (= 100%) and are means ± standard deviations ($n = 5$); n.d. = not detectable. $P \leqslant 0.001$ compared to a flow-rate of 0.39 ml/min for PteGlu, DHF and DCF and $P \leqslant 0.01$ for MTX as judged by a Student's *t*-test.

| Flow-rate (ml/min) | PteGlu | DHF | MTX | DCF |
|---|---|---|---|---|
| 0.39 | 100.0 ± 7.8 | 100.0 ± 10.1 | 100.0 ± 7.9 | 100.0 ± 3.1 |
| 0.45 | 50.3 ± 12.3 | 37.1 ± 7.9 | 99.1 ± 1.0 | 85.3 ± 2.6 |
| 0.58 | 17.5 ± 11.0 | n.d. | 86.8 ± 1.6 | 51.6 ± 9.1 |
| 0.79 | 7.1 ± 3.0 | n.d. | 75.4 ± 1.4 | 38.1 ± 5.8 |

*Characteristics of the method*

Table III lists the detection limits (signal-to-noise ratio 3:1) of the proposed method in comparison with other published data and Table IV gives the quantitative chromatographic parameters of the system described. Calibration graphs were constructed for each compound by plotting the peak area *versus* the amount injected and calculation by least-squares regression analysis.

## DISCUSSION

The aim of this study was to establish a rapid and sensitive technique for the separation and quantification of the main folacins and the internal standards MTX and DCF.

THF, 5-$CH_3$-THF, 5- and 10-CHO-THF are the most important naturally occurring folacins, whereas PteGlu and DHF are usually not found at all or are found

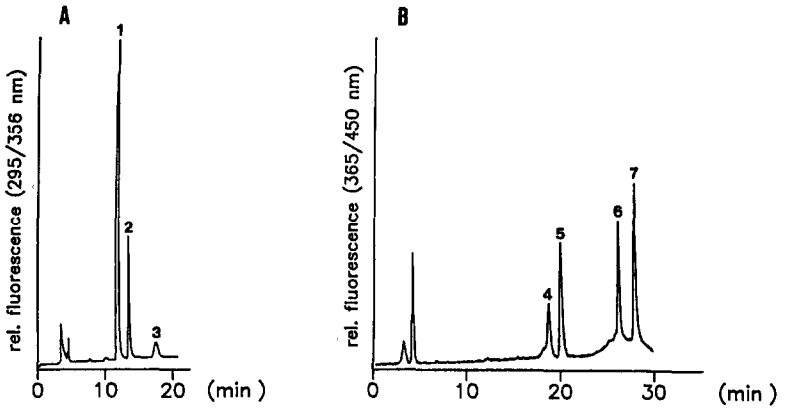


Fig. 2. Chromatograms of the main folacins using fluorimetric detection. Separation conditions as in Fig. 1; 25 pmol of each compound except 5-$CH_3$-THF (5 pmol). Detection: (A) native fluorescence of reduced folates at excitation and emission wavelengths of 295 and 356 nm, respectively; (B) fluorescence (365/450 nm) of the products obtained by oxidation of several folates using post-column derivatization with 1% potassium peroxodisulphate; reagent was pumped into the eluent stream leaving the first detector at a flow-rate of 0.3 ml/min. Peaks: 1 = THF; 2 = 5-$CH_3$-THF; 3 = 5-CHO-THF; 4 = DHF; 5 = PteGlu; 6 = MTX; 7 = DCF.

only in small amounts in biological materials [39]. 10-CHO-THF can be converted into the 5-CHO derivative by heat treatment during the extraction procedure and determined in this form [33].

The chromatographic system presented should also be suitable for studies of folacin metabolism, in which radiolabelled PteGlu is often used and DHF can be

TABLE III

DETECTION LIMITS (ng PER INJECTION) OF VARIOUS HPLC METHODS FOR THE DETERMINATION OF FOLACINS

Data represent the minimum levels detectable under the conditions described in each paper; –, not determined.

| Detection | PteGlu | DHF | THF | 5-$CH_3$-THF | 5-CHO-THF | Ref. |
|---|---|---|---|---|---|---|
| UV (254 nm) | 20 | 100 | 200 | – | 100 | 18 |
| UV (285 nm) | 25 | 15 | – | 25 | 50 | 23 |
| UV (284 nm) | 1.5 | 1.0 | 2.0 | 2.0 | 1.5 | 29 |
| UV (280 nm) | 2.2 | 4.4 | 2.2 | 2.4 | 0.5 | 43 |
| UV (280 nm) | 3 | 5 | 5 | 3 | 10 | 32 |
| Fluorimetry (365/450 nm) | 0.2 | 0.9 | 0.3 | – | – | 32 |
| Fluorimetry (295/356 nm)[a] | 1.0 | – | 0.07 | 0.014 | 0.15 | 33 |
| Fluorimetry (295/356 nm) | – | – | 0.89 | 0.18 | 9.5 | 35 |
| *L. casei* assay | 0.025 | – | 0.03 | 0.05 | 0.03 | 36 |
| Electrochemical (+0.75 V) | 0.08 | – | 0.003 | 0.001 | 0.15 | 34 |
| UV (295 nm) | 1.10 | 1.77 | 2.67 | 1.15 | 2.37 | This work |
| Fluorimetry (295/356 nm)[b] | 0.57 | 0.89 | 0.04 | 0.007 | 0.28 | This work |

[a] PteGlu and THF were detected at $\lambda_{ex}$ 365/$\lambda_{em}$ 450 nm.
[b] PteGlu, DHF and THF were detected at $\lambda_{ex}$ 365/$\lambda_{em}$ 450 nm.

TABLE IV

QUANTITATIVE CHROMATOGRAPHIC PARAMETERS FOR HPLC ANALYSIS OF FOLACINS USING FLUORIMETRIC DETECTION

| Compound | Retention time (min) | Range of linearity (pmol) | Detection limit (pmol) | Calibration graph parameters[a] | | | Precision[b] |
|---|---|---|---|---|---|---|---|
| | | | | *a* | *b* | *r* | |
| THF | 11.17 | 1.25–250 | 0.1 | 6.87 | −4.21 | 0.999 | 4.7 |
| 5-$CH_3$-THF | 12.82 | 0.016–50 | 0.015 | 11.34 | 0.23 | 0.999 | 1.6 |
| 5-CHO-THF | 16.67 | 1.25–250 | 0.6 | 0.64 | 1.79 | 0.999 | 1.7 |
| DHF | 18.53 | 2.5–250 | 2.0 | 0.15 | −0.61 | 0.981 | 11.0 |
| PteGlu | 19.59 | 2.5–250 | 1.3 | 0.28 | −0.78 | 0.995 | 5.8 |
| MTX | 25.14 | 2.5–250 | 1.4 | 0.18 | −0.10 | 0.998 | 7.8 |
| DCF | 26.64 | 2.5–250 | 1.0 | 0.26 | −0.27 | 0.999 | 3.1 |

[a] Parameters were determined using the equation $y = ax + b$, where $x$ = pmol injected and $y$ = peak area.

[b] Precision is the percentage deviation of the mean as obtained by repeated analysis of the same sample ten times; the sample contained 62.5 pmol per injection of each compound.

found as an intermediate [1]. Further, the system should include the main degradation products of folacins, *i.e.*, pABG, Pt and Pt-6-COOH, to prevent misinterpretation caused by co-chromatography of degradation products with natural folates. This difficulty occurs, for example, when the highly fluorescent pABG ($\lambda_{ex}$ 295 nm, $\lambda_{em}$ 356 nm) cannot be separated from THF, a problem which has been reported elsewhere [22].

Within the last few years, a number of HPLC procedures for the separation and quantification of folacins have been reported, usually based on anion exchange [18–21], ion-pair reversed-phase HPLC [21–31] or conventional reversed-phase HPLC [32–35]. None of these methods allows the separation of all the folacins and degradation products that are separated by our system. Many previously described chromatographic systems could not even separate some important folates, *e.g.*, 5-$CH_3$-THF and 5-CHO-THF [19,20], PteGlu and DHF or 5-$CH_3$-THF and PteGlu [26,27].

Although the use of internal standards might be very helpful in controlling the accuracy of an analytical system and in determining the yield during the sample preparation, their application has never been reported. Only a few attempts have been made to determine MTX in blood samples, but the procedures used were not able to separate all natural folates [23,40].

Pre-tests with ion-pair reversed-phase HPLC also revealed some of these problems: either THF and pABG or PteGlu and 5-$CH_3$-THF could not be separated completely. Further, according to previously published data [35], we found that the fluorescence of folacins was minimal at neutral pH and therefore not suitable for quantification. At pH 7 the relative fluorescence intensity was *ca.* 0.8% and 0.4% of that observed at pH 2 for 5-$CH_3$THF and THF, respectively.

For these reasons, we chose reversed-phase chromatography at pH 2.3, first described by Day and Gregory [32] and modified by Gregory *et al.* [33]. Pre-tests with the analytical column (10-$\mu$m $\mu$Bondapak Phenyl) and the mobile phase used by the latter [33] showed that PteGlu and 5-CHO-THF were only partially separated as shown by Gregory *et al.* [33]. It is possible to differentiate between these two

derivatives with the detection systems because 5-CHO-THF shows native fluorescence whereas PteGlu and DHF must be converted oxidatively to fluorescing products. However, such a chromatographic system neither allows UV detection nor can it be used for radiochromatographic studies. We noted that in the system described by Gregory *et al.* [33] DHF, which was not determined by them, co-chromatographed with 5-CHO-THF. Further, they did not examine the chromatographic properties of some degradation products. Resolution of all substances as narrow peaks (Fig. 1) could only be achieved with the use of 3-$\mu$m ODS Hypersil and reducing the $KH_2PO_4$ concentration of the buffer to 5 m*M*.

In many chromatographic systems for folacin analysis, 40–60 min for one separation and sometimes even longer are needed [20,24,25]. The present system is as fast as other methods [23,33], although it integrates more derivatives. The time factor might be of importance in routine analyses.

As can be seen in Table III, fluorimetric detection of folacins is much more sensitive than UV detection. Further, it is more specific compared with the absorption of folates, which occurs at 280–300 nm and therefore at a wavelength relatively non-specific for analyses of natural materials. PteGlu, DHF, MTX and DCF, showing no native fluorescence, can be split into highly fluorescent pterins.

The fact that the relative fluorescence intensity of the resulting pterine fragments depends greatly on the flow-rate of the oxidants (Table II) is probably due to incomplete splitting at low flow-rates and degradation of the products if the flow-rate is too high. In agreement with others [41], we recognized that 5-$CH_3$-THF was not converted to a fluorescent product. The fluorescence of the splitting products of 5-CHO-THF and THF was negligible compared with their native fluorescence (1% and 0.1% of the area, respectively). Hence a derivatization of all folates, as has been proposed for example, for thiamine derivatives [42] and which would allow the second detector to be omitted, is not possible. Attempts to detect the fluorescence of the resulting pABGs produced by the derivatization procedure failed. At excitation and emission wavelengths of 295 and 356 nm, respectively, only a minor fluorescence signal was observed, indicating that pABG was rapidly destroyed.

Another derivatization technique which has not yet been examined in folate analysis might be the use of an electrochemical cell to oxidize the folates. Placed in the effluent stream leaving the first detector, it should be as efficient as chemical oxidation with the advantage that no aggressive oxidants need be used and the derivatization pump could be omitted.

As Table III clearly indicates, the method proposed here is much more sensitive than procedures using UV detection. It is nearly as sensitive as microbiological assays and sufficient for the direct determination of folacins in biological samples, *e.g.*, plasma (5–20 ng/ml folate). On the other hand, fluorimetry does not reach the sensitivity of electrochemical detection [34].

The reproducibility and day-to-day precision of the method indicate that it is reliable. Only the detection of DHF shows large deviations, which is due to the extreme lability of this derivative. Concerning the reproducibility, it should be mentioned that we injected very small amounts of substrate. As can be seen from the results of others [43], reproducibility increases when larger amounts are chromatographed.

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REFERENCES

1 R. L. Blakley, *The Biochemistry of Folic Acid and Related Pteridines*, North-Holland, Amsterdam, 1969.
2 J. M. Scott and D. G. Weir, *Clin. Haematol.*, 5 (1976) 547.
3 C. L. Krumdieck, T. Tamura and J. Eto, *Vitam. Horm.*, 40 (1983) 45.
4 J. E. Ford, *J. Dairy Res.*, 34 (1967) 239.
5 D. Chippel and K. G. Scrimgeour, *Can. J. Biochem.*, 48 (1970) 999.
6 J. D. O'Broin, I. J. Temperley, J. P. Brown and J. M. Scott, *Am. J. Clin. Nutr.*, 28 (1975) 438.
7 T. Tamura and E. L. R. Stokstad, *Br. J. Haematol.*, 25 (1973) 513.
8 C. J. Bates, A. E. Black, D. R. Phillips, A. J. A. Wright and D. A. T. Southgate, *Hum. Nutr. Appl. Nutr.*, 36A (1982) 422.
9 I. H. Rosenberg and J. Dyer, in M. I. Botez and E. H. Reynolds (Editors), *Folic acid in Neurology, Psychiatry, and Internal Medicine*, Raven Press, New York, 1979, p. 19.
10 O. D. Bird, V. M. McGlohon and J. W. Vaithus, *Can. J. Microbiol.*, 15 (1969) 465.
11 B. Shane, T. Tamura and E. L. R. Stokstad, *Clin. Chim. Acta*, 100 (1980) 13.
12 S. Waxman, C. Schreiber and V. Herbert, *Blood*, 38 (1971) 219.
13 J. F. Gregory, III, B. P. F. Day and K. A. Ristow, *J. Food Sci.*, 47 (1982) 1568.
14 J. H. Copenhaver and K. L. O'Brien, *Anal. Biochem.*, 31 (1969) 454.
15 J. M. Scott, *Methods Enzymol.*, 66 (1980) 437.
16 K. U. Buehling, T. Tamura and E. L. R. Stokstad, *J. Biol. Chem.*, 249 (1974) 1081.
17 J. Kas and J. Cerna, *J. Chromatogr.*, 124 (1976) 53.
18 L. S. Reed and M. C. Archer, *J. Chromatogr.*, 121 (1976) 100.
19 R. W. Stout, A. R. Cashmore, J. K. Coward, C. G. Horvath and J. R. Bertino, *Anal. Biochem.*, 71 (1976) 119.
20 C. K. Clifford and A. J. Clifford, *J. Assoc. Off. Anal. Chem.*, 69 (1977) 1248.
21 S. K. Chapman, B. C. Greene and R. R. Streiff, *J. Chromatogr.*, 145 (1978) 302.
22 A. R. Branfman and M. McComish, *J. Chromatogr.*, 151 (1978) 87.
23 B. A. Allen and R. A. Newman, *J. Chromatogr.*, 190 (1980) 241.
24 R. N. Reingold, M. F. Picciano and E. G. Perkins, *J. Chromatogr.*, 190 (1980) 237.
25 D. W. Horne, W. T. Briggs and C. Wagner, *Anal. Biochem.*, 116 (1981) 393.
26 K. E. McMartin, V. Virayotha and T. R. Tephly, *Arch. Biochem. Biophys.*, 209 (1981) 127.
27 D. R. Briggs, G. P. Jones and P. Sae-Eung, *J. Chromatogr.*, 246 (1982) 165.
28 R. N. Reingold and M. F. Picciano, *J. Chromatogr.*, 23 (1982) 171.
29 D. S. Duch, S. W. Seaton and C. A. Nichol., *Anal. Biochem.*, 130 (1983) 385.
30 S. A. Kashani and B. A. Cooper, *Anal. Biochem.*, 146 (1985) 40.
31 A. Schulz, K. Wiedemann and I. Bitsch, *J. Chromatogr.*, 328 (1985) 417.
32 B. P. F. Day and J. F. Gregory, III, *J. Agric. Food Chem.*, 29 (1981) 374.
33 J. F. Gregory, III, D. B. Sartain and B. P. F. Day, *J. Nutr.*, 114 (1984) 341.
34 M. Kohashi, K. Inoue, H. Sotobayashi and K. Iwai, *J. Chromatogr.*, 382 (1986) 303.
35 J.-C. Gounelle, H. Ladijimi and P. Progmom, *Anal. Biochem.*, 176 (1989) 406.
36 S. D. Wilson and D. W. Horne, *Anal. Biochem.*, 142 (1984) 529.
37 P. A. Bristow, P. N. Brittain, C. M. Riley and B. F. Williamson, *J. Chromatogr.*, 131 (1977) 57.
38 H. Engelhardt and U. D. Neue, *Chromatographia*, 15 (1982) 403.
39 J. Perry, *Br. J. Haematol.*, 21 (1971) 435.
40 O. Van Tellingen, H. R. van der Woude, J. H. Beijnen, C. J. T. van Beers and W. J. Nooyen, *J. Chromatogr.*, 488 (1989) 379.
41 T. Maruyama, T. Shiota and C. L. Krumdieck, *Anal. Biochem.*, 84 (1978) 277.
42 J. Bontemps, P. Phillippe, L. Bettendorf, J. Lombet, J. Dandrifosse and F. Schoffeniels, *J. Chromatogr.*, 307 (1984) 283.
43 C. Wegner, M. Trotz and H. Nau, *J. Chromatogr.*, 378 (1986) 55.
44 A. Hahn, K. H. Flaig and G. Rehner, *J. Chromatogr.*, submitted for publication.