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Optimised extraction of folic acid from multivitamin-mineral preparations for liquid chromatographic analysis

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Abstract

Degradation of folic acid may occur during extraction of multivitamin-mineral preparations. The degradation may be caused by presence of ions such as Fe^{3+} and Cu^{2+}, however, the buffer composition may also be critical. This study presents an optimised extraction procedure tested on 24 different products of multivitamin-mineral tablets. The present method yielded mean recoveries of 97% ($n = 20$) for folic acid and prevented degradation of folic acid in at least 24 h in extracts from multivitamin-mineral tablets.

Keywords: Multivitamin-mineral tablets; Pteroic acid; High performance liquid chromatography

1. Introduction

Folates are a group of pteroic acid polyglutamate compounds with similar biological activity as folic acid. Folic acid is a synthetic pteroic acid monoglutamate, not naturally occurring, and due to high stability [1] often used as a folate source in supplements and food fortification. The bioavailability of folic acid by fortified foods is the same as in supplements and probably higher than folates naturally occurring in food [2].

Folate deficiency is associated with increased plasma homocysteine concentrations and thereby related to cardiovascular disease [3]. Folate deficiency prior to and during early pregnancy has also been related to neural tube defects in newborns [4].

Recently, several countries have raised the recommended daily intake of folate and as a consequence, producers of supplements have increased the content of folic acid in vitamin tablets. Until recently, we have analysed folic acid in vitamin tablets by means of a microbiological assay with *Lactobacillus casei* as the test organism. We experienced that among the number of tablets analysed, a few multivitamin-mineral products gave very low recoveries and analytical results in disagreement with the declared content. Preliminary experiments showed that addition of a chelating agent to the extraction buffer increased the amount of folic acid determined. However,

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the presence of a chelating agent during microbiological growth may remove essential metal ions from growth media and thereby disrupt the microbiological analysis.

Previous reports have showed good agreement between HPLC analysis and microbiological analysis of folic acid from multivitamin preparations [5] and in multivitamin-mineral preparations [6]. However, it was showed that the stability of folic acid in extracts from multivitamin-mineral preparations was significantly higher when a chelating agent was present [7].

The presence of minerals in vitamin tablets, particularly copper and iron, may cause oxidation or complex binding of folic acid during extraction [8–11]. It was shown that reaction between Cu^{2+} and tetrahydropteridin at a pH above 4 formed a transient, purple complex followed by a brown precipitate [9]. A similar reaction between Cu^{2+} and tetrahydrofolat or 5-methyltetrahydrofolat formed a more stable complex. Treatment of folic acid with ferricyanide at pH 5.6 at room temperature did not result in breakdown of folic acid [8]. At pH 9, however, small amounts of folic acid were destroyed. Dihydrofolate, on the other hand, was oxidised to folic acid at pH 9 and to a mixture of dihydroxanthopterin and 6-formyldihydropterin at pH 5.6, with yield and composition depending on buffer and temperature.

Our working hypothesis was that the presence of certain metal ions combined with high extraction temperature and neutral pH results in either complex formation of folic acid or oxidative attack on the *p*-aminobenzoylglutamat residual of folic acid.

The objective of this study was to optimise the extraction of folic acid from multivitamin tablets to ensure full conservation during extraction and to link the extraction procedure to a liquid chromatographic method.

2. Experimental

2.1. Apparatus

The liquid chromatograph system was a Waters 2670 alliance separation module (Waters Corp., Milford, MA) equipped with a Waters 2487 dual channel UV-detector and Millennium 32 chromatography manager data acquisition system (Waters Corp., Milford, MA). The column temperature was kept constant by an Iglo-sil column cooler (Cluzeau Info Lab, Sainte-Foy-La-Grande, France).

2.2. Reagents and materials

Folic acid, (WHO Centre for Chemical Reference Substances, Stockholm, Sweden). Methanol, LC grade (Fisher), L (+)-ascorbic acid (Merck), 2-mercaptoethanol (Sigma), sodium acetate, anhydrous (Merck), potassium dihydrogen phosphate (Merck), Na_2EDTA (Merck), *ortho*-phosphoric acid 85% (Merck), sodium hydroxide (Kebolab, Denmark), acetic acid, 100% (Merck).

2.3. HPLC buffer

The HPLC buffer was a 0.03-M potassium dihydrogen phosphate buffer adjusted to pH 2.20 with 85% *ortho*-phosphoric acid.

2.4. Extraction buffer

The extraction buffer was based on 0.5 M sodium acetate–5% ascorbic acid buffer with addition of 6 g l^{-1} Na_2EDTA and 0.7 ml l^{-1} 2-mercaptoethanol. The buffer was adjusted to pH 6.0 with NaOH.

2.5. Standard preparation

2.5.1. Standard stock solution (100 μg ml^{-1})

Folic acid (10.87 mg) was transferred to a 100-ml glass together with 20 ml Millie Q water. In order to promote solution of folic acid one to two drops of 1 M NaOH were added. Immediately after solvation, pH was adjusted to 7–8 with 0.1 M hydrochloric acid and quantitatively transferred to a 100-ml measurement flask and made up to 100 ml with Millie Q water.

In order to prevent oxidation of folic acid, the stock solution was transferred to a dark flask, two drops of toluene was added to it and stored at 5°C. Under these conditions folic acid was stable

in solution for 4 weeks. In order to control the resulting concentration of folic acid the solution was further diluted ten times in a 0.6 M acetate buffer (pH 4.7). The UV absorption of the 10 μg ml^{-1} solution and a blind solution was measured at 278, 280, 281, 282 and 284 nm. The difference between blank and stock solution at 281 nm was determined and the extinction, ε, was calculated to be 654 M^{-1} cm^{-2}.

2.6. Samples

Samples analysed in this study consisted of 30 different products of multivitamin tablets, 26 containing minerals and four without minerals. The products here were coded corresponding to the ID number in our laboratory database.

2.7. Sample extraction

Five tablets were crushed in a mortar, transferred quantitatively to a 300 ml conic flask together with 100 ml extraction buffer and autoclaved for 5 min at 121 ± 2°C. The supernatant was quickly cooled to room temperature, transferred to a 250-ml measurement flask and filled up to the mark with extraction buffer. After shaking, an aliquot of 10 ml was centrifuged at 8000 × *g* for 5 min at 5°C. Samples were filtered through a 0.45 μm filter prior to analyses and were kept cold and dark prior to and during analysis.

2.8. Extraction buffers during optimisation

During development of the present method, several combinations of buffers and additives, e.g. ascorbic acid, EDTA and 2-mercaptoethanol, were tested and compared. The letters refer to the following combinations:

(A) Microbiological method, extraction buffer, 0.1 M potassium dihydrogen phosphate–1% ascorbic acid, pH 6.0;
(B) HPLC analysis, extraction buffer, 0.1 M potassium dihydrogen phosphate–1% ascorbic acid, pH 6.0;
(C) HPLC analysis, extraction buffer, 0.1 M potassium dihydrogen phosphate–1% ascorbic acid–3 g l^{-1} Na_2EDTA, pH 6.0;
(D) HPLC analysis, extraction buffer, 0.5 M sodium acetate–1% ascorbic acid–6 g l^{-1} Na_2EDTA, pH 6.0;
(E) HPLC analysis, extraction buffer, 0.5 M sodium acetate–5% ascorbic acid–6 g l^{-1} Na_2EDTA–0.7 ml l^{-1} 2-mercaptoethanol, pH 6.0.

2.9. Chromatographic conditions

Samples and standards were analysed on a liquid chromatographic system equipped with an UV detector. The detector was set at 280 nm and samples and standards were injected in volumes of 100 μl. Chromatography was performed on a Ultrasphere ODS 250 × 4.6 mm id, packed with 5 μm silica particles (Beckman) at 22°C. The mobile phase was 78% HPLC buffer and 22% methanol. The mobile phase was isocratically applied to the column at 1.0 ml min^{-1} for the first 12 min. However, in order to flush out interfering compounds, the column was washed with an increasing amount of methanol and the total run time between injections was 25 min.

Isocratic analysis without a washing programme, on the other hand, required a total run time of 35 min to ensure stable baseline.

2.10. Validation

The method was 'intern' validated in agreement with the Nordic Committee on Food Analysis (NMKL) procedure NR 4 1996 [12].

2.11. Quantification

Samples were calculated as means of doublet injections. The folic acid amounts were quantified, based on external standard of 1 μg ml^{-1} and calculated in brackets with one standard per four samples.

2.12. Statistics

The different extraction procedures were compared with a two-tailed paired *t*-test for different mean values. *P* values below 0.05 were considered

significant. Regression analyses were performed by The SAS system, version 6.12 (SAS Institute Inc., Cart, NC).

3. Result and discussion

This method was developed in order to overcome problems with destruction of folic acid during extraction of multivitamin-mineral tablets. The chromatographic method was based on a C18-column with a low back pressure (2000 psi) and a simple methanol–phosphate-buffer eluent. The method gave a good separation of folic acid from compounds derived from the extraction buffer and from the tablets (Figs. 1 and 2).

In order to avoid thermal degradation of folic acid, cold extractions were attempted by stirring or shaking the crunched tablet in extraction buffer at room temperature. However, this procedure

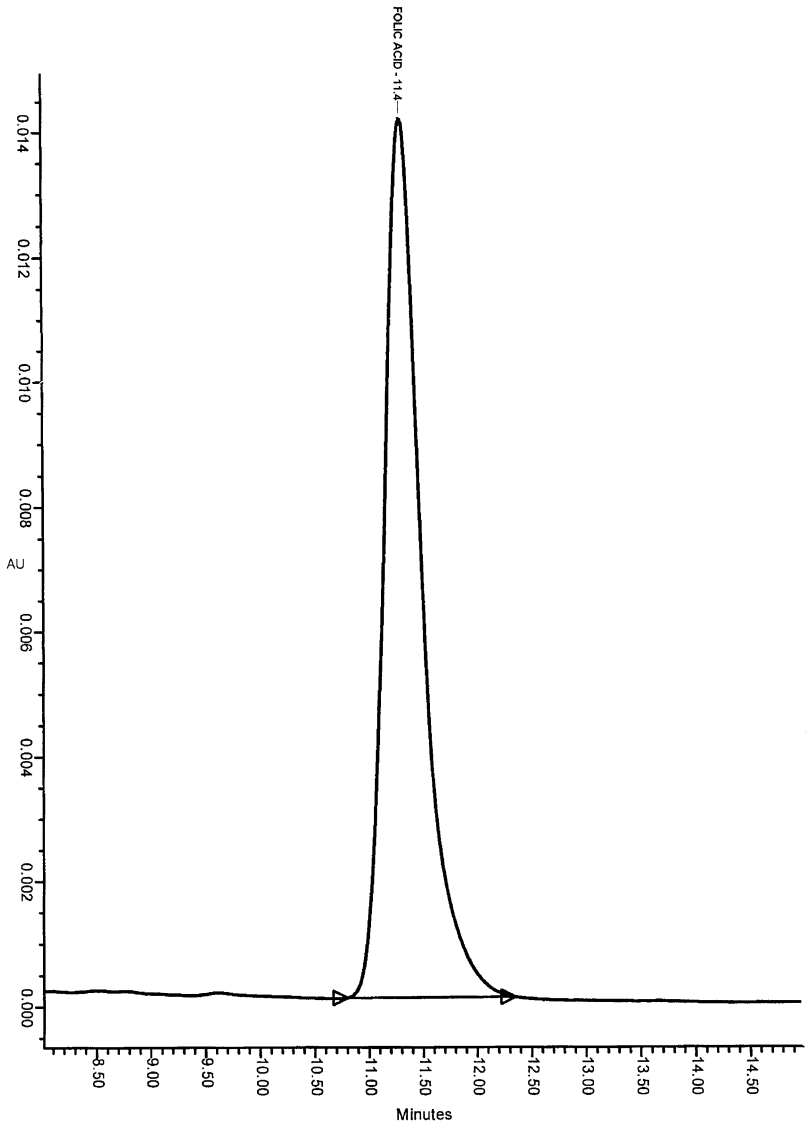

Fig. 1. HPLC chromatogram of 1.0 μg ml^{-1} folic acid standard.

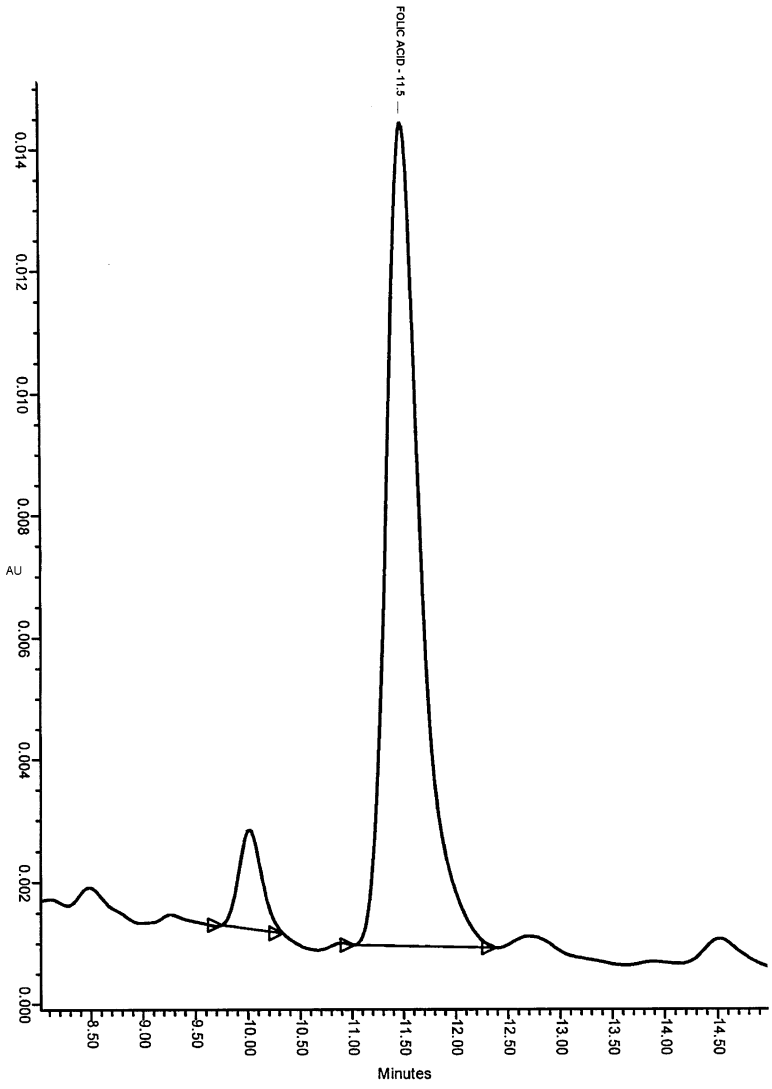

Fig. 2. HPLC chromatogram of five tablets (97-578) diluted in 250 ml of buffer E.

resulted in increased degradation of folic acid. The degradation was observed by peak splitting in the chromatograms and by low recoveries. The degradation was probably due to the large surface of the solution during stirring and shaking. We found that folic acid was not heat labile under the experimental conditions, in agreement with [13], and that treatment for 5 min at 121 ± 2°C in a closed bottle resulted in complete liberation of folic acid from the tablet material. Yet, the rate of the subsequent cooling to room temperature and filtration had a high impact on folic acid degradation. Thus, extracts were cooled to room temperature immediately, and subsequently diluted and centrifuged to precipitate the tablet material.

During the first attempts to optimise this analysis we used a 0.1 M potassium dihydrogen phosphate extraction buffer, pH 6. Addition of EDTA to this buffer increased the recovery significantly for several products. The addition of EDTA excluded the possibility of using a microbiological detection of folic acid. Some products, however,

still gave low recoveries; some resulted in broadening of the folic acid peak and for some the folic acid peak split into two during LC analysis. Further optimisation of the extraction involved buffers based on potassium dihydrogen phosphate–citric acid, sodium acetate–citric acid, sodium acetate, ammonia acetate and bis–tris. The buffers were compared with respect to buffer capacity and preservation of folic acid (Tables 1 and 2).

The results indicate that the stability of folic acid was independent of pH in the range from 6 to 9, as proposed by [1–13] and in particular for non-phosphate buffers, in agreement with [1]. Extracts from the ammonia acetate buffer caused a shoulder on the folic acid peak in the LC chromatograms, probably due to co-elution of a metal–iron complex of the $[M(NH_3)_l(H_2O)_m]^{n+}$ kind, or more likely due to a degradation product of folic acid formed in the presence of NH_4OH [14]. Despite the fact that the sodium acetate–ascorbic acid buffer had low capacity at pH 6, this buffer performed the best stabilisation of folic acid from tablets, previously difficult to analyse, compared with other buffer combinations tested. In addition, sodium acetate–ascorbic acid buffer extracts gave LC chromatograms with minor interference.

This work was initiated because of inadequate analytical results performed by our microbiological method (A). However, from Table 3 it was obvious that the problem was caused by the extraction rather than the detection system, since the HPLC analysis of the same extracts (B) gave concordant results ($P > 0.05$). Addition of EDTA to the phosphor buffer excluded the microbiological method; however, HPLC analysis of the phosphate extracts with added EDTA (C) did not change the performance of the analysis compared with microbiological analysis ($P > 0.05$). Replacement of potassium dihydrogen phosphate with sodium acetate (D) increased the performance of the method compared with (A) ($P < 0.05$), and (C) ($P < 0.05$) and the analytical results were not

Table 1
Effect of buffer, temperature and tablet material on pH in solution[a]

Product ID	Autoclave pH	Buffer			
		Phosphate buffer (0.1 M)	Ammonium acetate buffer (0.5 M)	Sodium acetate buffer (0.5 M)	Bis–tris buffer (0.1 M)
97-638	Before	6.0	6.2	6.0	6.0
	After	5.6	7.3	7.9	6.3
97-578	Before	6.3	6.4	7.2	6.1
	After	7.0	8.6	9.3	6.5

[a] To all buffers were added 1% ascorbic acid and 3 g l^{-1} EDTA and pH was adjusted to 6.0 before extraction. pH was measured after suspension of the crushed tablets and after 5 min of thermal treatment at 121°C.

Table 2
Comparison of four extraction buffers used on two different tablets difficult to analyse[a]

Product ID	Buffer				
	Declaration (μg U^{-1})	Phosphate buffer (0.1 M, μg U^{-1})	Ammonium acetate buffer (0.5 M, μg U^{-1})	Sodium acetate buffer (0.5 M, μg U^{-1})	Bis–tris buffer (0.1 M, μg U^{-1})
97-638	100	62	92	94	90
97-578	66	43	50	42	37

[a] Both tablets gave a broad or split folic acid HPLC peak and low recoveries when using a phosphate extraction buffer. To all buffers were added 1% ascorbic acid and 3 g l^{-1} EDTA, and pH was adjusted to 6.0.

Table 3
Comparison of results obtained during optimisation of the extraction procedure[a]

Product ID	Declaration (μg U^{-1})	Microbiological analysis (μg U^{-1})	LC analysis (μg U^{-1})		
		A	B	C	D
97-439	100	54	61	65	86
97-578	66.6	10	7	43	42
97-631	100	85	59	[b]	116
97-635	100	53	55	51	102
97-638	100	41	38	62	94
97-639	100	72	n.a.[c]	70	97
97-640	100	103	n.a.	84	90
97-641	100	101	n.a.	75	93
97-662	100	36	39	74	89
98-249	200	154	160	156	n.a.

[a] A and B are the same extracts of multivitamin-mineral tablets analysed by means of microbiological and HPLC analysis, respectively. C and D are the results of HPLC analysis from extractions with different buffers. A, 0.1 M potassium dihydrogen phosphate–1% ascorbic acid buffer, pH 6.0; B, 0.1 M potassium dihydrogen phosphate–1% ascorbic acid buffer, pH 6.0; C, 0.1 M potassium dihydrogen phosphate–1% ascorbic acid–3 g l^{-1} Na_2EDTA buffer, pH 6.0; D, 0.5 M sodium acetate–1% ascorbic acid–6 g l^{-1} Na_2EDTA buffer, pH 6.0.
[b] Peak split.
[c] Not analysed.

different from the declared amounts $P > 0.05$. However, the mean recovery was $90.6 \pm 2.8\%$ (mean $\pm$ S.E.M., $n = 6$) and not satisfying.

Further optimisation of the extraction procedure showed good protective effect if the concentration of ascorbic acid in the sodium acetate buffer was increased to 5% and by addition of 0.7 ml l^{-1} 2-mercaptoethanol.

Eighteen tablets were then extracted by the optimised method (E) and by method (A) and (D) (Table 4). The extraction procedure (D) and the microbiological method (A) gave different results ($P < 0.0005$), however, (D) also gave results different from the declared values ($P < 0.0005$). The optimised procedure (E) was different from (D) ($P < 0.05$), but did not give results different from the declared values ($P = 0.11$). The mean recovery was $96.8 \pm 1.3\%$ (mean $\pm$ S.E.M., $n = 20$). The performance of the method was evaluated and found to be adequate, and based on the development data the method was validated.

3.1. Repeatability

The repeatability was determined to be 2.3% as the R.S.D. of 18 single determinations of an in-house reference tablet.

3.2. Internal reproducibility

The internal reproducibility was determined to be 4.9% as the R.S.D. of the mean difference of doublet determinations of 19 authentic samples.

3.3. Accuracy

The accuracy was determined by spiking ten different tablets two times ($n = 20$). The method accuracy was calculated to be $96.8 \pm 1.3\%$ (mean $\pm$ S.E.M.).

3.4. Linearity

The linearity on a five-point standard curve, from 0.2–2 μg ml^{-1} was described by $y = 340183x + 2880$, $R^2 = 0.9998$. The intercept was not different from zero ($P > 0.05$). The linearity on an eight-point standard curve, from 0.05–2 μg ml^{-1} was described by $y = 343321x + 1746$, $R^2 = 0.9998$. The intercept was not different from (0.0)

Table 4
Comparison of results obtained from extraction of multivitamin-mineral tablets and multivitamin tablets by (A) the original microbiological analysis, (B) HPLC method (D in Table 3) and the final extraction method[a]

Product ID	Declaration (μg U^{-1})	Analysed amount (μg U^{-1})		
		Microbiological analysis	LC analysis	
		A	D	E
With minerals				
98-1297	66.7	26	47	58
98-1494	100	55	78	89
98-1499	100	58	92	100
98-1572	100	53	89	97
98-1683	75	n.a.[b]	63	66
98-1744	133	97	124	129
98-1775	100	33	57	70
98-1887	100	75	84	99
98-1889	200	200	191	201
98-1890	200	198	195	205
98-1894	75	87	87	89
98-1908	75	72	76	78
98-1911	200	168	178	192
98-1914	75	69	76	76
Without minerals				
98-1206	200	143	175	172
98-1291	200	140	172	164
98-1486	100	80	85	130
98-1766	400	320	352	337

[a] A, 0.1 M potassium dihydrogen phosphate–1% ascorbic acid buffer, pH 6.0; D, 0.5 M sodium acetate–1% ascorbic acid–6 g l^{-1} Na_2EDTA buffer, pH 6.0; E, 0.5 M sodium acetate–5% ascorbic acid–6 g l^{-1} Na_2EDTA–0.7 ml l^{-1} 2-mercaptoethanol buffer, pH 6.0.
[b] Not analysed.

($P > 0.05$). The quantification limit was determined to be 0.05 μg ml^{-1}.

3.5. Stability

The stability of folic acid was tested in an extract from a multivitamin-mineral tablet during 24 h storage in dark at 5°C. The R.S.D. of 12 determinations during the 24-h period was 0.7% with a difference of 2%.

In conclusion, we have experienced that there is a large variation in the formulation of tablet material in common multivitamin-mineral tablets. It is obvious from Tables 3 and 4 that some multivitamin-mineral tablets were analysed with identical results independent of the extraction buffer while the yield of several tablets differed more than two times, despite the fact that they all contained the same cascade of minerals in comparable amounts.

The optimised extraction procedure presented in this study was developed in order to avoid destruction of folic acid during extraction and analysis, and thus compensate for this difference in tablet matrices.

References

[1] J.D. O'Broin, I.J. Temperley, J.P. Brown, J.M. Scott, Am. J. Clin. Nutr. 28 (1975) 438–444.
[2] N. Colman, R. Green, J. Metz, Am. J. Clin. Nutr. 28 (1975) 459–464.
[3] M. Ward, H. McNulty, J. McPartlin, J.J. Strain, D.G. Weir, J.M. Scott, Q. J. Med. 90 (1997) 519–524.

[4] L.E. Daly, P.N. Kirke, A. Molloy, D.G. Weir, J.M. Scott, J. Am. Med. Assoc. 274 (1995) 1698–1702.
[5] C. Paveenbampen, D. Lamontanaro, J. Moody, J. Zarembo, C. Rehm, J. Pharm. Sci. 75 (1986) 1192–1194.
[6] I.J. Holcomb, S.A. Fusari, Anal. Chem. 53 (1981) 607–609.
[7] W.H. Tafolla, A.C. Sarapu, G.R. Dukes, J. Pharm. Sci. 70 (1981) 1273–1276.
[8] D. Chippel, K.G. Scrimgeour, Can. J. Biochem. 48 (1970) 999–1009.
[9] M.C. Archer, D.J. Vonderschmitt, K.G. Scrimgeour, Can. J. Biochem. 50 (1972) 1174–1182.
[10] D.J. Vonderschmitt, K.G. Scrimgeour, Biochem. Biophys. Res. Commun. 28 (1967) 302–308.
[11] J.A. Blair, A.J. Pearson, J. Chem. Soc. Perkin Trans. II (1974) 80–88.
[12] O.B. Jensen, NMKL-Prosedyre nr 4 Validering av kjemiske analysemetoder, 1996.
[13] B. Paine-Wilson, T.S. Chen, J. Food Sci. 44 (1979) 717–722.
[14] J. Krzek, A. Kwiecien, J. Pharm. Biomed. Anal. 21 (1999) 451–457.