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Short communication

Densitometric determination of impurities in drugs Part IV. Determination of *N*-(4-aminobenzoyl)-L-glutamic acid in preparations of folic acid

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1. Introduction

Folic acid (pteroylglutamic acid) belongs to water-soluble vitamins of the 'B' group that participate in cell divisions, protein synthesis and in other reactions appearing important for life. Folic acid deficiency often induces irreversible pathological changes designated jointly as a neural tube defect, among these lesions the most severe and most common are encephalopathy and encephalomyeloceles [1–3]. This capital importance of folic acid for regular and proper functioning of living organisms justifies the necessity to extensively study both folic acid and its metabolites [4–6].

In medical treatment practice, folic acid is usually applied in the form of 5 and 15 mg tablets, or in many compositions containing small doses ranging from 0.2 to 1 mg [7].

This paper is based on an investigation instituted with the intention of evaluating the purity of folic acid. In pharmacopial monographs on folic acid, contained in various Pharmacopeias such as the European Pharmacopea (Ph.Eur 3,1997), British Pharmacopea (BP, 1993), German Pharmacopea (DAB 10,1994) and Polish Pharmacopea (FP 5,1993), thin-layer chromatography (the so called TLC method) appears to be most useful for both the identification and evaluation of the purity. With this method, intensity of spots on chromatograms is visually compared. It should be noted that in all mentioned Pharmacopeias, a similar technique of sample preparation for investigation is recommended, i.e. a substance should be dissolved in an ammonia hydroxide and methanol mix. However, even at this stage of investigations, negative changes in the substance being analysed can occur in this alkaline medium.

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While proceeding with the quantitative determination of impurities in drugs [8–10] using densitometry, it was decided to elaborate a chromatographic–densitometric method (QTLC) for the identification and determination of *N*-(4-aminobenzoyl-)-L-glutamic acid (NABG) and 4-aminobenzoic acid (AB) contained in folic acid tablets. Attempts were made to select the most suitable determination conditions enabling the investigation of these tablets in both ultraviolet (UV) and visible range of waves (VIS). Thus, for the purpose of chromatographic separation, two phases were applied in parallel: a newly adopted (through experiments) mobile phase and a pharmacopeial phase (FP).

In the recent available literature there were found some papers dealing with the issue of determination of NABG and AB, which are the degradation products of folic acid in composed preparations [11] and in systemic fluids, using the HPLC method [12]; this technique was also used in a monograph about folic acid in the American Pharmacopea (USP-23, 1994). Also, in the literature, an isotopic [13] and densitometric methods are described with regard to 'Leucovorin Calcium' preparations [14].

2. Experimental

2.1. Apparatus, solutions and reagents

In experiments the following items and apparatus were used: ready-made plates of aluminium foil covered with a silica gel (DC-Alufolien Kieselgel 60) manufactured by Merck, TLC Scanner 3 densitometer with Cats 4 computer software (produced by Camag, Muttenz, Switzerland), and a Linomat IV (from the same manufacturer) to place samples on plates.

Tablets with folic acid preparations were powdered by grinding them in a mortar for 5 min. The preparatory work for making solutions involved the following: in order to obtain 0.15 g folic acid, the corresponding mass of powdered tablets was accurately weighed; next, 25 ml methanol was added to it; the obtained mix was shaken for 30 min and, finally, filtered. The folic acid preparations included in the investigation schedule were randomly selected from a series of preparations generally applied. They are: acidum folicum containing 5 mg of folic acid, series 000298 and 031297, as well as containing 15 mg, series 010398. For the purpose of investigations, the following standard substances were used: a 0.02% w/v solution of folic acid of properties complying with those as set in the American Pharmacopea (USP-23, 1994), a 0.003% w/v solutions of investigated impurities: *N*-(4-aminobenzoyl-)-L-glutamic acid (Fluka) and 4-aminobenzoic acid (Merck) dissolved in methanol.

The Ehrlich reagent: 1 g of 4-dimethylbenzoic aldehyde was used as a mix of 25 ml hydrochloric acid (concentration 36%) and 75 ml ethyl alcohol (concentration 95%).

2.2. Establishing the conditions for determination procedures

With use of Linomat, respective solutions were brought on plates, in amounts from 5 to 50 μl, and their chromatograms were developed on a route of 10–20 cm for various mobile phases. Those chromatograms were dried at room temperature and visually and densitometrically analysed. The results obtained from the two analyses allowed for setting the optimal conditions for both the chromatographic separation and densitometric measurements. The latter one was applied to settle the procedure of NABG determination, which is the impurity in folic acid preparations.

2.3. Carrying out the determination

On plates, 12 × 10 cm, with use of a Linomat IV, 20 μl each of the standard and experimental solutions were brought in the form of a 15-mm wide band. Chromatograms were developed on a route of 10 cm under application of an experimentally selected mobile phase: *n*-propanol–ammonia hydroxide–25%-ethanol (2:2:1, v/v/v) (F) and toluene–methanol–glacial acetic acid–acetone (14:4:1:1, v/v/v/v) (N). As soon as the chromatograms were dried at room temperature, they

were registered in the reflected light, by a densitometer, in UV at 278 nm, and in the visible light range at 450 nm after they had been coloured with an Ehrlich reagent. For this purpose, upon completing the determination in UV, the chromatograms were sprinkled by the Ehrlich reagent and then heated for 5 min at 105°C; as a result, yellow coloured spots on a white background were obtained.

The NABG content was calculated by comparing the peak areas of respective standard and experimental solutions; the content was computer calculated. In Fig. 1, chromatograms and relevant densitograms are presented.

3. Results

In the search for suitable conditions for the chromatographic separation of NABG, present as an impurity in folic acid preparations, two mobile phases were chosen:

1. *n*-propanol–ammonia 25%–ethanol (2:2:1, v/v/v) (F), and this phase, variously modified if necessary, is recommended to evaluate folic acid by some pharmacopeial monographs (Ph.Eur., BP, DAB, FP);
2. Toluene–methanol–glacial acetic acid–acetone (14:4:1:1, v/v/v/v) (N) which was experimentally selected.

On chromatograms, after their colouring with the Ehrlich reagent, yellow spots of components are visible that are derived from standard and experimental substance; the location of spots on the chromatograms was described by the R_f parameter.

In the 'F' mobile phase, the folic acid preparation showed two spots which are derived from NABG ($R_f \cong 0.60$) and from folic acid ($R_f \cong 0.52$). In this preparation, no spot deriving from 4-aminobenzoic acid was noted, which in the standard solution was visible on the chromatogram ($R_f \cong 0.80$). The presence of impurities in folic acid was shown by investigations carried out after the separation performed in the 'N' mobile phase and for the folic acid preparation two spots were obtained that derived from NABG ($R_f \cong 0.20$) and from folic acid ($R_f \cong 0.06$). In the standard solution of AB, there was a spot noted on a chromatogram ($R_f \cong 0.85$). Besides all the stated differences, confirmed by the R_f values in the 'F' and 'N' phases, essential variances were found in the chromatographic separation times. The separation time with the 'F' phase used was 3 h, whereas the chromatogram development time was definitely shorter when a new phase 'N' was applied, and it was about 30 min. The limits of detection and quantification were also set while determining a minimum quantity of NABG and AB found on a chromatogram which amounts to 0.15 μg for NABG and 0.10 μg for AB. Under the given determination conditions it was stated that no AB acid is present, thus, further investigation dealt only with NABG.

The chromatograms were densitometrically analysed and absorption spectrum of NABG was registered in the range from 200 to 400 nm. The outcome was a properly shaped absorption maximum at 278 nm and this length has been chosen as the analytical wavelength.

Analogous investigations were conducted for chromatograms coloured with the Ehrlich reagent, in the range from 400 to 800 nm and the result was an absorption spectrum with its maximum at 450 nm (Fig. 2).

The correlation between the peak areas and the concentration of determined components was studied, $P = f(c)$; it was noted that the correlation runs linearly within the range of investigated concentrations. This statement was the basis of further conducted densitometric measurements (Fig. 3).

The usefulness of the developed method compared with the routine analyses, was proved by the determination of NABG serving here as an example. The results of conducted investigations and statistical evaluation are given in Table 1.

While comparing the results of the determination of NABG contained in the folic acid tablets (presented in Table 1), some differences in the contents of this impurity were noted which depended on the applied mobile phases. The results of determination (after the chromatographic separation with the 'F' mobile phase used) were higher if compared with the results obtained on the chromatograms after the separation in the 'N'

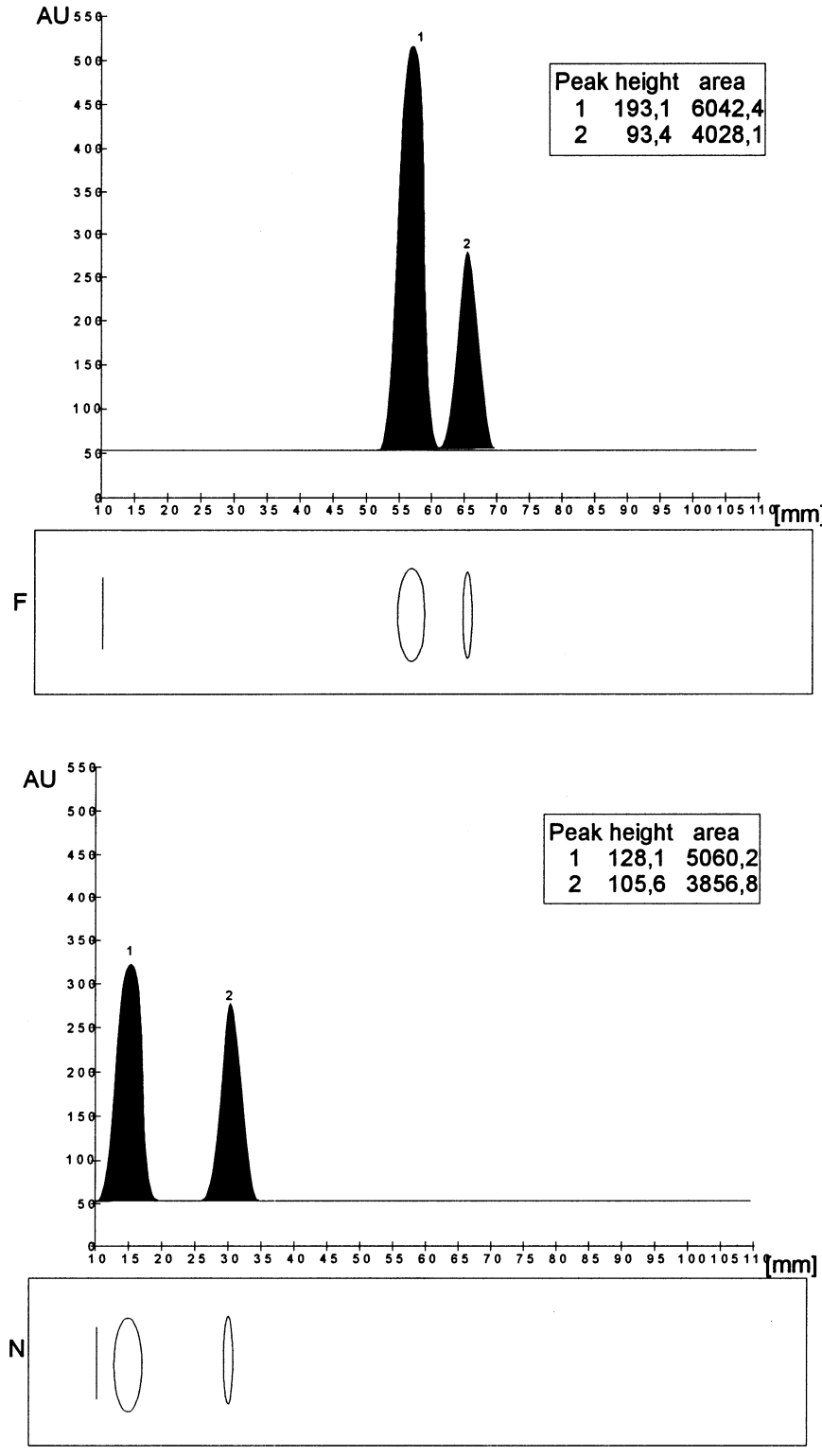

Fig. 1. Densitograms and chromatograms of the folic acid preparation (1) folic acid (2) *N*-(4-aminobenzoyl-)-L-glutamic acid. The separation was performed in 'F' and 'N' mobile phases while taking measurements in UV at $\lambda = 278$ nm.

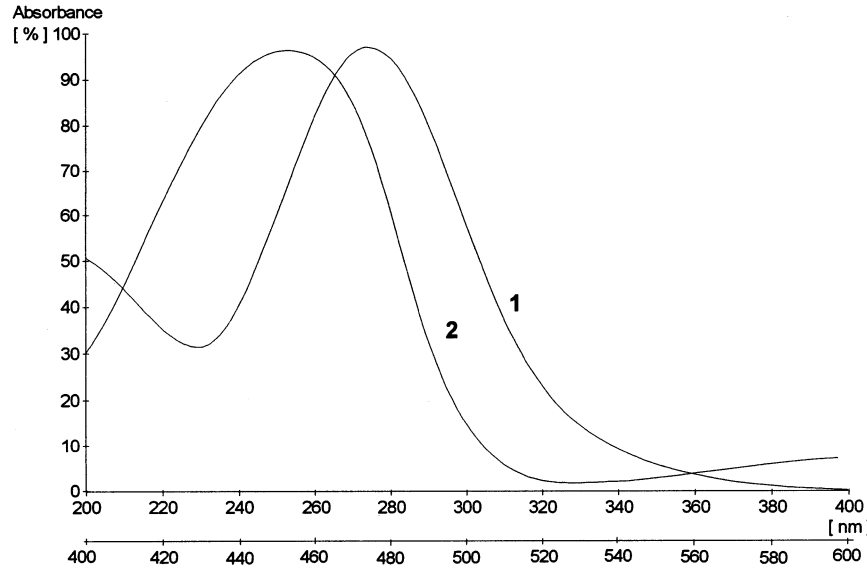

Fig. 2. The spectrum of *N*-(4-aminobenzoyl-)-L-glutamic acid, registered directly from the chromatogram in UV (1) and in the visible range (2).

mobile phase. With regard to this fact, the impact of the ammonium hydroxide — an important component of the 'F' mobile phase — on the NABG content was studied.

3.1. The impact of ammonium hydroxide on the NABG content

As far as the stage of preparing the solutions for investigation with use of ammonia hydroxide is concerned and also with regard to the separation stage with the applied mobile phase with ammonia hydroxide, the results obtained were higher than those achieved under the conditions as suggested by us, thus, it was decided to study the ammonia hydroxide impact on the NABG content. In order to explain remaining discrepancies in results obtained, folic acid solutions in ammonium hydroxide were prepared, and those preparations were studied with application of the 'N' mobile phase for separation. The preparation for investigations were made of three weighed samples of powdered tablets' mass that corresponded to 0.045 g folic acid with subsequently added 1 ml ammonia solutions, their concentrations being 6.25, 12.5 and 25% v/v respectively; 1 ml water was added to the fourth weighed sample. The samples were closed in small hermetic vessels, shaken for 30 min; next, 9 ml of methanol was added to them and they were left for 6 h at 30°C. After filtration, the NABG content was determined, using 'N' mobile phase for separation. The results as shown in Fig. 4 confirmed the authors' expectations, namely, the NABG content in the solution increased with the rise of the concentration of ammonium hydroxide.

4. Discussion

The conditions necessary to conduct a chromatographic separation of NABG, which appears as an impurity in folic acid preparations, were developed.

In the mobile phases investigated, it is possible to achieve a proper and good separation of impurities from folic acid, and this statement is evidenced by differences in the R_f parameter for

these components. Especially reliable outcomes are achieved with application of the 'N' mobile phase in which folic acid ($R_f \cong 0.06$) certainly moves slower in relation to the impurity being determined, i.e. to NABG ($R_f \cong 0.20$).

Under conditions of the chromatographic separation presented above, the densitometric measurements could be conducted both in the UV range at $\lambda_{278\ nm}$ and in the visible range at $\lambda_{450\ nm}$ because these two wavelengths ensure that accurate and reproducible results are obtained. The evidence thereof is data, presented in Table 1 and referring to the recorded NABG content values. There are negligent differences in results obtained at various wavelengths with only one of the described phases, and they are within the limits of tolerance (admissible errors).

The statistical evaluation of the results obtained confirmed the precision of the applied method — the evidence thereof is a sparse confidence interval and a standard deviation of the mean result. The high sensitivity of the method is noteworthy, because the smallest detectable and determinable concentration of the impurity on the chromatograms is 0.15 μg, and this appears essential when designing such investigation schemes.

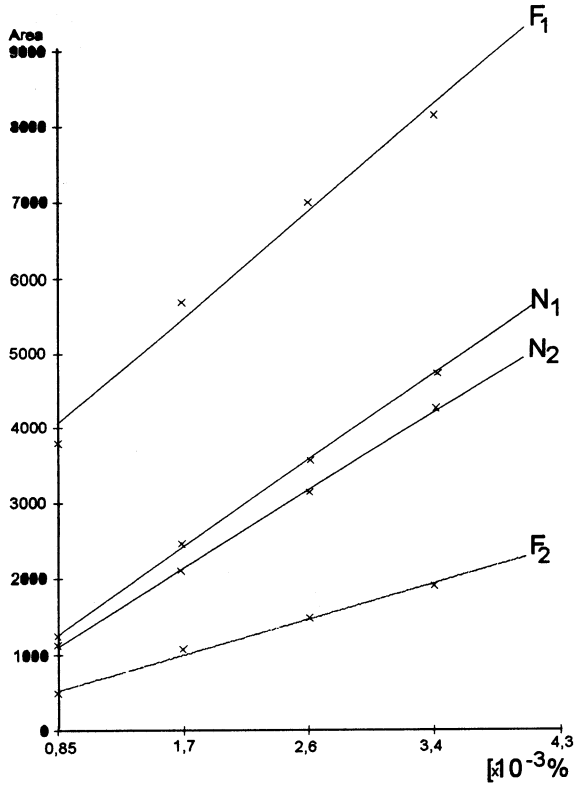

Fig. 3. The correlation between the peak areas and the concentration of *N*-(4-aminobenzoyl-)-L-glutamic acid. 'F' and 'N'-mobile phases; (1) wavelength 278 nm; (2) wavelength 450 nm.

Table 1
The *N*-(4-aminobenzoyl)-L-glutamic acid content [% w/w] in various series of the folic acid preparations, determined after the performed chromatographic separation in 'F' and 'N' mobile phases ($n = 5$; $\mu_{95\%}$)[a]

Series	F		N	
	λ278	λ450	λ278	λ450
000298				
$\bar{x}$	1.53	1.40	0.85	0.95
s	0.0725	0.0487	0.0469	0.1339
Sx	0.0324	0.0218	0.0210	0.0599
μ	1.53 ± 0.09	1.40 ± 0.06	0.85 ± 0.06	0.95 ± 0.17
010398				
$\bar{x}$	1.12	1.1	0.80	0.83
s	0.0604	0.0624	0.0464	0.0995
Sx	0.0270	0.0279	0.0207	0.0445
μ	1.12 ± 0.07	1.1 ± 0.08	0.80 ± 0.06	0.83 ± 0.12
031297				
$\bar{x}$	0.82	0.84	0.70	0.73
s	0.1107	0.1366	0.0918	0.0784
Sx	0.0495	0.0611	0.0410	0.0351
μ	0.82 ± 0.14	0.84 ± 0.17	0.70 ± 0.11	0.73 ± 0.10

[a] $\bar{x}$, arithmetic mean; s, standard deviation of single result; S_x, standard deviation of arithmetic mean; μ-confidence interval for the probability of 95%.

A significant observation resulting from the investigations performed is the documentation of the difference referring to the NABG content in folic acid tablets when individual mobile phases are applied.

For the 'F' mobile phase, recommended by some pharmacopoeias (Ph.Eur., FP, BP, DAB), the results gained are higher if compared with the similar determination procedures performed after the separation in the 'N' mobile phase, and this refers to both the determination in UV and in the visible range.

Attempts to interpret the stated inconsistencies reveal that the mobile phase and the method of

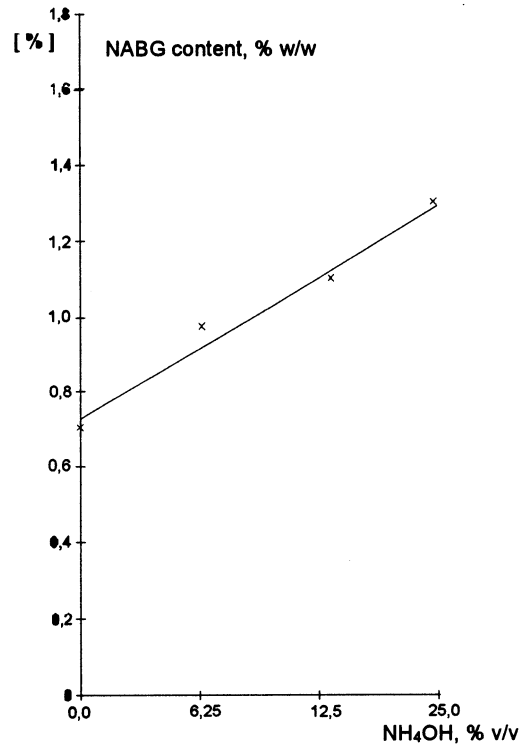

Fig. 4. The impact of ammonia hydroxide on the determined values of *N*-(4-aminobenzoyl-)-L-glutamic acid in folic acid tablets.

preparing samples for investigations might influence the folic acid decomposition. The studies on the impact of the ammonia solution on the NABG content (Fig. 4.) proved that the increase of its concentration caused the rise of the content of this impurity in the folic acid preparations.

Thus, it should be taken into consideration that the outcomes of the determination of the NABG content in the folic acid preparations are higher for the investigations performed under the pharmacopeial conditions.

5. Conclusions

The performed investigation shows that the developed QTLC method can be applied to evaluate the purity of the folic acid preparations for the final purpose of determination of the NABG content as an impurity. The main advantage of this method is that it ensures the achieving of reasonable and credible results, and the second feature is its quickness. The chromatographic separation time is about 30 min for the developed conditions whereas the same time under the pharmacopeial conditions is about 3 h. Under the elaborated conditions of the NABG determination, no impact of the 'N' mobile phase was stated, contrary to the 'F' phase. In the QTLC method, the results of the determination of the NABG content in individual investigated series of folic acid tablets, with the 'N' mobile phase applied, range between 0.70 and 0.85% w/w for measurement in UV, and from 0.73 to 0.95% w/w in the visible light; with the 'F' mobile phase used — from 0.82 to 1.53% w/w and from 0.84 to 1.4% w/w respectively.

References

[1] P. Jacobson, Acta Obstet. Gynaecol. Scand. 75 (1996) 620.
[2] A.E. Czeizel, J. Dudas, New Engl. J. Med. 327 (1992) 1832.
[3] P.S. Romano, N.J. Waitzman, R.M. Scheffler, Am. J. Public Health 85 (1995) 667.
[4] J. McPartlin, A. Halligan, J.M. Scott, M. Darling, D.G. Weir, Lancet 341 (1993) 149.
[5] M.A. Caudill, J.F. Gregory, A.D. Huston, L.B. Bailey, J. Nutr. 128 (1998) 204.
[6] E. Nagy, G. Mezey, E. Kemecsei, Acta Pharm. Hung. 66 (1996) 181.
[7] J.K. Podlewski, A. Chwalibogowska-Podlewska, Leki współczesnej terapii, Split trading Sp. z o.o., Warszawa, 1994.
[8] J. Krzek, A. Bielska, A. Kessler, Farm. Polska 39 (1983) 343.
[9] J. Krzek, A. Bielska, B. Wojlych, Farm. Polska 48 (1992) 543.
[10] J. Krzek, Acta Polon. Pharm. 55 (1998) 99.
[11] M.J. Akhtar, M.A Khan, I. Ahmad, J. Pharm. Biomed. Anal. 16 (1997) 95.
[12] H. McNulty, J. McPartlin, D. Weir, J. Scott, J. Chromatogr. 614 (1993) 59.
[13] P.A. Kownacki-Brown, C. Wang, L.B. Bailey, J.P. Toth, J.F. Gregory, J. Nutr. 123 (1993) 1101.
[14] D. White, P. Varlashkin, D.N. Rusch, J. Pharm. Sci. 81 (1992) 1204.