ELSEVIER

Journal of Chromatography B, 665 (1995) 71–78

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic determination of nicotinic acid and nicotinamide in biological samples applying post-column derivatization resulting in bathmochrome absorption shifts

J. Stein*, A. Hahn, G. Rehner

Institute of Nutrition, Justus Liebig University, Wilhelmstrasse 20, D-35362 Giessen, Germany

First received 7 December 1993; revised manuscript received 14 November 1994; accepted 14 November 1994

Abstract

An ion-pair reversed-phase high-performance liquid chromatographic procedure for the rapid separation and sensitive quantitation of nicotinic acid (NA) and nicotinamide (NAM) in biological samples was developed. The vitamers were separated within 10 min on an octadecylsilica column applying a linear gradient of tetrabutylammonium phosphate and methanol. NA and NAM were converted to highly absorbing derivatives by a modified König's reaction using a double post-column derivatization arrangement consisting of two pumps and two knitted tubular reactors. The proposed method is highly sensitive and specific and applicable to biological materials as was shown by the analysis of rat intestinal tissue.

1. Introduction

The niacin vitamers, nicotinic acid (NA, 3-pyridinecarboxylic acid) and its amide nicotinamide (NAM, 3-pyridinecarboxylic acid amide) belong to the vitamin B complex and can be considered to be both nutrient and drug. On the one hand the vitamin is part of different compounds such as NAD and participates in many physiological redox reactions; on the other, NA is known to be an effective agent in treating hyperlipidemia [1].

Procedures already established for analysing NA and NAM include microbiological assays mainly based on the growth of *Lactobacillus plantarum* or *Tetrahymena pyriformis* [1–3]. These procedures are sensitive and relatively easy to perform but often time-consuming, difficult to standardize and therefore sometimes less reliable. Furthermore, these methods lack in specificity for single derivatives.

A classical approach towards the determination of nicotinic acid is König's reaction [4]. The resulting complex can be determined colorimetrically [5]. The technique is suitable for NA, NAM and different degradation products and thus lacks specificity for single derivatives. It has therefore been combined with thin layer chromatography.

Within the last few years several techniques

* Corresponding author. Address for correspondence: Department of Internal Medicine, Division of Gastroenterology, J.W. Goethe University of Frankfurt, Theodor Stern Kai 7, D-60590, Frankfurt, Germany.

0378-4347/95/$09.50
SSDI 0378-4347(94)00529-X

for the determination of NA and some of its metabolites applying high-performance liquid chromatography (HPLC) have been developed [7–9]. Complete separation of the compounds of interest was not achieved in all cases [7,9]. HPLC determination of NA and NAM was mostly combined with UV detection at 254 nm, a wavelength at which many other substances also show absorbance and thus cause interference. Therefore these methods might be suitable for monitoring NA or NAM levels in pharmacological studies after applying high doses of NA but they are neither applicable for measurement of endogenous amounts of NA and NAM nor for studies on the metabolism of the vitamers.

The aim of this work was to establish a new rapid HPLC technique to determine NA and NAM in biological materials by applying a post-column derivatization arrangement that permits adaptation of König's reaction to HPLC.

2. Experimental

2.1. Chemicals

NA and NAM were purchased from Sigma (Deisenhofen, Germany), chloramine T (sodium N-chloro-4-toluenesulfonamide) from Serva (Heidelberg, Germany) and Tris [tris(hydroxymethyl)aminomethane] from Paesel (Frankfurt, Germany). [^{3}H]Nicotinic acid ([^{3}H]NA) was synthesized by New England Nuclear (Dreieich, Germany) and had a specific activity of 11.9 Ci/mmol. It was checked by HPLC and found to be 98% pure. All other chemicals and solvents were obtained from Merck (Darmstadt, Germany) and were of the highest purity available. Water was purified with a Millipore Q system (Waters, Eschborn, Germany). Vitamin standards were diluted in distilled water and kept at −80°C.

2.2. Chromatography

All analyses were carried out on a Merck-Hitachi HPLC system consisting of a gradient former L-5000, a solvent metering pump 655A-11, an UV detector 655A-23 and a loop injector (Rheodyne, Model 7125) with a 100-μl syringe. Separation of the vitamers was achieved on a 5-μm ODS Hypersil (Shandon, Frankfurt, Germany) analytical column (250 × 4.6 mm I.D.) which had been filled by the upward slurry technique [10] using 2-propanol for preparing the slurry. The analytical column was equipped with a C-135 B pre-column kit (Upchurch Scientific, Oak Harbour, WA, USA) containing dry-packed Shandon ODS Hypersil (10 μm). Nicotinic acid and its metabolites were separated with a gradient of methanol (100%) and 5 m*M* tetrabutylammonium phosphate (TBAP) (pH adjusted to 7.0). The flow-rate was 1.2 ml/min. Composition of the eluent (TBAP–methanol) was linearly changed from 90:10 (v/v) to 30:70 (v/v) in 10 min. All solvents were freshly prepared on the day of use, filtered through 0.45-μm filters (Schleicher & Schüll, Dassel, Germany) and degassed ultrasonically under vacuum.

2.3. Post-column derivatization arrangement

For the derivatization of NA and NAM the set-up as shown in Fig. 1 was used: The first reagent (2% chloramine T) was pumped into the eluent stream leaving the column through a T-junction by a Waters M-6000 HPLC pump at a flow-rate of 0.5 ml/min. After a reaction zone consisting of a 2-m length of PTFE tube (0.5 mm I.D.) the second reagent (0.25% potassium cyanide, 25 m*M* Tris, 40 m*M* HCl) was delivered at a flow-rate of 0.5 ml/min by a Waters M-6000 HPLC pump. Thereafter an 8-m length of PTFE tube (0.5 mm I.D.) was mounted to achieve complete derivatization within the necessary time of 60 s. Both tube reactors were coiled three-dimensionally to minimize peak broadening [11] and kept at 60°C in a water bath. Absorption of the resulting reaction products was measured at 410 nm. Calibration curves were calculated on the basis of peak area using least-squares regression analysis.

To investigate the derivatization kinetics standards of NA and NAM were derivatized in test

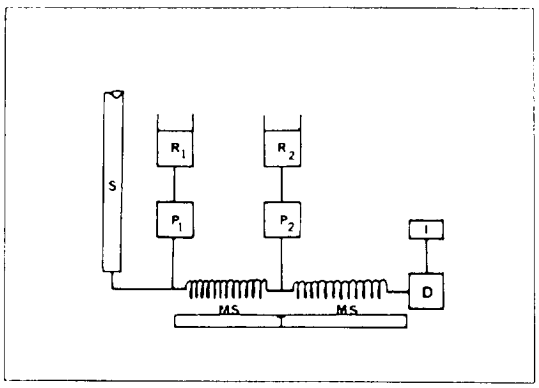

Fig. 1. Technical set-up: The first reagent (R_1; 2% chloramine T) was pumped into the eluent stream leaving the column through a T-junction by a Waters M-6000 HPLC pump (P_1) at a flow-rate of 0.5 ml/min. After a reaction zone consisting of a 2-m length of PTFE tube (MS; 0.5 mm I.D.) the second reagent (R_2; 0.25% potassium cyanide, 25 m*M* TRIS, 40 m*M* HCl) was delivered at a flow-rate of 0.5 ml/min by a Waters M-6000 HPLC pump (P_2). Thereafter an 8-m length of PTFE tube (MS; 0.5 mm I.D.) was mounted to achieve complete derivatization. Both reaction tubes were coiled three-dimensionally to minimize peak broadening [11] and kept at 60°C in a water bath (T); D = detector, I = integrator.

tubes and the amounts of the resulting products were measured over a 30-min period.

2.4. Extraction procedure

Extraction of NA and NAM from biological materials was performed by a minor modification of Carlson's [6] method: The sample was homogenized in physiological saline and 18 ml of acetone was added to an aliquot of 3 ml. The supernatant obtained after centrifugation (10 000 *g*, 10 min) was intensively mixed with 14 ml of chloroform and centrifuged again (1000 *g*, 5 min). A 500-μl volume of the upper, aqueous phase was filtered through a 0.45-μm filter and an aliquot of 100 μl was analyzed by HPLC. Loss of substance by the chloroform extraction was calculated by adding [^{3}H]NA to the homogenate and was found to be less than 10% of the amount added.

The extraction efficiency was determined by an endogenous labeling technique: "Everted sacs" from rat small intestine [12] were incubated for one hour in 25 ml of Krebs' bicarbonate solution containing 5 m*M* glucose and 5 μ*M* [^{3}H]NA. After incubations had been terminated the segments (approx. 10 cm in length) were opened, blotted on paper and extracted as described above. The radioactivity in the supernatant was measured by liquid scintillation counting and compared to that in the untreated homogenate. Pretests revealed that no measurable interconversion or loss of NA and NAM resulted from the treatment.

2.5. Application of the method to biological materials

Rat intestinal tissue analyzed as described above was obtained from male Wistar rats (Winkelmann, Borchen, Germany) with an average body weight of 240 ± 10 g. The animals were killed by cervical dislocation and the small intestine was removed and rinsed with ice-cold saline. The liver was coarsely cut with scissors. All tissues were extracted as mentioned above and analyzed immediately. Quantitation was performed using an external standard graph.

3. Results and discussion

3.1. Chromatography and detection

As can be seen from the standard chromatogram (Fig. 2a) the chromatographic system presented in this paper allows the complete separation of NA and NAM within 20 min. The next sample could be injected after about 25 min. When the extraction system and the sample pretreatment were applied as mentioned above, UV absorption at 260 nm resulted in the appearance of several impurities in the chromatogram of a gut sample that do not allow NA and NAM to be quantitated (Fig. 2b). Similar problems can be seen in chromatograms presented by others [7–9] with NA and NAM appearing as relatively small peaks on top of a high background noise.

Up to now only a few attempts have been made to determine NA and NAM by HPLC [7–9]. Such analytical procedures are still limited

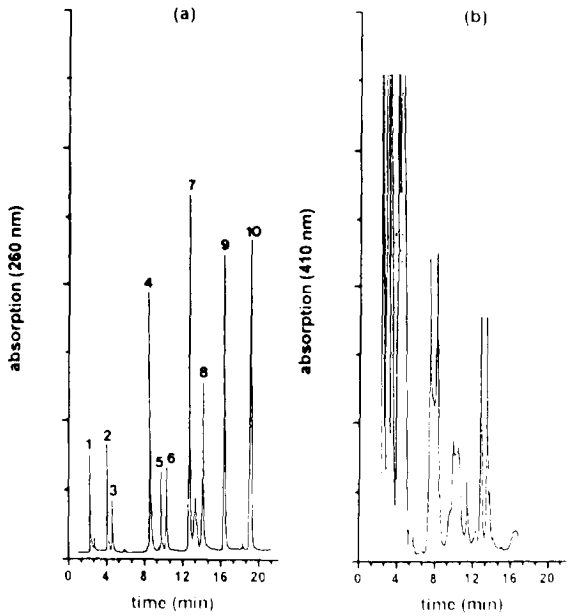

Fig. 2. Chromatographic separation of nicotinic acid, nicotinamide and derivatives by reversed-phase HPLC. (a) Standard chromatogram; Conditions: column, Shandon 5 μm ODS Hypersil (250 × 4.6 mm I.D.); mobile phase, gradient elution with 5 m*M* tetrabutyl ammonium phosphate (TBAP) and methanol (100%) at a flow-rate of 1.2 ml/min; composition of the eluent was linearly changed from 90:10 (TBAP–methanol) to 30:70 in 10 min at ambient temperature; detection wavelength 260 nm; 1 nmol of each compound was injected. Peaks: 1 = N-methyl-nicotinamide; 2 = NMN, nicotinamide mono-nucleotide; 3 = NAM, nicotinamide; 4 = NAD, nicotinamide adenine dinucleotide; 5 = NA, nicotinic acid; 6 = NARP, nicotinic acid ribonucleotide or nicotinic acid mononucleotide; 7 = nicotinic acid mononucleotide; 8 = NAAD, nicotinic acid dinucleotide; 9 = NADP, nicotinamide adenine dinucleotide phosphate; 10 = NAADP, nicotinic acid dinucleotide phosphate. (b) Typical chromatogram from rat intestinal tissue.

by the lack of a specific and sensitive detection system, UV absorption of NA and NAM being mainly used. Consequently, most methods were only applied to monitor the plasma levels of NA and/or NAM after administration of pharmacological doses of NA [8]. The suitability of these methods for determination of the endogenous status of NA and NAM under physiological conditions is therefore not proven; however, from the chromatograms given it must be assumed that neither the sensitivity nor the specificity is sufficient for this purpose. The investigators were only able to detect different niacin vitamers in plasma samples [9] after spiking the samples with the analyte.

An attempt to determine NA by HPLC with fluorimetric detection after derivatization with N,N′-dicyclohexyl-O-(7-methoxycoumarin-4-yl)-methylisourea (DCCI) has been described with NA appearing as a very small peak among several impurity peaks, indicating that the method is not suited for measuring physiological amounts of NA [13]. A distinct NA peak could only be observed after fortifying the sample with the analyte. Furthermore, it should be mentioned that the formation of 4-hydroxymethyl-7-methoxycoumarin esters is disturbed by the presence of water and thus requires intensive sample pretreatment, i.e. evaporation of the aqueous solvent to dryness. Therefore, it is not possible to perform this reaction on-line applying post-column derivatization.

A well-established detection system is provided by the classical König's reaction [5] in which NA forms a colored complex with aromatic amines in the presence of an oxidant. The reaction has also been combined with thin layer chromatography which increases the specifity but requires several handling steps and leads to low recoveries. To improve the sensitivity and specifity of the König's reaction for single derivatives we developed the HPLC method presented in this paper. Preliminary tests revealed three problems that had to be solved: first of all we found that delivery of the reagents had to be done separately to achieve reproducible and fast formation of the reaction products. It was not possible to use premixed reagents. Under such conditions the derivatization rate as well as the reproducibility decreased within two or three analyses due to reactions between the reagents themselves. To avoid such problems the post-column derivatization arrangement was applied as described. Furthermore, suitable concentrations and flow-rates of the derivatization reagents had to be tested in order to avoid crystalline deposits within the reaction tubes. Under the conditions described no technical problems were observed.

As can be seen from Fig. 3 maximal color intensities for the derivatives of NA and NAM are reached at different times, leading to a decrease in NAM color intensity while absorption of NA still increases. As a consequence of the differences in derivatization kinetics the sensitivity for the detection of NAM is lower than that for NA. Similar observations were made by others [6]. Taking into account that NA is rapidly converted to NAM under physiological conditions [14], resulting in much higher levels of NAM compared to NA, a higher sensitivity is required for NA. The lengths of the derivatization tubes as used in this method result in a higher sensitivity for NA than for NAM and were chosen because of the improvements described. Nevertheless, changing the lengths of the tubes affects the difference between the sensitivities for the two derivatives and thus can be adapted according to the analytical problem.

Since König's reaction is highly specific for NA and NAM (see above) an appropriate standard could not be found. All analogues (methylnicotinamide, chloronicotinic acid derivatives) which were tested for use as possible internal standards also did not react with the reagent (other analogues of nicotinic acid are not currently available). On the other hand, the high specificity and reproducibility of this method [6] reduces the need for an internal standard.

The linearity, and intra- and inter-assay precision and accuracy of the method were assessed using tissue spiked with known amounts of NA and NAM up to 500 pg; these were then analysed as described above. Both curves were found to be linear using a computer-based least-

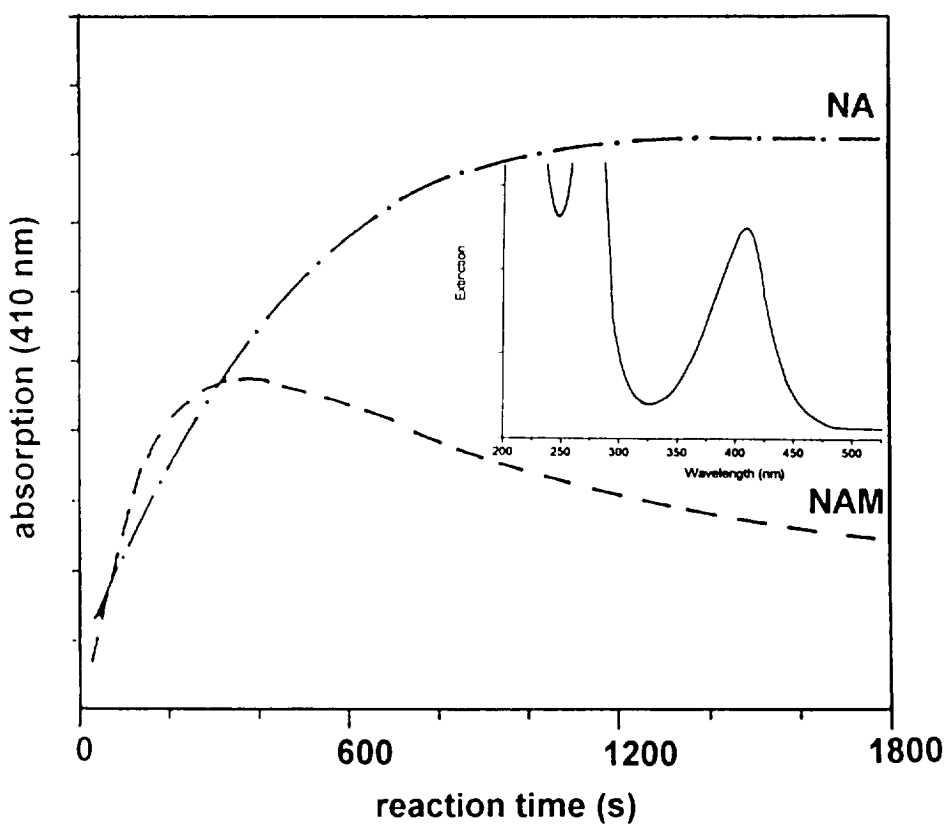

Fig. 3. Postcolumn conversion of nicotinic acid (NS) and nicotinamide (NAM) by the modified König's reaction (1 m*M* each); derivatization conditions see Fig. 1. Inset: UV spectrum of derivatized NA (1 m*M*).

Table 1
Quantitative chromatographic parameters for HPLC-UV analysis of nicotinic acid nicotinamide

Compound	Retention time (min)	Range of linearity (pmol)	Detection limit (pmol)	r^a
Nicotinamide	4.95	20–500	20.75 ± 0.53	0.994
Nicotinic acid	9.25	10–500	11.75 ± 0.33	0.995

[a] Based on $n = 5$ determinations each.

squares program (Table 1). Table 2 shows the precision and accuracy data. For both vitamins relative standard deviations (R.S.D.) were found to be $< 5\%$. The detection limits determined at a signal-to-noise ratio of 3:1 are at much lower than those observed by other authors [7–9]. Concerning the reproducibility of both chromatographic procedures presented here it should be mentioned that we injected very low quantities of the substrate. As can be seen from the results of others [15], reproducibility increases when higher amounts are chromatographed.

3.2. Extraction procedure

Fig. 4 shows that only minor impurities could be detected in the chromatograms of biological samples, indicating the specifity of the analytical procedure. This becomes even more evident when compared with the chromatograms presented by other investigators [9,13]. Recording UV spectra of the peaks and comparing them to those obtained from derivatized standards confirmed the identity of the peaks.

Other extraction techniques used for HPLC require the supernatant to be evaporated to dryness, even several times [9,13,15] or require the application of solid-phase extraction columns [8,17] or conventional ion-exchange chromatography on Dowex prior to the analysis [18]. The procedure applied here is much easier and faster to perform than the those mentioned above.

An important factor for the precision of a method is the efficiency by which the analyte is extracted from the sample. This efficiency is difficult to determine [19] and was evaluated by an endogenous labeling technique using intestinal tissue as a model matrix. Chromatographic studies revealed that the majority of [^{3}H]NA was converted to other vitamers indicating that the substrate equilibrated with the endogenous metabolites. Extraction efficiency as judged by this technique was 92.4 ± 3.7% ($n = 11$).

We favor the endogenous labeling technique over the generally given "recovery rates" which are easier to determine but which present several problems. For example, they do not reflect the efficiency with which protein bounds are broken because substrates given to tissue homogenate just prior to the extraction cannot be expected to behave like the endogenous substances as has already been shown [20].

Table 2
Intra- and inter-assay precision and accuracy of the HPLC-UV procedure of nicotinic acid and nicotinamide

Compound	Intra-assay ($n = 4$)		Inter-assay ($n = 5$)	
	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)
Nicotinamide	3.5	96.7	4.1	95.7
Nicotinic acid	2.9	97.2	3.3	96.2

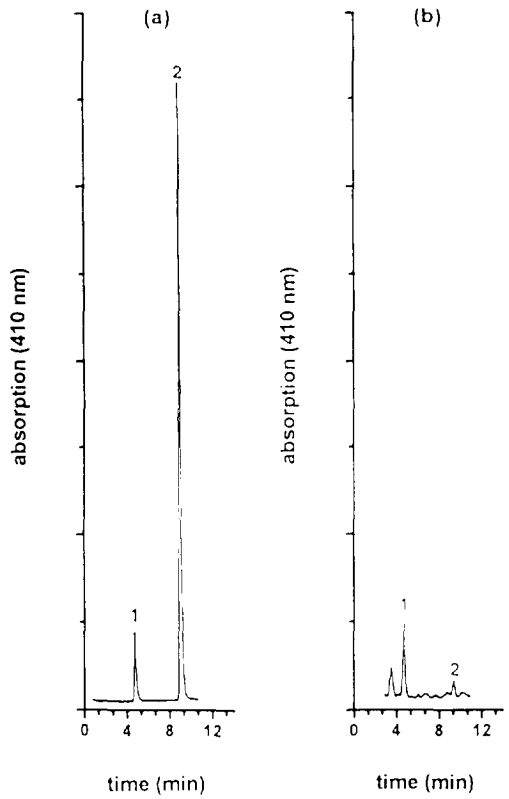

Fig. 4. Chromatographic separation of nicotinic acid and nicotinamide after postcolumn derivatization. (a) Standard chromatogram; Conditions: column, Shandon 5 μm ODS Hypersil (250 × 4.6 mm I.D.); mobile phase, gradient elution with 5 m*M* tetrabutyl ammonium phosphate (TBAP) and methanol (100%) at a flow-rate of 1.2 ml/min; composition of the eluent was linearly changed from 90:10 (TBAP–methanol) to 30:70 in 10 min at ambient temperature; detection wavelength 410 nm; 1 nmol of each compound was injected. Peaks: 1 = nicotinamide; 2 = nicotinic acid. (b) Typical chromatogram from rat intestinal tissue.

3.3. Analysis of rat intestinal tissue

Table 3 gives the amounts of NA and NAM as determined by our method and Fig. 4 show a typical chromatogram. It is evident that only minor amounts of niacin are detectable as NA and most of the vitamin is found as NAM. These findings can be explained by the specific biological pathway of niacin in the intestine, which involves a rapid postabsorptive conversion of nicotinic acid as recently demonstrated [14].

In summary our results clearly indicate that the method presented in this paper allows the fast separation and quantitation of NA and NAM in biological samples.

Acknowledgements

The authors are grateful to Prof. Dr. E.L. Sattler for providing the opportunity to work in the laboratories of the Radiation Center (Central Department) of the Justus Liebig University, and also to L.R. Hagey (USCD) for helpful discussions. This study was supported by a Grant of the German Research Foundation Re 437/5-1.

References

[1] L.V. Hanks, in L.J. Machlin (Editor), *Handbook of Vitamins*. Marcel Dekker, New York and Basel, 1984, pp. 329–378.
[2] H. Baker, O. Frank, I. Pasher, S.H. Hutner and H. Sobotka, *Clin. Chem.*, 6, (1960) 572.

Table 3
Nicotinic acid nicotinamide content of rat intestinal tissue (nmol/g wet weight ± S.D.)

Compound	Duodenum	Prox. jejunum	Distal jejunum
Nicotinamide	291 ± 53	283 ± 47	286 ± 49
Nicotinic acid	10.6 ± 2.6	9.8 ± 2.1	9.4 ± 2.1
Ratio	27.1:1	28.1:1	29.9:1

[3] M.N. Voigt, R.R. Eitenmiller and G.O. Ware, *J. Food Sci.*, 43 (1978) 418.
[4] M.N. Voigt, R.R. Eitenmiller and G.O. Ware, *J. Food Sci.*, 44 (1979) 29.
[5] König, *J. Prakt. Chem.*, 69 (1904) 105.
[6] L.A. Carlson, *Clin. Chim. Acta*, 13 (1969) 349.
[7] N. Hengen, V. Seiberth and M. Hengen, *Clin. Chem.*, 24 (1978) 1740.
[8] J.X. de Vries, W. Günthert and R. Ding, *J. Chromatogr.*, 221 (1980) 161.
[9] K. Takikawa, K. Miyazaki and T. Arita, *J. Chromatogr.*, 233 (1982) 343.
[10] P.A. Bristow, P.N. Brittain, C.M. Riley and B.F Williamson, *J. Chromatogr.*, 31 (1977) 57.
[11] H. Engelhardt and U.D. Neue, *Chromatographia*, 15 (1982) 403.
[12] G. Wiseman, *Methods in Medical Research*, 9 (1961) 287.
[13] Y. Tsuruta, K. Kohashi, S. Ishida and Y. Ohkura, *J. Chromatogr.*, 309 (1984) 309.
[14] J. Stein, E. Daniel, H., Whang, U. Wenzel, A. Hahn and G. Rehner, *J. Nutrition*, 124 (1994) 61.
[15] C. Wegner, M. Trotz and H. Nau, *J. Chromatogr.*, 378 (1986) 55.
[16] J.S. Sandhu and D.R. Fraser, *Int. J. Vit. Nutr. Res.*, 51 (1981) 139.
[17] A. Durrer, B. Walther, A. Racciatti and B. Testa, *J. Chromatogr.*, 495 (1989) 256.
[18] G.M. McCreanor and D.A. Bender, *Br. J. Nutr.*, 56 (1986) 577.
[19] J.F. Gregory, *Food Technol.*, 13 (1983), 75.
[20] S. Sander, A. Hahn, J. Stein, G. Rehner, *J. Chromatogr.*, 558 (1991) 115.