

**THIN-LAYER CHROMATOGRAPHIC ANALYSIS
OF HYDROPHILIC VITAMINS IN STANDARDS
AND FROM *Helisoma trivolvis* SNAILS**

*E. L. Ponder*¹, *B. Fried*², and *J. Sherma*^{1,*}

¹Department of Chemistry, Lafayette College, Easton, Pennsylvania 18042, USA

²Department of Biology, Lafayette College, Easton, Pennsylvania 18042, USA

SUMMARY

Separations of the hydrophilic vitamins thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), cobalamin (B12), ascorbic acid (C), and folic acid have been compared using 14 mobile phases and commercially available, precoated silica gel and chemically bonded silica gel thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) plates. The best separations of individual and mixed vitamin standards was achieved on silica gel plates with 1-butanol–chloroform–acetic acid–ammonia–water, 7:4:5:1:1, benzene–methanol–acetone–acetic acid, 70:20:5:5, and chloroform–ethanol–acetone–ammonia, 2:2:2:1, as mobile phases. Previously reported detection reagents selective for the hydrophilic vitamins were also tested and compared. To determine the usefulness of these chromatographic systems for analysis of biological samples, hydrophilic vitamins in *Helisoma trivolvis* snails (Pennsylvania strain) were identified and B2 was quantified by videodensitometry. Because of the amount of non-vitamin, water-soluble substances that gave fluorescence-quenched zones in the snail extracts analyzed, it was necessary to use natural color or fluorescence or a selective vitamin spray-reagent, in combination with R_F values, to identify vitamins in biological samples.

INTRODUCTION

Hydrophilic vitamins include thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), cobalamin (B12), ascorbic acid (C), folic acid, biotin (H), and pantothenic acid (B5). These vitamins play essential roles in normal biological functions as coenzymes and antioxidants [1,2]. Dietary deficiencies of the B vitamins are associated with several diseases, inclu-

ding degeneration of peripheral nerves and cerebral ataxia in humans [3,4]. The association of hydrophilic vitamin levels with important biological functions and diseases has led to numerous attempts to quantify these vitamins in foods, including milk, enriched pasta, shellfish, clams, oysters, and muscles [5–7].

Microbiological and chemical assays are available for identification and quantification of several water-soluble vitamins from biological samples, but most of these methods are complicated and limited to the analysis of a single vitamin [3,8–10]. Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) have been used alone or in combination with high-performance column liquid chromatography (HPLC) to provide alternatives to the complex biological and chemical assays used to separate and quantify analogs of single hydrophilic vitamins or multiple hydrophilic vitamins [5–7]. Although previous studies have reported use of thin-layer chromatographic stationary and mobile phases for separation of multiple hydrophilic vitamins, few studies have addressed the potential of TLC for analysis of biological samples containing large amounts of non-vitamin material.

Rizk et al. [11] quantified hydrophilic vitamins in medically important freshwater snails of the genera *Biomphalaria*, *Bulinus*, and *Lymnaea* by using chemical assays to determine the effects of starvation on vitamin content. These snails are important intermediate hosts for several parasitic flatworms, including *Schistosoma mansoni* and *S. haematobium*, that cause schistosomiasis in humans [11]. Use of TLC methods to analyze the effects of larval trematode infection on hydrophilic vitamins in these snails would offer a way to separate and quantify several vitamins simultaneously in place of chemical assays previously used to analyze one vitamin at a time. Analysis of hydrophilic vitamins in medically important snails might provide useful basic information enabling development of more rational chemotherapy strategies against snails infected with larval trematodes.

This study compares separation of the vitamins thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), cobalamin (B12), ascorbic acid (C), and folic acid by use of 14 TLC or HPTLC systems previously reported in the literature for analysis of these seven vitamins individually or in different combinations. Although multiple hydrophilic vitamins have been separated by use of transition metal-impregnated TLC plates [12] and by forced flow TLC [13], this study examined selected mobile phases using only commercially available, unmodified plates and ascending unidimensional capillary-flow development. Vitamins in mixed standards and tissue

from *Helisoma trivolvis* (Pennsylvania strain) snails were studied to determine the usefulness of previously reported systems for simultaneous identification of multiple hydrophilic vitamins in biological samples. Detection reagents reported to be specific for vitamins were also tested. The use of selective detection reagents might be necessary to distinguish vitamins from non-vitamin hydrophilic substances in chromatograms of extracts from biological samples. The potential for quantitative analysis is demonstrated by the HPTLC–videodensitometric determination of vitamin B2.

EXPERIMENTAL

Preparation of Standards

Standards of vitamins B1, B2, B3, B6, B12, C, and folic acid were purchased from Sigma (St Louis, MO, USA) and prepared at a concentration of $1.00 \mu\text{g } \mu\text{L}^{-1}$ in methanol–deionized water (1:3) [1], except for B2 and folic acid. Because folic acid and B2 at this concentration were not soluble in methanol–deionized water (1:3), these vitamin standards were prepared at a concentration of $0.100 \mu\text{g } \mu\text{L}^{-1}$. A vitamin mixture was prepared by combining 1000 μL each of the B3, C, and folic acid standard solutions and 500 μL each of the B1, B2, B6, and B12 solutions. The mixture was evaporated to dryness at 60°C with a light stream of air and reconstituted in 1000 μL methanol–deionized water (1:3). All standards and the vitamin mixture were prepared in subdued light and containers were wrapped in aluminum foil, because most of the vitamins are light-sensitive.

Chromatographic Analysis

Separations of vitamin standards were compared on: 20 cm \times 10 cm silica gel 60Å HPTLC plates with a pre-adsorbent sample application zone and 19 channels (LHPKDF #4806-711; Whatman, Clifton, NJ, USA); 20 cm \times 10 cm silica gel 60 CF₂₅₄ HPTLC plates with a pre-adsorbent sample-application zone (#13153; EM Science, Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany); 10 cm \times 10 cm amino-bonded silica gel 60 CF₂₅₄ HPTLC plates (#15647, EM Science); 20 cm \times 20 cm chemically bonded reversed phase TLC plates with pre-adsorbent zone (LKC_{18F} #4800-820; Whatman); and 10 cm \times 20 cm chemically bonded reversed phase RP-18F_{254S} HPTLC plates with pre-adsorbent zone (#15498; EM Science). Plates were prewashed by development with dichloromethane–methanol (1:1) before applying samples. The mobile phases evaluated

in this study are summarized in Table I. Plates were developed 6 cm beyond the origin in a Camag (Wilmington, NC, USA) HPTLC or TLC twin-trough chamber lined with a saturation pad (Analtech, Newark, DE, USA).

Table I

Mobile phases studied for separation of individual hydrophilic vitamin standards

No.	Components	Volume ratio	Ref.
1	1-Butanol–chloroform–acetic acid–ammonia–water	7:4:5:1:1	1
2	<i>n</i> -Propanol–chloroform–acetic acid–ammonia–water	7:4:5:1:1	1
3	Ethanol–chloroform–acetic acid–ammonia–water	7:4:5:1:1	1
4	Acetone–methanol–benzene	1:2:8	14
5	Acetone–methanol–toluene	1:2:8	This study
6	Benzene–methanol–acetone–acetic acid	70:20:5:5	15
7	Toluene–methanol–acetone–acetic acid	70:20:5:5	This study
8	Chloroform–ethanol–acetone–ammonia	2:2:2:1	16
9	Methanol–water	70:30	17
10	Ethanol–water	2:1	18
11	Water	–	19
12	1-Butanol–pyridine–water	50:35:15	13
13	Acetonitrile–water	70:30	20
14	25% Methanol–borate buffer (5 mM, pH 7.0)–acetonitrile	14:2:2	1

Water = deionized water

Plates were spotted with 5–10 μ L of each vitamin standard. Samples and standards in both qualitative and quantitative analysis were applied with a 10- μ L digital microdispenser (Drummond, Broomall, PA, USA). Standard and sample solution volumes of more than 5 μ L were difficult to apply to the Merck plates with pre-adsorbent zone and required use of a light stream of air while spotting. Whatman and Merck silica gel plates were developed with mobile phases 1 to 11 listed in Table I. Benzene in mobile phases 4 and 6 was replaced with toluene in systems 5 and 7 to evaluate the effectiveness of substituting a less toxic substance for benzene. Whatman silica gel plates were also developed with mobile phase 12. Merck amino-bonded silica gel plates were developed with mobile phase 13, and the reversed-phase plates were developed with mobile phase 14. Systems providing clear separations of the standards were used to separate the vitamin mixture and samples from snails.

Vitamins were detected by color in visible light, by fluorescence quenching under 254-nm ultraviolet (UV) light, or by fluorescence using excitation with 366-nm UV light. Vitamins B2 and B12 were detected as yellow and pink colored zones, respectively, in visible light [1,18]; B1, B3, B6, B12, C, and folic acid appeared as dark zones under 254-nm UV light [19]; and B1, B2, and B6 were visible as light blue, yellow [17], and dark blue fluorescent zones, respectively, under 366-nm UV light.

Evaluation of Detection Reagents

Standards of folic acid and vitamins B1, B2, B3, B6, B12, and C were separated by use of mobile phase 1 on Whatman silica gel plates and sprayed with previously reported spray reagents: 0.5% ether solution of iodine–Dragendorff reagent for visualization of B1 [18]; 1% methanol solution of 1-chloro-2,4-dinitrobenzene followed by 3 M NaOH for visualization of B3 and B6 [21]; 0.4% methanol solution of 2,6-dichloroquinone-4-chloroimide for visualization of vitamin B6 [18]; or a 1:1 mixture of 2% H₂SO₄–EtOH and 0.2% ethanol solution of *p*-dimethylaminocinnamaldehyde for visualization of biotin [22]. Although biotin was not analyzed in this study, this detection reagent was evaluated to determine if it could be used to detect other water-soluble vitamins. All chemicals were purchased from Sigma, and the selectivity of each reagent was evaluated.

HPTLC Analysis of Vitamins in *H. trivolvis* Snails

Whole-body samples of *Helisoma trivolvis* (PA) snails were prepared by removing the snail bodies from the shells and discarding the shells. For each of three samples, 7–10 snail bodies were pooled to obtain individual samples of approximately 1 g snail tissue. Each sample was homogenized in 10 mL methanol–deionized water (1:3) by use of a ground-glass homogenizer. Samples were then centrifuged for 15 min at 2000 × g and the supernatant was filtered through glass wool. Each sample was evaporated to dryness at 60°C with a light stream of air and reconstituted in 500 µL methanol–deionized water (1:3). Each sample was vortex-mixed for 5 min. All snail-tissue samples were prepared with the laboratory lights off, wrapped in aluminum foil, and stored at 4°C to reduce degradation of light-sensitive vitamins.

The hydrophilic vitamin content of *H. trivolvis* snails was evaluated by use of mobile phases 1, 6, and 7 on Whatman silica gel plates. Separated zones from the *H. trivolvis* samples using each TLC system were evaluated for visible color, fluorescence quenching, fluorescence, and *R_F*

values. Spray reagents were used to confirm the identity of zones with R_F matching those of vitamin standards. Separations were repeated as needed to enable spraying with different reagents for identification purposes.

Mobile phase 6 with the Whatman silica gel plate enabled the clearest separation of the *H. trivolvis* samples, so this HPTLC system was used to quantify vitamin B2. The B2 standard solution was diluted to $0.0250 \mu\text{g } \mu\text{L}^{-1}$ and 2.00–8.00- μL volumes (0.0500 to 0.200 μg) of the diluted solution were used to bracket the amount of B2 in the *H. trivolvis* samples. The plates were photographed under 366-nm UV light using a Camag Reprostar 3 videoscanner with VideoStore software. Quantitative analysis was performed using VideoScan software with a minimum peak width of five pixels, a minimum peak height of 100 pixels, and a minimum area of 300 pixels. The filter width was 5. The weights of B2 in sample zones were interpolated on the basis of the polynomial regression calibration curve created from the scans of the standard zones, and the concentration ($\mu\text{g g}^{-1}$) of B2 in snail samples was calculated.

RESULTS AND DISCUSSION

Chromatographic Analysis

R_F values of vitamin standards are summarized in Table II. Mobile phases 5 and 7 are not listed for the Whatman silica gel plate, because these mobile phases failed to run evenly in each of two trials. Mobile phases 11 and 14 are not listed for the Merck silica gel and reversed phase plates, respectively, because the water in these mobile phases dissolved the pre-adsorbent zones of these plates before separation occurred. Whatman silica gel plates developed with mobile phases 1, 6, and 8 yielded the most clearly resolved vitamin zones. Using mobile phase 1, vitamins B1, B2, and B12 were clearly resolved from each other and from other hydrophilic vitamins, and using mobile phase 6, B2, B3, B6, and C were clearly resolved from each other and from other hydrophilic vitamins. Mobile phase 8 worked best in the absence of B1 (because this zone was elongated) to separate B2, B3, B6, B12, and folic acid. Even in the presence of B1, this system might be useful, because folic acid was not detected by use of many of the chromatographic systems evaluated.

Separations of individual and mixed vitamin standards were compared for the Whatman silica gel HPTLC systems that gave the most promising single-vitamin separations (Table III). Although most vitamins from

Table II

R_F values of vitamins obtained from separation of vitamin standards with a variety of combinations of mobile phases and plate types. TLC system numbers correspond to the mobile phases listed in Table I, and the letters refer to the layers listed below.

TLC system*		R_F						
		B1	B2	B3	B6	B12	C	Folic acid
1	a	0.40	0.71	0.71	0.71	0.64	0.60	0.45
	b	0.32	0.51	0.63	0.61	0.45	0.45	nd
2	a	0.46	0.73	0.75	0.75	0.66	0.71	nd
	b	0.39	0.53	0.64	0.59	0.53	0.51	nd
3	a	0.51	0.83	0.81	0.81	0.73	0.81	nd
	b	0.38	0.62	0.71**	0.62	0.56	0.57	nd
4	a	0	0.08**	0.12**	0.33	0	0	nd
	b	0***	0.11***	0.03***	0.29***	0***	0***	nd
5	b	0***	0.05***	0.02***	0.09***	0***	0***	nd
6	a	0.03	0.22	0.47	0.30	0	0.15	nd
	b	0.05***	0.14***	0.32	0.20	0***	0.08***	nd
7	b	0.06	0.06	0.31	0.19	0.02	0.10	nd
8	a	0.44**	0.28	0.30	0.42	0.13	nd	0.20
	b	0.40**	0.30	0.36	0.49	0.13	nd	0.39**
9	a	0.55	0.91	0.94	0.87	0.83	0.94	0.85
	b	0.45	0.80	0.80	0.78	0.68	0.80	0.69
10	a	0.08**	0.83**	0.82	0.77	0.60	0.83	0.80**
	b	0.08	0.89	0.87	0.86	0.70***	0.88	0.88
11	a	nd	0.46**	0.64	0.31**	0.41	nd	nd
12	a	0.11	0.73	0.60**	0.65	0.55**	0.49	0.24**
13	c	0.29	0.14	0.08	0.27	0	0	0
14	d	0.09	0.39	nd	nd	0.53	nd	nd

*a, Whatman silica gel; b, Merck silica gel; c, amino-bonded silica gel; d, Whatman reversed-phase

** R_F from center of an elongated zone

***Large amount of sample remaining at the origin

0 denotes no movement from the origin; nd denotes not detected

the mixture were detected with R_F values similar to those of individual vitamin standards in each system, folic acid from the mixture was not detected after separation using 1-butanol–chloroform–acetic acid–ammonia–water, 7:4:5:1:1, as mobile phase. It is possible that some of this vitamin was lost during sample preparation, resulting in a lower than detectable level of folic acid separated from the mixture.

Table III

R_F values of vitamin standards alone and in mixtures separated using the three HPTLC mobile phases enabling the clearest separation of standards on Whatman silica gel plates

Mobile phase	Sample	Vitamin R_F values						
		B1	B2	B3	B6	B12	C	Folic acid
1-Butanol–chloroform–acetic acid–ammonia–water, 7:4:5:1:1	Standards	0.44	0.67	0.70	0.70	0.64	0.56	0.52
	Mixture	0.43	0.66	0.69	0.69	0.62	nd	nd
Benzene–methanol–acetone–acetic acid, 70:20:5:5	Standards	0.02	0.18	0.43	0.27	0	0.15	nd
	Mixture	nd	0.19	0.46	0.25	0	0.14	nd
Chloroform–ethanol–acetone–ammonia, 2:2:2:1	Standards	0.22–0.66	0.28	0.30	0.42	0.13	nd	0.20
	Mixture	0.67	0.30	0.34	0.44	0.11	nd	0.25

0 denotes no movement from the origin; nd denotes not detected

Evaluation of Detection Reagents

Vitamin standards developed using the Whatman silica gel plate and mobile phase 1 were used to evaluate the selectivity of each detection reagent for sub-microgram levels of hydrophilic vitamins. Spraying with a 0.5% solution in ether of iodine–Dragendorff reagent caused vitamin B1 to appear as a visible purple zone. This reagent stained the rest of the plate yellow and interfered with visualization of the fluorescence of B6 under 366-nm UV light. Spraying successively with a 1% methanol solution of 1-chloro-2,4-dinitrobenzene followed by 3 M NaOH resulted in visualization of B3 and B6 as visible yellow zones but interfered with detection of all fluorescence quenching zones under 254-nm UV light, although B2, B3, and folic acid all remained visible as fluorescent zones under 366-nm light. A 0.4% solution in methanol of 2,6-dichloroquinone-4-chloroimide visualized B6 as a blue–gray zone. Although previous use of this reagent indicated that the plate should be placed in a tank saturated with ammonia vapor after spraying, for visualization [18], the colored zone appeared without treatment with ammonia vapor in all the trials in this study. No interference with the visualization of other standards as colored, fluorescence-quenched, or fluorescent zones occurred.

Use of a 1:1 mixture of ethanolic 2% H_2SO_4 and 0.2% ethanolic *p*-dimethylaminocinnamaldehyde as spray reagent enabled detection of vita-

min C and folic acid as bright pink–orange zones but stained the background of the layer a light yellow–orange color. This reagent interfered with detection of all other vitamin standards except the visible pink zone for B12. Although this reagent has previously been reported as being specific for biotin [22], our study shows it can also be used to detect C and folic acid.

HPTLC Analysis of Vitamins in *H. trivolvis* Snails

Separation of hydrophilic vitamins from samples of *H. trivolvis* snails was evaluated using mobile phases 1, 6, and 8 with the Whatman silica gel plate. Separation using mobile phase 1 revealed three zones with R_F values of 0.69, 0.67, and 0.66 and an additional long streaking zone from R_F 0.60 to the origin. The zone at 0.67 was confirmed to be B2 by its yellow fluorescence. Spraying with the 1-chloro-2,4-dinitrobenzene reagent successfully turned the B3 and B6 standards yellow, but had no effect on the zone at R_F 0.69, indicating that this zone was not B3 or B6 despite its similar R_F value.

Separation using mobile phase 6 revealed seven clearly separated zones with R_F values of 0.71, 0.68, 0.39, 0.20, 0.17, 0.12, and 0.07 from the *H. trivolvis* samples. The zone at 0.20 was identified as B2 from its yellow fluorescence and matching R_F value. Although the zone at R_F 0.17 or 0.12 could have been vitamin C, spraying with the *p*-dimethylaminocinnamaldehyde reagent revealed that C and folic acid were not present in the sample.

Separation using mobile phase 8 revealed six zones with R_F values of 0.48, 0.45, 0.30, 0.22, 0.11, and 0.05. The zone at 0.30 was identified as B2 from its yellow fluorescence and similar R_F value. Spraying with the 1-chloro-2,4-dinitrobenzene and *p*-dimethylaminocinnamaldehyde detection reagents confirmed the other zones were not the hydrophilic vitamins being examined in this study.

Evaluation of these mobile phases using a biological sample containing non-water-soluble substances indicated that R_F values alone are not sufficient to confirm the presence of hydrophilic vitamins. Specific natural coloration of visible or fluorescent zones or a selective spray reagent should be used in combination with TLC separation to ensure accurate identification of these vitamins from biological samples.

Because vitamin B2 was positively identified by its R_F value and yellow fluorescence in the *H. trivolvis* samples by use of three mobile phases, this vitamin was quantified (Fig. 1). On the basis of interpolation of weights from a polynomial regression calibration curve ($R^2 = 0.994$), *H. tri-*

volvis snails used in this study contained $0.0766 \pm 0.0122 \mu\text{g B2 g}^{-1}$ body weight. In a previous study of hydrophilic vitamins in freshwater snails, Rizk et al. [11] found much higher values of 10.54, 12.04, and $16.62 \mu\text{g B2 g}^{-1}$ snail tissue in *Biomphalaria alexandrina*, *Bulinus truncatus*, and *Lymnaea truncatula*, respectively, using traditional chemical assays for quantification of this vitamin. Rizk et al. [11] also provided quantitative data for levels of B1, B3, and B6 in the three species of fresh water snails they worked with. Comparison of their study and ours is difficult, because of differences between the methods used and the snails species investigated in the two studies.

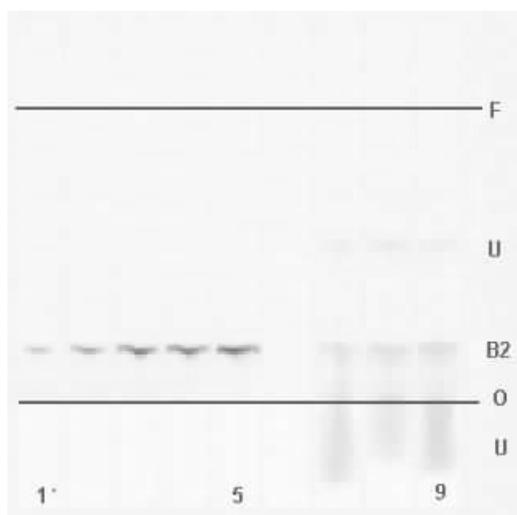


Fig. 1

Chromatograms obtained after separation of B2 standards and *H. trivolvis* samples by use of mobile phase 6 (Table I) on a Whatman channeled silica gel plate with pre-adsorbent zone, as described above. The naturally fluorescent zones on the plate were photographed under 366-nm UV light with a Camag VideoStore image-documentation system. F, mobile phase front; O, origin; lanes 1–5, B2 standards (0.0500 to $0.200 \mu\text{g}$); lane 6, blank; lanes 7–9, *H. trivolvis* samples. Fluorescent zones marked U are unidentified.

TLC and HPTLC systems have been developed and used for simultaneous determination of several classes of biochemical compound, including pigments, phospholipids, and amino acids, in uninfected freshwater snails and in those infected with larval trematodes [23–26]. Analysis of hydrophilic vitamins by TLC and HPTLC is a new tool enabling exploration of the basic biology of medically important snails and the effects of

their parasitic infections. Separation, detection, and quantification of B1, B2, B3, B6, B12, C, and folic acid using the mobile phases 1-butanol–chloroform–acetic acid–ammonia–water, 7:4:5:1:1, benzene–methanol–acetone–acetic acid, 70:20:5:5, and chloroform–ethanol–acetone–ammonia, 2:2:2:1 in combination with detection reagents and natural visible and fluorescent colors is an alternative to using individual chemical assays. The methods developed in this research, which have the potential for simultaneous qualitative and quantitative analysis, will be used in future studies to determine the effects of larval trematode infections on the hydrophilic vitamin content of *Biomphalaria glabrata* snails.

REFERENCES

- [1] R. Bhushan and M. Arora, *Sci. Lett.*, **25**, 159 (2002)
- [2] C.P. Earnest, K.A. Wood, and T.S. Church, *J. Am. Coll. Nutr.*, **22**, 400 (2003)
- [3] W.C. Ellefson in A. Jorgy, P. Barbara, B. Deborab, and B. Paul (eds), *Methods of Vitamin Assay*, John Wiley and Sons, New York, 1985, pp. 349–363
- [4] S. Morita, H. Miwa, T. Kihira, and T. Kondo, *J. Neurol. Sci.*, **216**, 183 (2003)
- [5] A. Rizzolo and S. Polesello, *J. Chromatogr.*, **624**, 103 (1992)
- [6] F. Watanabe and E. Miyamoto in J. Sherma and B. Fried (eds), *Handbook of Thin-Layer Chromatography*, Dekker, New York, 2003, pp. 589–605
- [7] J.C. Linnell in J. Sherma and B. Fried (eds), *Handbook of Thin-Layer Chromatography*, Dekker, New York, 1996, pp. 1047–1054
- [8] E.P. Frenkel, R. Prough, and R.L. Kitchens, *Methods Enzymol.*, **67**, 31 (1980)
- [9] J.J. Shah in A. Jorgy, P. Barbara, B. Deborab, and B. Paul (eds), *Methods of Vitamin Assay*, John Wiley and Sons, New York, 1985, pp. 365–383
- [10] H.B. Chin in A. Jorgy, P. Barbara, B. Deborab, and B. Paul (eds), *Methods of Vitamin Assay*, John Wiley and Sons, New York, 1985, pp. 497–514
- [11] M. Rizk, I. Nabin, T. Elmamlouk, and N. Said, *Bull. NRC, Egypt*, **23**, 437 (1998)

- [12] R. Bhushan and V. Parshad, *Biomed. Chromatogr.*, **8**, 196 (1994)
- [13] E. Postaire, M. Cisse, M.D. Le Hoang, and D. Pradeau, *J. Pharm. Sci.*, **80**, 368 (1991)
- [14] T.A. Konimtzis and I.N. Papadoyannis, *Mikrochim. Acta*, **1**, 147 (1979)
- [15] K. Randerath. *Thin-Layer Chromatography*, Academic Press, New York, 1963, p. 157
- [16] R. Zhang and Z.H. Ma, *Chin. J. Chromatogr. (Sepu)*, **7**, 243 (1989)
- [17] A. Navas Díaz, A. Guirado Paniagua, and F. García Sánchez, *J. Chromatogr. A*, **655**, 39 (1993)
- [18] M. Joneidi, M. Koleva, and O. Budevsky, *Pharmazie*, **30**, 453 (1975)
- [19] B. Bauer-Petrovska and L. Petrushevska-Tozi, *Int. J. Food Sci. Technol.*, **35**, 201 (2000)
- [20] Application #177 in TLC Cat. e2/5/0/1.98 PD, Machery–Nagel GmbH & Co. KG, Düren, Germany
- [21] *Dyeing Reagents for Thin Layer and Paper Chromatography*, E. Merck, Darmstadt, Germany, 1975, p. 20
- [22] K. Shimada, Y. Nagase, and U. Matsumoto, *J. Pharm. Soc. Jpn*, **89**, 436 (1968)
- [23] J. Sherma, C.M. O’Hea, and B. Fried, *J. Planar Chromatogr.*, **5**, 343 (1992)
- [24] J. Aloisi, B. Fried, and J. Sherma, *J. Liq. Chromatogr.*, **14**, 3269 (1991)
- [25] M.K. Perez, B. Fried, and J. Sherma, *J. Planar Chromatogr.*, **7**, 340 (1994)
- [26] E. Norfolk, S.H. Khan, B. Fried, and J. Sherma, *J. Liq. Chromatogr.*, **17**, 1317 (1994)