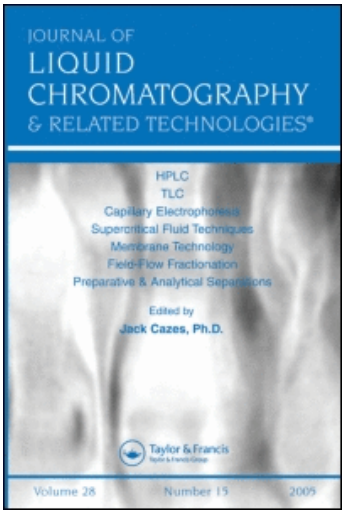

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 29 July 2004

To cite this Article Stroka, Joerg , Dasko, Lubomir , Spangenberg, Bernd and Anklam, Elke(2005) 'Determination of the Mycotoxin, Sterigmatocystin, by Thin-Layer Chromatography and Reagent-Free Derivatisation', Journal of Liquid Chromatography & Related Technologies, 27: 13, 2101 — 2111
To link to this Article: DOI: 10.1081/JLC-120039421
URL: http://dx.doi.org/10.1081/JLC-120039421

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 13, pp. 2101–2111, 2004

Determination of the Mycotoxin, Sterigmatocystin, by Thin-Layer Chromatography and Reagent-Free Derivatisation

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ABSTRACT

This paper describes a thin-layer chromatographic (TLC) method, which allows the determination of sterigmatocystin at a level of 2 μg/kg in various cereal grains of interest. Sterigmatocystin is extracted from the food matrix and further purified by phenyl-bond solid-phase extraction (SPE). The

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DOI: 10.1081/JLC-120039421 1082-6076 (Print); 1520-572X (Online)

separation and identification is performed on an amino-derivatised high-performance TLC (HP-TLC) plate. The derivatisation for densitometric measurement and visual inspection is achieved reagent free by heating the plate. Sterigmatocystin results in highly fluorescent spots. The method has shown good recovery values for the various cereals analysed (e.g., around 80% for wheat). The method was applied for monitoring the sterigmatocystin content in 85 cereal samples, purchased from the local (Italian) market in 2002. However, none of the samples was found to be positive, indicating that this mycotoxin was not a problematic contaminant in products of this particular region and in this specific year.

Key Words: Mycotoxin; Sterigmatocystin; TLC; Reagent-free derivatisation; Cereal grains.

INTRODUCTION

Sterigmatocystin (Fig. 1) is a mycotoxin produced by fungi of many *Aspergillus* species.[1] Its molecular structure is similar to those of aflatoxin B_1.[1] It is a precursor of aflatoxin B_1 in the biological transformation.[2] The steps involved in the biosynthesis of sterigmatocystin, as well as of aflatoxins, and the molecular characterisation of genes involved in the pathway are known and described in the literature.[3] Carcinogenic properties of sterigmatocystin were already studied and published.[4] The negative impact of sterigmatocystin on the DNA and on the tumor suppressor gene *p53*, in particular, was also studied.[5]

Relatively high levels (ppm range) of sterigmatocystin were detected in dwellings contaminated by *Aspergillus versicolor* caused by water flooding.[6]

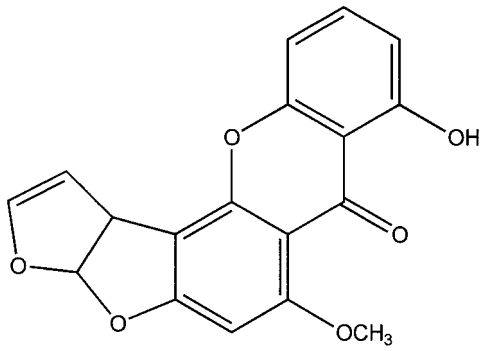

Figure 1. Structural formula of sterigmatocystin.

Contamination of cereals with *Aspergillus* fungi is a serious health risk, due to the potential of sterigmatocystin production by these fungi.

Very little data is available concerning monitoring of foodstuffs for sterigmatocystin. One of the few surveys in Europe was carried out in 1983 in the United Kingdom. Out of 523 analysed samples (about 3%) 17 were found positive.[7] The drawback of this survey was that the method had a relatively high limit of detection (LOD) of 20 μg/kg. This fact did not allow any conclusion concerning lower contamination levels, which are also of interest. Another larger survey was done in Brazil,[8] in the same decade, where sterigmato-cystin could be detected in none of the samples. However, again the LOD of the method applied was high (35 μg/kg).

These rather high LOD values that were obtained with conventional (non-mass spectrometric) methods are rather unsatisfactory, since some countries have set already relatively low maximum levels for sterigmatocystin (e.g., Czech Republic and Slovakia at the level 5 μg/kg for rice, vegetables, potatoes, flour, poultry, meat, milk, and 20 μg/kg for other foods).[9]

In order to evaluate human exposure to this mycotoxin and, more importantly, to monitor food products for existing or future legal compliance, suitable and simple analytical procedures are required that allow the precise determination of sterigmatocystin.

Methods have been described in the literature, which allow the detection of sterigmatocystin at levels ranging from 100 to 2 μg/kg.[10–15] However, it has been recognised that the surveillance of sterigmatocystin below 20 μg/kg was difficult in the past due to the weak fluorescence of this mycotoxin, and that a precise determination below this level requires mass-spectrometric detection.[10]

The most frequently applied analytical methods so far, are based on thin-layer chromatography (TLC) with fluorescence detection.[8,11–13] Because of its weak native fluorescence, a derivatisation procedure is generally used to visualise the sterigmatocystin on the developed TLC plates.[11] The most common derivatisation approach is spraying of the TLC plate with aluminium chloride ($AlCl_3$) solution, after development and heating of the plate. With such procedures, LODs were reported to be 50 μg/kg[11] and 140 μg/kg,[12] in cereal grains. A validated official method is also based on TLC with $AlCl_3$ spraying,[13] and is reported to have a limit of quantification (LOQ) of 100 μg/kg. Other methods are based on high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, and were applied for determination of sterigmatocystin in rice inoculated by *A. versicolor* at lower mg/kg levels.[14,15] Application of HPLC with post column derivatisation with $AlCl_3$ has also been reported,[15] with results comparable to the official TLC method.[13] The lowest detection limit of 1.7 μg/kg for maize and 1.9 μg/kg for bread was reported when liquid chromatography with mass spectrometric detection (LC–MS) was applied.[10] A method based on gas

chromatography with mass spectrometric detection (GC–MS), reported an LOD of 5 μg/kg for sterigmatocystin in wheat.[16]

In conclusion, currently the only methods with sufficient LOQs of sterigmatocystin at levels below 5 μg/kg are based on LC–MS and GC–MS.

For routine and monitoring purposes, it would be of interest to have at hand a simple, ecologically and economically friendly method.

Several publications have demonstrated simple derivatisation procedures for detection of substances after TLC. Simple heat treatments or spraying with acids or alkali has been shown to generate fluorescence in various substances, e.g., certain pesticides.[17] Fluorescent derivatives of steroid hormones were induced by thermal treatment of TLC plates in the presence of ammonium hydrogen carbonate.[18] A simple and effective method was developed for fluorometric detection of glucose, fructose, and other carbonylic compounds after TLC separation on amino-silica gel.[19] A reagent-free derivatisation procedure was also successfully applied for fluorescence induction in various carbohydrates,[20] uric acid, and creatinine[21] or catecholamines.[22]

Therefore, the aim of this paper was to develop a simple TLC-based method with reagent-free derivatisation, suitable for routine analysis of sterigmatocystin at levels of interest in the lower μg/kg range. In addition, the application of the method developed to cereal-based food products purchased from the local (Italian) market was shown.

EXPERIMENTAL

Materials

All solvents were of HPLC grade. Reagents, such as potassium chloride (Merck, Darmstadt, Germany) and aluminium chloride (Merck) were of analytical grade.

Sterigmatocystin was purchased from Sigma-Aldrich (Italy). Food samples (40 cereals) for investigation were purchased from local food markets (Italy) in 2002.

Based on the information on solubility and stability of sterigmatocystin in different solvents,[23] the stock solutions were prepared with 875 μg/mL in toluene : acetonitrile mixture 90 : 10 (v/v). Daily working standards with a concentration of 8.75 μg/mL were prepared by evaporation of an aliquot of the stock solution and re-dissolving in neat acetonitrile.

For separation, 20 × 10 cm high-performance TLC (HP-TLC) plates with NH_2-chemically bonded phase (with and without fluorescence indicator F_{254}), were used (Merck). The mobile (developing) phase was a mixture of toluene (100 mL) and acetone (5 mL).

Sample Preparation

An amount of 25 g of homogenised sample material was extracted with 95 mL of acetonitrile and 5 mL of 4% aqueous potassium chloride solution for 4 min, by means of a high-speed Ultraturrax® blender (IKA, Germany). For fortification experiments, the blank sample material was spiked with the sterigmatocystin working standard at levels of 1.75, 3.5, 7.0, 10.5, 14.0, and 17.5 μg/kg. This was done the day before analysis to allow the solvent to evaporate. Extracts were filtered through Whatman® No.1 folded filter and an aliquot of 50 mL of the filtrate was taken to dryness with a rotary evaporator at 150 mbar at 48°C (Buchi, Germany).

The residue was removed from the evaporation flask with toluene (3 × 500 μL), and the combined toluene layers were subsequently evaporated in a small amber vial under a gentle stream of nitrogen. The resulting residue was then re-dissolved in 200 μL of methanol.

Sample Clean-Up

Solid-phase extraction (SPE) phenyl columns of 3 mL syringe capacity with 500 mg SPE material (Sigma-Aldrich) were conditioned with 3 mL of methanol, followed by 5 mL of water prior to use. The re-dissolved methanol extract was quantitatively transferred on the SPE column by washing the vial with an additional 2 × 100 μL of methanol : water 40 : 60 (v/v). The SPE column was then washed with 3 mL of methanol : water 40 : 60 (v/v) and the sterigmatocystin was eluted with 10 mL of methanol : water 55 : 45 (v/v). The resulting eluate was evaporated to dryness under nitrogen at 60°C, and then re-dissolved quantitatively with toluene (3 × 500 μL) and transferred into a smaller (2 mL) amber vial. The toluene was evaporated under nitrogen and the vial with the residue was sealed with a cap. An amount of 200 μL of acetonitrile was injected through the sealed vial with a micro-litre syringe to avoid evaporation of the solvent, and shaken for 10 min.

TLC

A volume of 40 μL of this solution was then sprayed on the HP-TLC plate with a TLC-spraying device (Linomat IV, CAMAG, Switzerland) at 1 cm from the bottom of the plate. Spots were dried with warm air prior to developing the plate in a horizontal unconditioned and unlined tank for a distance of at least 7 cm. Developed plates were dried with warm air to remove the mobile phase and were subsequently heated in an oven for 15 min at 150°C.

The resulting yellowish fluorescent spots were quantified with a TLC scanner (CAMAG) at an excitation wavelength of 366 nm (mercury lamp), and using a 400 nm cut-off filter for emission or the diode-array scanner for spectrum elucidation.

For spectral scans (absorption and fluorescence) a diode-array TLC-Scanner (J&M, Aalen, Germany) was used with either the standard lamp for UV absorption or a UV-light emitting diode (LED) with a peak emission line of 370 nm (Nichia, Germany) for fluorescence measurement.

RESULTS AND DISCUSSION

The separation of sterigmatocystin on amino-bond TLC plates was found to be suitable with the above-mentioned mobile phase, resulting in R_f-values of 0.47–0.50.

For the optimisation of the derivatisation parameters different temperatures ranging from 100°C up to 200°C and heating times between 5 and 15 minutes were tested. Already, at 100°C within 5 min, significantly enhanced fluorescence was observed for sterigmatocystin. Further increase in fluorescence was observed when heating for 15 min at 150°C, while additional heating time up to 30 min had no further positive effect. Also, there was no observed improvement when heating at higher temperatures up to 200°C for 15 min, while heating at 200°C for 1 hr resulted in the extinction of fluorescence.

After optimisation of the derivatisation conditions as described, analytical results were obtained with this method and compared with the commonly used separation on silica gel plates and derivatisation with $AlCl_3$ solution.[13] For this reason, different sterigmatocystin amounts (1–10 ng absolute) were spotted on two different TLC plates (silica gel 60 and amino plate), developed, and derivatised (Fig. 2). The amino plate method revealed a much better performance such as homogeneity of the derivatisation, improved spot form, and stability of R_f-values within one TLC plate. Especially, the problem of a non-linear solvent front, as it was observed for the silica gel plates, did not occur with the amino TLC plates. In addition, the developed and derivatised plates were stable for several days, without any loss in fluorescence intensity, showing that the stability of the formed fluorescent derivative is excellent. For further elucidation of the optical properties of the formed derivative, spectral absorption and fluorescence scans were made (Fig. 3).

An interesting observation was that calibration curves established with the here-described method revealed several advantages. In fact, for both derivatisation methods, calibration curves were found not to be linear, while the slope on amino plates was found to be bigger at lower concentrations

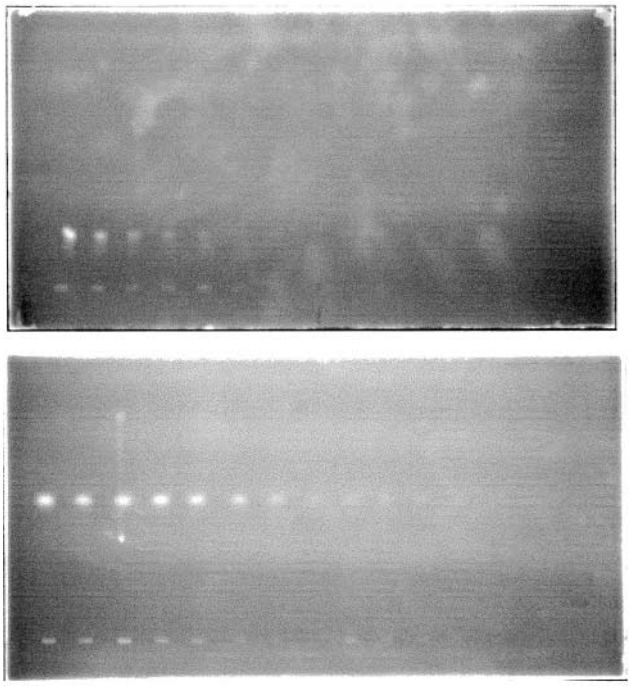

Figure 2. Derivatisation of sterigmatocystin on silica gel HP-TLC plates with $ALCl_3$ (upper plate) and on amino HP-TLC plates after heating (lower plate).

compared with the slope obtained with $AlCl_3$-solution. As a result, this offers a lower detection limit (Fig. 4).

In order to apply this new separation method for the determination of sterigmatocystin in cereal grains, a new sample clean-up procedure has been tested and applied. The main goal of the phenyl SPE clean-up procedure was not only to provide a sufficiently clean extract, but also to avoid chlorinated solvents such as chloroform, as it has been used in most methods previously published.[7,8,12–14,16]

Method performance criteria were obtained by analysing fortified samples (wheat and rice). The LOD and LOQ were found to be 2 and 6 $\mu g/kg$, respectively, for wheat flour, as well as 4 and 11 $\mu g/kg$, respectively, for rice. These parameters were calculated from the confidential interval of the calibration curve. The recovery for both matrices was 80% at 20 $\mu g/kg$.

A total number of 85 samples (various cereal-based products) from northern Italy were analysed with this method. A typical densitogram from such a material (fortified and a blank) is shown in Fig. 5. Despite the fact that this

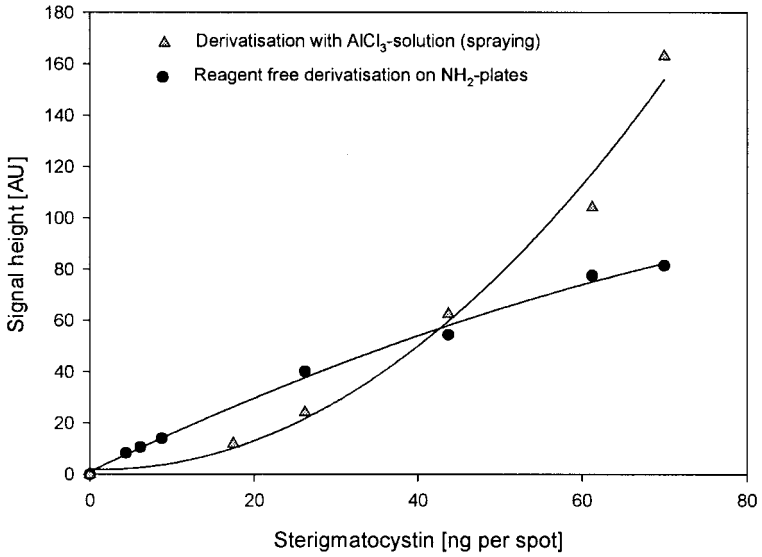

Figure 3. Direct comparison of the calibration graphs obtained with the different derivatisation methods.

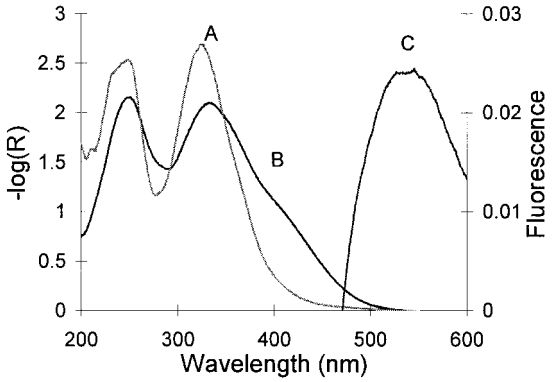

Figure 4. Absorption and fluorescence spectra of sterigmatocystin on amino HPTLC plates. Line (A), prior to heating; line (B), after 15 min at 150°C; line (C), fluorescence after heating for 15 min at 150°C. The characteristically intense yellow colour under UV light is due to the absorption in the range 400–500 nm. Lines A and B are recorded on different plates and are, therefore, not directly comparable in intensities. The fluorescence spectrum has been recorded with a 800-μW LED.

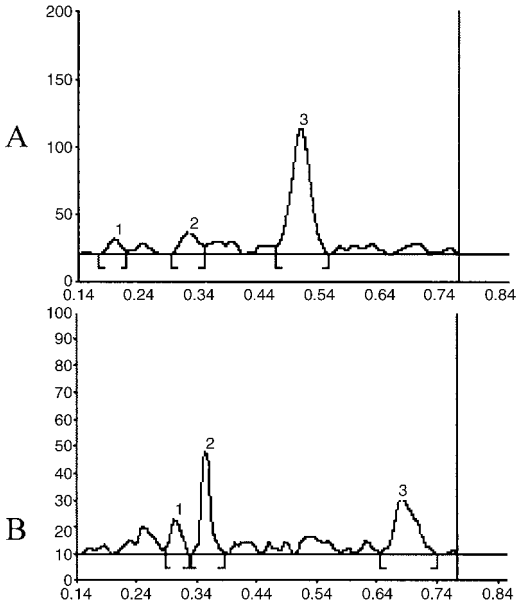

Figure 5. Chromatograms of fortified (A) and blank (B) samples. Sterigmatocystin is identified as peak (no. 3) with an R_f-value of 0.49. The level of fortification was 10 μg/kg.

simple method allowed determining considerably low levels of sterigmatocystin, in none of the tested samples could sterigmatocystin found.

CONCLUSION

Separation and derivatisation of sterigmatocystin on amino bonded TLC plates is a suitable tool for the fluorimetric quantification of sterigmatocystin at low levels, due to the formation of a highly fluorescent derivative. It was shown that this separation and derivatisation system reveals better results then the commonly used derivatisation with $AlCl_3$ solution on silica gel TLC plates, as has been previously published.

The suggested method provides comparable results from the point of view of LOD and LOQ, with the applied LC–MS method[10] yielding an average recovery of 80%. The clean-up SPE used allowed avoiding chlorinated solvents.

The fact that none of the tested samples from the local (Italian) market was found to be positive for sterigmatocystin indicates that this mycotoxin was not a frequent contaminant in the products of this particular region and in this specific year (2002). This situation, however, can only be regarded as a "snapshot," as geographical and environmental conditions such as temperature or rainfall have an influence on the mycotoxin occurrence.

REFERENCES

1. Mucke, W.; Lemmen, Ch. *Schimmelpilze–Vorkommen–Gesundheitsgefahren–Schutzmassnahmen, Ecomed.*; Landsberg, Germany, 1998.
2. Betina, V. *Mycotoxins: Chemical, Biological, and Environmental Aspects. Bioactive Molecules*; Elsevier: Amsterdam, The Netherlands, 1998; Vol. 9.
3. Sweeney, M.J.; Dobson, A.D.W. Mini review. Molecular biology of mycotoxin biosynthesis. FEMS Microbiol. Lett. **1999**, *175*, 149–163.
4. Web page: http://www.who.int (accessed February 2002).
5. Tong-Xi, X.; Junichi, M.; Kazuo, A.; Wen-Yuan, Z.; Shu-Ying, L. Absence of p53-mediated G1 arrest with induction of MDM2 in sterigmatocystin-treated cells. Int. J. Oncology **2000**, *17*, 737–742.
6. Nielsen, K.F.; Gravesen, S.; Nielsen, P.A.; Andersen, B.; Thrane, U.; Frisvad, J.C. Production of mycotoxins on artificially and naturally infested building materials. Mycopathologia **1999**, *145*, 43–56.
7. Buckle, A.E. The occurrence of mycotoxins in cereals and animal feedstuffs. Veterinary Res. Comm. **1983**, *7*, 171–186.
8. Valente Soares, L.M.; Rodriguez-Amaya, D.B. Survey of aflatoxins, ochratoxin A, zearalenone and sterigmatocystin in some Brazilian foods by using multi-toxin thin-layer chromatographic method. J. Assoc. Off. Anal. Chem. **1989**, *72*, 22–26.
9. *Food Codex of Slovak Republic on Food Additives and Contaminants*; Part 2, 29 April 2003; Chapter 3.
10. Scudamore, K.A.; Hetmanski, M.T.; Clarke, P.A.; Barnes, K.A.; Startin, J.R. Analytical methods for determination of sterigmatocystin in cheese, bread and corn products using HPLC with atmospheric pressure ionization mass spectrometric detection. Food Add. Contamin. **1996**, *13*, 343–358.
11. Athnasios, A.K.; Kuhn, G.O. Improved thin-layer chromatographic method for the isolation and estimation of sterigmatocystin in grains. J. Assoc. Off. Anal. Chem. **1977**, *60*, 104–106.
12. Gimeno, A. Thin-layer chromatographic determination of aflatoxins, ochratoxins, sterigmatocystin, zearalenon, citrinin, T-2 toxin, diacetoxyscirpenol, penicillic acid, patulin and penitrem A. J. Assoc. Off. Anal. Chem. **1979**, *62*, 579–585.

13. AOAC official method 973.38 sterigmatocystin in barley and wheat. In *AOAC Official Methods of Analysis*; 1995; Chapter 49, 43.
14. Schmidt, R.; Mondani, J.; Ziegenhagen, E.; Dose, K. High performance liquid chromatography of the mycotoxin sterigmatocystin and its application to the analysis of mouldy rice for sterigmatocystin. J. Chromatogr. **1981**, *207*, 435–438.
15. Neely, F.L.; Emerson, C.S. Determination of sterigmatocystin in fermentation broths by reversed-phase high performance liquid chromatography using post-column fluorescence enhancement. J. Chromatogr. **1990**, *523*, 305–311.
16. Salhab, A.S.; Russell, G.F.; Coughlin, J.R.; Hsieh, D.P.H. Gas–liquid chromatography and mass spectrometric ion selective detection of sterigmatocystin in grains. J. Assoc. Off. Anal. Chem. **1976**, *59*, 1037–1044.
17. Mallet, V.; Surette, D.P. Fluorescence of pesticides by treatment with heat, acid or base. J. Chromatogr. **1974**, *95*, 243–246.
18. Segura, R.; Gotto, A.M., Jr. A new fluorimetric procedure for the detection and quantitation of organic compounds in thin-layer chromatography. J. Chromatogr. **1974**, *99*, 643–657.
19. Klaus, R.; Fischer, W.; Hauck, H.E. Use of a new adsorbent in the separation and detection of glucose and fructose by HPTLC. Chromatographia **1989**, *28*, 364–366.
20. Klaus, R.; Fischer, W.; Hauck, H.E. Application of a thermal in situ reaction for fluorometric detection of carbohydrates on NH_2-layers. Chromatographia **1990**, *29*, 467–472.
21. Klaus, R.; Fischer, W.H.; Hauck, E. Qualitative and quantitative analysis of uric acid, creatine and creatinine together with carbohydrates in biological material by HPTLC. Chromatographia **1991**, *32*, 307–316.
22. Klaus, R.; Fischer, W.; Hauck, H.E. Extension of the application of the thermal in-situ reaction on NH_2 layers to the detection of catecholamines in biological materials. Chromatographia **1993**, *37*, 133–143.
23. Septien, I.; Cutuli, M.T.M.; Garcia, E.; Suarez, G.; Blanco, J.L. Solubility and stability of sterigmatocystin in different organic solvents. Toxicon **1993**, *31*, 1337–1340.

Received January 5, 2004
Accepted January 19, 2004
Manuscript 6297