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Short communication

Determination and validation of a simple high-performance liquid chromatographic method for simultaneous assay of iprodione and vinclozolin in human urine

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Abstract

A method based on solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) was developed for the simultaneous determination of 3-(3,5-diclorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione (vinclozolin) and 3-(3,5-diclorophenyl)-*N*-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide (iprodione) in human urine. Urine samples containing vinclozolin and iprodione were collected by solid phase extraction using C_{18} cartridges. The chromatographic separation was achieved on a Spherisorb ODS2 (250 mm × 4.6 mm, 5 μm) column with an isocratic mobile phase of acetonitrile–water (60:40, v/v). Detection was UV absorbance at 220 nm. The calibration graphs were linear from 30 to 1000 ng/mL for the two fungicides. Intra- and inter-day R.S.D. did not exceed 2.9%. The quantitation limit was 50 ng/mL for vinclozolin and 30 ng/mL for iprodione, respectively.

Keywords: Pesticides; HPLC; Human urine; SPE

1. Introduction

Vinclozolin or 3-(3,5-diclorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione (Fig. 1a) and iprodione or 3-(3,5-diclorophenyl)-*N*-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide (Fig. 1b) are two agrochemical fungicides widely used in agriculture [1–3] for the control of fungal diseases in a variety of vegetables and fruits in Italy 1981. Even low-level exposures may be associated with adverse health effects such as endocrine disruption, chemical sensitivity, and cancer [4]. Vinclozolin, for instance, is a proven endocrine disruptor [5,6] causing anti-androgenic effects. Following prenatal and perinatal exposure to low doses of vinclozolin, male rats developed sex organ changes including retained nipples, reduced ejaculated sperm numbers, and reduced ventral prostate weight [7,8]. Young male rats exposed to vinclozolin showed delayed puberty [9]. These anti-androgen effects are caused by two of its metabolites, which are able to bind to the androgen receptor and block its activity [10,11]. Due to the fact that vinclozolin was found to develop tumours in animal experiments, its genotoxicity is also discussed [12–15]. Moreover iprodione is an anti-androgen with anti-maleness properties similar to vinclozolin. It is able to block androgen binding to the human androgen receptor. Male offspring of rats exposed to iprodione e during pregnancy and early lactation showed a range or reproductive deformities such as permanent nipples and malformed penises [15]. The growing toxicological concern over vinclozolin and iprodione have prompted studies on analytical techniques for better monitoring these pesticides in agricultural products. Methods used to analyse fungicides and their residues are similar to those used for other pesticides. Gas chromatography (GC) is a classical method, and presently GC–MS is often used [16–19] and liquid chromatography (LC) is more widely recommended for thermally and unstable labile compounds, usually in conjunction with UV, fluorimetric detection or with a mass spectrometry system [20–28]. Rodriguez et al. have developed a micellar electrokinetic capillary chromatography method for the simultaneous determination several residues

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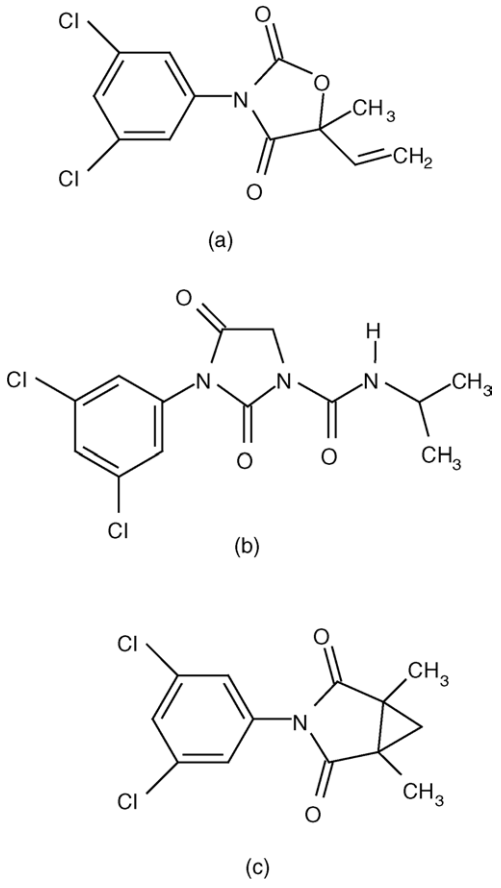

Fig. 1. Chemical structures of vinclozolin (a), iprodione (b) and internal standard (c).

of fungicides from vegetables [29]. The determination of vinclozolin metabolites has also been achieved in human urine by HPLC with electrochemical detection [30]. So far no method available, for the simultaneous determination of these xenobiotics in biological fluids, has been described. The aim of the present work was to develop and validated an analytical methods for the determination vinclozolin and iprodione in human urine of greenhouse operators.

2. Experimental

2.1. Chemicals and materials

Iprodione, vinclozolin and procymidone (internal standard; Fig. 1c) were purchased by Dr. Ehrenstorfer GmbH. The purity of the supplied iprodione, vinclozolin and procymidone were confirmed to be 99.5% by HPLC analysis. Acetonitrile (HPLC grade) was obtained from Carlo Erba Reagenti (Milan, Italy). Methanol, chloroform, all analytical-grade reagents were purchased from Fluka Chemika-BioChemika (Buchs, Switzerland). Water (HPLC grade) was obtained by passage through the ELIX 3 and Milli-Q Academic water purification systems (Millipore, Bedford, MA, USA). Octadecyl 200 mg Isolute cartridges from Argonaut (Stepbio, Bologna, Italy) were used for SPE.

2.2. Standard solutions and samples

Stock solutions of vinclozolin, iprodione and procymidone (internal standard) were prepared by dissolving 10 mg of each compound in 10 mL of acetonitrile. These solutions could be stored at −20 °C for over one month no evidence of decomposition. Standard solutions, each containing the three pesticides, were obtained by diluting the stock solutions with blank urine in the range 10–1000 ng/mL. For each solution the internal standard was added at a constant level of 100 μL of a 100 μg/mL acetonitrile solution. These standards were treated concurrently in the same manner as the samples to be analysed. Calibration graphs were obtained by plotting the peak-area ratios of vinclozolin or iprodione to internal standard against the drug concentrations obtained after extraction.

2.3. Equipment

HPLC analysis was carried out using a chromatographic system composed of the following: a model 515 pump and a model 996 diode array detector (Waters, Milford, MA, USA). A model 7725i sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20 μL loop was used. Chromatographic data management was automated using a software Millennium[32] (Waters, Milford, MA, USA).

The analysis was performed on an analytical 250 mm × 4.6 mm i.d. reversed-phase Spherisorb ODS2 5 μm particle size) column (Waters, Milford, MA, USA), protected by a 20 mm × 4.6 mm i.d. (40 μm particle size) disposable Pelliguard precolumn (Supelco, Bellefonte, PA, USA). The mobile phase consisted of a mixture of acetonitrile and water (60:40, v/v). Water prior to use was filtered through WCN 0.45 μm filter, while acetonitrile through WTP 0.5 μm filter (Whatmann, Ltd., Maidstone UK). The mobile phase was prepared daily, degassed using an in-line degasser (Waters, Milford, MA, USA) and delivered at a flow rate of 1.0 mL/ min. Column eluate was monitored at 220 nm. All measurements were carried out at room temperature.

2.4. Urine collection and storage

Urine samples from human volunteers were collected and kept frozen at −20° until use. After the urine samples had been thawed, they were shaken for homogenisation. The required volume was then sampled as quickly as possible to avoid sedimentation of any solids.

2.5. Sample preparation

Samples were thawed just before the extraction procedure, thoroughly agitated and centrifuged at 1000 × *g* for 10 min. The Isolute cartridges, mentioned above, were placed in a luer that fitted the top of the Supelco vacuum manifold, which may be loaded with up to 12 cartridges. A vacuum of 250–500 Torr was applied to the manifold to carry out the various steps of the extraction. A 5.0 mL chloroform–methanol mixture (9:1, v/v) rinse followed by 5 mL of methanol served to desorb any organic

impurities from the cartridges and to wet the silica packing before introducing of the urine samples. One hundred microliters of internal standard added to 20 mL of urine were passed through the cartridge, followed by 10 mL of water. The effluent was discarded. Three milliliters of chloroform–methanol mixture (9:1, v/v) were then applied to the cartridge and the eluate collected. This fraction was finally centrifuged, transferred to a new tube and evaporated to dryness with a nitrogen stream under vacuum utilizing the Supelco Drying Attachment. After reconstitution of the residue with 500 μL of mobile phase, the sample was mixed on a vortex agitator filtered using filter with pore size of 0.22 μm and 20 μL was injected into the HPLC system.

3. Result and discussion

A series of studies were conducted in our laboratory in order to develop a convenient and easy-to-use method for quantitative analysis of iprodione and vinclozolin. Several high-performance liquid chromatography (HPLC) method variables with respect to their effect on the separation of iprodione, vinclozolin and internal standard from the matrix were investigated. In our extensive preliminary experiments a set of column packing including C_8, C_{18}, and SymmetryShield with different lengths and particle size was tested. The final choice of the stationary phase giving satisfactory peak shape, resolution and run time was a Spherisorb ODS2 80 Å (250 mm × 4.6 mm i.d.; 5 μm particle size). Also, a series of aqueous mobile phase containing different modifiers including acetonitrile, methanol with different fractions were tested. The elution was monitored in the whole UV region and the wavelength of 220 nm exhibited the best detection. The amount of organic modifier present in the mobile phase influences analytes that are retained predominantly by adsorption onto the stationary phase. The optimal amount of organic modifier that should be used for the separation of iprodione, vinclozolin and internal standard (good separation between three peaks, short time of analysis) can be obtained when the percentage of acetonitrile in the mobile phase is 60%. From these data it was determined that mobile phase consisted of 40% water and 60% acetonitrile would provide good retention for iprodione vinclozolin and internal standard as well as acceptable run time of less than 12 min for the separation. In addition different cartridges for solid phase extraction (Isolute C_{18} and Isolute 101) were tested in order to obtain satisfactory values for recovery of iprodione, vinclozolin and internal standard were obtained when human urine were extracted with Octadecyl Isolute cartridges. When solid-phase extraction with Octadecyl Isolute cartridge was used for sample preparation, values for recovery for iprodione, vinclozolin and internal standard ranged from 89 ± 1.6 to 96 ± 2.5%. On other hand, when solid-phase extraction was performed on Isolute 101 cartridges, values for recovery ranged from 67.8 to 70.4% for xenobiotics.

Recoveries of vinclozolin and iprodione from spiked samples were calculated by comparing the peak area of human urine with standards at low medium and high concentration levels, and submitted to the sample preparation procedure; with those obtained from the analysis of corresponding directly-injected standards

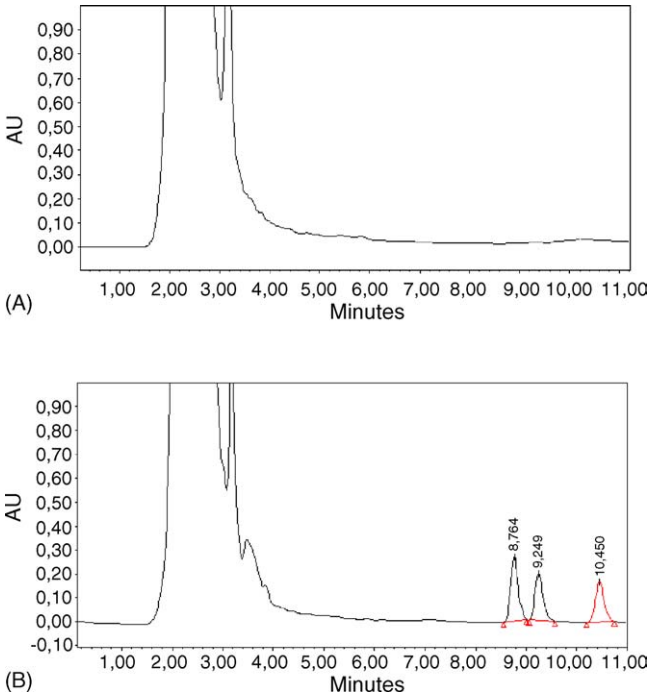

Fig. 2. Typical chromatograms of human urine samples after extraction: (A) blank human urine; (B) blank human urine spiked with iprodione (50 ng mL^{-1}), internal standard (30 ng mL^{-1}), and vinclozolin (200 ng mL^{-1}).

(n = 5). The extraction recoveries of iprodione and vinclozolin in human urine were 89 ± 1.6 and 96 ± 2.5%.

Typical chromatograms of the blank human urine (A) and urine sample spiked with iprodione, vinclozolin and internal standard (B) are shown in Fig. 2A and B, respectively .The elution peaks do not show any interferences deriving from other human urine components and are characterized by retention times of 8.8 (iprodione), 9.2 (internal standard) and 10.5 (vinclozolin) min. Typical chromatograms of the human samples urine obtained from greenhouse operators are shown in Fig. 3C and D.

Figs. 2 and 3 illustrates representative chromatograms of a blank human urine, blank human urine spiked with vinclozolin and iprodione, and human urine samples obtained from greenhouse operators. The selectivity of the assay was determined by analysis of blank human urine from seven different subjects, with e without internal standard. Under these chromatographic conditions no endogenous sources of interference were observed in human urine, and the resolution between vinclozolin and iprodione was satisfactory (Fig. 2).

The detection limit (LOD) for each fungicide was determined with spiked samples analysis at a signal-to-noise of three after the SPE extraction. The estimated limit of detection for iprodione and vinclozolin was 10 ng/mL. The limit of quantification was defined as the lowest amount detectable with a precision of less than 15% (n = 3) and an accuracy of ± 15% (n = 3). The limit of quantification (LOQ) for vinclozolin and iprodione were 50 ng/mL for vinclozolin and 30 ng/mL for iprodione, respectively.

Calibration curves for HPLC analysis were obtained by plotting the peak-area ratio of each analyte to internal standard

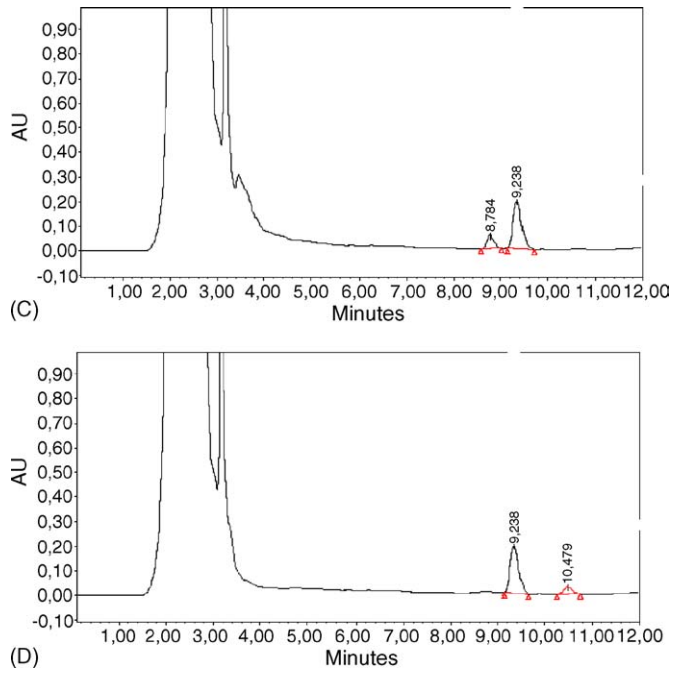

Fig. 3. Chromatograms of human urine samples of two greenhouse operators after extraction. (C) after applications of iprodione and (D) vinclozolin, respectively. The concentration calculated are (42.5 ng mL^{-1}) and (64.4 ng mL^{-1}) for iprodione and vinclozolin.

versus its concentration. The equations, obtained through regressional analysis of data for the above standard solutions (each datum average of a minimum of five determinations) were: for iprodione $y = 74494x - 743.32$ ($r = 0.9986$) and for vinclozolin $y = 49992x - 132.73$ ($r = 0.9972$), where y is the peak-area ratio in the arbitrary units of the detector used and x is the analyte concentration (ng/mL). The accuracy of the assay method was defined as the percentage of the systematic error, which is calculated as the standardised agreement between the measured value and the true value. To be acceptable, the measures should be within ±15% at all concentrations. The accuracy values in intra-day variation studies at low, medium and high concentrations for vinclozolin and iprodione in human urine were into acceptable limits (Table 1). The precision of a method is expressed as the relative standard deviation (R.S.D.) of replicate measurements. To be acceptable, the measures should be within ±15% at all concentrations. In this work precision of the method was tested by both intra- and inter-day reproducibilities in human urine.

3.1. Intra-day variability of the assay

The intra-day variability of the assay was determined by repeated analysis of quality control samples at low, medium and high concentrations on the same day. Results are shown in Table 2. These data indicate that the assay method is reproducible within the same day.

3.2. Inter-day variability of the assay

The inter-day variability of the assay was determined by repeated analysis of quality control samples at low, medium and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at −20 °C until analysis. Results are showed in Table 2. These data indicate that the assay method is reproducible within different days.

Figs. 2 and 3 illustrates representative chromatograms of a blank human urine, blank human urine spiked with vinclozolin and iprodione, and human urine samples obtained from greenhouse operators. The elution peaks are lacking in interferences deriving from other human urine components and are characterized by retention times of 8.8 (iprodione), 9.2 (internal standard) and 10.5 (vinclozolin) min.

The optimisation of the analytical procedure has been carried out by varying the following: reversed-phase column used, mobile phase composition, flow rate and wavelength. The degree of reproducibility of the results, obtained through small deliberate variations in method parameters changing instruments and operators, has been very satisfactory. In fact, the percent of recovery for iprodione and vinclozolin was acceptable under most conditions and did not show any significant change when the critical parameters were modified. The tailing factor for vinclozolin and iprodione was always less than 1.4 and the components were well separated under all the changes. Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying the experimental at room temperature would conclude that the method conditions are robust. The solid-phase extraction procedure eliminates endogenous interference, which is frequently present in biological sample. The filtration of extracts before injection onto chromatographic column avoids rapid obstruction of the pre-column, increasing its life. The main advantage of this method in comparison with previous papers report in literature [28,29] is the rapid SPE procedure for the preparation of the biological

Table 1
Accuracy of HPLC method for determining vinclozolin and iprodione in human urine samples

Concentration added (ng/mL)	Vinclozolin		Iprodione	
	Concentration found (mean ± S.D.) (ng/mL)	Accuracy (%)	Concentration found (mean ± S.D.) (ng/mL)	Accuracy (%)
50	48.6 ± 0.5	−2.8	49.3 ± 1.2	−1.4
500	489.1 ± 0.9	−2.1	497.8 ± 0.4	−0.4
1000	997.6 ± 0.8	−0.2	996.8 ± 0.8	−0.3

$n = 5$; S.D.: standard deviation.

Table 2
Intra- and Inter-day variability of HPLC method for determining vinclozolin and iprodione in human urine samples

Concentration added (ng/mL)	Vinclozolin urine samples		Iprodione urine samples	
	Concentration found (mean ± S.D.) (ng/mL)	R.S.D. (%)	Concentration found (mean ± S.D.) (ng/mL)	R.S.D. (%)
Intra-day				
50	48.9 ± 0.8	1.6	48.3 ± 0.6	1.2
500	497.2 ± 0.6	0.1	497.6 ± 0.7	0.1
1000	996.5 ± 0.9	0.1	998.7 ± 0.6	0.1
Inter-day				
50	48.9 ± 1.4	2.9	48.2 ± 1.4	2.9
500	497.2 ± 0.8	0.2	490.8 ± 0.9	0.2
1000	995.5 ± 0.5	0.1	998.1 ± 0.8	0.1

$n = 5$; R.S.D.: relative standard deviation (%); S.D.: standard deviation.

sample, which gives high extraction yields. The proposed HPLC method employing solid-phase extraction for sample preparation is simple and convenient for the determination of iprodione and vinclozolin in urine samples. The typical assay time is about 12 min. The relatively short retention time of iprodione and vinclozolin in our technique allows the analysis of a large number of samples in a short time. The proposed method is simply, rapid and provides efficient clean up of the complex biological matrix and high recovery of iprodione, vinclozolin and internal standard. In conclusion, the developed HPLC method with UV detection shows a good sensitivity and selectivity, and is suitable for reliable determination of iprodione, vinclozolin in human urine of greenhouse operators. The HPLC assay method presented here, is sensitive, specific and robust and should be of value for the quantification of iprodione and vinclozolin in human urine of greenhouse operators.

References

[1] M.L. Gullino, C. Alio, A. Garibaldi, Gent 55 (1990) 967.
[2] H. Yunis, Y. Elad, Y. Mahrer, Pestic. Sci. 31 (1991) 325.
[3] V. Solel, Y. Oren, K. Miriam, Crop. Protect. 16 (1997) 659.
[4] N.A. Ashfond, C.S. Miller, Chemical Exposure: Low Levels and High Stakes, Wiley, New York, 1988.
[5] W.R. Kelce, E.M. Wilson, J. Mol. Med. 75 (1997) 198.
[6] L.E. Gray, J. Ostby, W.R. Kelce, Toxicol. Appl. Pharmacol. 129 (1994) 46.
[7] L.E. Gray, J. Ostby, E. Monosson, W.R. Kelce, Toxicol. Ind. Health 15 (1999) 48.
[8] J. Ashby, P.A. Lefebre, Regul. Toxicol. Pharmacol. 26 (1997) 330.
[9] E. Monosson, W.R. Kelce, C. Lambright, J. Ostby, L.E. Gray, Toxicol. Ind. Health 15 (1999) 65.
[10] L.E. Gray, Toxicol. Lett. 102–103 (1998) 331.
[11] M. Vinggaard, E.C. Joergensen, J.C. Larsen, Toxicol. Appl. Pharmacol. 155 (1999) 150.
[12] P. Hrelia, C. Fimognari, F. Maffei, F. Vivagni, R. Mesirca, L. Pozzetti, G. Cantelli Forti, Mutagenesis 11 (1996) 445.
[13] V. Stadie, Umweltmedizin 17 (1997) 84.
[14] C.G. Smith, Am. J. Ind. Med. 4 (1983) 107.
[15] J. Ostby, W.R. Kelce, C. Lambright, C.J. Wolf, P. Mann, L.E. Gray, Toxicol. Ind. Health 15 (1999) 80.
[16] L.M.L. Nollet (Ed.), Handbook of Food Analysis, Marcel Dekker, New York, 1996, p. 1461.
[17] C.M. Torres, Y. Picó, J. Mañes, J. Chromatogr. A 754 (1996) 301.
[18] G.R. van der Hoff, P. van Zoomen, J. Chromatogr. A 843 (1999) 301.
[19] J.L. Bernal, M.J. Del Nozal, J.M. Rivera, J.J. Jimenez, J. Atienza, J. Chromatogr. A 754 (1996) 507.
[20] E. Hogendoorn, P. van Zoonen, J. Chromatogr. A 892 (2000) 435.
[21] Y. Picó, G. Font, J.C. Molto, J. Mañes, J. Chromatogr. A 882 (2000) 153.
[22] F. Menzinger, Ph. Schmitt-Kopplin, D. Freitag, A. Kettrup, J. Chromatogr. A 891 (2000) 45.
[23] P. Cabras, P. Diana, M. Meloni, F.M. Pirisi, R. Pirisi, J. Chromatogr. 256 (1983) 176.
[24] M. Sandahl, E. Ulfsson, L. Mathiasson, Anal. Chim. Acta 424 (2000) 1.
[25] E. Viana, J.C. Molto, G. Font, J. Chromatogr. A 754 (1996) 437.
[26] J. Oliva, S. Navarro, A. Barba, G. Navarro, J. Chromatogr. A 833 (1999) 43.
[27] P. Sandra, B. Tienpont, J. Vercammen, A. Tredoux, T. Sandra, F. David, J. Chromatogr. A 928 (2001) 117.
[28] F. Rouberty, J. Fournier, Chromatographia 41 (1995) 693.
[29] R. Rodriguez, Y. Picó, G. Font, J. Mañes, J. Chromatogr. A 924 (2001) 387.
[30] K. Wittke, H. Hajimiragha, L. Dunemann, J. Bergerow, J. Chromatogr. B 755 (2001) 215.