ELSEVIER

Journal of Chromatography A, 915 (2001) 209–216

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Improving gas chromatographic determination of residual solvents in pharmaceuticals by the combined use of headspace solid-phase microextraction and isotopic dilution

Silvia A. Coran*, Valerio Giannellini, Sandra Furlanetto, Massimo Bambagiotti-Alberti, Sergio Pinzauti

Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via Gino Capponi 9, I-50121 Firenze, Italy

Received 20 October 2000; received in revised form 12 February 2001; accepted 13 February 2001

Abstract

Cyclohexane and toluene were gas chromatographically determined via headspace solid-phase microextraction both in ketoprofen drug substance and ketoprofen capsules by a procedure relying on isotopic dilution (ID), an analytical tool derived from mass spectrometry (MS). This approach, using an internal standard method, gave mean precision and accuracy (RSD 2.56%, 2.97% and bias 0.21%, −0.99% for cyclohexane and toluene, respectively) not obtainable by the more commonly used external standard ones in the presence of real sample matrices. Optimisation of the operative conditions was also supported by experimental design. More generally, the proposed method, exploiting ID without resort to the costly MS instrumentation, could be recommended whenever opportune deuterated analogues of the target analytes and GC capillary columns able to separate all the peaks involved are ready available on the market.

Keywords: Pharmaceutical analysis; Experimental design; Isotopic dilution; Ketoprofen; Profens

1. Introduction

In manufacturing drug substances, residual solvents arising from the final purification by recrystallization, and also from one or more steps of the whole synthetic process, can be retained in the end products. Very often these solvents, referred to as organic volatile impurities (OVIs), are transferred to the pharmaceutical preparation concerned, making their determination mandatory. In fact, as the major part of OVIs are recognised to be toxic to various degrees, methods for their quantification are reported by the USP and European Pharmacopoeia, where inter alia the widespread but relatively expensive automated headspace gas chromatography (HS-GC) instrumental set-up is indicated. With these methods relative standard deviations (RSDs) as high as 15% are allowed [1,2].

From a qualitative point of view, in the last years headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography (HS-SPME–GC) has gained a sturdy reputation as a valid alternative to HS-GC. This is mainly because simplicity of execu-

*Corresponding author. Fax: +39-55-240-776.

E-mail address: coran@farmfi.scifarm.unifi.it (S.A. Coran).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00658-6

tion and low cost of the hardware involved are paramount among the many advantages of this solvent-free technique [3,4]. From a quantitative point of view, the most recently reported applications favour the external standard approach by the spiking procedure [5].

As is well known, SPME operates on the basis of an equilibrium established between the analytes contained in the sample matrix and the stationary phase coated on fibres [3]. The equilibrium is controlled by a number of parameters all crucial in obtaining the best analytical results. In these conditions, the choice of an internal standard procedure to set up a quantitative analytical method is highly recommended and should rely on the availability of an internal standard with a stationary phase affinity as close as possible to that of the corresponding analyte. A deuterated homologue complies with this requirement. In other words, the isotopic dilution (ID) strategy, typical of mass spectrometry (MS) analytical methods, could be applied in order to ensure that the HS-SPME absorption behaviour of the analytes and their internal standards are equal.

To test this approach we chose to determine cyclohexane and toluene present in ketoprofen (bulk drug substance and pharmaceutical preparation) as manufacturing residual solvents by comparing the performance of SPME–GC both in external and internal standard calibration modes. The ID internal standard procedure was carried out using [$^2H_8$]toluene and [$^2H_{12}$]cyclohexane as the internal standards. The aim was to improve the accuracy and RSD with respect to the currently available HS-GC official methods. HS-SPME optimisation was performed by using experimental design methodology.

2. Experimental

2.1. Chemicals and materials

Toluene (standard for GC; ≥99.9%) and cyclohexane (standard for GC; ≥99.7%) were purchased from Fluka (Buchs, Switzerland). [$^2H_8$]Toluene (100.0 atom % ^2H) and [$^2H_{12}$]cyclohexane (99.6 atom % ^2H) were obtained from Aldrich (Steinheim, Germany). Analytical-grade anhydrous ethanol and Na_2CO_3 were from Carlo Erba (Milan, Italy). The deionized water used was 18 MΩ from a Milli-Q filtration system (Millipore, Bedford, MA, USA).

Ketoprofen (drug substance) was obtained from SIMS (Reggello, Firenze, Italy).

Ketoprofen, 50 mg capsules (lactose and magnesium stearate as excipients), is a product currently marketed in Italy and was purchased locally.

SPME holder and polydimethylsiloxane (PDMS, 100 μm film) coated fibres were obtained from Supelco (Bellefonte, PA, USA).

A Capilettor five-step microliter piston pipette (Labora, Mannheim, Germany) was used to measure the chemical and deuterated standard amounts.

Crimp-top HS glass vials (10 ml) with PTFE-lined septa were selected for volume consistency.

2.2. Instrumental analysis

A Model 8320 Perkin-Elmer gas chromatograph (Perkin-Elmer Beaconsfield, UK), with a flame ionization detection (FID) system was used. A programmed-temperature vaporizer (PTV), fitted with a narrow bore (2 mm I.D.) quartz liner, was employed in the splitless mode. Analyses were carried out in 35°C isothermal runs on a Supelco MDN-1 fused-silica capillary column (30 m×0.25 mm I.D., 1.00 μm film thickness), carrier gas helium at 14 p.s.i. (1 p.s.i.=6894.76 Pa). Data handling was performed by Chromatographic 3 (version 3.0) software running on a Model 7500 Professional Computer (both from Perkin-Elmer, Norwalk, CT, USA).

Confirmation of the analyte identities in real samples was obtained by GC–MS in the electron impact mode (EI, electron energy 70 eV) using a Turbomass instrument (Perkin-Elmer) and the above described column.

2.3. SPME procedure

Extraction was performed in the headspace for 16 min without magnetic stirring. Reproducible extraction temperature at 20°C was assured using a water bath thermostat with a setting precision of ±0.1°C. Desorption was prolonged for 4 min into the gas chromatograph injection port set isothermally at 200°C in order to assure complete release of the analytes and avoid carry-over. The fibre was then let into the PTV to the end of the GC run.

2.4. Standard and sample preparations

Two stock solutions, one containing 43.1 mg of toluene and 19.5 mg of cyclohexane (as the chemicals stock solution) and the other 17.26 mg of [$^2H_8$]toluene and 7.79 mg of [$^2H_{12}$]cyclohexane (as the internal standards solution), were made in 100-ml volumetric flasks by dissolving in anhydrous ethanol the corresponding calculated volume of each compound (toluene 50.0 μl, cyclohexane 25.0 μl, [$^2H_8$]toluene 20.0 μl and [$^2H_{12}$]cyclohexane 10.0 μl).

The working chemicals solution containing 4330 ng ml^{-1} of toluene and 1950 ng ml^{-1} of cyclohexane was obtained by diluting 1 ml of the chemicals stock solution to 100 ml with aqueous 0.1 *M* Na_2CO_3.

The working standards solution containing 1890 ng ml^{-1} of [$^2H_8$]toluene and 890 ng ml^{-1} of [$^2H_{12}$]cyclohexane was obtained with the same procedure. These solutions could be preserved for a few days in zero-headspace glass vials at 4°C.

The ketoprofen drug substance solution was also prepared in 0.1 *M* Na_2CO_3 by dissolving and diluting 500.0 mg of this material to a volume of 100 ml.

The ketoprofen capsule solution was prepared in a 100-ml volumetric flask by dissolving the equivalent content of 10 capsules (average from 15 capsules) in 0.1 *M* Na_2CO_3, the resulting concentration being 5.0 mg ml^{-1}.

2.5. Calibration solutions

Volumes of 400, 300, 200, 100 and 50 μl of the chemicals working solution were pipetted into a series of vials, each containing 500 μl of the internal standard working solution. Opportune volumes of 0.1 *M* Na_2CO_3 were then added in order to obtain five 1-ml calibration solutions.

2.6. Accuracy and assay procedure

Volumes of 50, 200 and 400 μl of the chemicals working solution were pipetted into a series of vials, each containing 500 μl of the internal standard working solution. Opportune volumes of 0.1 *M* Na_2CO_3 were then added in order to give the samples used to assess the accuracy.

Volumes of 50, 200 and 300 μl of the working chemicals solution were added to a series of vials, each containing 500 μl of the internal standard working solution, 100 μl of ketoprofen drug substance solution and opportune volumes of 0.1 *M* Na_2CO_3, to obtain a spiking of 216, 866, 1299 ng of toluene and 97, 389, 583 ng of cyclohexane per ml.

The ketoprofen drug substance assay required three vials each containing 500 μl of the internal standard working solution, 250 μl of 0.1 *M* Na_2CO_3 and 250 μl of the ketoprofen drug substance solution.

For the ketoprofen capsule assay, three vials were similarly prepared.

3. Results

3.1. Optimisation of headspace microextraction

Preliminary results indicated that extracting ketoprofen in the simplest mode, i.e., the headspace–solid matrix system, gave both low sensitivity and high RSD. Some unsatisfactory data were collected by suspending ketoprofen in water under various operative conditions. Reasonably this occurred in spite of the large headspace–water partition coefficients of toluene and cyclohexane, because the solvents remain strongly embedded into the matrix. Consequently, in order to maximise the gas phase transfer in the headspace and to improve the method precision, we chose to set up the extraction procedure by dissolving ketoprofen in mild alkaline medium producing a homogeneous system favouring solvent release. The hydrocarbon nature of the analytes considered suggested the use of an absorption-type fibre [6] which ensures a wide linear dependence between the amount of an analyte extracted and its concentration in a sample. Consequently, the rugged PDMS fibre coating was selected with 100 μm phase thickness.

The development of a SPME method generally consists of a number of stages. In this study, we paid particular attention to the most critical factors such as the extraction time and extraction temperature. Agitation, which usually speeds up the absorption

Table 1
Experimental plan of the full factorial design 2^2

Experiment No.	U_1 (°C)	U_2 (min)	Peak area count
1	20	1	42 024
2	50	1	15 338
3	20	15	63 589
4	50	15	24 512
5	35	8	36 626
6	35	8	34 504

process at the expense of some experimental complication, was omitted since in our experiments the equilibrium in static conditions was obtained in only 13 min.

An experimental design strategy was then set up in order to reduce the number of experiments required to optimise peak areas. Since, under our experimental conditions, cyclohexane and toluene behaved similarly, this study was restricted to toluene only.

As is well known, statistical design is a way of choosing experiments efficiently and systematically to give reliable and coherent information. In particular, experimental design helps the researcher to verify if a change in factor values produces a statistically significant variation of the observed response.

In this case the considered factors were extraction temperature (U_1) and extraction time (U_2). The experimental domain ranged for temperature and time from 20 to 50°C and from 1 to 15 min, respectively.

The hypothesised empirical relationship between factors and response was linear and a full factorial design 2^2 was used to estimate the model coefficients. The experimental matrix included also two experiments at the central level. The experiments were carried out in a randomised order according to the experimental plan reported in Table 1 in which the obtained responses are also reported. The analysis of variance (ANOVA) pointed out that the regression model assumed was significant (Table 2), thus indicating that the change in the observed response was due to the level change of factors. Besides, ANOVA indicated that the postulated model fitted well the data in the experimental domain under study and that no curvature was present [7–9].

Table 3
Statistical analysis of effects

	Coefficient
b_0	36 098.833*
b_1	−16 440.750*
b_2	7684.750*
b_{12}	−3097.750*

*Statistical significant effect.

In order to identify the active factors, the change in level of which determined a statistically significant variation of the response, the statistical analysis of coefficients was considered (Table 3). Statistically significant coefficients were those with an absolute value greater than zero, with a probability level of 95%. The confidence interval was calculated starting from the estimate of coefficients, the estimate of standard error for each coefficient b_i, and the value of Student's t for a 95% probability level and $n-1$ degrees of freedom, where n is the number of replications. The estimate of standard error for each coefficient was given by $SD/\sqrt{k}$ where k is the number of experiments and SD is an estimate of standard deviation σ of the experimental response y [7]. In this case, all effects, including the interaction effect, were to be statistically significant for the response (Table 3). From this analysis, it was

Table 2
ANOVA for the linear model assumed

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio
Regression	$1.355 \cdot 10^9$	3	$4.519 \cdot 10^8$	290.97[a]
Residuals	$3.106 \cdot 10^6$	2	$1.553 \cdot 10^6$	
Lack-of-fit	$8.549 \cdot 10^5$	1	$8.549 \cdot 10^5$	0.3797[b]
Pure error	$2.251 \cdot 10^6$	1	$2.251 \cdot 10^6$	
Total	$1.358 \cdot 10^9$	5		

[a] $290.97 > F^{crit.} = 99.17$ (with 3 and 2 degrees of freedom and $\alpha = 0.001$).
[b] $0.3797 < F^{crit.} = 40.52$ (with 1 and 1 degrees of freedom and $\alpha = 0.001$).

possible to point out that to maximise the response it is important to move the extraction temperature factor toward the low level of the considered experimental domain, and extraction time toward the high level. The same conclusions can also be obtained from the response surface (Fig. 1). In particular, the effect of the interaction can be very easily seen in Fig. 1, where it is evident that the effect of the time is more relevant when the temperature is at the lower level. The optimal conditions found, in order to produce a high peak area, were temperature 20°C and time 15 min. This result is a clear demonstration of the superiority of the multivariate approach over the one-variable-at-a-time approach. In fact, in the latter case, the interaction would not have been detected.

As previously reported [10], these results confirmed that, in spite of a concentration increase in the headspace, higher temperatures lead to a greater decrease in the partition coefficient between the coating and the headspace. Thus, the equilibration time for the analytes was determined at 20°C. Since in 13 min both analytes were steady on the plateaux of their absorption profiles, the extraction time was finally set at 16 min to minimise analysis time without affecting the reproducibility of the whole procedure.

3.2. Calibration method

As a first step linearity, precision and accuracy were determined by an external standard procedure. The calibration points were obtained in triplicate at five levels over a range of 97–779 ng ml^{-1} for cyclohexane and 218–1732 ng ml^{-1} for toluene. The equations for the curves were calculated by linear regression analysis assuring method linearity over the mass range studied with satisfactory precision (not beyond the 5% RSD typical for SPME determination [11]). To assess the method reliability we decided to test the accuracy proceeding both by analysing aqueous solutions of the analytes within the concentration range of the calibration curve and by accuracy study on the real matrix (ketoprofen drug substance). This last study was carried out with the standard additions technique, as a solvent-free ketoprofen drug substance was not available. While the accuracy assessed by analysing the above mentioned solutions of known concentration gave acceptable results (for cyclohexane, bias = −2.87%, RSD = 3.23% and for toluene, bias = −3.07%, RSD = 5.67%), the accuracy was very unsatisfactory when the analytes were spiked into ketoprofen alkaline solutions (for cyclohexane bias = 10.32%, RSD = 8.27% and for toluene bias = 14.81%, RSD = 12.75%). The assay procedure also failed to determine the targets. The method built on the external standard procedure showed to be precise but not accurate when the matrix was more complex than pure water.

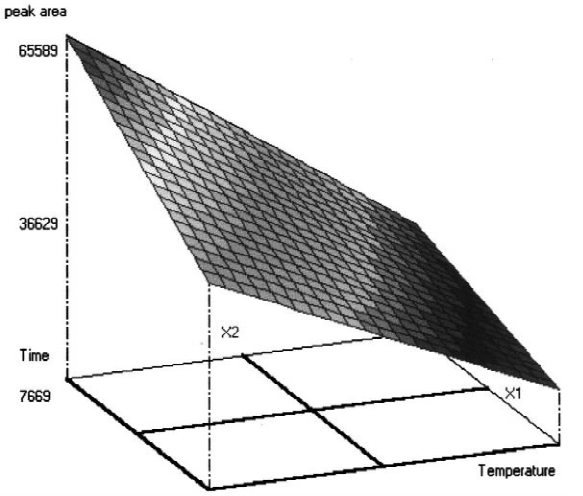

Fig. 1. Peak area response surface obtained plotting temperature against time.

As the high resolution power of the selected column allowed the gas chromatographic separation of toluene and cyclohexane from their labelled analogues [$^2H_8$]toluene and [$^2H_{12}$]cyclohexane used as internal standards, the ID calibration procedure was set up to improve the recovery. Fig. 2 shows the headspace-SPME–GC–FID chromatogram.

3.2.1. Linearity

Both cyclohexane and toluene gave linear calibration curves of peak area ratios (analyte/deuterated standard) vs. the corresponding concentration ratios in the same range of the external standard method. The calibration equations are $y = 1.185204x + 0.057642$ ($n = 15$) for cyclohexane and $y = 1.28055x + 0.031474$ ($n = 15$) for toluene, with $R^2 =$

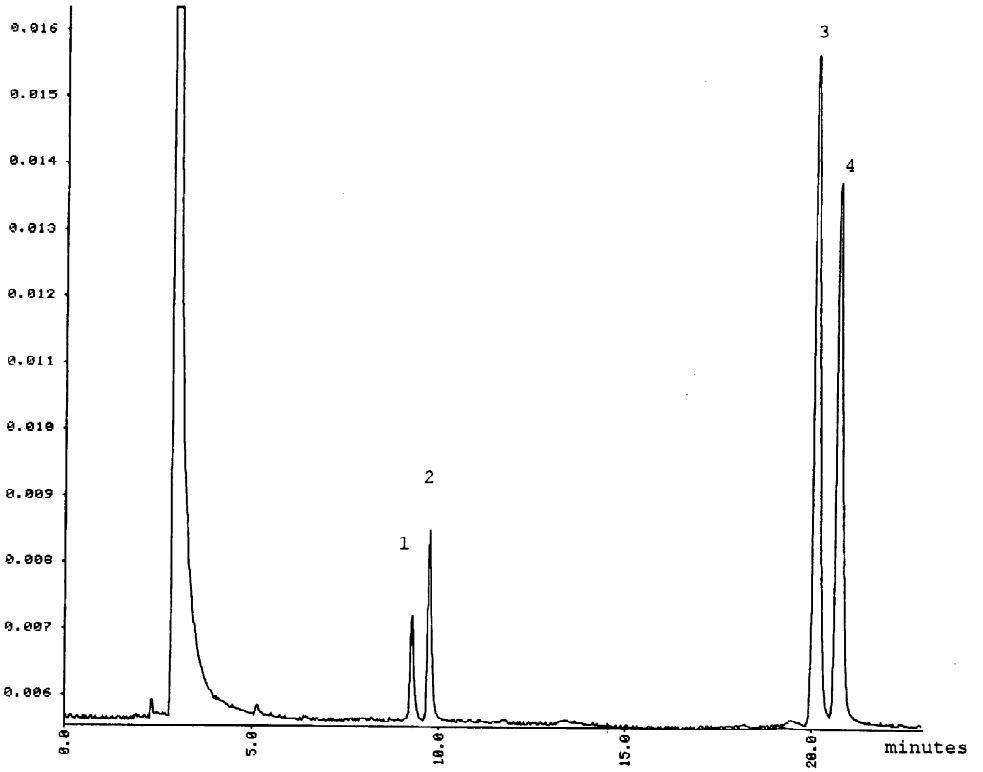

Fig. 2. Typical GC–FID trace showing separation of analytes and internal standards. 1=[$^2H_{12}$]Cyclohexane; 2=cyclohexane; 3=[$^2H_8$]toluene; 4=toluene.

0.99279 and 0.99761, SE=0.06059 and 0.04035, slope SD=0.07494 and 0.05469, intercept SD=0.05836 and 0.05007, respectively. No significant day-to-day variability was observed.

The limits of quantitation (LOQs) were determined in the aqueous system using the signal-to-noise approach. The LOQs ($S/N \geq 10$) for cyclohexane 60 ng ml^{-1} and toluene 50 ng ml^{-1} were subsequently validated by independent analyses of a suitable number (six replicates) of samples prepared at the quantitation limits.

3.2.2. Accuracy and precision

The accuracy and precision data, obtained from a total of nine determinations at three concentration levels (three replicates each) covering the linearity range, are summarised in Table 4. The bias for cyclohexane ranged from 3.1 to 2.3%, with a mean of 2.7%, RSD 1.3% and for toluene from 3.1 to −0.6%, mean 1.6%, RSD 2.1%.

The accuracy assessed with the method of standard additions at three concentration levels (three replicates each) gave good results with bias ranging for cyclohexane from −0.26 to 1.03% and for toluene from −1.85 to −0.21% (Table 5).

3.3. Analysis of real samples

The developed procedure was applied to the analysis of ketoprofen drug substance and ketoprofen capsule. The concentrations (mg kg^{-1}) of cyclohexane and toluene found in these samples and statistical data are reported in Table 6. The data for capsules are consistent with those obtained from the manufacturer using a HS-GC USP reference method [1].

Table 4
Precision and accuracy within the calibration curve concentration range

Analyte	Concentration (ng ml^{-1})		Bias (%)	RSD (%)
	Calculated	Found (mean, n=3)		
Cyclohexane	97	100	2.7	0.58
	389	401	3.1	1.98
	779	797	2.3	1.45
Toluene	216	243	3.1	2.70
	866	861	0.6	2.74
	1732	1774	2.4	1.59

Table 5
Precision and recovery of theoretical spike in ketoprofen drug substance

Analyte	Added (ng)	RSD (%) (mean n=3)	Bias (%)
Cyclohexane	97	2.00	1.03
	389	2.88	−0.13
	583	2.80	−0.26
Toluene	216	2.82	−1.85
	866	3.72	−0.21
	1299	2.37	−0.93

4. Conclusions

Achieving accuracy and precision, according to pharmaceutical analysis requirements, is a matter of considerable concern with SPME when the usual GC external standard procedure is applied to matrices of various complexity. In these instances, isotopic dilution GC–MS (GC–ID-MS) becomes mandatory to attain a reliable procedure. However this approach implies the availability of an expensive instrumental set-up in contrast with the low cost of the SPME technique. To overcome this difficulty here we have developed the present method.

While the GC separation of the analyte and its deuterated internal standard is not required in GC–ID-MS, in this case, where quantitation is based on peak area measurements, a complete resolution of all the GC peaks involved must be achieved. At present, many 30–50 m×0.20–0.25 mm I.D. capillary columns of appropriate film thickness on the market give the plate number required for the task, pending the commercial availability of highly deuterated homologues of the analytes considered (increasing the deuterium content of the internal standard homologue increases its physical difference with the analyte and then the possibility of GC separation).

The results of this study illustrate that GC conditions producing complete resolution of cyclohexane, [$^{2}H_{12}$]cyclohexane and toluene, [$^{2}H_{8}$]toluene pairs were readily found, and provide a method with high relative recoveries (99.6 and 100.7%) for the analytes in real samples, with RSDs (3.18 and 3.82%) consistently lower than 15% [1,2]. On the other hand, it should be noted that the proposed ID procedure call for an internal standard for each

Table 6
Assay for residual solvents in real samples

Analyte	Ketoprofen drug substance		Ketoprofen capsule			
	Found (mg kg^{-1})	RSD (%) (n=3)	Present method		Reference method	
			Found (mg kg^{-1})	RSD (%) (n=3)	Found (mg kg^{-1})	RSD (%) (n=3)
Cyclohexane	136.3	2.81	56.6	4.10	51.8	13.90
Toluene	655.1	2.87	583.2	4.40	563.4	7.25

analyte, doubling de facto the number of peaks to be managed by the GC system. Of course, this might exceed the column resolving capability when too many analytes are present

Acknowledgements

Financial support by Italian Ministry of University and Scientific Research (MURST) is gratefully acknowledged.

References

[1] US Pharmacopoeia, 24th ed., US Pharmacopoeial Convention, Rockville, MD, 2000, p. 1877.
[2] European Pharmacopoeia Supplement 1999, European Directorate for the Quality of Medicines – European Pharmacopoeia, Strasbourg, France, 1999, Section 2.4.24, p. 14 and Section 5.4, p. 205.
[3] H. Lord, J. Pawliszyn, J. Chromatogr. A 885 (2000) 153, and references cited therein.
[4] S. Scypinski, A.-M. Smith, in: S.A. Scheppers Wercinski (Ed.), Solid Phase Microextraction, Marcel Dekker, New York, 1999, p. 111.
[5] C.C. Camarasu, M. Mezei-Szuts, G. Bertòk Varga, J. Pharm. Biomed. Anal. 18 (1998) 623.
[6] T. Gorecki, X. Yu, J. Pawliszyn, Analyst 124 (1999) 643.
[7] G.A. Lewis, D. Mathieu, R. Phan-Tan-Luu, Pharmaceutical Experimental Design, Marcel Dekker, New York, 1999.
[8] R. Carlson, Design and Optimization in Organic Synthesis, Elsevier, Amsterdam, 1992.
[9] D.C. Montgomery, Design and Analysis of Experiments, Wiley, New York, 1997.
[10] Z. Zhang, J. Pawliszyn, Anal. Chem. 67 (1995) 34.
[11] J. Pawliszyn, Solid Phase Microextraction – Theory and Practice, Wiley–VCH, New York, 1997.