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Validation of an HPLC method on short columns to assay ketoconazole and formaldehyde in shampoo

A. Nguyen Minh Nguyet [a], L. Tallieu [a], J. Plaizier-Vercammen [b], D.L. Massart [a], Y. Vander Heyden [a,*]

[a] *Department of Pharmaceutical and Biomedical Analysis, Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium*

[b] *Department of Pharmaceutical Technology and Physical Pharmacy, Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium*

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Abstract

An HPLC method to determine simultaneously ketoconazole and formaldehyde in an anti-dandruff shampoo, originally developed on a long column, was transferred to two short columns with similar stationary phase properties, but with a length of at the most 30% of the initial one. Using the conventional column as reference, the fast HPLC methods on the short columns were validated. The validation characteristics consisted of selectivity, linearity range, precision (repeatability and time-different intermediate precision), bias and robustness. For the ketoconazole assay, linearity for peak area was found in the concentration range up to 0.20 mg/ml. For formaldehyde, two calibration ranges ($0–10 \times 10^{-5}$ and $0–10 \times 10^{-4}$%) were linear, both for peak area and height. The assays for both ketoconazole and formaldehyde in these ranges showed no bias and an acceptable precision, although the precision found with the short columns was slightly worse than with the long one. The robustness tests were performed applying a Plackett-Burman design. For the ketoconazole assay, 6 factors were examined in a 12 experiments design and for formaldehyde, 11 factors in 16 experiments. The methods were found to be robust. Despite the somewhat less good precision the transfer seems to be successful and the obtained assays on the short columns are applicable for fast routine analysis.

Keywords: Short column HPLC; Method validation; Robustness test; Plackett-Burman design; Ketoconazole; Formaldehyde

1. Introduction

In the pharmaceutical, biomedical and food analysis, a tendency can be observed to develop miniaturised and fast methods for high-throughput screening. Short columns HPLC gives shorter analysis times, lower sample and solvent consumption, faster re-equilibration, high mass sensitivity

* Corresponding author. Tel.: +32-2-477-4723; fax: +32-2-477-4735.
E-mail address: yvanvdh@vub.vub.ac.be (Y. Vander Heyden).

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(since smaller column volume results in less dilution of the analyte peaks), precise quantitation and high resolution [1–3]. Furthermore, the efficiency—expressed as the number of theoretical plates—of a column is inversely proportional to the particle size, so that for instance a shorter column (e.g. 75 mm) with smaller particles (e.g. 3.5 μm) can be a substitute for a longer one (e.g. 150 mm) with larger particles (e.g. 5 μm) without sacrificing too much in resolution, performance and reliability [2,4]. The main reason in pharmaceutical analysis to apply these short columns is that they allow fast analysis.

Nowadays we are frequently dealing with situations where a method was developed on a classical long column (e.g. one of 25 cm) and that one wants to transfer this method to a short one. The European Pharmacopoeia [5], for instance, allows a number of adjustments to the initially prescribed conditions, among which also to the stationary phase properties. The column length might be changed with ±70%, the column internal diameter with ±25% and for the particle size a reduction of 50% is permitted. However, at the moment, columns with dimensions exceeding these limits also are on the market. One then might wonder what are the consequences for the method validation characteristics, of the direct transfer of a method previously developed on a classical column to such a short column.

Ketoconazole is known as a broad-spectrum antifungal agent [6,7]. It is the active ingredient in an anti-dandruff shampoo which also contains imidurea or imidazolidinylurea as a formaldehyde donor preservative. Formaldehyde can harm the human health (causing eye, nose and throat irritation, insomnia, chest pains, rashes, asthma attacks,...) and a maximum allowable indoor exposure level to formaldehyde is set at 0.1 parts per million [8]. National, European and Food and Drug Administration regulations exist which regulate the maximum free formaldehyde content [9–12]. The use of formaldehyde as a preservative in cosmetic products, e.g. cosmetic hair products, is allowed up to a maximum concentration of 0.2% (except for nail hardeners for which a concentration up till 5% is allowed), but if the concentration exceeds 0.05% the product has to be labelled 'contains formaldehyde' [9–11,13]. Thus its concentration should be restricted in shampoo, while on the other hand a minimal concentration is required as preservative.

In the need of a fast and convenient tool to determine both ketoconazole and formaldehyde in the anti-dandruff shampoo, a method was developed earlier using one HPLC system (i.e. same stationary and mobile phase) for quantifying both substances [14]. Ketoconazole is UV absorbing and measured directly, while formaldehyde needs a pre-column derivatisation using 2,4-dinitrophenylhydrazine (2,4-DNPH) as reagent [15]. This method was transferred to two short columns with different particle sizes, 3.5 and 5 μm, and with different lengths, 75 and 50 mm, respectively. It can be noticed that the first column has a length equal to the original column length (25 cm) minus 70%, which is the limit allowed for monograph analyses, while the second column is exceeding this lower limit. The other column properties are such that all columns used would be considered as acceptable alternatives according to the European Pharmacopoeia prescriptions [5].

Using the conventional column as a control, these fast methods were validated in order to verify if their performance parameters are acceptable for the quantitative determination of ketoconazole and formaldehyde in shampoo and to ensure the reliability of the methods. The validation characteristics examined consist of selectivity, precision, linearity range, bias and robustness [16–26].

2. Experimental

2.1. Chemicals

The anti-dandruff shampoo was prepared in-house by the Pharmaceutical Technology and Physical Pharmacy Department [27]. It contains 2% ketoconazole, 0.3% imidureum, Cocamide DEA (Comperlan KD), Glucamate DOE-120, sodium-laurylethersulphate (NaLES) (28% aqueous solution), di-sodium-lauryl-(3)-ether-sulphosuccinate (Na_2LESS), NaCl, Orange–Yellow S, HCl 1 M to adjust the pH to 6.5 and distillated

water till 100%. All percent values mentioned above are m/m% values.

Ketoconazole was obtained from Kraemer & Martin (St. Augustin-Buisdorf, Germany), imidurea, imidazolidinylurea or Germall 115 from ISP (St. Niklaas, Belgium), sodium-laurylether sulphate or LES 28 (NaLES) as a 28% aqueous solution from Eur-O-Compound (Oudenaarde, Belgium), disodium laurylether-sulfosuccinate or Euranaat LS3 (Na_2LESS) from Eur-O-Compound, Comperlan KD or coconut fatty acids diethanolamide from Henkel (Düsseldorf, Germany), macrogol 120 methylglucose dioleate or Glucamate DOE-120 from Amerchol (Edison, New Jersey), sodium chloride (NaCl) from Merck (Darmstadt, Germany), Orange–Yellow S or Sunset Yellow FCF from BASF (Ludwigshafen, Germany), hydrochloric acid (HCl) 1 M and formaldehyde 37% m/m solution from Merck, acetonitrile from BDH Supplies (Poole, England), sodium dihydrogenphosphat monohydrat ($NaH_2PO_4 \cdot H_2O$), 2,4-DNPH, H_3PO_4 85%, HCl 32% and NaOH 1 M solution, all were of pro analysis grade and were supplied by Merck. Water for preparation of buffer and reagent solutions was produced in-house by the Milli-Q water purification system (Millipore, Milford, MA). All buffer solutions were filtered through a 0.2 μm membrane filter from Schleicher & Schuell (Dassel, Germany). The mobile phase was degassed in an ultrasonic bath before use.

2.2. Standard and sample preparations

Stock solutions of 1 mg/ml of ketoconazole and 10^{-1}% (m/V) of formaldehyde were prepared in the mobile phase (27.0 ml of 37% formaldehyde solution was diluted to 100.0 ml with mobile phase to obtain a 10% intermediate stock solution). Since formaldehyde is volatile, the exact concentration of the formaldehyde 37% standard solution should be determined prior to analysis. An assay for formaldehyde is described in the European Pharmacopoeia [28]. Formaldehyde reacts with iodine solution and the excess of iodine was back-titrated with sodium thiosulphate using starch as indicator. The exact concentration of sodium thiosulphate was determined by using the reaction with potassium bromate, and the iodine solution was standardised with the use of the sodium thiosulphate solution, according to the procedures described in Ref. [28]. The concentration of the formaldehyde solution used was found to be 37.33%.

Working solutions of ketoconazole and formaldehyde were obtained by diluting the stock standard solution with the same solvent. The dilutions of shampoo were prepared using the following scheme: weigh accurately 1.0 g of shampoo (in a volumetric flash containing already some mobile phase) and dilute to 10.0 ml with mobile phase (= shampoo stock solution). The final dilution was then obtained by further diluting this stock solution. Samples and standards were prepared daily prior to injection.

For formaldehyde, a pre-column derivatisation was required, in which 0.4 ml of 2,4-DNPH 0.1% solution (0.05 and 0.01% when using short columns) is added to 1.0 ml sample or standard. This mixture is vortexed for 1 min and allowed standing at room temperature during 2 min. The solution is then stabilised by adding 0.4 ml of a phosphate buffer 0.1 M (pH 6.8) and 0.7 ml of NaOH 1 M. This mixture is then injected.

The reagent solution 2,4-DNPH was prepared in a mixture of HCl 32% and water 40:60 (v/v). The phosphate buffer pH 6.8 was an aqueous 0.1 M NaH_2PO_4 solution adjusted to pH by NaOH 1 M.

2.3. Instrumentation

The chromatographic system comprised a Merck-Hitachi L-6200 Intelligent Pump (Tokyo, Japan) equipped with a Rheodyne injector (Cotati,CA), a Merck T-6300 Column Thermostat, a Merck-Hitachi L-4000 UV Detector and a Merck-Hitachi D-2500 Chromato-Integrator. The columns used were an Alltima C_8 (250 × 4.6 mm ID 5 μm) (Alltech, Laarne, Belgium) (column A), a Zorbax SB C_8 (75 × 4.6 mm ID 3.5 μm) (Hewlett-Packard, Waldbronn, Germany) (column B) and a Discovery C_8 (50 × 4.6 mm ID 5 μm) (Supelco, Bellefonte, USA) (column C).

2.4. Chromatographic conditions

The mobile phase contains a mixture 45:55 (v/v) of acetonitrile and 0.01 M $NaH_2PO_4 \cdot H_2O$ aqueous solution, the latter is adjusted to pH 4.0 with H_3PO_4 1 M solution. At nominal conditions, analyses were performed at a flow rate of 1 ml/min, at room temperature and at a detection wavelength of 250 nm for ketoconazole and 345 nm for formaldehyde. The HPLC conditions with the two short columns were kept the same as for the long column except for the injection volume, which was 20 μl with the long column but only 5 μl with the short ones. The reason is that the short columns yielded bad shaped peaks when using a 20 μl injection loop, which may be due to sample overload.

3. Results and discussion

3.1. Ketoconazole

3.1.1. Selectivity

The selectivity of the method, on the short columns, towards the excipients of the shampoo was tested by injecting a standard solution containing 0.10 mg/ml of ketoconazole (A), a 200 times diluted shampoo sample (B) and a blank shampoo sample. The chromatograms obtained with column B (75 × 4.6 mm ID, 3.5 μm) at nominal conditions are shown in Fig. 1. In the blank shampoo solution, no interfering peaks were found at the retention time of ketoconazole. The same selectivity was found with column C (50 × 4.6 mm ID, 5 μm). Compared to a long column, for column B the analysis times are reduced with a factor 4–6 depending on the kind of long column used [14]. For column C the reduction is even larger.

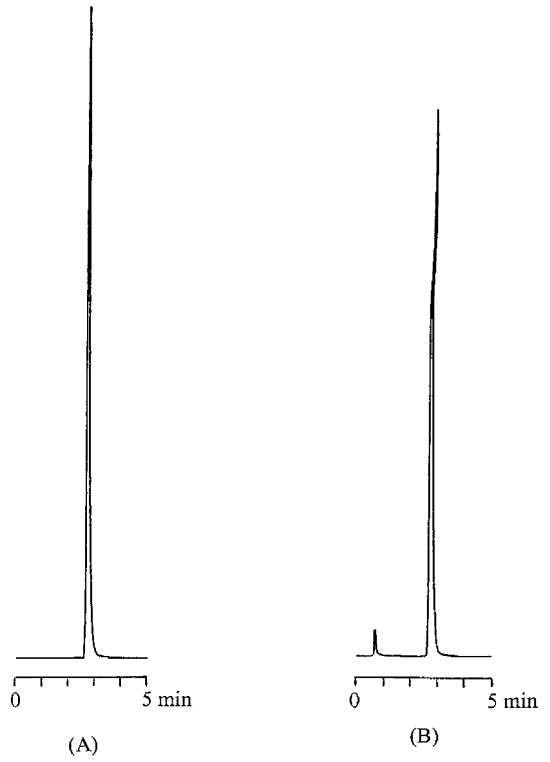

Fig. 1. Chromatograms at nominal conditions obtained on column B (75 × 4.6 mm ID). (A) 0.10 mg/ml ketoconazole solution, and (B) 200 times diluted shampoo, t_R (ketoconazole) = 2.8 min.

3.1.2. Linearity and range

In a previous study [14], it was found that on a long column (250 × 4.6 mm ID) the peak area is linearly proportional to concentrations up to 0.30 mg/ml. On the two short columns, the concentration range till 0.20 mg/ml could also be regarded as linear, as was observed visually, by calculating the correlation coefficient (r) and the quality coefficient (QC) of the calibration line [16]. Each calibration line was established based on a blank and five different standards. The straight line equations found in two calibration ranges 0–0.05 and 0–0.20 mg/ml on the three columns are shown in Table 1. The reason for the differences in slope between the long and the short columns is that the injected amounts are different, due to the different loops used. The correlation coefficients all are larger than 0.999, which is sometimes considered as an indication of acceptable fit of the data to the regression line [17]. Furthermore, the quality coefficient, which characterises the quality of the fit of the data to the straight line calibration model, was calculated. It is defined as

Table 1
The linearity in two calibration ranges of ketoconazole on the three columns

Column	0–0.025–0.05–0.10–0.15–0.20 mg/ml			0–0.01–0.02–0.03–0.04–0.05 mg/ml		
	Equation	r	QC (%)	Equation	r	QC (%)
A	$y = 307074.4x - 12475.7$	0.99999	0.403	$y = 302237.6x - 752.7$	0.999999	0.173
B	$y = 106163.1x + 10781.7$	0.99969	2.603	$y = 104342.2x + 8012$	0.99980	1.924
C	$y = 106327.5x + 8330.6$	0.99996	0.979	$y = 108929x + 398.2$	0.999997	0.253

$$\mathrm{QC} = \sqrt{\frac{\sum\left(\frac{\hat{y}_i - y_i}{\bar{y}}\right)^2}{n-1}}100\%$$

where $\hat{y}_i$ represents the response for standard i predicted by the model, y_i the response measured for standard i, $\bar{y}$ the mean of the measured responses and n the number of data points, including the blank.

The smaller the quality coefficient, the better the experimental points fit the line [16]. The quality coefficient value can be considered as acceptable with all three columns.

Visual inspection of the plots of peak height versus concentration showed curvature on all columns.

3.1.3. Precision

3.1.3.1. Repeatability. The repeatability (within-day precision) was determined by analysing six independently prepared samples of 250 times diluted shampoo. Since the concentration of ketoconazole in the shampoo is about 20 mg/ml, the estimated concentration in the dilution is then equal to about 0.08 mg/ml. The relative standard deviations (RSD) of the estimated concentrations found were 1.3, 2.3 and 2.5% on columns A, B and C, respectively. Bartlett's test for homogeneity of variances considers all variances to be equal at $\alpha = 0.05$ ($T = 1.97$, $\chi^2_{2\mathrm{df}} = 5.99$). An F-test comparing the most extreme variances also was not significant ($P = 0.089$).

3.1.3.2. Time-different intermediate precision. The time-different intermediate precision (between-days precision) was assessed by analysing one sample of 250 times diluted shampoo daily, during 11 days [18]. The standards and blank to establish the calibration lines were also prepared and measured daily. The time-different intermediate precision was found to be 1.5, 2.5 and 2.9% on columns A, B and C, respectively. Bartlett's test considered the variances to be equal at $\alpha = 0.05$ ($T = 3.98$, $\chi^2_{2\mathrm{df}} = 5.99$). However, the most extremes are considered to be significantly different at $\alpha = 0.05$ level (F-test, $P = 0.025$).

Precision, both repeatability and time-different intermediate precision, seems to be less good on the short columns, but still acceptable according to Ref. [19] in which a RSD value of 5.3% is still regarded acceptable for the within or between-days precision if the analyte concentration is around 0.1 mg/ml. Another way to evaluate the obtained precision estimates is based on the equation proposed by Horwitz et al. [20], $\mathrm{RSD} = 2^{(1-0.5\log x)}$ where x is the concentration in g/g expressed in negative powers of 10. With the 250 times diluted shampoo, x is equal to 8000 μg/100 g or 8×10^{-5} and RSD is 8.3%. This value is considered as acceptable value for the time-different intermediate precision and half of it (meaning 4.2%) as the limit for repeatability.

Taking into account these acceptance limits, the repeatability and time-different intermediate precision found indicate an acceptable precision of the method on all three columns, even though the precision on the short columns seems to be somewhat less good than on the long one.

3.1.4. Bias

The bias of the method is expressed as percentage recovery and was determined in three spiked samples with different concentrations of ketoconazole (0.04, 0.08 and 0.12 mg/ml) added to the

sample of 250 times diluted blank shampoo. The fortified samples were analysed in triplicate. A diluted blank shampoo was also injected. The percentage recovery was calculated as $\%R = [(C_F - C_U)/C_A] \times 10$ where C_F represents the concentration of analyte measured in fortified sample, C_U the analyte concentration measured in unfortified sample (here zero) and C_A the added concentration of analyte in the fortified sample [29]. The mean recovery rates were found to be 100.4, 98.1 and 101.2% with columns A, B and C, respectively. The bias of the procedure can be regarded as acceptable because the recoveries are between 90 and 107% which is the limit defined in [19] for an analyte concentration of 0.01%. It can be concluded that no systematic positive or negative bias was found with either column. As for the precision, the bias of the evaluated method also largely remained within the literature limits.

3.1.5. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [22,30]. A robustness test is the experimental set-up to evaluate the robustness of a method. Six factors (parameters) were selected from the analytical procedure. The extreme factor levels were usually defined symmetrically around the nominal ones and formed an interval that slightly exceeds the variations, which can be expected when the method is transferred [30–32]. The first 6 factors and their levels, described in Table 2, are those examined for the ketoconazole assay. The levels of the first 5 factors were chosen based on the uncertainty (absolute error) with which a factor can be set and reset [30]. For example, to define the levels for the concentration of NaH_2PO_4 in the mobile phase, the uncertainties on the mass of NaH_2PO_4 weighed and on the prepared volume are taken into account. For a more detailed explanation, we refer to Refs. [30,31]. The extreme levels for the detection wavelength were chosen based on the authors' experience and on literature knowledge. For the temperature an asymmetric interval relative to the nominal interval was selected. The (−1) level can be considered as a standardised 'room temperature' level. The (+1) level was selected to be 35 °C. This allows to evaluate whether working at a standardised temperature above room temperature is not affecting the assay.

Table 2
Factors and their levels in the robustness test on the ketoconazole and formaldehyde assays

Factors	Levels		
	−1	0	1
(1) NaH_2PO_4	3.40 g/l	3.45 g/l	3.50 g/l
(2) pH	3.8	4.0	4.2
(3) ACN	0.43	0.45	0.47
(4) Flow	0.9 ml/min	1 ml/min	1.1 ml/min
(5) Temperature	25 °C	Room temperature	35 °C
(6) Wavelength			
(a) Ketoconazole	249 nm	250 nm	251 nm
(b) Formaldehyde	344 nm	345 nm	346 nm
(7) Fraction of HCl	38%	40%	42%
(8) pH of buffer	6.6	6.8	7.0
(9) Volume DNPH	0.35 ml	0.40 ml	0.45 ml
(10) Volume buffer	0.35 ml	0.40 ml	0.45 ml
(11) Volume NaOH	0.65 ml	0.70 ml	0.75 ml

Abbreviations: NaH_2PO_4, concentration of NaH_2PO_4 in aqueous part of the mobile phase; pH, pH of the aqueous part of the mobile phase; ACN, fraction of acetonitrile in mobile phase; flow, flow rate of mobile phase; temperature, column temperature; wavelength, wavelength of the detector; fraction of HCl, fraction of HCl in mixture HCl:H_2O to prepare 2,4-DNPH solution; pH of buffer, pH of buffer used in derivatisation reaction; volume DNPH, volume of 2,4-DNPH solution used in derivatisation reaction; volume buffer, volume of buffer solution in derivatisation reaction; volume NaOH, volume of NaOH added to this reaction.

The ruggedness test strategy (RTS) program was used to define the experimental set-up [32]. A Plackett-Burman design for 11 factors requiring 12 experiments was chosen in which 5 dummy factors were included, because at least three dummies are recommended for the statistical interpretation of the effects [33]. The robustness test on column C was executed without examining factor temperature, for practical reasons on the one hand and because of its non-significant effect on the quanti-

tative response observed with the other columns on the other.

For practical reasons, experiments were sorted (blocked) by 2 factors: NaH_2PO_4 and pH. Within the blocks the experiments are randomised. For more information we refer to [30–32]. After every fourth design experiment (Table 3), an experiment at nominal levels was performed to check if the nominal response is drifting as a function of time and occasionally to correct the design results for such drift [33]. When a drift occurs, it namely can contribute to wrong effect estimates for the design factors.

The solutions injected for each experiment were the calibration line 0, 0.025, 0.05, 0.10, 0.20 mg/ml of ketoconazole and two dilutions of the shampoo, 500 and 200 times. When mobile phase or column temperature was changed between two consecutive experiments, the system was stabilised for at least 30 min.

The following responses were determined for each experiment: the content of ketoconazole in the shampoo calculated from peak area, the retention factor (k') and the tailing factor (Asf) of the ketoconazole peak, calculated as defined by the USP XXIII [34].

No drift or time effect was found for any of the responses. The average nominal results and the average design results for the different responses on the three columns are shown in Table 4. The sample considered was the 200 times diluted shampoo. It can be seen that both averages on a given column are similar, indicating that either no significant factor effects occur or that the considered response as a function of the factor levels has a linear behaviour (linear effects). If the effect(s) of the significant factor(s) as a function of the levels would be non-linear then both averages are not expected to be comparable. The results found with the 500 times diluted shampoo were similar.

The retention time of ketoconazole decreased substantially with column length, from 8.7 min (column A) to 2.8 min (column B) and 1.3 min (column C). The number of theoretical plates was calculated to be 8300, 3100 and 1200 on columns A, B and C, respectively. The reason for the higher number of plates in column B, in comparison with

Table 3
Experimental set-up (0, −1 and +1 are the factor level) of the robustness test for ketoconazole

Exp.	Factor					
	NaH_2PO_4	pH	ACN	Flow	Temperature	Wavelength
0	0	0	0	0	0	0
6	−1	−1	−1	1	−1	1
12	−1	−1	−1	−1	−1	−1
5	−1	−1	1	−1	1	1
4	−1	1	−1	1	1	−1
0	0	0	0	0	0	0
2	−1	1	1	−1	1	1
10	−1	1	1	1	−1	−1
7	1	−1	−1	−1	1	−1
3	1	−1	1	1	−1	1
0	0	0	0	0	0	0
11	1	−1	1	1	1	−1
1	1	1	−1	1	1	1
8	1	1	−1	−1	−1	1
9	1	1	1	−1	−1	−1
0	0	0	0	0	0	0

The dummy factor columns of the Plackett-Burman design are not shown. Exp. 1, 2,. . ., 12, design experiments; Exp. 0, experiment at nominal conditions.

Table 4
The average nominal results and the average design results from the robustness test of ketoconazole on the three columns

Responses	Average nominal results			Average design results		
	A	B	C	A	B	C
[C]area	1.84	1.95	1.95	1.85	1.92	1.98
Asf	1.08	1.06	1.26	1.09	1.16	1.32
k'	3.30	2.97	0.91	3.56	3.09	1.02

Abbreviations: [C]area, content of ketoconazole in shampoo (m/m%) calculated from peak area. Sample: 200 times diluted shampoo.

column C, is the smaller size of particles (3.5 μm) packed in the former one which gives more interaction sites and which compensates partly for the shorter length compared to column A. As a consequence, the retention factor was highest on column A, slightly smaller but still comparable on column B and obviously reduced on column C.

The RTS program was used to calculate and to interpret the factor effects. To identify statistically significant effects, a t-test was performed. The absolute value of the effect of a factor X is considered to be significant if it is larger than a critical effect (E_{critical}) [30,35].

$$|E_X| \Leftrightarrow E_{\text{critical}} = t_{\text{critical}}(\text{SE})_e$$

The standard error $(\text{SE})_e$ was estimated from the dummy factor effects [35], the critical t-value (t_{critical}) is the tabulated t-value with n degrees of freedom at $\alpha = 0.05$ or $\alpha = 0.01$, and n the number of dummies. In case the effects are normalised relative to the average nominal result (y_n), the absolute value of the normalised effect of a factor ($\%E_X = E_X 100/y_n$) is compared with the normalised critical effect ($\%E_{\text{critical}}$)

$$|\%E_X| \Leftrightarrow \%E_{\text{critical}} = \frac{E_{\text{critical}} 100}{y_n}$$

The statistically significant effects on the different responses from columns A, B and C are shown in Table 5. The RTS software also provided normal probability plots. In such plot, non-significant effects on a response tend to fall on a straight line and significant ones deviate from it. The results found with the statistical and graphical interpretations agreed. The assay can be considered robust on the three columns because none of the studied factors has a significant effect on the determination of the content of ketoconazole in the shampoo. However, the latter requires some additional comments. It can be observed that the critical effects on columns B and C are considerably higher than those on column A. This confirms the worse precision that was observed earlier with the short columns and it leads to relative high dummy effect estimates. On the other hand, none of the factor effects seems to affect the content determination significantly and that is why from the robustness test result it was concluded that the method is robust.

On the responses tailing factor and retention factor, the $\%E_{\text{critical}}$ are comparable on the three columns and some significant effects are found. It was observed that on the three columns, the factors 'flow rate' and 'detection wavelength', as well as the dummy factors never were significant. The effect of the factor 'temperature' on the retention factor was, on column B, indicated as borderline significant at 5% level. The other 3 factors, pH, amount of NaH_2PO_4 and fraction of acetonitrile in the mobile phase, are rather important as they affect the response on most columns. The response tailing factor was not always influenced by the same factors. For example, it was affected by the pH and the fraction of acetonitrile on column B, but only by acetonitrile on column A, and by no factor on column C. However, from the robustness test, it is observed that under all design conditions the ketoconazole peak remains acceptably symmetric and clearly resolved from the solvent peak.

System suitability tests (SST) are established as a step following the robustness test 'to ensure that the validity of the analytical procedure is maintained whenever used' [22]. The use of the results

Table 5
Effects (E_X), normalised effects ($\%E_X$), critical effects ($E_{critical}$, $\%E_{critical}$) and significance (SF) of the factors on the responses measured for the ketoconazole assay

Factors	[C]area			Asf			k'		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(a) Column A									
NaH_2PO_4	−0.003	−0.13	–	−0.010	−0.92		−0.440	−14.11	[b]
pH	0.007	0.39	–	−0.007	−0.61	–	1.062	34.04	[b]
ACN	0.010	0.51	–	−0.208	−18.36	[b]	−0.467	−14.96	[b]
Flow	−0.008	−0.46	–	−0.025	−2.30	–	−0.040	−1.27	–
Temperature	−0.006	−0.31	–	−0.045	−4.13	–	0.046	1.46	–
Wavelength	−0.006	−0.30	–	−0.040	−3.67	–	0.080	2.58	–
d1	0.004	0.19	–	0.023	2.14	–	−0.120	−3.84	–
d2	−0.002	−0.08	–	−0.013	−1.22	–	0.020	0.64	–
d3	−0.001	−0.05	–	−0.018	−1.68	–	0.160	5.12	–
d4	0.013	0.71	–	0.092	8.41	–	0.031	0.99	–
d5	−0.008	0.42	–	0.033	3.06	–	−0.107	−3.42	–
Significance level	$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$	
5%	0.018	0.98		0.118	10.85		0.264	8.45	
1%	0.028	1.54		0.185	17.01		0.413	13.25	

Factors	[C]area			Asf			k'		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(b) Column B									
pH	0.003	0.15	–	−0.173	−13.80	[a]	1.459	50.03	[b]
ACN	0.013	0.67	–	−0.132	−11.47	[a]	−0.854	−29.28	[b]
Flow	−0.039	−2.03	–	0.071	6.17	–	0.133	4.57	–
Temperature	0.031	1.62	–	−0.128	−11.10	–	0.254	8.71	[a]
Wavelength	−0.040	−2.07	–	−0.062	−5.35	–	0.036	1.23	–
d1	−0.054	−2.83	–	0.001	0.073	–	−0.013	−0.45	–
d2	−0.107	−5.56	–	−0.049	−4.25	–	0.132	4.52	–
d3	0.065	3.37	–	0.091	7.91	–	0.046	1.59	–
d4	0.002	0.11	–	0.045	3.90	–	−0.107	−3.67	–
d5	0.052	2.73	–	0.010	0.88	–	−0.064	−2.20	–
Significance level	$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$	
5%	0.168	8.74		0.130	11.30		0.216	7.41	
1%	0.264	13.70		0.204	17.72		0.339	11.62	

Factors	[C]area			Asf			k'		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(c) Column C									
NaH_2PO_4	0.004	0.25	–	0.108	8.50	–	−0.162	−17.98	[b]
pH	0.030	1.72	–	−0.041	−3.23	–	0.354	39.25	[b]
ACN	0.026	1.48	–	0.036	2.83	–	−0.150	−16.69	[b]
Flow	−0.018	−1.03	–	0.008	0.59	–	−0.018	−2.00	–
Wavelength	0.041	2.31	–	−0.036	−2.83	–	0.031	3.40	–
d1	0.045	2.53	–	−0.024	−1.91	–	0.017	1.92	–
d2	−0.040	−2.25	–	0.066	5.20	–	−0.019	−2.15	–

Table 5 (*Continued*)

Factors	[C]area			Asf			k'		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(c) Column C									
d3	0.018	0.99	–	−0.058	−4.55	–	0.029	3.26	–
d4	−0.005	−0.27	–	−0.054	−4.28	–	0.042	4.62	–
d5	−0.056	−3.14	–	0.071	5.60	–	0.013	1.48	–
d6	0.002	0.08	–	−0.066	−5.20	–	−0.045	−4.99	–
Significance level	$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$	
5%	0.084	4.73		0.143	11.31		0.074	8.20	
1%	0.127	7.16		0.217	17.13		0.112	12.43	

–, not significant.
[a] Significant at 5% level.
[b] Significant at 1% level.

of the worst-case situations to define SST-limits has been proposed elsewhere [36]. The worst-case conditions are the factor level combinations which give the least desired, i.e. the worst result, e.g. for resolution the lowest one, for retention factor the smallest one and for tailing factor, in general, the highest one. To select the worst-case conditions, the non-significant factors are kept at nominal level while the significant ones are at the levels which cause the worse result for that response. For instance with column A, the worst-case condition for the retention factor is the one with factor NaH_2PO_4 at +1 level, pH at −1 level, fraction of acetonitrile at +1 level and the remaining factors at the nominal level. The SST limit for a response can be predicted from the theoretical model as the value Y estimated at the worst-case conditions:

$$Y = b_0 + \frac{E_{F_1}}{2}F_1 + \frac{E_{F_2}}{2}F_2 + \ldots\frac{E_{F_k}}{2}F_k$$

with b_0 the average design result, E_{F_i} the effect of the factor considered and F_i the level (−1 or +1) causing the worst result. For non-significant factors, the F_i value is 0.

The SST-limits can also be determined from the results of replicate experiments at the worst-case conditions. The SST-limits are then defined as the lower or upper limit from the one-sided 95% confidence interval around the worst-case mean $\bar{Y}_{\text{Worst-case}}$, $[\bar{Y}_{\text{Worst-case}} - t_{\alpha,m-1}s/\sqrt{m}, \infty]$ or $[0, \bar{Y}_{\text{Worst-case}} + t_{\alpha,m-1}s/\sqrt{m}]$ with m the number of replicates, s the standard deviation of the replicates and $t_{\alpha,m-1}$ the tabulated t-value with $m-1$ degrees of freedom at significance level α. The worst-case experiment was carried out in three independent replicates.

The obtained SST-limits from both approaches, which were comparable, are summarised in Table 6. They are the most extreme values of these responses on the considered system that still allow a quantitative determination under conditions comparable to those at which the method validation is conducted. The SST limit for the tailing factor on column C was determined in a different way from the others. This response was not

Table 6
The SST-limits predicted from the worst-case experimental situations and from the theoretical model in the assay of ketoconazole

Column	From worst-case experimental results		From theoretical model	
	Asf	k'	Asf	k'
A	1.12	2.68	1.19	2.57
B	1.28	2.09	1.31	1.80
C	1.30	0.75	(–)	0.69

(–), cannot be calculated.

influenced by any of the examined factors. Therefore, the replicate experiments were executed at nominal levels under time-different intermediate precision conditions and the SST limit was derived from these results. On the other hand, the SST limit from the theoretical model cannot be predicted. Occasionally, the worst result observed in the design could be used here as an alternative, when no replicated (worst-case) experiments are performed.

In summary, the assay to determine ketoconazole has been validated on the three columns. The method showed a good selectivity towards the excipients. The linearity, precision and bias were acceptable in the examined concentration range. Precision is less good on the short columns, while the robustness tests did not indicate any factor to affect the assay.

3.2. Formaldehyde

3.2.1. Selectivity

Typical chromatograms for (A) blank solution, (B) standard solution containing 4×10^{-4}% of formaldehyde and (C) 100 times diluted shampoo, after the derivatisation reaction, obtained with column B (75×4.6 mm ID 3.5 μm) at nominal conditions, are shown in Fig. 2. The small peak of formaldehyde observed in the blank solution can be explained by the fact that an amount of formaldehyde occurring in the air [8] is absorbed by the solution. Apart from the main peaks of reagent 2,4-DNPH and of formaldehyde, no interference was present in the chromatograms, which confirms the selectivity of the method found earlier [14]. Compared to analyses on a long column a reduction in analysis times with a factor of about 4–5 was observed.

3.2.2. Linearity and range

Previously, with the long column [14], linearity of the calibration lines from peak area and height was observed in three ranges: (a) $0–2 \times 10^{-5}–4 \times 10^{-5}–6 \times 10^{-5}–8 \times 10^{-5}–10 \times 10^{-5}$%, (b) $0–2 \times 10^{-4}–4 \times 10^{-4}–6 \times 10^{-4}–8 \times 10^{-4}–10 \times 10^{-4}$% and (c) $0–1 \times 10^{-3}–2 \times 10^{-3}–3 \times 10^{-3}–4 \times 10^{-3}–5 \times 10^{-3}$% in which always a 2,4-DNPH concentration of 0.1% has been used for the derivatisation reaction.

Using short columns, this 2,4-DNPH concentration does not result in well-resolved peaks between reagent and formaldehyde due to an excess of the former. As a result of a number of preliminary experiments, the 2,4-DNPH concentration was decreased for the short columns. Linearity was observed in concentration ranges (a) and (b) on column B (75×4.6 mm) when using 0.05% 2,4-DNPH solution. With column C (50×4.6 mm^2) the concentration of 2,4-DNPH to be applied is 0.01% for range (a) and 0.05% for range (b). Range (c) could not be regarded as linear on the short columns, regardless whether peak area or height was considered. The straight line equations for calibration ranges (a) and (b), their correlation coefficients and quality coefficients are shown in Table 7. Again, the differences in slope between columns can be explained by the variation in reagent concentrations (see above, lower concentration in range (a) for column C seems to have affected the reaction kinetics) and injection volumes. The correlation and quality coefficients which are all larger than 0.9997 and smaller than 2.3, respectively, can be considered as an indication of the acceptable fit of the data to the regression lines.

Therefore on the three columns, both peak area and height can be used to derive the content of formaldehyde in shampoo in the calibration ranges (a) and (b).

3.2.3. Precision

3.2.3.1. Repeatability. The repeatability was determined at two concentration levels of formaldehyde, namely in the 500 and in the 100 times diluted shampoo, by analysing six independently prepared samples at each level. The concentration of formaldehyde in the diluted shampoo was estimated around 8×10^{-5} and 4×10^{-4}%, respectively, i.e. about 4×10^{-2}% in undiluted shampoo. On columns A, B and C, the RSD of the concentration calculated from peak area was found to be 2.28, 2.33 and 2.58%, respectively for the 500 times diluted shampoo and 1.98, 2.22 and 2.35%, respectively for the 100 times diluted

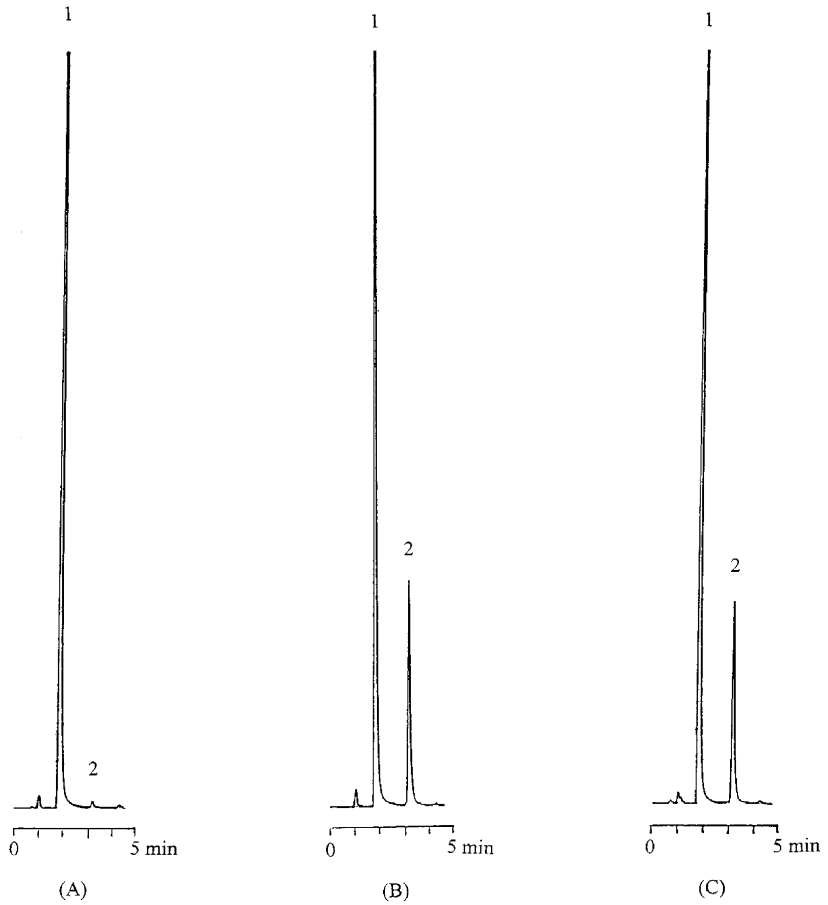

Fig. 2. Chromatograms obtained, after the derivatisation reaction, on column B (75 × 4.6 mm ID) at nominal conditions; (A) blank solution (mobile phase), (B) 4×10^{-4}% formaldehyde solution and (C) 100 times diluted shampoo; (1) 2,4-DNPH, $t_R = 2.0$ min and (2) formaldehyde, $t_R = 3.4$ min.

Table 7
The linearity of two calibration ranges of formaldehyde on the three columns

Column	Range (a)			Range (b)		
	Equation	r	QC (%)	Equation	r	QC (%)
Peak area						
A	$y = 35211.5x + 18975.5$	0.99982	1.73	$y = 34313.2x + 21281.2$	0.999997	0.35
B	$y = 21166.7x + 7044.2$	0.99974	2.12	$y = 19946.1x + 11442$	0.99998	0.83
C	$y = 9927.9x + 2005.8$	0.99972	2.27	$y = 20982.9x - 7020$	0.99982	1.89
Peak height						
A	$y = 2333.2x + 585$	0.99996	0.88	$y = 2210.8x + 1577.8$	0.99986	1.63
B	$y = 2337.5x + 966.5$	0.99991	1.27	$y = 2217.5x + 2063.8$	0.99983	1.82
C	$y = 1542.3x + 463.8$	0.99986	1.59	$y = 2587.2x + 2344.5$	0.99978	2.08

shampoo. Bartlett's test indicates equality of variances. The most extreme variances are very similar too (F-test, $P = 0.39$ for 500 times diluted shampoo, and $P = 0.36$ for 100 times diluted one). Thus, in contrast to the ketoconazole determination, the repeatability of the formaldehyde concentration can be considered more comparable on the three columns. The RSD values between both dilutions are also comparable, indicating heteroscedasticity in the concentration range.

3.2.3.2. Time-different intermediate precision. The time-different intermediate precision was assessed by analysing daily two samples of the two above shampoo dilutions, during 11 days. The calibration lines were also constructed daily. The RSD was 3.40, 4.53 and 4.93% with the 500 times diluted and 3.06, 3.72 and 3.91% with the 100 times diluted shampoo on columns A, B and C, respectively. Bartlett's test was not significant in both cases, while also the most extreme variances are not significantly different (F-test, $P = 0.13$ and $P = 0.23$ for 500 and 100 times diluted shampoo, respectively). Again the RSD values on the three columns are more comparable than for the ketoconazole determination where significant differences were observed. From the above results it is also observed that RSD values from the 100 times diluted shampoo were systematically slightly smaller than those from the 500 times diluted shampoo.

The RSD values for repeatability and time-different intermediate precision can be considered as acceptable when consulting Ref. [19] or using the equation proposed by Horwitz et al. [20] for the corresponding concentration level. Therefore the precision of the developed method can be considered as agreeable on the three columns.

3.2.4. Bias

To determine the bias of the method, three different concentrations of formaldehyde (2×10^{-4}, 4×10^{-4} and 6×10^{-4}%) were added to samples of 100 times diluted shampoo. The diluted shampoo and each spiked sample were injected three times. The mean recovery rates were found as 101.8, 96.6 and 99.2% from the peak area and 99.5, 95.3 and 99.0% from the peak height on columns A, B and C, respectively. Referring to Ref. [19], these values are indications of an acceptable bias for the formaldehyde assay.

3.2.5. Detection limit

Earlier, it was found that formaldehyde concentrations below 2×10^{-5} g/100 ml still were derivatised but the resulting peak was not proportional to the concentration anymore [14]. The detection limit was at least 4×10^{-6} g/100 ml since this concentration still causes an important increase in the peak compared to the one observed in a blank. Here, no additional experiments to evaluate the detection limit were performed, since our first intention was to transfer the existing method and it was found that quantitative measurements could be performed in the same range as on the long column.

3.2.6. Robustness

To investigate the robustness of the method, 11 factors were selected. The first six were the same as in the robustness test on the assay of ketoconazole. The other five were related to the derivatisation reaction of formaldehyde. These factors (Table 2) were examined in a Plackett-Burman design for 15 factors with 16 experiments. The experiments were sorted by 3 factors: NaH_2PO_4, pH and fraction of acetonitrile [30,35]. To check for drift, a nominal experiment was added at every fourth design experiment [30,35]. The experimental set-up is shown in Table 8.

Due to the different amounts of 2,4-DNPH, of phosphate buffer and of NaOH added to the reaction mixture, the total volume was not the same for every experiment. Therefore, for each derivatisation reaction, the volume was adjusted with water to 2.65 ml which is the highest volume required by the design. In each experiment, the solutions injected were two calibration lines (0, 2.5×10^{-5}, 5×10^{-5}, 10×10^{-5}% formaldehyde and 0, 2.5×10^{-4}, 5×10^{-4}, 10×10^{-4}% formaldehyde) and a shampoo at two dilutions, 500 and 100 times. The HPLC system was stabilised for 30 min before performing the first injection at new chromatographic conditions. The responses determined were the retention and the tailing factors of the formaldehyde peak, the resolution (Rs) between the peaks of reagent and formaldehyde, all

Table 8
Experimental set-up of the robustness test for formaldehyde

Exp	Factors										
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
0	0	0	0	0	0	0	0	0	0	0	0
4	−1	−1	−1	1	1	1	1	−1	1	−1	1
16	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1
3	−1	−1	1	1	1	1	−1	1	−1	1	1
7	−1	−1	1	−1	−1	−1	1	1	1	1	−1
0	0	0	0	0	0	0	0	0	0	0	0
6	−1	1	−1	−1	−1	1	1	1	1	−1	1
12	−1	1	−1	1	1	−1	−1	1	−1	−1	−1
2	−1	1	1	1	1	−1	1	−1	1	1	−1
10	−1	1	1	−1	−1	1	−1	−1	−1	1	1
0	0	0	0	0	0	0	0	0	0	0	0
5	1	−1	−1	−1	1	1	1	1	−1	1	−1
8	1	−1	−1	1	−1	−1	−1	1	1	1	1
11	1	−1	1	1	−1	−1	1	−1	−1	−1	1
13	1	−1	1	−1	1	1	−1	−1	1	−1	−1
0	0	0	0	0	0	0	0	0	0	0	0
9	1	1	−1	−1	1	−1	−1	−1	1	1	1
14	1	1	−1	1	−1	1	1	−1	−1	1	−1
1	1	1	1	1	−1	1	−1	1	1	−1	−1
15	1	1	1	−1	1	−1	1	1	−1	−1	1
0	0	0	0	0	0	0	0	0	0	0	0

The factors (1), (2),. . ., (11) are described in Table 2. The dummy factor columns of the Plackett-Burman design used are not shown. Exp. 1, 2,. . ., 15, design experiments; Exp. 0, experiment at nominal conditions.

calculated as defined by the USP XXIII [34], and the content of formaldehyde in the shampoo calculated from peak area and height. For none of the responses, a time effect was found. The average nominal and the average design results for the different responses on the three columns can be seen in Table 9, the sample considered was the 100 times diluted shampoo. Again, as in Table 4, both averages on a given column were similar leading to the same conclusions about occasional factor effects, i.e. either linear effects or no significant effects occur. The results found with the 500 times diluted shampoo, were similar.

The tailing factor of the formaldehyde peak on column A (close to one) was obviously better than on columns B and C (around 1.5). This trend is

Table 9
The average nominal and the average design results for the three columns from the robustness test on the formaldehyde assay

Responses	Average nominal results			Average design results		
	A	B	C	A	B	C
[C]area	3.44	3.42	3.59	3.41	3.44	3.52
[C]height	3.42	3.40	3.53	3.44	3.43	3.51
Asf	1.04	1.53	1.58	1.01	1.52	1.55
k'	5.26	3.42	1.81	4.88	3.29	1.67
Rs	18.81	8.64	4.28	17.74	8.61	4.13

Abbreviations: [C]area, content in shampoo calculated from peak area ($\times 10^{-2}$ m/V%); [C]height, content calculated from peak height ($\times 10^{-2}$ m/V%). Sample: 100 times diluted shampoo.

different from the observed in the assay of ketoconazole (Table 4) where the tailing factor on column A was comparable to that on column B and better than on column C. The small size particles packed in column B also enhance its number of theoretical plates, which were 18400, 5900 and 2200 on columns A, B and C, respectively when calculated from the formaldehyde peak. Consequently, the retention factors and the resolutions found were highest on column A, smaller on column B and still smaller on column C. The differences observed between columns A and B are larger than for the ketoconazole assay.

The statistically significant factor effects on the different responses are shown in Table 10 for the three columns. Regardless whether peak area or height was used to calculate the content of formaldehyde in the shampoo, these responses were not influenced by any of the examined factors at $\alpha = 5\%$. Therefore, the assay of formaldehyde on the three columns can be regarded as robust. Concerning the critical effects, the same remarks as for the ketoconazole assay can be made.

It can also be seen that none of the factors from the derivatisation reaction, nor the factor 'detection wavelength' or the four dummies are indicated as significant for any of the responses on the three columns ('volume of phosphate buffer' is on the limit of significance on column C for resolution). The factors from the derivatisation reaction are expected to have occasionally an influence on the content determination, not really on the other parameters, as was indeed observed from the robustness test.

Several factors were found to have significant effects on the responses retention factor and resolution on the three columns and on the response tailing factor on column A. The latter response, on columns B and C, was not affected by any of the examined factors. However, the response also showed a much higher variability on these columns. It can, for instance, be observed that for the response tailing factor, the dummy effects on column A are considerably smaller than that on columns B and C which leads to a lower critical effect on the former (Table 10). As a consequence, more factors were found to be significant on the former but not on the two latter. For retention factor and resolution, a different trend was seen. The critical effects on columns A and B were considerably higher than that on column C which results in more significant factors found on the latter. However, from the robustness test, it was observed that the peaks of reagent and of formaldehyde remain well separated and are reasonably symmetric under all design conditions.

The SST-limits for the three latter responses were then determined from experiments at the worst-case conditions and from the theoretical model. The predicted SST-limits are presented in Table 11. The values for the tailing factor on columns B and C were obtained in the same manner as described for column C in the ketoconazole assay.

In summary, it can be concluded that on the three columns this method is robust in respect of the examined factors since the content determination of formaldehyde was not affected by the introduced factor changes. Furthermore, the other validation characteristics as linearity, precision and bias, as well as resolution between the peaks of reagent and formaldehyde can be regarded as acceptable.

4. Conclusion

Validation was performed on two short column assays for ketoconazole and formaldehyde in an anti-dandruff shampoo, using the original long column method as a reference. The columns examined were, as far as their length was considered, on or below the lower limit of what is considered acceptable for a European Pharmacopeia monograph analysis. In comparison with the classical method, these fast ones—after some minor modifications such as smaller injection volumes and reduced concentration of reagent in the derivatisation reaction of formaldehyde—can also be regarded as acceptable for their intended use. Good selectivity and resolution was reached using these short columns while analysis times are considerably reduced. Linearity was found in a calibration range from 0 to 0.20 mg/ml for ketoconazole and in two concentration ranges ($0–10 \times 10^{-5}$ and $0–10 \times 10^{-4}$%) for the assay

Table 10
Effects (E_X), normalized effects ($\%E_X$), critical effects ($E_{critical}$, $\%E_{critical}$) and significances (SF) of the design factors on the different responses of the formaldehyde assay

Factors	[C]area			[C]height			Asf			k'			Rs		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(a) Column A															
NaH_2PO_4	0.087	2.54	–	0.012	0.34	–	−0.005	−0.55	–	0.240	4.50	–	0.624	3.38	–
pH	0.054	1.59	–	0.110	3.21	–	0.063	6.48	[b]	−0.293	−5.49	–	−0.209	−1.13	–
ACN	0.130	3.81	–	0.016	0.46	–	0.079	8.18	[b]	−0.974	−18.25	[a]	−1.929	−10.45	[a]
Flow rate	0.056	1.64	–	−0.019	−0.56	–	0.006	0.66	–	−0.127	−2.38	–	−0.269	−1.46	–
Temperature	−0.118	−3.46	–	−0.098	−2.86	–	−0.066	−6.77	[b]	−0.744	−13.94	[a]	−1.069	−5.79	–
Wavelength	−0.085	−2.48	–	−0.029	−0.85	–	−0.018	−1.89	–	0.101	1.90	–	0.349	1.89	–
Fraction of HCl	−0.004	−0.10	–	0.030	0.86	–	0.023	2.41	–	0.110	2.06	–	0.356	1.93	–
pH of buffer	−0.053	−1.54	–	−0.011	−0.33	–	0.017	1.77	–	−0.284	−5.32	–	−0.416	−2.26	–
Volume DNPH	0.094	2.73	–	0.065	1.90	–	0.003	0.35	–	0.160	3.00	–	0.086	0.47	–
Volume buffer	−0.018	−0.51	–	−0.051	−1.47	–	−0.022	−2.23	–	0.227	4.26	–	0.536	2.91	–
Volume NaOH	−0.006	−0.19	–	0.033	0.97	–	−0.005	−0.55	–	0.038	0.71	–	0.384	2.08	–
d1	0.037	1.09	–	−0.023	−0.67	–	0.009	0.89	–	−0.189	−3.55	–	−0.466	−2.53	–
d2	0.017	0.49	–	0.067	1.96	–	0.013	1.33	–	−0.366	−6.86	–	−0.834	−4.52	–
d3	0.038	1.12	–	0.061	1.76	–	0.012	1.25	–	−0.251	−4.70	–	−0.586	−3.18	–
d4	0.113	3.31	–	0.040	1.16	–	0.007	0.06	–	−0.051	−0.96	–	−0.311	−1.69	–
Significance level	$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$	
5%	0.175	5.12		0.141	4.11		0.027	2.82		0.673	12.62		1.615	8.75	
1%	0.291	8.50		0.234	6.81		0.045	4.67		1.117	20.93		2.678	14.51	

Factors	[C]area			[C]height			Asf			k'			Rs		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(b) Column B															
NaH_2PO_4	0.020	0.63	–	−0.020	−0.61	–	0.047	3.18	–	0.387	11.13	–	1.090	12.82	–
pH	0.188	5.83	–	0.143	4.45	–	0.016	1.07	–	0.191	5.50	–	0.786	9.25	–
ACN	0.037	1.16	–	0.053	1.65	–	0.022	1.49	–	−0.582	−16.75	[a]	−1.588	−18.69	[a]
Flow rate	0.008	0.25	–	0.010	0.30	–	−0.008	−0.51	–	0.148	4.24	–	0.761	8.96	–
Temperature	−0.164	−5.10	–	−0.118	−3.67	–	0.012	0.78	–	−0.522	−15.02	–	−1.091	−12.83	–
Wavelength	0.088	2.74	–	0.032	1.00	–	−0.016	−1.10	–	0.101	2.91	–	−0.089	−1.05	–
Fraction of HCl	−0.003	−0.09	–	−0.022	−0.69	–	−0.003	−0.21	–	−0.092	−2.65	–	−0.907	−10.67	–
pH of buffer	−0.170	−5.27	–	−0.047	−1.46	–	−0.072	−4.83	–	0.171	4.93	–	1.229	14.46	–
Volume DNPH	0.109	3.40	–	0.085	2.64	–	0.014	0.95	–	0.099	2.84	–	0.570	6.71	–
Volume buffer	0.068	2.12	–	−0.058	−1.81	–	0.047	3.19	-	−0.383	−11.01	–	−1.095	−12.88	–
Volume NaOH	−0.200	−6.21	–	−0.083	−2.57	–	−0.006	−0.38	–	−0.132	−3.78	–	−0.542	−6.38	–
d1	0.015	0.47	–	0.081	2.53	–	0.027	1.85	–	0.147	4.22	–	0.608	7.16	–

Table 10 (*Continued*)

Factors	[C]area			[C]height			Asf			k'			Rs		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(b) Column B															
d2	0.084	2.60	–	0.090	2.78	–	−0.027	−1.85	–	−0.209	−6.01	–	−0.120	−1.42	–
d3	0.132	4.09	–	0.074	2.29	–	0.011	0.75	–	−0.195	−5.62	–	−0.630	−7.41	–
d4	0.167	5.19	–	0.061	1.90	–	−0.053	−3.60	–	−0.254	−7.31	–	−0.386	−4.54	–
Significance level	$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$	
5%	0.318	9.87		0.214	6.66		0.093	6.25		0.569	16.37		1.339	15.75	
1%	0.527	16.38		0.356	11.04		0.154	10.37		0.944	27.14		2.220	26.11	

Factors	[C]area			[C]height			Asf			k'			Rs		
	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF	E_X	$\%E_X$	SF
(c) Column C															
NaH_2PO_4	0.374	10.07	–	0.172	5.13	–	−0.144	−9.70	–	−0.044	−2.26	–	0.225	5.27	[a]
pH	0.157	4.23	–	0.088	2.62	–	−0.129	−8.70	–	−0.001	−0.03	–	0.035	0.82	–
ACN	0.228	6.14	–	0.015	0.46	–	−0.024	−1.62	–	−0.507	−26.06	[b]	−1.437	−33.73	[b]
Flow rate	−0.146	−3.95	–	0.027	0.79	–	−0.016	−1.08	–	−0.032	−1.62	–	0.296	6.96	[b]
Temperature	0.063	1.70	–	0.102	3.05	–	−0.030	−2.01	–	−0.191	−9.80	[b]	−0.096	−2.26	–
Wavelength	−0.073	−1.97	–	−0.077	−2.30	–	−0.075	−5.08	–	0.005	0.28	–	0.072	1.70	–
Fraction of HCl	0.167	4.51	–	0.186	5.52	–	0.013	0.89	–	0.001	0.04	–	−0.047	−1.11	–
pH of buffer	0.131	3.53	–	0.069	2.04	–	−0.057	−3.83	–	0.021	1.06	–	−0.076	−1.78	–
Volume DNPH	0.038	1.03	–	−0.008	−0.23	–	0.020	1.32	–	0.009	0.44	–	0.095	2.22	–
Volume buffer	0.088	2.38	–	0.091	2.71	–	0.016	1.06	–	0.035	1.82	–	0.153	3.60	[a]
Volume NaOH	0.136	3.68	–	0.109	3.25	–	0.037	2.50	–	−0.001	−0.04	–	−0.010	−0.24	–
d1	0.109	2.94	–	0.046	1.37	–	0.045	3.05	–	−0.020	−1.05	–	−0.052	−1.23	–
d2	−0.125	−3.37	–	0.006	0.19	–	0.101	6.83	–	0.011	0.55	–	0.024	0.57	–
d3	0.218	5.87	–	0.118	3.51	–	−0.118	−7.96	–	−0.017	−0.85	–	0.076	1.78	–
d4	−0.067	−1.81	–	−0.017	−0.51	–	0.017	1.15	–	−0.018	−0.93	–	−0.026	−0.62	–
Significance level	$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$		$E_{critical}$	$\%E_{critical}$	
5%	0.391	10.54		0.178	5.28		0.226	15.25		0.047	2.40		0.137	3.22	
1%	0.649	17.48		0.294	8.76		0.375	25.29		0.078	3.98		0.228	5.34	

–, not significant.
[a] Significant at 5% level.
[b] Significant at 1% level.

Table 11
The SST-limits predicted from the worst-case experimental situations and from the theoretical model in the assay of formaldehyde

Column	From worst-case experimental results			From theoretical model		
	Asf	k'	Rs	Asf	k'	Rs
A	1.11	4.36	17.02	1.11	4.02	16.78
B	1.57	3.13	8.24	(–)	3.00	7.82
C	1.62	1.35	3.41	(–)	1.32	3.15

(–), cannot be calculated.

of formaldehyde. The precision and bias were acceptable on the three columns for both assays. However, the short columns seem to have a slightly less good precision than the long column method, especially for the ketoconazole assay. Furthermore, all methods were considered robust since none of the contents was influenced by any of the examined factors. Anyway, despite the somewhat less good precision the validation showed promising application possibilities of the fast short column methods for simultaneous quantifying ketoconazole and formaldehyde in the shampoo during routine analysis. A reduction in analysis times with a factor of at least five can be obtained by using these columns.

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