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Application of experimental design to the development of an HPLC method for the analysis of ochratoxin A in *Triticum aestivum* grain

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Received May 8, 2003, accepted August 19, 2003

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Pharmazie 59: 175–177 (2004)

Ochratoxin A is a mycotoxin, a natural product of *Aspergillus* and *Penicillium* species. It can be present in grain from *Triticum aestivum*, (Graminae) and other starch-abundant cereals. This paper describes the investigation of ochratoxin A in grain from *Triticum aestivum* using a statistically optimized HPLC method. The assay was developed using two mathematical statistical models: factorial design and response surface mapping. The final step was to optimize the values of variables by response surface design. The analysis of variance 'ANOVA' method was applied to the analytical results in order to construct an adequate model. The optimal experimental conditions obtained by the response surface diagram method were: pH = 2.5, composition of the mobile phase acetonitrile: water 55 : 45 v/v and flow rate 1.0 ml/min. with a C18 column. Retention time and capacity factor for ochratotoxin A were 7.46 min. and 1.19, respectively.

1. Introduction

Ochratoxin A is one of the mycotoxins which are toxic secondary metabolites produced by fungi growing on agricultural commodities in the field or during storage. It causes nephrotoxicity, hepatotoxicity and carcinogenicity in animals, (Delacruz and Bach 1990; Kuiper and Scott 1989). Several analytical methods for the quantification of ochratoxin A in biological samples (Pascale and Visconti 2000), agricultural commodities (Scudamore and MacDonald 1998; Kwak and Shon 2000; Entwisle et al. 2000; Eskola et al. 2002) and food (Gareis and Scheuer 2000; Markaki et al. 2001) have been reported previously. All of these have been developed by traditional methodology. The aim of this work was the application of the experimental design technique to the development and optimisation of an RP HPLC method for the determination of ochratoxin A in different wheat. A full factorial design, '2^3' was used to obtain information about the effects of different factors on response resolution and analysis time. Response surface methods are useful optimization tools because the global optimum can be found (Massart et al. 1988; Lough and Wainer 1996). Retention mapping is designed to completely describe or "map" the chromatographic behavior of solutes in the design space by its response surface, which shows the relationship between the chromatographic response factor, i.e. the capacity factor of the solute, and several input variables, e.g. the components of the mobile phase. The chromatographic response factor of every solute in the sample can then be predicted, rather than performing many separations and simply choosing the best one obtained.

2. Investigations, results and discussion

This work presents the results of an experimental study designed to determine the combined effect of pH, mobile phase composition and flow rate on the RP HPLC behavior of ochratoxin A. The chromographic parameters examined were the percentage of acetonitrile as organic modifier in the mobile phase, pH and flow rate of the mobile phase. The design matrix (Table 1) shows the eight treatment combinations of low (−) and high (+) levels of the factors.

From the estimate of the effects of the various factors (Table 2) the pH and percentage of acetonitrile in the mo-

Table 1: Factorial design

No.	Factor level*			Capacity factor (k)
	A	B	C	
1	−	−	−	6.66
2	+	−	−	7.66
3	−	+	−	0.56
4	+	+	−	0.80
5	−	−	+	4.55
6	+	−	+	4.71
7	−	+	+	0.50
8	+	+	+	0.34

Low (−) and high (+) levels of the following factors

Factor	Level	(−)	(+)
A	Flow, ml/min.	1	1.5
B	% of ACN in mobile phase	50	80
C	pH mobile phase	2.0	3.5

Table 2: Estimates of factor effects

M	3.2231
A	0.3087
B	−5.3437
AB	−0.2712
C	−1.3962
AC	−0.3087
BC	1.1337
ABC	0.1112

M – meet value of all combined effects
AB, BC – interactions of first order
ABC – interactions of second order

bile phase have the main effect. A response surface diagram was used to optimise the experimental conditions for HPLC analysis of ochratoxin A (Vanbel et al. 1996; Attwood and Florence 1985). Applying model fitting methods to the experimental data gave the following equation for the relationship between pH and percentage of acetonitrile in mobile phase, where K is a capacity factor dependent variable.

$$K = 69.598 - 0.761X - 1.824Y - 0.984X^2 + 0.011Y^2 + 0.077XY$$

$X = \text{pH}; \qquad Y = (\%)$ acetonitrile in mobile phase

The optimal experimental conditions obtained by the method response surface diagram were: pH 2.5, composition of the mobile phase acetonitrile: water 55 : 45 v/v and flow rate 1 ml/min (Fig. 1) Figure 2 presents representative chromatograms of ochratoxin A (standard solutions – **a** and sample solutions **b**).

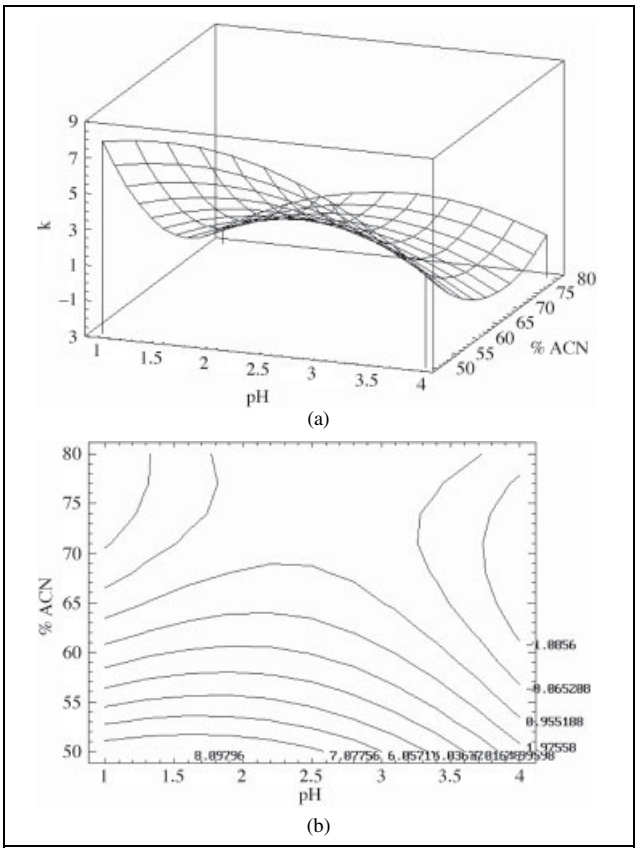

Fig. 1: Predicted capacity factor of ochratoxin A (a) and contours of constant response (b) for the retention surface as a function of pH and % acetonitrile in mobile phase

Table 3: Analysis of variance for variables and for the full regression for capacity factor (k) of ochratoxin

Source of variation	Sum of squares	d.f.	Mean square	F-ratio
pH	44.550681	1	44.550681	145.43
ACN (%)	17.797776	1	17.797776	58.06
pH^2	60.439814	1	60.439814	197.18
ACN $(\%)^2$	12.437982	1	12.437982	40.58
ACN × pH	4.0445149	1	4.0445149	13.19
Model	139.271	5	27.8542	90.871
Error	3.98479	13	0.30652	
Lack of fit	3.53956	12	0.294963	0.6625
Purely experimental uncertainty	0.44523	1	0.445230	
Total (corr)	143.256	18		

The analysis of variance – ANOVA method was used to analyze the results in order to obtain an adequate elution model (Van der Veen and Pauwels 2000). Since the factors chosen had a significant effect on response, the variance in the data set accounted for by the factors was larger than the variance of the residuals. It was confirmed by the Fisher variance ratio for significance of the regression i.e. the factor effect. $F = 90.87$, ($F_{crit} = 3.025$) was significant at the 95% level of confidence. The test for lack of fit was used to compare the variance due to the lack of fit with the variance due to purely experimental uncertainty. $F_{lof} = 0.6625$ ($F_{crit} = 243.91$) was not significant. We concluded that there was no significant amount of variation in the measured responses and that the measured responses could be explained by the model. As expected, the residuals were very small. The coefficient of multiple determination $R^2 = 0.9722$ indicates that the factors explained the data very well. Taking the degrees of freedom into account, the adjusted $R^2 = 0.9615$ (Table 3).

3. Experimental

3.1. Chemicals and reagents

Sigma Chemical, Co.USA, donated ochratoxin A pure samples.
Methanol and acetonitrile (Merck, Darmstadt, Germany) were of HPLC grade.

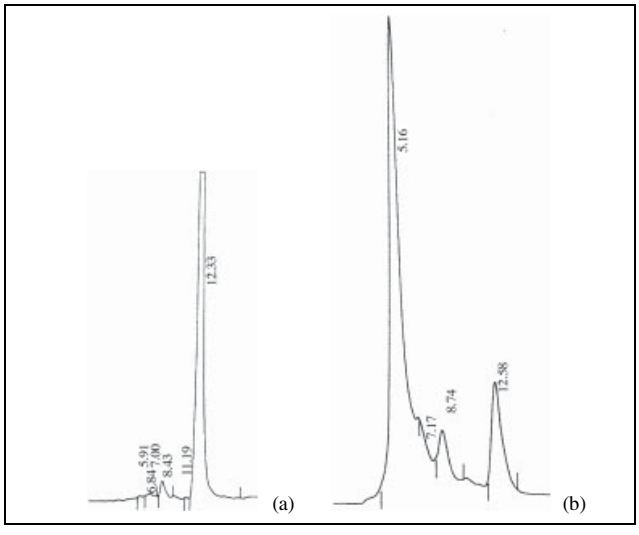

Fig. 2: Chromatogram of ochratoxin A elute of standard (a) and chromatogram of ochratoxin A elute of contaminate of the wheat (b) after optimization. Eluent: acetonitrile : water 55 : 45 v/v, pH 2.5, and flow rate 0.5 ml/min

3.2. Standard solution

A stock solution of ochratoxin A (1 mg/ml) was prepared in methanol. This solution was diluted to 0.01 mg/ml with distilled water.

3.3. Extraction procedure for standard

To 10 ml aqueous solution of ochratoxin A, concentration 0.01 mg/ml, 5 ml 10% acetic acid and 50 ml of toluene were added and shaken well. The organic phase was separated and evaporated under nitrogen gas. The residue was reconstituted in 1.0 ml of mobile phase and 100 µl of this solution was injected into the HPLC column.

3.4. Extraction procedure from sample of wheat

Twenty grams of contaminated wheat were transferred to an Erlenmeyer flask and 50 ml 0.4 M $MgCl_2$, 30 ml 2 M HCl and toluene were added. The flasks were shaken well for 60 min and filtered. The filtrate was evaporated and the residue reconstituted in 1.0 ml of mobile phase and 100 µl of this solution was injected into the HPLC column.

3.5. Chromatographic conditions

The HPLC analysis was performed with a Biorad Bio-Sil C18HL 90-5S column (250 × 4.6 mm, 5 µm, Varian Star System, USA). The mobile phase consisted of different ratios of water and acetonitrile (70 : 30, 55 : 45, 50 : 40, 35 : 65, 20 : 80 v/v). The pH was adjusted to 2.0, 2.5, 3.0 and 3.5 with glacial acetic acid. The mobile phase was filtered and degassed before use. The injection volume was 200 µl, elution was performed at a flow rate of 1.0, 1.25, 1.5 ml min^{-1} and the column was maintained at ambient temperature. The absorbance was monitored at $\lambda = 256$ and 330 nm with a LKB Bromma 2140 diode array detector.

3.6. Statistical methods

Statistic StatSoft version 5.0 and Statgraphics version 4.2 programs were used for statistical analysis of the results in this work. Calculations were performed on a Pentium IV personal computer.

Acknowledgement: These results are the part of project No. 1458 "Molecular structures, chemical transformations, physicochemical characterization, pharmaceutical purity, and analysis of pharmacologically active compounds" financed by the Ministry of Science and Technology of the Republic of Serbia.

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