Brazilian Journal of Microbiology (2007) 38:304-308
ISSN 1517-8382

INTRALABORATORY OPTIMIZATION AND VALIDATION OF A METHOD FOR PATULIN DETERMINATION IN GRAPES BY THIN-LAYER CHROMATOGRAPHY

Renata Cabrera de Oliveira; Érika Bando; Miguel Machinski Junior*

Universidade Estadual de Maringá, Departamento de Análises Clínicas, Laboratório de Toxicologia, Maringá, PR, Brasil.

Submitted: June 05, 2006; Returned to authors for corrections: September 21, 2006 Approved: February 12, 2007

ABSTRACT

The aim of this work was to optimize and validate, by intralaboratorial procedures, a method for the determination of patulin in grapes by thin-layer chromatography. The steps of extraction, cleanup, detection and quantification were optimized. For the validation of the method, recovery assays with standard solutions and artificially contaminated samples were carried out. The mean recovery and the variation coefficient were 65.4% and 7.58%, respectively. The optimized conditions were: 50 mL of grape juice, three extraction stages (with 100 mL of ethyl acetate in the first stage and 50 mL in second and third stages), and 100 µL of ethanol to solubilize the extract. The solvent-system used was toluene - ethyl acetate - formic acid (6:3:1), and 0.5% MBTH in 5% formic acid was sprayed on the plates to intensify the fluorescence. The visual detection and quantification limits were 7.44 ng and 15.87 $\mu g \cdot kg^{-1}$, respectively. The optimized and validated method demonstrated sufficient efficiency for adoption in the monitoring of patulin in grape.

Key words: patulin; mycotoxins; grape; thin-layer chromatography

INTRODUCTION

Patulin is a secondary metabolite produced by several species of filamentous fungi belonging to the genera *Penicillium*, *Aspergillus*, *Byssochlamys*, *Gymnoascus* and *Paecilomyces*, with *Penicillium expansum* considered to be the most important patulin producer (13). Many authors consider this mycotoxin as a promoter of mutagenesis (16,18,19,22,28,31,33) carcinogenesis (4,9,24) and teratogenesis (6,16,29).

Patulin has been found to contaminate various fruits, such as apricots, cherries, grapes, pears, peaches and apples, and also products based on these fruits (14,21). There are, however, few studies on the contamination of grapes and grape juice by patulin, as mentioned by Altmayer *et al.* (1), Czerwiecki (8), Frank *et al.* (15), Kubrak *et al.* (20), Meyer (23), and Woller and Majerus (35).

Many studies have been conducted to monitor and control the development of toxigenic mould and mycotoxin production in foods and feeds, as well as to develop simple and efficient methods for the determination of these metabolites, evaluating toxicant doses and maximum limits of tolerance for these substances in various products (5,12,25).

Several countries have established the maximum limit of 50 $\mu g \cdot kg^{-1}$ for patulin in products for human consumption. However, other countries have been recommending the limits of 20 $\mu g \cdot kg^{-1}$ and 30 $\mu g \cdot kg^{-1}$ for baby and infant foods, respectively (11,27). In 1995, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) modified the Provisory Tolerable Weekly Intake (PTWI) for patulin of 7 µg/kg body weight/week for a Provisory Maximum Tolerable Dairy Intake (PMTDI) of 0.4 µg/kg of body weight/day (34).

Thin-layer chromatography (TLC) is the traditional technique for mycotoxin determination. Its low cost and simplicity are the main advantages of this analytical procedure. The significant separation of the mycotoxins from the non-desired compounds in this technique confers specificity and sensitivity to the method. The main disadvantage of it, however, is the low repeatability related to the process of applying the samples to the plates, development of the plates, which can be improved by the analyst's experience (7).

*Corresponding Author. Mailing address: Universidade Estadual de Maringá - Dep. de Análises Clínicas - Lab. de Toxicologia - Av. Colombo, 5790. Cep: 87020-900 Maringá, PR - Brasil. Tel.: (44) 3261-4489. E-mail: mmjunior@uem.br

The objective of this study was to optimize and validate, by intralaboratorial procedures, the method described by Tanner and Zanier (32) for determining patulin in grapes, to make it an accessible and applicable technique for a large majority of laboratories and, at the same time, to make it capable of working within the limits of tolerance established by international legislation.

MATERIALS AND METHODS

Reagents

Standard patulin was purchased from Sigma Co. (St. Louis, MILLSTONE, USA), kept under freezing temperature and protected from light until the time of the preparation of the solutions. The standard working solutions were prepared and standardized according to AOAC (3). About 0.5 mg of patulin was dissolved in 50 mL of chloroform, and 5 mL of this solution was transferred to a beaker and submitted to a water bath at 40ºC. Immediately after drying, the residue was dissolved in 5 mL of ethanol and the concentration of the standard working solution was determined spectrophotometrically by ultraviolet light at 275 nm. It was then stored in an amber flask under refrigeration.

The stability of these solutions was monitored, repeating the standardization procedures throughout the period of the experiment. The concentrations of the standard working solutions were: 3.72, 9.92, 10.85, 11.50, 14.94, 15.14 and 16.95 $\mu g \cdot mL^{-1}$ in this period. The pro-analysis-grade reagents, sodium sulfate anhydrous, 3-methyl-2-benzothiazolinone hydrazone hidrocloride (MBTH) and the TLC aluminum sheets (20x20 cm) with silica gel 60 G were purchased from Synth (Brazil), Merck (Germany), Sigma (USA) and Macherey-Nagel (Germany), respectively.

Equipment

The equipment used was: a waterbath (Quimis), an ultrasonic bath (Unique), a spectrophotometer (Shimadzu model UV-1601PC), a pressure and vacuum pump (Marconi), an incubator (Fanem) and an ultraviolet cabinet.

Samples

Acquired commercial grape samples were blended and filtered in a sieve with a mesh size of 20. The obtained juice was stored at -20ºC. The samples were spiked with known quantities of patulin by the addition of the standard solution.

Optimization of the method

The method optimized was described by Tanner and Zanier (32), which quantifies the patulin in apple juice by thin-layer chromatography (TLC). The procedure consists of using 50 mL of the sample, extracted with 50 mL of ethyl acetate in the first and second stages, followed by the cleanup of the extract with 20 mL of 1.5% sodium carbonate solution. After drying, the residue was dissolved in chloroform (100 µL) and applied to the TLC plates.

a) Extraction step

Recovery assays were carried out with grape samples spiked with 33.9 and 67.8 $\mu g \cdot L^{-1}$ of patulin to which the extraction solvent, ethyl acetate. In order to optimize the extraction step, the quantity of extractor solvent and the number of extractions were evaluated.

b) Cleanup step

The efficiency of the cleanup step using sodium carbonate, the precipitation of proteins by the Carrez reagent and silica column chromatography was evaluated.

Glass columns were manually packed in the laboratory with 7.5 g of silica gel 60 (Merck, Germany) suspended in toluene. The elution was carried out with 100 mL of toluene - ethyl acetate (3:1) or benzene - ethyl acetate (3:1), and the influence of this step on the recovery process of the method was determined.

Some samples of grape juice were deproteinized with different volumes of Carrez Reagent (potassium ferrocyanide and zinc acetate - Synth, Brazil) to evaluate the recovery process of this method before extraction with ethyl acetate.

c) Detection and quantification steps

The chromatographic conditions evaluated were: the application of spiked samples in quantities ranging from 1 to 10 µL; solvent-system in the following ratios: toluene - ethyl acetate - formic acid (5:4:1), toluene - ethyl acetate - formic acid (6:3:1), toluene - ethyl acetate - formic acid (4:5:1), chloroform - methanol (95:5), chloroform - acetone (9:1), chloroform - methanol - acetone (1:1:1), toluene - ethyl acetate - chloroform - formic acid (38:28:26:10) and toluene - ethyl acetate (1:3); and the chromogenic reagent sprays: phenylhydrazine and MBTH for intensifying the fluorescence.

Analytic procedure

The established analytic procedure in the optimized method was as follows:

a) Extraction and Cleanup

Aliquots of 50 mL of homogenized grape juice were extracted with 100 mL of ethyl acetate by shaking manually for 1 minute. The aqueous and organic phases were then separated into different beakers. The extraction of the aqueous phase was repeated 2 more times with 50 mL of ethyl acetate. The total organic extract was transferred to a separation funnel and submitted to vigorous shaking with 40 mL of 1.5% sodium carbonate solution. The organic phase was then filtered with anhydrous sodium sulfate, and the filtrate was evaporated in a waterbath at 40ºC until dry. The dried residue was then transferred to a small vial, redissolved ultrasonically in 3 mL of

ethyl acetate for 3 minutes (i.e. one minute for each ml added), and evaporated under the same conditions again. The final dry residue was stored at -20ºC.

b) Thin- layer chromatography (TLC)

To dissolve the residue, 100 µL of ethanol was added and the mixture was shaken in a vortex for 1 minute. Aliquots of 5, 7 and 10 µL of the sample extract and 1 to 10 µL of the standard patulin working solution were applied on chromatographic plates. The solvent-system used was toluene - ethyl acetate - formic acid (6:3:1). The plates were then dried, MBTH was applied to them, and visualization in ultraviolet 366 nm was carried out. The fluorescence intensity of stained samples was compared with those of the standards.

Validation of the optimized method

For the validation of the method, standard solutions and spiked samples (2,10) were used, according to the analytic procedures established during the optimization of the method. Validation of the analytical method was based on the following criteria: selectivity, accuracy, precision and visual detection and quantification limits.

a) Limit of visual detection

The limit of visual detection was determined by using volumes of 1 to 10 µL of the standard working solutions for verification of the lowest detectable quantity of patulin by visual analysis.

b) Specificity, accuracy, precision and limit of quantification of the method

The specificity of the method was evaluated through the use of grape juice samples spiked with the standard working solutions in two concentration levels (33.9 and 67.8 $\mu g \cdot L^{-1}$) and blank samples, with all tests carried out in triplicate. The resolution of the patulin stain, the presence of non-desired compounds and the agreement among the retention factors (Rf) of the patulin and of the standard working solution and spiked samples were verified.

For the evaluation of precision, accuracy and the quantification limit of the method, blank and spiked samples, in four concentration levels, were analyzed, with at least two repetitions per level.

The precision of the method was evaluated through the variation coefficients of the results for each of the concentration levels. Repeatability and reproducibility were evaluated by the comparison of the results obtained in assays carried out on the same day, and on different days, of extraction, respectively.

Accuracy was evaluated by the comparison of the theoretical values of the patulin additions and the values obtained in the tests, expressed in terms of recovery percentage. The criteria of acceptability determined by Horwitz *et al*. (17) (60 to 100% accuracy and 30% precision) were used as a reference for the evaluation of the variation coefficients and mean percentages of recovery obtained in the tests.

The quantification limit of the method was determined as being the smallest patulin concentration that could be detected throughout all the assay repetitions, by considering the values of the mean recovery percentages and the variation coefficients defined as being acceptable, (60 to 100% accuracy and 30% precision).

RESULTS AND DISCUSSION

Optimization of the method

a) Extraction step

The use of ethyl acetate, according to the original method, i.e. 50 mL in the first and second extraction, did not present good recovery due to emulsification of the sample. Recoveries between 30 and 38% were obtained. The amount of solvent extractor and the number of extractions were increased to 100 mL of ethyl acetate in the first extraction and 50 mL in the second and third, according to Smith and Stewart (30). The mean recovery obtained was around 66.9%. This modification contributed to the obtainment of appropriate results, according to the criteria of Horwitz *et al.* (17).

b) Cleanup step

The cleanup step in a silica column presented recoveries between 30% and 40%, with either the toluene - ethyl acetate (3:1) or benzene - ethyl acetate (3:1) solvent-systems. These results were not acceptable.

The use of Carrez reagent, proposed by Pogosyan and Gelfand (26), was also not satisfactory, presenting recovery values between 40% and 50%.

In the original method, Tanner and Zanier (32) proposed a cleanup stage using 20 mL of 1,5% sodium carbonate solution to 100 mL of ethyl acetate. In the present optimized method, 40 mL of 1,5% sodium carbonate solution was used because the amount of ethyl acetate used had increased to 200 mL. Therefore, in effect, no modification of the original method was made to this stage of the process.

c) Detection step

In the TLC, detection efficiency was observed in the elution of patulin when using toluene - ethyl acetate - formic acid (6:3:1) as the solvent-system. It presented good resolution and reproducibility of the chromatogram. The use of a non-saturated chromatographic cube enabled fast development - approximately 30 minutes.

The chromogenic agent chosen was MBTH as it presented better sensitivity, as can be seen in Table 1. The advantage of using of MBTH is that, under white light, light yellow round and compact stains are detected, and under UV light, the patulin emits orange fluorescence.

Validation of the optimized method

The detection limit for the standard patulin solution was 7.44 ng, a mass corresponding to the smallest amount of standard working solution tested, detected visually under UV light (Table 1). The method also showed sufficient specificity in the tests using spiked samples. Non-desired compounds present in the samples could be detected as not being patulin.

The mean recoveries and variation coefficients obtained in the assays with spiked samples using the optimized method are illustrated in Table 2. The method presented recoveries between 60% and 85%, with an average of 65.4%.

The variation coefficients for repeatability and reproducibility obtained for the concentrations studied ranged from 7.58 to 13.51% and 0.36 to 18.76%, respectively. The mean recoveries and variation coefficients were within the established limits of acceptability according to Horwitz *et al*. (17), thereby demonstrating accuracy and precision.

The quantification limit was 15.87 $\mu g \cdot kg^{-1}$, since the mean recovery and variation coefficient were 62.5% and 7.58%, respectively.

CONCLUSIONS

The optimized method showed the best performance when carrying out three extractions with ethyl acetate, cleanup with 40 mL of sodium carbonate, the use of toluene - ethyl acetate - formic acid (6:3:1) as a solvent-system and the use of 0.5% MBTH in 5% formic acid as a chromogenic agent. Specificity was demonstrated by the separation of patulin from the non-desired matrix compounds, by the resolution of the stains and by the satisfactory recovery percentage. Accuracy and precision were observed to be in the range from 15.87 to 67.80 $\mu g\ kg^{-1}$. The visual detection and quantification limits of 7.44 ng and 15.87 $\mu g\ kg^{-1}$ were within the maximum limits permitted by international legislation. The validated method demonstrated efficiency for use in the monitoring of patulin in grape products.

RESUMO

Otimização e validação intralaboratorial de método para a determinação de Patulina em uva por Cromatografia em Camada Delgada

O objetivo deste trabalho foi otimizar e validar, por procedimentos intralaboratoriais, um método de determinação de patulina em uva por cromatografia em camada delgada. Foram realizados testes de otimização das etapas de extração, limpeza, detecção e quantificação. Para validação do método foram realizados ensaios de recuperação com soluções padrões e amostras artificialmente contaminadas. A recuperação e o coeficiente de variação foram 65,4% e 7,58%, respectivamente. As condições otimizadas foram: 50 mL do suco da uva; três etapas de extração, 100 mL de acetato de etila na primeira etapa e 50 mL na segunda e terceira etapas; 100 µL de etanol para

Table 1. Detection limit for patulin using MBTH and phenylhydrazine, under visible and ultraviolet (366 nm) ligh..

	PATULIN (3.72 ng µL^{-1})							
	(µL)	1	2	3	4	5	6	7
Chromogenic agents	(ng)	3.72	7.44	11.16	14.88	18.6	22.32	26.04
Phenylhydrazine	VIS	-	-	-	+	+	++	+++
MBTH	VIS	-	-	-	+	++	+++	+++
MBTH	UV	+/-	+	+	++	++	+++	+++

(+) visible; (+ / -) weakly visible; (UV) ultraviolet; (VIS) visible light.

Table 2. Recoveries and variation coefficients of patulin concentration in spiked grape samples.

Added concentration ($mg \cdot kg^{-1}$)	Mean concentration found ($mg \cdot kg^{-1}$)	MeanRecovery (%)	Variation coefficient – repeatability (%)	Variation coefficient – reproducibility (%)
15.87	9.92	62.5	7.58	-
22.99	15.00	65.3	13.51	-
33.90	24.90	73.4	-	18.76
67.80	40.87	60.3	-	0.36
Mean		**65.4**		

solubilizar o extrato; a fase móvel tolueno-acetato de etila-ácido fórmico (6:3:1) e o revelador o MBTH 0,5% em ácido fórmico 5%. O limite de detecção visual foi de 7,44 ng e o de quantificação de 15,87 µg/kg. O método otimizado e validado apresentou eficiência para ser adotado em atividades de monitoramento de patulina em uva.

Palavras-chave: patulina; micotoxinas; uva; cromatografia em camada delgada.

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