DETERMINATION OF PATULIN IN APPLE JUICE BY HPLC USING A SIMPLE AND FAST SAMPLE PREPARATION METHOD

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Abstract: The goal of this work was to develop a simple and rapid preparation method for patulin analysis in apple juice without previous clean-up. This method combined sonication and liquid extraction techniques and was used for determination of patulin in 37 commercial apple juices available on the market in the South of Brazil. The method performance characteristics were determined using a sample obtained in a local market fortified at five concentration levels of patulin and done in triplicates. The coefficient of variation for repeatability at the fortification level of $20.70 \mu g.L^{-1}$ was 3.53 % and the recovery 94.63 %, respectively. The correlation coefficient was 0.9996 and agrees with the requirements for a linear analytical method value. The detection limit was $0.21 \mu g.L^{-1}$ and the quantification limit $0.70 \mu g.L^{-1}$. Only three of the analyzed samples were upper the allowed level of $50.00 \mu g.L^{-1}$ recommended for the World Health Organization.

Keywords: patulin, HPLC/UV, apple juice, fast sample preparation method, ultrasonic extraction

Introduction

Many fungi found in spoiled food such as *Aspergillus* spp. and *Penicillium* spp., including *A.clavatus, A. giganteus, A. terreus, P. urticae, P.expansum* and *Byssochlamys nivea*, produce patulin frequently when they invade apples, pears, peaches and berries. Patulin contamination is primarily associated with fruit juices made from poor quality fruits (damaged and rotting fruits). A toxicological concern about apple product, such as apple juice is made particularly because of the apple-rotting produced by fungus *P. expansum* [1-4]. From studies conducted over the past twenty years, only apple products, especially apple juice were found to be easily contaminated by patulin, therefore, the patulin level has been regarded as a quality indicator for fruit used to made apple juice[5].

For human beings, patulin has caused nausea, vomiting, and gastrointestinal disturbances. The International Agency for Research on Cancer (IARC) has listed patulin as a potential carcinogen [6]. The U.S. Food and Drug Administration in its presentation to the Food Advisory Committee (FAC) recommended that patulin in apple juice should be reduced to minimum level and suggested a maximum permitted level at $50 \mu g.L^{-1}$

in apple juice, other countries like Switzerland, Sweden, Belgium and Norway that are concerned about this issue, have set a maximum permitted concentration at 50 $\mu g.L^{-1}$ as recommended by the World Health Organization [7,8].

In many laboratories, ethyl acetate has been the universal extraction solvent, and purification by sodium carbonate solution has been widely used in the sample pretreatment. Otherwise, liquid–liquid extraction are considerably expensive and time consuming due to the use of large amounts of organic solvents and the clean-up procedures using sodium carbonate can make patulin deactivate since it is more stable in an acidic medium [9,10]. Some articles using solid-phase extraction for analysis of patulin in apples and apple juices had been reported recently [11,12]. Reverse phase liquid chromatography/UV is considered more convenient and sensitive technique for analysis of patulin and methods to confirm the presence of patulin usually include detection techniques such as mass spectrometry (MS) after liquid or gas chromatography separations [9-14].

Few works reported reduction in time and solvent for sample preparation for patulin analysis in apple juice and no works about sonication extraction of patulin was found besides of the power of this extraction technique [15]. The ultrasonic technique described here, used a combination of two non-miscible solvents together (water in the apple juice and ethyl acetate), that permitted a rapid extraction of patulin from apple juice using a reduced amount of sample and extraction solvent in only one step.

Experimental

Reagents and chemicals

Patulin standard, 98% pure (Sigma-Aldrich, Steinheim, Germany); acetonitrile HPLC grade (Carlo Erba Reagenti, Milan, Italy); ethyl acetate HPLC grade (Tedia Company Inc., Fairfield, CA, USA) and acetic acid obtained from Vetec Quimica Fina Ltda.(Rio de Janeiro, RJ, Brazil).

Apparatus

Shimadzu UV-Visible Spectrophotometer, Model 1203 (Kyoto, Japan), with wavelength range from 190 to1100 nm. Sonication bath, Model USC 1400A (240x137x100 mm, 40 kHz and 100 W) produced by Unique Sistemas Automáticos de Limpeza (Indaiatuba, SP, Brazil). Agilent high performance liquid chromatograph, Model HP-1100 LC/MSD series produced by Agilent Technologies (Waldbronn, Germany), equipped with UV and MS detectors and automated sample injector. Synergi Fusion RP column (250 mm x 2.0 mm ID, 4 μm Fusion- RP80 stationary phase) obtained from Phenomenex Inc. (Torrance, CA, USA). Sample concentration system consisted of hot plate, vacuum and nitrogen lines developed by LAMIC for private laboratory use (Santa Maria, RS, Brazil).

Standard stock solution and Standard working solutions

Five milligrams of pure crystal patulin was accurately weighted into a 5 mL volumetric flask and dissolved in ethyl acetate. The flask was tightly wrapped with aluminum foil, identified as stock solution and kept in freezer (-18 ºC).

Two hundred and fifty micro liters of patulin standard stock solution was transferred into a 5 mL volumetric flask, evaporated to dryness under a nitrogen stream, and then homogenized and diluted to volume with absolute alcohol. The concentration was determined by spectrophotometry, using 276 nm and ε=14600 parameters.

Samples

Thirty-seven samples purchased from supermarkets and local stores in cities from Rio Grande do Sul State (Brazil). The samples included 100% pure apple juice (6), Soya added-apple juice (13) and apple nectar drink (18) packed in easy open cans or tetra pack box.

Sample extraction

A 5 mL apple juice sample and 20 mL of ethyl acetate were added into a 50 mL flask and capped. This flask was introduced in the sonication bath and allowed to extract for 15 minutes. The ethyl acetate phase was separated and transferred to another flask and placed to a hot plate (40°C) apparatus settled with vacuum and a gentle nitrogen stream to evaporate the solvent. When the residue was completely dry, it was immediately dissolved in 500 μL of acetonitrile/water/acetic acid (840:160:5, v/v/v) solution and analyzed by liquid chromatography.

High performance liquid chromatography

The final solution was analyzed under the following conditions: the analytical column was set at 30°C and isocratic mode. Mobile phase was 1% acetic acid solution-acetonitrile (99:1, v/v), with flow rate at 0.3 ml/min; UV detector wavelength set at 276 nm; sample injection of 2 μl. Patulin retention time obtained at 12.48 minutes.

Method validation

To validate the method, the analytical curve was constructed with standard patulin solutions at five concentration levels and in triplicates: 20.7, 31.0, 51.8, 103.6 and 207.2 $\mu g \cdot L^{-1}$. Samples of apple juice were fortified with the patulin solution at the same five levels of concentration used for the analytical curve construction (20.7, 31.0, 51.8, 103.6 and 207.2 $\mu g.L^{-1}$), in triplicates and the chromatographic analysis was performed in duplicates. They were compared with the blank sample (juice without fortification) to obtain the method recovery. All samples including the standard patulin solutions (used for construction of the analytical curve) were diluted in a mixture of acetonitrile/water/acetic acid (840:160:5, v/v/v) for the HPLC analysis.

Results and discussion

The method

This method was simple just because the sample preparation involves only three steps: extraction by sonication bath, phase separation and concentration. An extraction method with few steps allows a good recovery, as can be seen at Table 1. Also in this case, it is possible to sonicate several samples at the same time which depends only of the sonication bath size. In our case, we did ten samples by time (15 minutes). To separate the phases, it is not necessary to use separation funnel and no need of other clean up process. It can be done using a Pasteur pipette (as shown at Figure 1). The amount of sample and extraction solvent was determined in previous extractions tests using fortified sample at the low concentration level. The sonication extraction time used was defined based in author experience with this technique in other application [16].

However, some caress is needed, the prepared sample can't be exposed at temperature higher than 45°C due to loss or degradation of patulin and need to be dissolved in the working solution as soon as it dryers. The chromatographic analysis need to be done just after that. That was the reason why the fortified level of 50.80µg.L^{-1} had a high variation coefficient (10.37 %); in this case one of the samples was allowed to exceed the state of dryness.

The total time of the chromatographic analyses was 17 minutes and the patulin peak appears at 12.48 minutes. Each run consumes approximately 6 mL of acetonitrile. The Figure 2 shows the chromatogram of patulin (Fig.2a), apple juice without fortification(Fig.2b) and apple juice fortified with 20.70 µg.L^{-1} of patulin (Fig. 2c) respectively. Besides of the patulin peak broadening due to isocratic mode, it is possible to detect the analyte at low levels of quantitation.
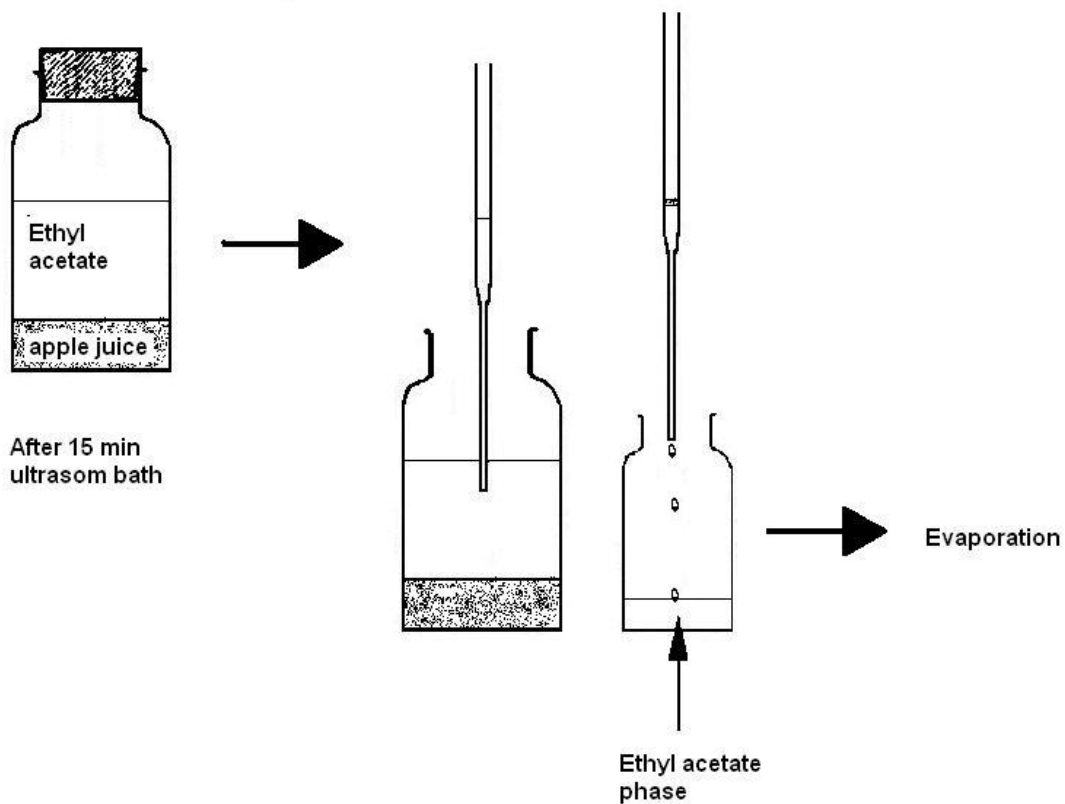
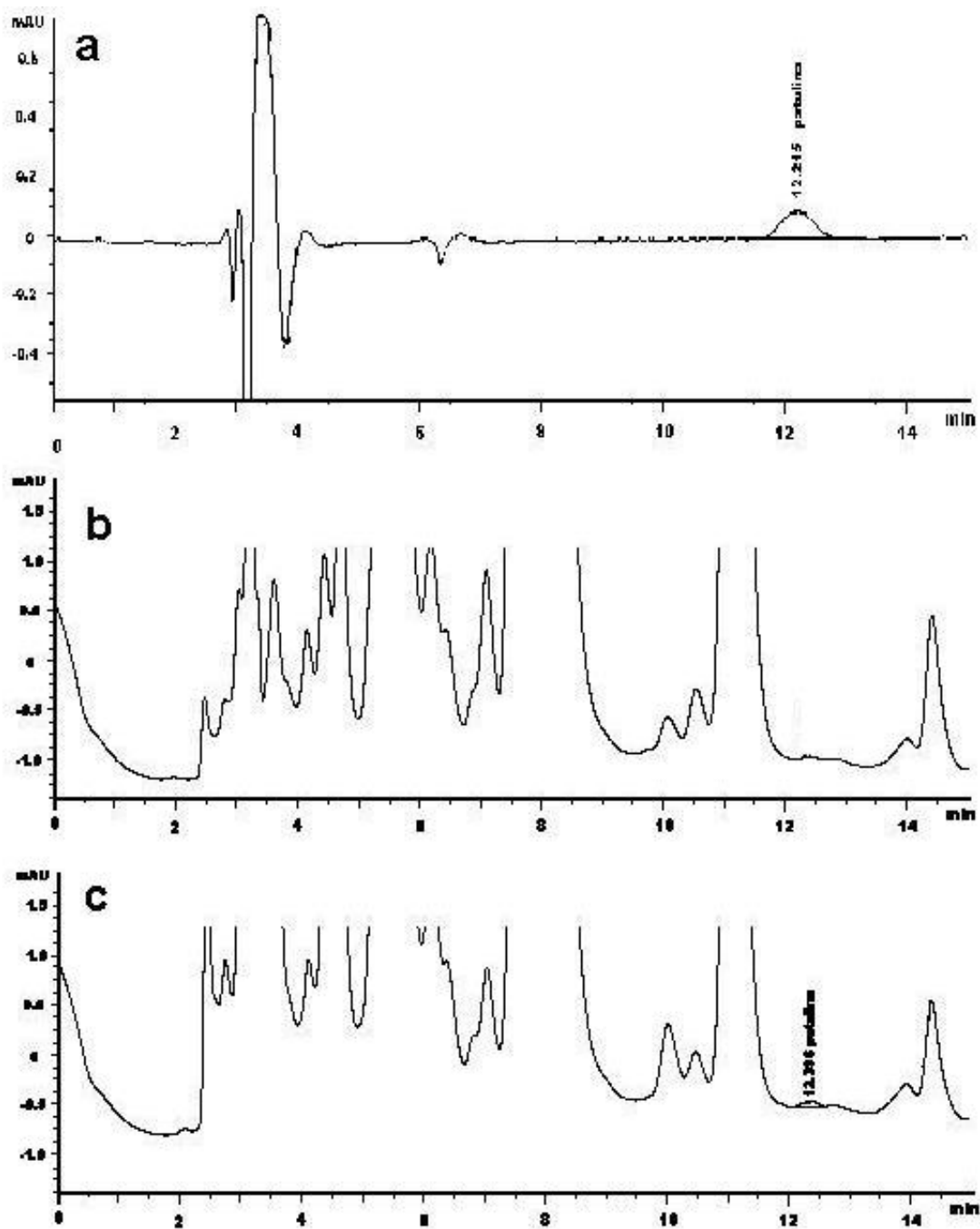**Figure 1.** Phase separation after sonication extraction.

Figure 2. Chromatogram referring to the determination of patulin in apple juice after extraction: (a) Patulin standard (100 μg.L^{-1}); (b) blank apple juice; (c) apple juice spiked with Patulin (20 μg.L^{-1}). Chromatographic conditions: Synergi Fusion RP column; mobile phase: acetic acid solution 1% and acetonitrile (99:1), isocratic mode; flow rate, 0.5 mL.min^{-1}; UV detection (276 nm), oven at 30°C.

Table 1. Recoveries of patulin from spiking.

Spiking level (μg.L-1)	RSD (%) (n=6)	Recovery (%)
20.70 ± 0.07	3.53	94.63
31.00 ± 0.11	4.35	82.58
51.80 ± 0.43	10.37	80.22
103.60 ± 0.14	1.54	86.83
207.20 ± 0.45	2.31	94.65

LOD = 0.21 μg.L^{-1}
LOQ = 0.70 μg.L^{-1}

Performance characteristics

As it can be seen at Table 1, the recovery for the method varies from 80.22 to 94.65% and is considered as a goodmethod. The standard deviation is between 0.07 and 0.45 with a variation coefficient from 1.54% (spiking of 20.70μg.L^{-1}) to 10.37% (spiking of 51.80μg.L^{-1}), at this level, we considered that this value was due patulin degradation in one sample that was allowed to exceed the state of dryness

The standard deviations (SD) were determined based on the areas obtained from each compound. The values of limit of detection (LOD) and limit of quantification (LOQ) were calculated using the formula 3×SD and 10×SD for the low fortification level (20.7 μg.L^{-1}).[17] The values obtained were: 0.70 μg.L^{-1} and 0.21 μg.L^{-1}, respectively. The linearity was studied for the patulin over a range of spiking levels from 20.70μg.L^{-1} to 207.00μg.L^{-1}. The correlation coefficient was 0.9996 and fulfils the requirement for a linear method.

Analysis of apple juice samples

From the 37 commercial apple juices available on the market of the South of Brazil (Rio Grande do Sul State), only three samples were upper the amount of 50 μg.L^{-1} recommended by the World Health Organization. From these samples, the contaminated samples were of Soya added-apple juice (2) and 100% pure apple juice (1) with amounts of 53.5, 54.9 and 51.6 μg.L-1 respectively. This contamination tax corresponds to 8.1 % of the total samples analyzed.

Conclusions

This method was successfully applied to the extraction and analyze of patulin from apple juice (Soya-added, nectar or 100% apple juice) and only three samples (8.1% of the total) had patulin contamination level higher than 50.μg.L^{-1}.

The proposed method is simple, cheaper and can be used for routine analyses because allows to extract several samples at the same time using a short period of time. In this work, it was possible to extract 10 to 12 samples in less than one hour, and it depends on the size of the ultrasonic bath and sample extraction vials. Only 6 mL of acetonitrile and 20 mL of ethyl acetate was used by sample, what contributes to minimize the chemical residue when compared to liquid-liquid extraction in the official method [18,19].

The ultrasonic technique described here, using a combination of two non-miscible solvents together (water in the apple juice and ethyl acetate), permitted a rapid extraction in only one step, solvent reduction and good reproducibility and recovery, as seen in the validation parameters obtained. Finally, the low quantification limit permits analysis of reduced quantities of samples.

Acknowledgements

Resumo: O objetivo deste trabalho foi desenvolver um método simples e rápido para preparação de amostras para análise de patulina em sucos de maçã sem "clean-up" prévio. Este método combina os métodos de extração líquida com o ultrassom e foi utilizado para a determinação de patulina em 37 amostras de suco de maçã disponíveis no mercado do sul do Brasil. Os parâmetros estatísticos para validação do método foram obtidos através de fortificação de suco de maçã, obtido no mercado local, em cinco níveis de concentração de patulina e realizadas em triplicatas. O coeficiente de variação para a repetibilidade no nível de fortificação de 20,70 µg.L^{-1} foi de 3,53 % e a recuperação de 94,63 %. O coeficiente de correlação foi de 0,9996 e este valor está de acordo com os requisitos de linearidade de um método analítico. O limite de detecção obtido foi de 0,21 µg.L^{-1} e o limite de quantificação de 0,7 µg.L^{-1}. Somente três das amostras analisadas tiveram valores superiores ao limite de 50,00 µg.L^{-1} recomendado pela Organização Mundial da Saúde.

Palavras-chave*:* patulina, CLAE/UV, suco de maçã, extração por ultrassom, método rápido de preparação de amostra.

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