Development of HPTLC method for determination of patulin in apple products

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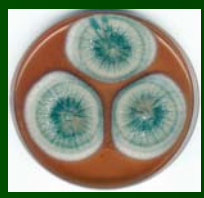
Introduction

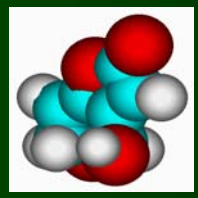
Patulin, 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one, is a lactone containing secondary metabolite of several fungal species of ***Penicillium***, ***Aspergillus*** and ***Byssochlamys*** growing on fruit, including **apples**, **pears**, **grapes** and other **fruit**. It has also been reported in **vegetables**, **cereal grains** and **silage**. In whole fruits, visual inspection will usually identify poor-quality items. The principal risk arises when unsound fruit is used for the production of juices and other processed products.

Penicillium expansum appears to be the mould usually responsible for **patulin** in **apple juice**. Thermal processing appears to cause only a moderate reduction in patulin levels. Thus the patulin present in apple juice will survive the usual pasteurization processes, addition of ascorbic acid, irradiation, and addition of sulfur dioxide. Although, its toxic effects on human are not clear yet, the **Joint FAO/WHO Expert Committee on Food Additives (JECFA)** suggests a provisional tolerable weekly intake of **7 micrograms/kg** body weight.

Consequently, a variety of methods have been study to rapidly determine the presence of patulin in apples and apple products. The majority of them employ an extraction step, normally using ethyl acetate, followed by **HPLC** separation by reversed phase chromatography.



Penicillium expansum



Patulin

Experimental

Extraction and clean-up procedure

A. The **sample** of apple product (5 g) was diluted with water (8 mL) and three times shaken with addition of **ethyl acetate** (8 mL) at high speed. The solution was centrifuged, ethyl acetate extracts were collected and evaporated to dryness under vacuum. The residue was dissolved in **ethyl acetate-benzene** (5:20, v/v; 2.5 mL). The **solid-phase extraction** on **silica-gel columns** (Merck,Si 500 mg) was used for cleaning procedure. Patulin was eluted with **benzene-ethylacetate** (9:3, *v/v*; 10 mL).

B. The **sample** of apple juice (2.5 mL) was shaken with addition of **acetonitril-water** (84:16, v/v; 10 mL). After centrifugation the upper phase (4 ml) was purified on **MycoSep Patulin column Romer**, where matrix and other interfering substances were bound. The analyte (patulin) was not bound by the packing material of the MycoSep column.

C. The **sample** of apple juice (2.5 mL) was three times shaken with addition of **ethyl acetate** (10 mL), ethyl acetate extracts were collected and evaporated to dryness. The **MycoSep Patulin column Romer** was used for cleaning procedure of extract (2 mL) dissolved in **acetonitril-water** (84:16, v/v; 7 mL).

The purified extracts were evaporated to dryness under nitrogen, and dissolved in **chloroform** (100 µL) for **HPTLC** analysis.

Analytical method

Chromatography was performed on 20 cm x 10 cm **silica gel 60** HPTLC plates (Merck). Diluted calibration standard of patulin in chloroform (5 µg/mL), (1, 5, and 10 µL corresponding to **5**, 2**5**, and **50** ng patulin), and cleaned samples were applied by **spot technique** with a Camag model III automatic TLC sampler; spots were 1 cm from the edge of plate, distance between samples was 5 mm. The plates were **developed** with **toluene-ethyl acetate-formic acid** (6:3:1, v/v), in the dark, in a saturated 20 cm x 10 cm vertical development chamber. After drying in a stream of cold air the plate was **sprayed** with solution of **3-methyl-2-benzothiazoline hydrazone** (MBTH) in water (0.5 %) and heated (105 ^{0}C) for 10 min.

Patulin was measured by **fluorescence** densitometry by means of a Camag TLC Scanner II with **Deuterium lamp** and K 400 secondary filter. The excitation wavelength was **275 nm**, the emission wavelength **420 nm**, and the SENS and SPAN parameters were **160** and **65**, respectively. The R_F value of patulin under these conditions was **0.56**. **Calculation** of sample content was performed by comparision of peak heights and areas with those of standard. **Validation** of the method was performed according to the principles of the **ICH Guideline for pharmaceutical analysis**.

Results and Conclusions

In summary, compare single steps, in point of rapidity and simplicity was the best the second extraction and clean-up procedure with MycoSep Patulin column Romer, while in term of limit of quantification (LoQ) the best results were from the first procedure with silicagel column (Si 500 mg) Merck. The found values of **limit of quantification** and **recovery** are presented in Table.

Mycotoxin patulin	Clean-up procedure		
	A	B	C
LoQ (µg/kg)	10.0	16.0	11.5
Recovery (%)	80 - 90	85 - 90	85 - 90