Improvements in the Quantitation of Patulin in Apple Juice by High-Performance Liquid Chromatography†

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A method is described for Sep-Pak silica cartridge purification of patulin from apple juice followed by reversed-phase high-performance liquid chromatographic (HPLC) determination. The method has a rapid sample preparation and offers a good separation of patulin from other constituents. The 80–87% recovery at 3–100 ppb levels is on the order of the best described methods for patulin analysis. The method has been applied to 10 different apple juice concentrates and 8 commercial apple juices, showing a fairly good reproducibility and a detection level of 2 ppb.

Patulin is a secondary metabolite produced by a large number of fungi (Davis and Diener, 1978) including *Penicillium expansum*, a causal organism of apple rot. The main source of patulin in the human diet is probably apple juice prepared from apples contaminated with molds (Mortimer et al., 1985). Patulin is toxic and produces tumors in rats at the place of injection when injected subcutaneously (Dickens and Jones, 1961), but there are no published toxicological or epidemological data to indicate whether consumption of patulin is harmful to humans (IARC, 1986). However, health authorities in Switzerland, Sweden, Belgium, Russia, and Norway apparently regard patulin contamination of food as a problem and have set a maximum permitted concentration (MPC) of 50 ppb for patulin in apple juice (Watkins et al., 1990). Patulin has also become a quality indicator of fruit used in the processing of apple juice. Because of this, a rapid and reliable method for the quantitation of patulin in apple juice is important to processors and governments interested in monitoring the quality of apple juice and concentrate.

Many methods have been developed for the analysis of patulin in apple juice. A fast method for the analysis of patulin by reversed-phase HPLC using sodium carbonate as the only purification step has been described (Möller and Josefsson, 1980; Kubacki and Goszcz, 1988), although some doubts exist about the stability of this toxin in such a basic system (Windholz, 1983). Many other methods using either reversed-phase HPLC or GLC have been published, though all require relatively long analysis times or lack good sensitivity for residue analysis (Engel and Teuber, 1984; Ehlers, 1986; Lehmann and Wald, 1990).

The purpose of this study was to make improvements in the analysis of apple juice for patulin using Sep-Pak cartridge and reversed-phase HPLC.

EXPERIMENTAL PROCEDURES

Juice Samples. Apple juice concentrates were received from several Catalan producers, and apple juices were bought in different local stores.

Reagents and Standards. Water was doubly distilled and purified by the TRABSA RO-2000 system (Trabsa, Barcelona, Spain). The solvents used for extraction were distilled in glass, and the tetrahydrofuran (THF) used in the HPLC analysis was of HPLC grade. The solvent for HPLC analysis was filtered through a 0.45-μm filter and degassed in vacuum just prior to use. Patulin was purchased from Sigma Chemical Co. (St. Louis, MO), and stock 1000 and 100 ppm patulin standards were prepared by dissolving 10.00 and 1.00 mg of patulin in 10 mL of acetonitrile each and storing at −20 °C. Working standards of 10, 1, and 0.1 ppm of patulin were prepared by dilution of stock solutions with water–THF (99 + 1).

A stock apple juice solution was prepared before each run analysis by weighing 20.0 g of concentrate juice and 120.0 g of water. The contents were stirred until homogenization. Spiked test samples from the apple juice were then prepared to contain 100, 20, or 3 ppb of added patulin. Spiked test samples to study the effect of some steps on recovery were prepared by spiking the corresponding solution at each step with an equivalent amount of patulin.

Sample Preparation. Fifty milliliters of apple juice bought or prepared from apple juice concentrate as described above was extracted three times with an equal volume of ethyl acetate. The organic solutions were combined and evaporated to dryness under reduced pressure. The dried residue was redissolved in 20 mL of chloroform plus 0.5 mL of water and dried for 1 h over ca. 1 g of anhydrous sodium sulfate. After that, 5 mL of the chloroform solution was introduced into a Sep-Pak SiO_2 cartridge (Waters Chromatography Division, Millipore Corp., Milford, MA), previously conditioned with 5 mL of chloroform. The cartridge was washed with 1 mL of chloroform, 1 mL of chloroform–ethyl acetate (8 + 2), and 1 mL of chloroform–ethyl acetate (5 + 5). All of these fractions were discarded. Then 2 mL of ethyl acetate–chloroform (8 + 2) was passed through the cartridge. The eluent was collected, and the solution was evaporated under a gentle stream of nitrogen.

The dried residue was dissolved in 1 mL of water–THF (99 + 1), the sample was filtered through a 0.45-μm filter, and 50 μL was injected into the HPLC.

HPLC Conditions. An Applied Biosystems Series 400 pumping system (ABI Analytical Kratos Division, Ramsey, NJ), an Applied Biosystems 491 dynamic mixer/injector with a 20-μL loop, an Applied Biosystems 783 A variable-wavelength absorbance detector set at 272 nm, and a Hewlett-Packard 3396 Series II integrator (Hewlett-Packard, Avondale, PA) were used. All extracts were analyzed with duplicate injections.

A mobile phase consisting of water–THF (99 + 1) was used at a flow rate of 1 mL/min. A Spherisorb ODS-2 10-μm (25 × 0.4 cm) analytical column (Teknokroma, Sant Cugat del Vallés, Spain) preceded by a C_{18} guard column (Waters Chromatography) were used.

Statistical Analysis. The data were subjected to analysis of variance utilizing the Statgraphics program (Statistical Graphics Corp., Rockville, MD). The Scheffe test was applied to determine the significance of differences between mean values.

RESULTS AND DISCUSSION

Studies were made to determine the influence of drying time, evaporating system, and Sep-Pak fractionation on

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Table I. Loss of Patulin from a 10 ppm Chloroform Solution after Drying under a Nitrogen Stream

sample	nitrogen stream	drying period	mean, ppm	SD (n = 3)	% recov[a]
1	gentle	to dryness	9.9	0.3	99[a]
2	strong	to dryness	8.5	2.0	85[b]
3	strong	15 min after dryness	7.2	5.0	72[c]
4	strong	60 min after dryness	6.3	3.0	63[d]

[a] Means in the same column bearing different superscripts are significantly different ($P < 0.05$).

Table II. Recovery of Patulin in Three Consecutive Washes Used in Sep-Pak Cartridge Purification

ethyl acetate–chloroform (5 + 5)		ethyl acetate–chloroform (8 + 2)		
mean,[a] ppb	SD (n = 5)	mean,[a] ppb	SD (n = 5)	ethyl acetate mean,[a] ppb
3.2	1.2	91.8	0.9	0

[a] Concentration related to 100 ppb in apple juice.

patulin recoveries. To carry out those studies, as mentioned under Experimental Procedures, spiked solutions were prepared just before the corresponding step was carried out. Once the effect of each step had been determined, the whole method was checked out and finally applied to commercial apple juice and apple concentrate.

Inert Gas Drying. Because of some erratic results at the beginning of our studies, we decided to carry out an experiment to determine the influence on the recovery of patulin of the stream of inert gas used. A solution of 10 ppm of patulin in chloroform was prepared, and four different sets of experiments were carried out. In each of them, three replicates of 1 mL were evaporated using either a gentle or strong stream of dry nitrogen and different evaporating times. Residues were redissolved in 1 mL of mobile phase and analyzed by HPLC using the method described under Experimental Procedures.

Table I shows that a strong nitrogen stream and a long drying period were able to produce a significant loss of patulin. Thus, whereas 99% of patulin is recovered using a gentle nitrogen stream, recovery decreases to 85% or lower when other drying conditions are used. Consequently, care should be taken during the nitrogen drying step.

Sep-Pak Purification. Since the two last solutions used in the Sep-Pak purification were very close in polarity, a study was carried out to determine the possibility of losing patulin in the ethyl acetate–chloroform (5 + 5) fraction.

An apple juice blank was extracted and a chloroform solution obtained, as described under Experimental Procedures. This organic solution was spiked with patulin to an equivalent concentration of 100 ppb in apple juice and dried over anhydrous sodium sulfate. The corresponding aliquot was introduced into the cartridge and eluted as described under Experimental Procedures. To study whether some patulin could remain in the cartridge, an additional 3 mL of ethyl acetate was finally passed through the cartridge and a new fraction was recovered. The three fractions were dried under a gentle current of nitrogen, and the residues were dissolved in the mobile phase and analyzed by HPLC.

Table II shows that the toxin was mainly recovered in the second fraction, no patulin was recovered in any of the five replicates of the last fraction, and less than 4% appeared in the first fraction. These results, together with the presence of different components in the ethyl acetate–chloroform (5 + 5) fraction which could produce interferences, when a low detection level was required, led us to use the fractionating system described under Experimental Procedures.

Table III. Effect of Drying Extract over Sodium Sulfate on Recovery of Patulin

drying agent	period, h	mean,[a] ppb	SD (n = 3)	% recov[b]
no		77.5	2.1	77.5[a]
yes	1	90.0	1.4	90.0[b]
yes	12	80.5	0.7	80.5[a]
yes	24	55.5	2.1	55.5[c]

[a] Concentration related to apple juice. [b] Recoveries related to 100 ppb in apple juice. Means in the same column bearing different superscripts are significantly different ($P < 0.05$).

Table IV. Recovery of Patulin from Spiked Apple Juice

sample[a]	mean, ppb	SD (n = 6)	% recov[b]
100	87.7	7.5	87.7[a]
20	16.8	0.5	84.0[a,b]
3	2.4	0.1	80.0[b]

[a] Concentration in spiked apple juice (ppb). [b] Means in the same column bearing different superscripts are significantly different ($P < 0.05$).

Table V. Patulin Contamination in Apple Juice from Concentrate

sample	mean, ppb	SD (n = 3)	sample	mean, ppb	SD (n = 3)
1	ND[a]		6	26.3	1.2
2	16.3	1.2	7	3.7	0.6
3	3.3	0.6	8	2.3	0.6
4	2.0	0.1	9	34.0	3.5
5	26.3	1.2	10	28.3	5.1

[a] ND, not detected.

Sodium Sulfate Drying. After ethyl acetate extraction of apple juice, the solvent was evaporated and the residue was redissolved in a mixture of chloroform–water (20 + 0.5). A spiked solution of patulin was prepared from this solvent at an equivalent concentration in apple juice of 100 ppb. Water helps to improve the patulin dissolution in the chloroform phase, but it can produce some losses during Sep-Pak purification. Consequently, a drying step seems necessary.

Table III shows the effect of anhydrous sodium sulfate on patulin recovery. If a drying agent were not used, recovery would decrease to 77%, whereas after 1 h of drying the recovery was 90%. After 12 and 24 h, the recoveries decreased again to 80% and 55%, respectively.

Recovery. Patulin recovery rates from our procedure were determinated by addition of known amounts of patulin to an apple juice prepared from concentrate which had previously shown no patulin contamination within our level of detection (2 ppb; signal:noise, 3:1). Table IV shows recoveries are 80% or higher in the three levels studied.

Applicability. The method has been used to analyze 10 different apple juice samples from concentrates supplied by local industries, and 8 different apple juices bought in several local stores. Juices were extracted in triplicate with duplicate injections of each extract. Tables V and VI show the level found in each juice together with the standard deviation (SD) of the three replicates. Figure 1 presents the chromatogram of an apple juice with a contamination level of 3 ppb, and Figure 2 corresponds to an apple juice without patulin within the level of detection.

Conclusion. The method described here was found to show good recoveries for patulin from apple juice. These

Table VI. Patulin Contamination in Apple Juice Bought in Different Local Stores

sample	mean, ppb	SD ($n = 3$)	sample	mean, ppb	SD ($n = 3$)
1	17.0	0.1	5	ND[a]	
2	78.0	9.6	6	20.0	1.0
3	25.0	1.0	7	18.0	0.1
4	3.0	0.1	8	ND[a]	

[a] ND, not detected.

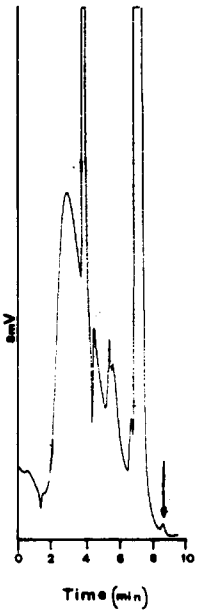

Figure 1. Chromatogram of an apple juice with a contamination level of 3 ppb (arrow shows patulin pic).

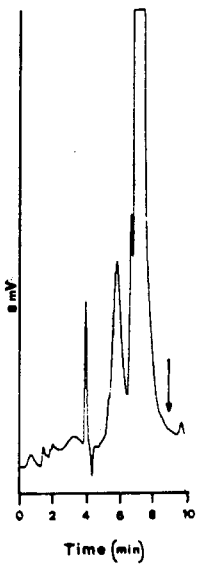

Figure 2. Chromatogram of an apple juice without patulin within our level of detection (arrow shows retention time of patulin).

recoveries were higher than those of the method of Möller and Joseffson (1980) and similar to those of the collaborative study of Kubacki and Goszcs (1988), both using a sodium carbonate step as the only purification step. Furthermore, our method showed a better detection limit than those claimed by these authors. Relative to other methods which use one or more columns for purification, our method is faster, has similar or better recovery levels, and has the same or better sensitivity (Leuenberger et al., 1978; Stray, 1978, Forbito and Babsky, 1985; Kubacki and Goszcz, 1988; Lehmann and Wald, 1990; Tarter and Scott, 1991).

Only one of the different apple juices analyzed showed a contamination level higher than 50 ppb (Table VI), the maximum value permitted for patulin by the health authorities of some countries (Watkins, 1990). Rates of positive samples (90% in apple juice from concentrate and 75% in apple juice bought in local stores) are higher than the ones found in other countries. Thus, Giepel et al. (1981) analyzed apple juice from Germany and found 64% of positive samples with contamination levels of 2–42 ppb, whereas Watkins (1990) found in Australia 67% of positive samples with contamination levels of 5–625 ppb. However, in this case the detection limit (5 ppb) was slightly higher than our own.

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