Micellar Electrokinetic Capillary Electrophoresis for Rapid Analysis of Patulin in Apple Cider

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A micellar electrokinetic capillary chromatography (MECC) mode was applied to a capillary electrophoresis (CE) method, which was developed for detection and quantitation of patulin in apple ciders. This method used a small sample amount (2 mL) and consumed minimal organic solvent compared to the most commonly used HPLC methods. The sample preparation procedure of the CE method was also simpler than other chromatographic techniques developed for patulin analysis. Patulin was detected with a photodiode array detector at 273 nm. The standard curve was linear ($r^2 = 0.9984$) from 75 μg/L to 121 μg/mL with patulin working solutions corresponding to 3.8 μg/L to 6.1 μg/mL patulin in the sample. The linearity was better in a narrower range of concentrations ($r^2 = 0.9999$) from 75 μg/L to 24.1 μg/mL. The limit of detection of the method was 3.8 μg/L. Patulin recoveries at 4 levels in spiked samples (10−121 μg/L) ranged from 95.2 to 105.4%. The recoveries were 96.9% and 99.2% for 2 levels (22.3 and 223 μg/L, respectively) of patulin in infected apple samples. This method represents a unique alternative method for rapid and sensitive analysis of patulin in apple ciders.

Keywords: *Patulin; capillary electrophoresis; micellar electrokinetic capillary electrophoresis; micellar electrokinetic capillary chromatography; apple juice; apple cider; Penicillium expansum*

Patulin (Figure 1) is a naturally occurring mycotoxin produced in apples and other fruits by several Penicillium and Aspergillus species, especially *Penicillium expansum* Link (Brian et al., 1956; Scott and Bullerman, 1975). It has been shown to cause a variety of adverse effects in toxicological studies, including nausea, vomiting in humans (Walker and Wiesner, 1944), induction of tumors in rats at the site of subcutaneous injection (Dickens and Jones, 1961; Brackett and Marth, 1979), mutagenicity to *Saccharomyces cerevisiae* (Mayer and Legator, 1969), and teratogenicity to chicken embryos (Ciegler et al., 1976). Levels of patulin found in apple juice have caused concerns for some populations of people, especially young children (Prieta et al., 1994). The adverse problems caused by patulin as well as by other mycotoxins such as aflatoxins could become even more serious if organic farming becomes more popular in the future. It was reported that conventional apple juice (from apples which had been sprayed with synthetic fungicides pre- and/or postharvest) had patulin levels ranging from 244 μg/L up to 3993 μg/L, whereas juices from organically grown apples had patulin at rates up to 45 000 μg/L (Lovejoy, 1994). Because of health-related concerns, it has been suggested that patulin be used as a quality indicator for apples used in food and drink (Rovira et al., 1993; Prieta et al., 1994). The World Health Organization (WHO), and many European countries, such as Switzerland, Sweden, Belgium, Russia, and Norway, have set the maximum acceptable level of patulin in apple juice at 50 μg/L (Moller and Josefsson, 1980; Forbito and Babsky, 1985; Van Egmond, 1989; Stoloff et al., 1991, Rovira et al., 1993).

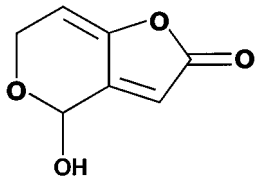

Figure 1. Chemical structure of patulin.

Analysis of patulin in apple juice or cider has long been a difficult task. Methods using TLC, GC, GC-MS, and HPLC have been developed (Stray, 1978; Williams, 1984; Moller and Joseffson, 1980; Forbino and Babsky, 1985; Tarter and Scott, 1991; Rovira et al., 1993; Bartolome et al., 1994; Prieta et al., 1994; Gokmen and Acar, 1996; Herry and Lemetayer, 1996; Machinski and Midio; 1998). The HPLC method has gained popularity in recent years over the others because of its lower detection limit, better precision, and ease of automation (Brause et al., 1996). A reverse-phase HPLC method was used by Moller and Josefsson (1980) for rapid analysis of patulin. Rovira et al. (1993), however, argued that using only sodium carbonate, a very basic material, in the purification step might affect the stability of patulin. More recently, Rovira et al. (1993) developed an HPLC method using Sep-Pak cartridges in sample preparation. The limit of detection of HPLC methods has been in the range of 1−5 μg/L using an ultraviolet detector set at 272−280 nm. Recovery of patulin from spiked apple juice in those methods was 71−92% at concentrations between 3 and 240 μg/L (Stray, 1978; Moller and Josefsson, 1980; Rovira et al., 1993; Herry and Lemetayer, 1996). These HPLC methods involved

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extensive extraction and cleanup procedures for good chromatographic separation. Despite the many efforts in simplifying the extraction and cleanup procedures for rapid analysis of patulin, many of them are still cumbersome (Moller and Josefsson, 1980; Forbito and Babsky, 1985; Rovira et al., 1993).

Capillary electrophoresis (CE) is a new separation and analytical technique, which has been increasingly used in analysis of many different compounds in various food samples (Zeece, 1992; Cancalon, 1995; Colon et al., 1997). In CE, a fused silica capillary is filled with an aqueous run buffer and an electric field is applied to the capillary. Separation is achieved by migration of charged particles in the run buffer. Cations migrate to the cathode and anions migrate to the anode under the influence of an electroosmotic flow. Neutral compounds or a mixture of neutral and charged compounds are usually analyzed with the micellar electrokinetic capillary electrophoresis or micellar electrokinetic capillary chromatography (MECC) mode, which is performed by addition of micelle-forming compounds such as SDS (sodium dodecyl sulfate) in the run buffer at a concentration above their critical micelle concentration (Zeece, 1992; Weston and Brown, 1997). CE offers many advantages over conventional chromatographic techniques, including reduction in use of organic solvents, small sample volume, and increased efficiency and resolution (Cancalon, 1995; Weston and Brown, 1997).

This paper reports the use of CE as a rapid and sensitive analytical method for the mycotoxin patulin in apple cider.

MATERIALS AND METHODS

Chemicals. Reverse osmosis water (in-house) was further purified by the Milli-Q system (Millipore Canada Ltd., Mississauga, ON, Canada). All solvents used in preparation of standard solutions and extraction of samples were of HPLC grade from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Patulin standard was purchased from Sigma Chemical Co. (St. Louis, MO); sodium dodecyl sulfate (SDS), sodium tetraborate, and acetic acid were of reagent grade from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Run Buffer, SDS, and Standard Solutions. Sodium tetraborate solution was prepared at 0.1 M. SDS was made at 0.2 M. The borate buffer and the SDS solution were then mixed and diluted so they were at 25 and 50 mM, respectively, in the final run buffer. The pH value of the run buffer was 9.0. The run buffer was filtered through a 0.45-μm Gelman Acrodisk GHP (Gelman Sciences, Ann Arbor, MI) prior to use in CE analysis. Run buffer in vials at both electrodes was replaced with freshly prepared buffer after every 10−15 runs. A stock solution of patulin standard was prepared in acetonitrile (ACN) at a concentration of 121 μg/mL (w/v). Working standard solutions of patulin (24.1, 4.8, 1.0, 0.6, 0.3, 0.15, and 0.075 μg/mL) were prepared by dilution of stock solution with acidified water (pH 4.0, adjusted with acetic acid). Spiked samples from a patulin-free apple cider were fortified to contain 121, 50, 25, and 10 μg/L of patulin. A cider (with patulin concentration at 38.9 μg/mL) made from a "Red Delicious" apple completely rotten by *P. expansum* was diluted with a patulin-free cider to 223 and 22.3 μg/L and used in an additional recovery test.

Sample Preparation. "Red Delicious" apples free of *P. expansum* infection were pressed with a hydraulic press into a cider which was then filtered through a sheet of cheesecloth and centrifuged for 15 min at 10 000 rpm (Sorvall Superspeed RC2-B, Ivan Sorvall Inc., Newtown, CT). The supernatant was stored in a freezer (−75 °C) before being used in the spike recovery tests. Partially or completely rotten apples by *P. expansum* were pressed similarly into ciders. A completely rotten "Red Delicious" apple was obtained by incubating a wounded and inoculated apple (with *P. expansum* at 10^3 conidia/mL) in cold storage (1 °C) for 6 months. The cider prepared from this apple was first analyzed by direct injection for its patulin concentration and then diluted and used in the recovery test as a "naturally contaminated apple cider". Partially rotten apples (Red Delicious, Jonagold, Empire, MacIntosh, Northern Spy) were obtained by incubating wounded and inoculated apples (with *P. expansum* at 10^3 conidia/mL) at 20 °C) for 9 days. All cider samples were filtered through a 0.45-μm filter before being directly injected for analysis. Ciders with patulin concentrations higher than the highest concentration in the calibration curve (24.1 μg/mL) were diluted with water to a concentration that fell to the linear dynamic range before being reinjected. For samples with concentrations lower than the minimum detectable concentration (75 μg/L), 2 mL of cider was extracted (vortexed at 2500 rpm for ca. 30 s) three times with an equal volume of ethyl acetate in a 4-mL dram vial with a Teflon-lined screw cap. The ethyl acetate layers were carefully removed with a pipet, combined and passed through a 1-mL disposable pipet containing 1.5 g anhydrous sodium sulfate into a 10-mL conical test tube with a screw cap. The sodium sulfate was rinsed with additional 0.5−1 mL of ethyl acetate prior to drying. The dried ethyl acetate solution was evaporated under a gentle stream of nitrogen to dryness. The dried residue was reconstituted in 0.1 mL acidic water solution (pH 4) and subjected to immediate CE analysis. Spiked samples and naturally contaminated samples followed the same extraction procedures as stated above.

CE Apparatus and Operating Conditions. The capillary electrophoresis instrument used in this study was a Beckman P/ACE 5500 model with a photodiode array detector, an autosampler with two rotating trays, and a cartridge interface with cooling option (Backman Instruments, Inc., Fullerton, CA). The run buffer (pH 9.0) contains 25 mM borate (see above) and 50 mM SDS. Buffers and sample solutions were filtered through a 0.45-μm syringe filter before analysis, with the exception of samples of less than 0.1 mL. The fused silica capillary used for separation was kept at a constant temperature of 25 °C and had an effective length of 50 cm with 75 μm i.d. The capillary was conditioned by flushing at high pressure (20 psi) with the capillary regenerator solution A (Beckman Instruments, Inc., Fullerton, CA) for 2 min, distilled water for 1 min, and finally with run buffer for 1 min, prior to each run. A separation was carried out at a constant potential of 15 kV, and the maximum current was set at 200 μA. UV absorption at 273 nm was monitored for patulin. A pressurized mode of injection was used. Injection was made at low pressure (0.5 psi) for 5 s, corresponding approximately to an injection volume of 30 nL. The total run time of the method was 10 min.

Data Analysis. All data were analyzed using SAS/STAT 6.12 (SAS Institute Inc., Cary, NC). General linear model procedure was used for the analysis of variance and mean separations. Differences between treatments were determined by Fisher's protected LSD test.

RESULTS AND DISCUSSION

The low recovery rates of patulin (<60%) obtained at the early stage of this study were found to be caused by the strong streams of nitrogen; therefore, care was taken during the sample preparation process, and only a gentle stream of nitrogen gas was used to dry the ethyl acetate extract. A strong stream of nitrogen and longer drying time were found to significantly reduce the recovery rate of patulin (Rovira et al., 1993). Early in the method development process, run buffer was used to dissolve patulin standard or ethyl acetate extract of samples. A disadvantage of this procedure, however, is that patulin dissolved in the run buffer (pH 9.0) must be analyzed immediately after sample preparation to avoid degradation, because this mycotoxin is chemically labile in such an alkaline solution (Forbito and Babsky,

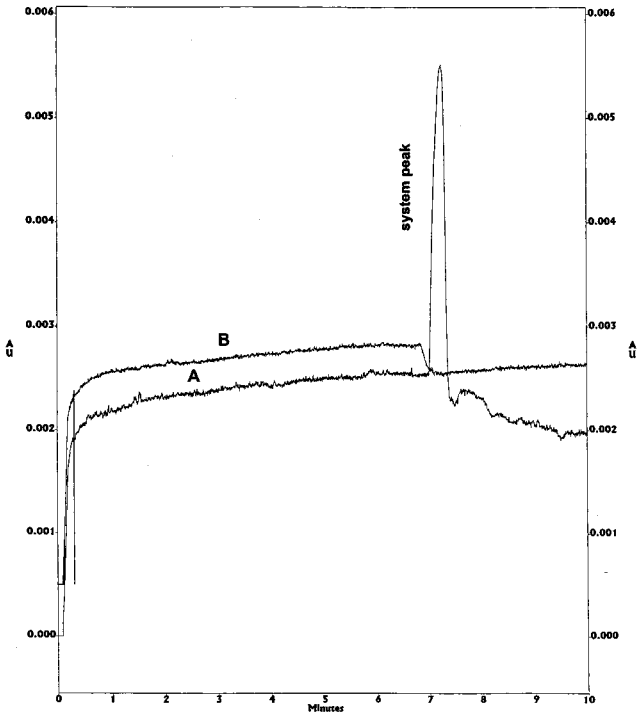

Figure 2. Electropherograms of a control sample prepared from patulin free cider (within our level of detection) and reconstituted in acidified water (A) and run buffer (B).

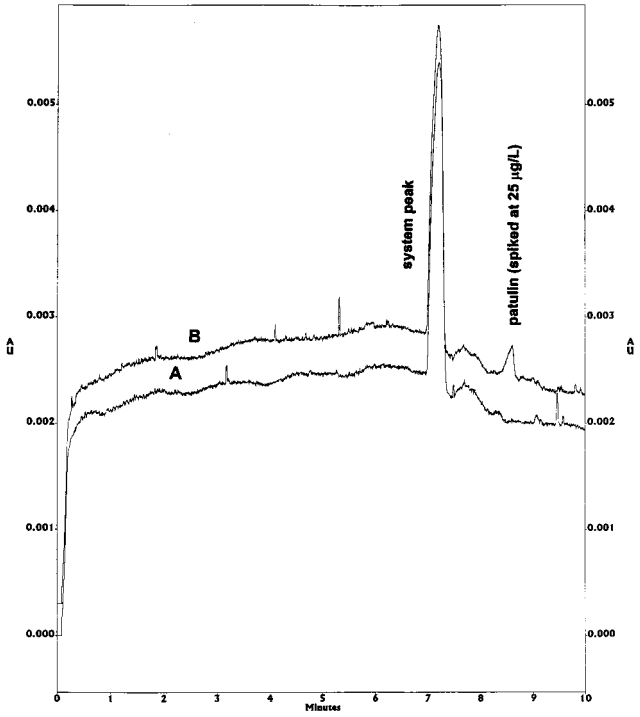

Figure 3. Electropherograms of an apple cider sample without patulin (A) and the same sample spiked with patulin at 25 μg/L (B).

1985). We found that up to 26% of patulin (1−24 μg/mL) was lost 80 min after it was dissolved in a run buffer and left under ambient laboratory conditions. Patulin dissolved in a pH 4.0 acetic acid solution was more stable (9% loss of a 0.5 μg/mL solution after 130 min). Although the acetic acid solution did not completely prevent patulin from degradation, acidified water has been used in many methods to stabilize patulin before being injected into the HPLC system (Forbito and Babsky, 1985; Brause et al., 1996). The only advantage of dissolving samples in run buffer was that it resulted in a smoother chromatogram (Figure 2). Chromatograms generated from acidified water usually had a system peak at 7.2 min, though the peak did not interfere with that of patulin (Figure 3). Chromatographic reproducibility of injections of patulin standard solutions was high with a RSD of 0.6% by retention time and 8.8% by peak area (Figure 4).

A standard curve for patulin was established by plotting the peak area against patulin concentration. The curve was linear in the range of 75−24 100 μg/L, with a linear correlation coefficient r^2 of 0.9999. Even in a wider range of 75−121 000 μg/L, r^2 was 0.9984; this is a linear dynamic range over 3 orders of magnitude. Peak height also exhibited good linearity, with r^2 = 1.0000 and 0.9993 of the above-mentioned two ranges, respectively. Concentrations from the sample were calculated using standard curves based on the peak area of patulin in the range of 75−24 100 μg/L. The lower end of the standard curve (75 μg/L) represents the minimum detectable concentration of patulin standard (signal/noise, or S/N, = 3 at 273 nm); therefore, the limit of detection of this CE method is 3.8 μg/L (concentration factor 20, i.e., from 2 mL of sample to the final preparation of 100 μL).

The recovery rates of patulin from our procedure were determined by spiking known amounts of patulin to an apple cider freshly pressed from healthy apples (free of

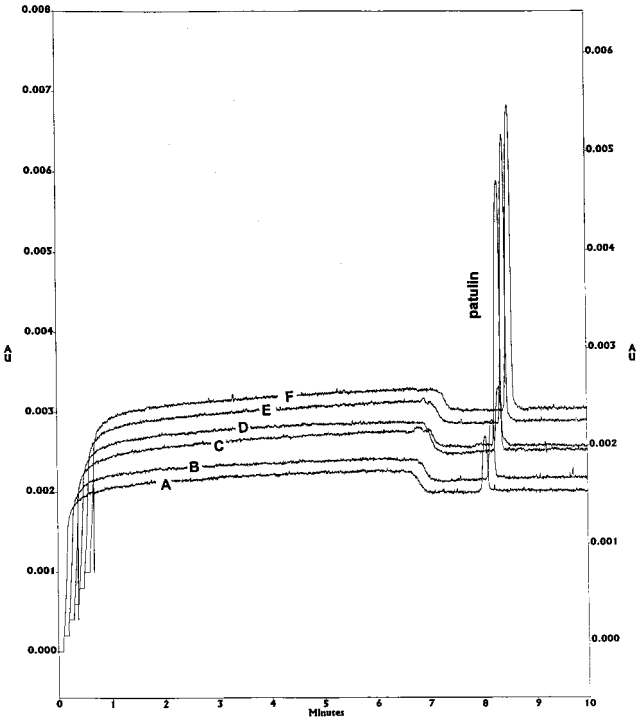

Figure 4. Electropherograms of two concentrations of patulin standard solutions. (A−C) 1.0 μg/mL; (D−F) 4.8 μg/mL. The relative standard deviations (RSD = SD/Mean × 100) among the retention times of three injections of the same concentration were 0.4% and 0.1%, respectively. The RSD among the peak areas of three injections of the same concentration were 9.1% and 8.4%, respectively. The pooled RSD of the six injections was 0.6% by retention time, and the average of the RSD by peak area was 8.8%.

rot) which had shown no patulin contamination within our limit of detection (3.8 μg/L, S/N = 3/1) (Figure 3). Results of the spike recovery experiments with different levels of patulin in the apple cider are presented in

Table 1. Spike Recovery of Patulin from an Apple Cider Prepared from *Penicillium expansum* Free Healthy Apples ("Red Delicious")

patulin spiked (μg/L)	recovery (μg/L)[a]	recovery (%)	RSD[b](%)
121	114.2 ± 18.0	95.2	15.8
50	52.7 ± 4.1	105.4	7.8
25	22.9 ± 2.3	91.6	10.0
10	9.9 ± 0.4	99.0	4.0

[a] Mean ± standard deviation. n=3. [b] Relative standard deviation.

Table 2. Recovery of Patulin from an Apple Cider Prepared from *Penicillium expansum* Infected Apple ("Red Delicious")

patulin (μg/L)	recovery (μg/L)[a]	recovery (%)	RSD[b] (%)
223	238.9 ± 30.5	107.1	12.8
22.3	21.6 ± 2.5	96.9	11.6

[a] Mean ± standard deviation. n = 3. [b] Relative standard deviation.

Table 1. Recovery rates were 91% or higher at all four levels studied. All recovery experiments were followed by the same extraction procedures as for the samples. Reproducibility of the recovery test was generally good, with the relative standard deviation (RSD) less than 10.0%, except for the highest concentration in the spike recovery test (Table 1). Figure 3 presents an electropherogram of an apple cider sample in which patulin was undetectable (A) and an electropherogram of a sample corresponding to 25 μg/L patulin (B). Recovery tests were also performed using a cider prepared from a *P. expansum* infested and fully rotten apple (see the next paragraph for preparation). The highly contaminated apple cider pressed from this rotten apple was diluted with noncontaminated "Red Delicious" cider to contain 23 and 233 μg/L of patulin and recovered using the same extraction procedures used in spike recovery test. Results from this recovery test were shown in Table 2. The rates of recovery with the naturally contaminated apple cider were similar to those found with the spiked samples (Tables 1 and 2).

Cider from a completely rotten "Red Delicious" apple infected by *P. expansum* and stored for ca. 6 months at 1 °C was diluted 100× with water and directly injected to the CE system for patulin analysis. The concentration of the diluted cider was determined as 38.9 μg/mL using the standard curve generated with standard solutions of patulin. This concentration, representing 3890 μg/mL of patulin in the original apple cider (Table 3), is extremely high considering the maximal acceptable level of patulin in apple cider is 50 μg/L in many countries (Moller and Josefsson, 1980; Forbito and Babsky, 1985; van Egmond, 1989; Stoloff et al., 1991). One such "bad" apple in 77 800 would cause the maximal acceptable level of patulin to be exceeded in cider or juice. Table 3 also shows the percentage of diseased tissue and patulin concentrations in the lesion tissues of different apple varieties that were inoculated with *P. expansum* and incubated for 9 days at 20 °C before being analyzed by CE. The percentage of the diseased tissue caused by *P. expansum* differed among the tested varieties; Empire apples were the most susceptible to the pathogen with 10.0% lesion (percentage of rotten tissues) in the apple, whereas Northern Spy was the most resistant with 4.0% lesion (Table 3). The highest concentration of patulin was found in the rotten tissue of MacIntosh with an average of 138.0 μg/mL; whereas the lowest was in the rotten tissue of Empire with an average of 46.7 μg/mL (Table 3).

Several sample preparation procedures for analyzing patulin in apple cider were evaluated by others and found to work well; however, there are increasing concerns over waste solvent disposal and cost reductions. This has prompted evaluation of other procedures using less organic solvent and not requiring solid-phase extraction columns (Brause et al., 1996). CE is an attractive technique for several reasons. It offers all the advantages of a miniaturized separation technique, including low solvent consumption, low sample-volume requirement, increased mass sensitivity, high separation efficiency, and low operational cost (Colon et al., 1997). We did not encounter any problems with direct injection of the reconstituted apple cider extracts; i.e., we did not find it necessary to filter the reconstituted extracts through a 0.45-μm filter prior to the injection to CE as used by Rovira et al. (1993) in their HPLC method. This is especially helpful considering the small total sample volume in this method (0.1 mL), because the syringe-filtration process may cause loss of the sample. The simplified procedure of this method could further lower the cost associated with the HPLC methods. The fused silica capillary column used in CE costs significantly less than a typical column for HPLC, and because it is simply a hollow tubing, column-related maintenance and problems such as high back pressure are minimal. In addition, because CE has a completely different mode of separation than that of HPLC, interference from other sample components, such as positively or negatively charged ions, could be of less concern. The current CE method only required 2 mL of sample, a smaller amount compared to 5−50 mL used in most HPLC methods (Brause et al., 1996; Moller and Josefsson, 1980; Forbito and Babsky, 1985; Herry and Lemetayer, 1996; Stray, 1978).

CONCLUSIONS

This study represents the first effort in developing a capillary electrophoretic procedure for the analysis of patulin in apple cider or juice and demonstrates the superiority of this CE method over other analytical techniques for this application.

Table 3. Percentage of Rotten Tissue and Patulin Concentration (μg/mL) in the Lesion of Apples Infected with *Penicillium expansum*

	Red Delicious	Jonagold	Empire	MacIntosh	Northern Spy	Red Delicious[c]
% lesion[a]	4.9 ± 1.2	7.2 ± 2.3	10.0 ± 1.0	5.5 ± 1.2	4.0 ± 1.3	100
patulin (μg/mL)[b]	82.6 ± 62.5 (ab)	95.6 ± 60.4 (ab)	46.7 ± 13.6 (b)	138.0 ± 103.5 (a)	122.3 ± 42.3 (a)	3890 ± 910

[a] Percentage of rotten tissue in a whole treated apple. Numbers are averages of 5 apples inoculated and incubated for 9 days at 20 °C. [b] Mean ± standard deviation of patulin concentration in the cider pressed from the lesion tissues. Each sample (apple) was analyzed by CE in duplicates. Numbers in the same row (except for the column on the far right) sharing the same letter are not significantly different from one another according to Fisher's protected LSD test (P = 0.05). [c] The completely rotten apple was obtained by inoculating a "Red Delicious" apple with *P. expansum* and incubating at 1 °C for 6 months. Patulin concentration was not included in the LSD test.

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