49.7.03

AOAC Official Method 2000.02
Patulin in Clear and Cloudy Apple Juices and Apple Puree
Liquid Chromatographic Method
First Action 2000

(Applicable to determination of patulin at >25 ng/g in clear apple juice, cloudy apple juice, and apple puree.)

See Table 2000.02 for results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Apple juice or puree is extracted with ethyl acetate and then cleaned up by extraction with sodium carbonate solution. (Cloudy apple juice and apple purees are pretreated with pectinase enzyme.) The ethyl acetate extract is dried with anhydrous sodium sulfate. After evaporation of the solvent, patulin is quantitatively determined by LC with UV detection.

B. Apparatus

(a) LC apparatus.—(1) Pump.—LC pump(s) and eluent reservoir. (2) Injection system.—Test sample applicator. (3) Separation columns.—A 4.3 mm id octadecylsilane (ODS) precolumn with 5 µm particle stationary phase. An analytical reversed-phase LC column such as ODS, fully end capped with 5 µm particle stationary phase, 25 nm pore size, and 12% carbon loading, or ODS super end capped with 5 µm particle stationary phase, 12 nm pore size, and carbon loading of 17%. (4) Detector.—UV detector at 276 nm and data integration system.

(b) Spectrophotometer.—350–250 nm. Calibrate as follows: Determine absorbance (A) of the 3 solutions of K₂Cr₂O₇ in H₂SO₄, C(b), C(c), and C(d), at maximum absorption near 350 nm, against 0.009M H₂SO₄ as solvent blank. Calculate molar absorptivity (ε) at each concentration as follows:

\[
\epsilon = \frac{A \times 1000}{C}
\]

where \(A\) = absorbance at maximum near 350 nm, \(C\) = mM concentration of K₂Cr₂O₇ solution.

If the 3 values vary by more than guaranteed accuracy of A scale, check either technique or instrument. Average 3 ε values to obtain \(\bar{\epsilon}\). Determine correction factor (CF) for particular instrument and cells by substituting in equation:

\[
CF = \frac{3160}{\bar{\epsilon}}
\]

where 3160 = value for ε of K₂Cr₂O₇ solutions, \(\bar{\epsilon}\) = average of the 3 ε values calculated above. If CF is <0.95 or >1.05, check either technique or instrument to determine and eliminate cause. [Use same set of cells in calibration and determination of purity of patulin, C(r).]

(c) Quartz cells.—Optical path length 1 cm.

(d) Centrifuge.—4500 × g.

(e) Centrifuge tubes.—50 mL with screw cap.

(f) Rotary evaporator.

(g) Round bottomed flasks.

(h) Hand-held pipets.—25, 50, 1000 µL.

(i) Syringe filters.—0.45 µm pore size, 13 mm, PTFE.

C. Reagents

(a) Ethanol.—≥99.7% (v/v), LC grade.

(b) Potassium dichromate, ca 0.25 mM.—Accurately weigh ca 78 mg K₂Cr₂O₇ (primary standard) and dissolve in 1.0 L 0.009M

| Table 2000.02. Interlaboratory study results for patulin in clear and cloudy apple juices and apple puree |
|-------------------------------------------------|----------------|----------------|----------------|----------------|----------------|
| ID     | \(\xi\), ng/g | No. of labs\(^{ab}\) | \(S_\xi\) | RSD\(_\xi\), % | \(S_R\) | RSD\(_R\), % | HORRAT | Rec., % |
| Clear apple juice |
| 75 ng/g (a)\(^c\) | 67 | 12 (0) | 8.4 | 13 | 15.3 | 23 | 0.95 | 89 |
| nc (b) | 26 | 12 (0) | 3.7 | 14 | 8.4 | 33 | 1.18 |
| nc (c) | 54 | 12 (0) | 6 | 11 | 13.6 | 25 | 1.02 |
| nc (d) | 128 | 10 (2) | 9.9 | 8 | 14 | 11 | 0.50 |
| Cloudy apple juice |
| 75 ng/g (a) | 60 | 11 (1) | 7.8 | 13 | 12.5 | 21 | 0.85 | 80 |
| nc (b) | 26 | 12 (0) | 8.9 | 35 | 8.9 | 35 | 1.25 |
| nc (c) | 69 | 9 (2) | 4.3 | 6 | 10 | 14 | 0.61 |
| nc (d) | 106 | 10 (2) | 10.2 | 10 | 12.9 | 12 | 0.54 |
| Apple puree |
| 75 ng/g (a) | 69 | 9 (1) | 7.5 | 11 | 9.2 | 13 | 0.56 | 92 |
| nc (b) | 23 | 8 (1) | 6.4 | 27 | 8.5 | 36 | 1.23 |
| nc (c) | 38 | 9 (2) | 3.8 | 10 | 12.6 | 33 | 1.27 |
| nc (d) | 121 | 10 (0) | 23.6 | 19 | 34.8 | 29 | 1.31 |

\(^{ab}\) a = number of labs retained after eliminating outliers; (b) = number of labs removed as outliers.

\(^c\) Laboratories received 4 sets of duplicate test samples labeled a, b, c, and d.

\(^d\) nc = naturally contaminated.
H₂SO₄ (ca 1 mL H₂SO₄ diluted to 2 L); calculate mM to 3 significant figures (MW K₂Cr₂O₇ = 294.2).

(c) Potassium dichromate, ca 0.125 mM.—Dilute 25 mL 0.25mM K₂Cr₂O₇ (b) to 50 mL with 0.009M H₂SO₄ in volumetric flask.

(d) Potassium dichromate, ca 0.0625 mM.—Dilute 25 mL 0.125mM K₂Cr₂O₇ (e) to 50 mL with 0.009M H₂SO₄ in volumetric flask.

(e) Glacial acetic acid.—≥99.5% (v/v).

(f) Acetonitrile.—≥99.5% (v/v), LC grade.

(g) Ethyl acetate.

(h) Pectinase enzyme solution, endogalacturonase.—Typical activity 1400 U/g. Unit definition: the amount of enzyme which catalyzes the decrease in viscosity of 1% pectin solution by 20% in 5 min at pH 3.4 and 25°C. Macer8 FJ supplied by Biocatalysts Ltd., (Main Ave, Treforest Industrial Estate, Pontypridd CF37 5UT, Wales, UK) is suitable.

(i) Perchloric acid.—60% (v/v).

(j) 5-Hydroxymethyl furfural (HMF).

(k) Sodium carbonate.—Anhydrous.

(l) Sodium sulfate.—Anhydrous.

(m) Sodium carbonate solution.—1.5%. Dissolve 1.5 g sodium carbonate (k) in 100 mL H₂O.

(n) pH 4 water.—Adjust water with acetic acid (e) to pH 4.

(o) Elution solution for LC.—Add 3–10% acetonitrile (f) to water containing 0.095 parts per volume perchloric acid 60% (l). Exact amount of acetonitrile used will depend on test portion extract and LC column chosen for analysis. Degas this solution before use.

(p) Patulin.—(4-Hydroxy-4H-furo(3,2-c)pyran-2(6H)-one), ≥99%.

(q) Patulin stock solution.—Dissolve 5 mg patulin (p) in 5 mL ethyl acetate (g). Transfer to 25 mL volumetric flask and dilute to volume with ethyl acetate (g). Stock solution stored in freezer at -20°C, is stable for several months.

(r) Patulin calibrant solution, ca 10 μg/mL patulin.—Evaporate 1000 μL stock solution (q) to dryness under N, and immediately dissolve residue in 20 mL ethanol (a). Solution stored at 4°C is stable for several months.

To determine exact mass concentration in calibrant solution, record absorption spectrum between 350 and 250 nm in 1 cm quartz glass cell (c) in spectrophotometer (b) with ethanol (a) in reference path. Calculate patulin mass concentration (μg/g) using the following equation:

\[ \mu g/g \text{ Patulin} = \frac{A \cdot MW \cdot 1000 \cdot CF}{\varepsilon} \]

where A = absorbance of patulin solution at 276 nm, MW = molecular mass of patulin (154 Dalton), CF = correction factor for quartz cells and spectrophotometer obtained by following procedure in (b) (c) (e) = molecular absorbance coefficient of patulin solution at the wavelength maximum (276 nm) of absorption spectrum (14 600 L mol⁻¹ cm⁻¹ in ethanol).

(s) Patulin working calibrant solution, 1 μg/g patulin.—Evaporate 500 μL calibrant solution (r) or aliquot, which is equivalent to absolute amount of 5 μg patulin, to dryness, dissolve in 5 mL pH 4 water (n), stopper, and shake vigorously. Use the same day to make patulin LC calibration standard solutions.

(t) Patulin LC calibration calibrant solutions.—Into a series of 2 mL volumetric flasks transfer by pipet B(h) 1000, 800, 500, 200, and 100 μL patulin calibrant standard solution (s). Dilute to mark with pH 4 water (n), stopper, and shake vigorously to mix. These solutions contain 0.5, 0.4, 0.25, 0.1, and 0.05 μg/mL patulin, respectively. Transfer standards to vials for LC analysis and use on same day as preparation.

(u) HMF solution.—Dissolve 5 mg HMF (j) in 25 mL ethyl acetate (g).

(v) HMF-patulin solution.—Transfer by pipet B(h) 100 μL patulin calibrant solution (r) and 100 μL HMF solution (u) to 10 mL volumetric flask and evaporate to dryness under stream of N. Dissolve residue and dilute to volume with pH 4 water (n).

D. Procedure

Preparation of test portion.—For clear apple juice, no preparation is required. For cloudy juices, measure 20 mL test portion into centrifuge tube B(e) and add 150 μL pectinase enzyme solution C(h). Leave overnight at room temperature or for 2 h at 40°C; then centrifuge at 4500 × g for 5 min. For apple puree, weigh 10 g test portion into centrifuge tube B(e), add 150 μL pectinase enzyme solution C(h) followed by 10 mL H₂O, and mix thoroughly. Leave solution at room temperature overnight or for 2 h at 40°C; then centrifuge at 4500 × g for 5 min.

Extraction of patulin from the test solution.—Pipet 10 mL clear juice (or cloudy juice or puree as prepared above) into 100 mL separating funnel. Add 20 mL ethyl acetate C(g) and shake 1 min. Let layers separate and drain them into 2 separate conical flasks. Transfer aqueous layer back into same separating funnel and re-extract with second 20 mL portion of ethyl acetate. Let the layers separate and drain lower aqueous layer into empty conical flask and top layer into conical flask containing ethyl acetate layer from first extraction. Repeat this extraction procedure for a third time. After layers separate, drain lower aqueous layer to waste. Combine the 3 ethyl acetate phases in separating funnel. Rinse conical flask used to collect ethyl acetate phases with additional 5 mL ethyl acetate; add this to ethyl acetate extract in separating funnel.

Add 4 mL Na₂CO₃ solution C(m) to separating funnel and shake 0.5 min. Let layers separate; then drain the lower aqueous layer into conical flask. Pour top layer into round-bottomed flask B(g) through a funnel and filter paper containing 15 g anhydrous Na₂SO₄ C(l). Transfer aqueous layer back into separating funnel, rinse conical flask with 10 mL ethyl acetate C(g), add this to separating funnel, and shake 0.5 min. Let layers separate, drain the lower layer to waste, and pour top layer through the Na₂SO₄ into the round-bottomed flask, wash with 2 × 10 mL ethyl acetate C(g), and collect in round-bottomed flask.

Note: Patulin is not stable in alkaline solutions; therefore, perform this stage as quickly as possible to avoid losses.

Preparation of extract for LC analysis.—Evaporate extract to dryness and redissolve in final volume of 1 mL (500 μL for puree) pH 4 water C(n). Transfer to LC vial. If necessary, filter solution through a syringe filter B(i) before analysis by LC. Check filter with standard solution to assess any loss of patulin before filtering test extracts.

LC operating conditions.—See below for typical LC operating conditions. Note: It may be necessary to wash LC system with 100% acetonitrile after each test extract injection to ensure that no materials...
are retained on column. After the wash, re-equilibrate system with mobile phase before next injection.

**Column evaluation.**—Using chosen LC conditions, inject 50 µL HMF-patulin solution \( C(\nu) \). HMF and patulin should elute as 2 separate peaks with baseline separation. It may be necessary to raise acetonitrile content of LC eluent \( C(\omega) \) (≤10%) and decrease flow rate to 0.75 mL/min if HMF and patulin do not separate. On some columns, reduction of acetonitrile at fixed flow rate will also improve separation.

**E. Calculation**

Inject 50 µL each patulin working standard solution \( C(\tau) \). By using measured peak areas (or peak heights) from recorder, prepare standard curve by plotting peak areas vs concentrations of patulin working standard solutions.

Inject 50 µL extract. Read patulin concentration in extract directly from plotted graph. If peak area of extract is outside range of standard curve, dilute extract with pH 4 water, reinject, and re-analyze diluted extract solution.

Calculate concentration of patulin in test sample (ng/g) as follows:

\[
\text{ng/g Patulin} = \frac{C_T}{10} \times \frac{1000}{d}
\]

where \( C_T \) = concentration of patulin in extract (ng/g), 10 = ratio of test portion in test solution (5 g apple juice or apple puree is represented by 0.5 mL test solution); \( d \) = dilution factor, which = 1 for undiluted test portion.