# Quantitative Analysis of Patulin in Apple Juice

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A method for the quantitative analysis of patulin in apple juice has been developed. The technique is based on mass fragmentographic detection of the parent (P) ion of the silylated patulin derivative. Sensitivity of the method is estimated at 1 ppb and no cleanup is required. Confirmation of identity at the 10 ppb but not the 1 ppb is achieved by simultaneous monitoring of the P and P + 1 ions.

Interest in the development of a quantitative, confirmative method of analysis for 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one (patulin, I) has been high recently because of its known carcinogenic (Dickens and Jones, 1961) and mutagenic (Mayer and Legator, 1969) properties and because of its detection in commercial apple juice (Scott et $al.$, 1972; Ware et $al.$, 1973). The two most recent methods for the determination of patulin are thin-layer (Scott and Kennedy, 1973) and high-pressure liquid (Ware et $al.$, 1973) chromatography. Both methods claim lower limits of sensitivity at approximately 10 ppb but require further analysis for confirmation at these levels. The method we will describe is also $confirmative$ at the 10-ppb level without further laboratory manipulation. This method may also be used for analysis at 1 ppb but confirmation at this level probably cannot be achieved because of interfering materials.

I; R = H
II; R = Si(CH₃)₃
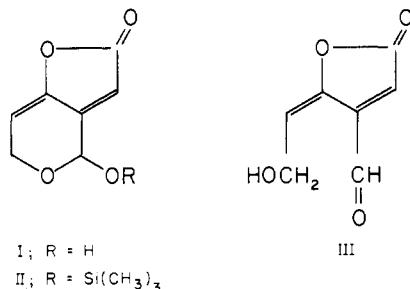
Our method is based on mass fragmentography, which in addition to high sensitivity also offers a high degree of specificity because identification is based on the mass spectroscopic behavior of the material in addition to its glc retention time. In a previous publication (Pareles and Rosen, 1974) we have described a special form of mass fragmentography and named it, for want of a better name, dynamic single ion detection. The mass spectrometer is made to repeatedly scan back and forth across a specific ion instead of focusing on this ion directly as with commercially available mass fragmentography accessories. In this manner, the focus instability problems associated with our mass spectrometer are overcome.

EXPERIMENTAL SECTION

Instrumentation. A Du Pont Model 21-490 mass spectrometer equipped with a digital mass marker and interfaced to a Varian Model 2740 gas chromatograph (equipped with flame-ionization detector) via a glass single-stage jet separator was used. Focus of the ions under investigation was monitored with an RCA Model 158 cathode ray oscilloscope. An accessory circuit for dynamic

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ion detection has been described in detail (Pareles and Rosen, 1974). An operator-adjustable reference voltage has now been added to the accelerating voltage sweep control section of this circuit. In effect, the operator can now focus on the parent peak (P) of a material in one channel (A) and offset the accelerating voltage to focus on the P + 1 peak in the other channel (B). The focus of both ions is thus set and the operator can determine the P or P + 1 ion by simply activating channel A or B. Addition of another sample/hold amplifier, attenuator, bucking control, and strip-chart recorder allows for simultaneous recording of the P and P + 1 peaks. Details of the electronic modifications and timing changes may be obtained by writing to the authors.

Gc–Mass Spectral Conditions. A 6 ft × 0.25 in. o.d. glass column (2 mm i.d.) containing 3% OV-17 on Gas-Chrom Q, 80–100 mesh, was used. Carrier gas (99.9999% helium) flow was 30 cm³/min. Column, detector, and injector temperatures were 175, 210, and 210°, respectively. Jet separator, glass transfer line, and ion source temperatures were 250, 210, and 225°, respectively. Ionizing voltage was 20 eV and filament current was 70 μA. Retention times (4.4 min for trimethylsilylpatulin, II) were measured from the start of the solvent front (as determined by FID). The solvent and gross impurities (mainly silylated carbohydrates) were not allowed to enter the mass spectrometer as the valve controlling access to the mass spectrometer was not opened until 3.25 min after the start of the solvent front.

Procedure. A stock solution (kept frozen) containing 1.44 mg/l. of patulin (obtained from the Food and Drug Administration) in ethyl acetate was used to spike 100-ml apple juice samples by the addition of 1, 2, 6, and 10 ml. The spiked juice was then extracted three times with 50 ml of ethyl acetate and the combined extracts were washed once with 50 ml of distilled water. Sodium chloride was used to break emulsions. The ethyl acetate extract was dried over 5 g of anhydrous magnesium sulfate and evaporated to near-dryness on a rotary evaporator. The concentrate was transferred, together with ethyl acetate rinses, to a 2-ml volumetric flask and the solution was concentrated to $ca.$ 1 ml under a stream of nitrogen. To this was added 0.5 ml of bistrimethylsilylacetamide (BSA) and enough ethyl acetate to fill to volume. The solution was then heated on the steam bath for 1.5 hr. Injections (5 μl) of this solution were made into the gc–mass spectral system. This method of extraction and derivative (II) preparation is essentially a modification of the method prescribed by Pohland et $al.$ (1970).

Determination of Calibration Curve. Standard solutions (5 μl) were injected. In each case the injection of standard was preceded by injection of silylated extract to ascertain that the glc column was saturated.

RESULTS AND DISCUSSION

The apple juice purchased at the supermarket contained 12 ppb of patulin. Finding patulin-free apple juice

Table I. Recovery of Patulin Added to Apple Juice

Patulin added, μg/l.	Patulin recovd,[a] μg/l.	Calcd, μg/l.	Recovery,[a] %
0	12.1		
14.4	30.2	26.5	114
28.8	46.0	40.9	112
86.4	98.6	98.5	100
144.0	159.9	156.1	102

[a] Average of one determination each from two different samples.

Figure 2. Simultaneous monitoring of unspiked apple juice extract (12 ppb of II). Note: attenuator for m/e 226 set at 16× instead of 2× to simulate 1-ppb level.
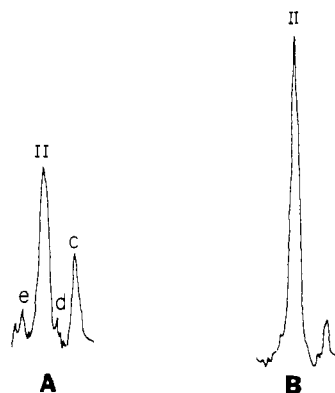
Figure 1. Mass fragmentograms of apple juice extracts at m/e 226: (A) 12.35 ppb, attenuator = 2×; (B) 49 ppb, attenuator = 4×; recorder = 50 mV in both.

Figure 3. Mass fragmentograms of apple juice extract at (A) m/e 211; (B) m/e 183.

is difficult as shown by Ware *et al.* (1973) who detected patulin in 8 of 13 samples in concentrations ranging from 44 to 309 ppb. Table I shows the recoveries obtained after spiking 100-ml samples of this juice with 1.44–14.4 μg of patulin. The excellent recoveries demonstrate the reliability of the method.

The type of mass fragmentograms obtained is illustrated in Figure 1. The unspiked sample (12 ppb) gave a recorder response of 23%. By comparison, the liquid chromatography method gave a recorder response of 2.5% for 11 ppb. We thus estimate that mass fragmentography can be used to determine patulin in apple juice at the 1 ppb level. Peaks of even greater size may be obtained by concentrating the 2-ml derivatized solution to 200 μl.

One of the advantages of mass fragmentography is greater assurance of compound identity because the analyst is making use of at least one specific ion of the compound in addition to its glc retention time. Obviously, one specific ion does not a confirmation make and it is most useful to use at least two, especially if the ions are the P and P + 1 peaks. From the known isotopic natural abundances, the P/(P + 1) (226/227) ratio for compound II is 6.21. By recording the 226 and the 227 mass fragmentograms simultaneously (Figure 2), it was shown that only for compound II was the measured ratio close to the calculated values. The eluents immediately preceding and succeeding II had 226/227 ratios of approximately 0.04 and 2, respectively. The 227 contribution of the eluent immediately preceding II (peak d in the illustrations) would make it difficult to confirm the presence of II in apple juice samples where patulin was present in concentrations of less than 10 ppb because it would essentially swamp the 227 contribution of II. We therefore examined the mass fragmentograms at other known fragments of II, i.e., m/e 183 and 211 (Figure 3). While peak d is no longer a problem at these specific ions, we judged that the large contributions made by peak e would not allow us to con-
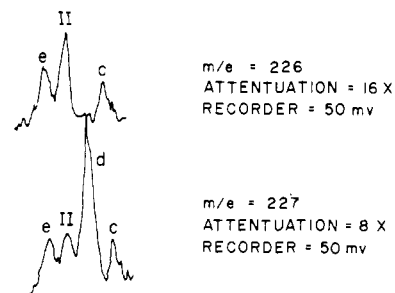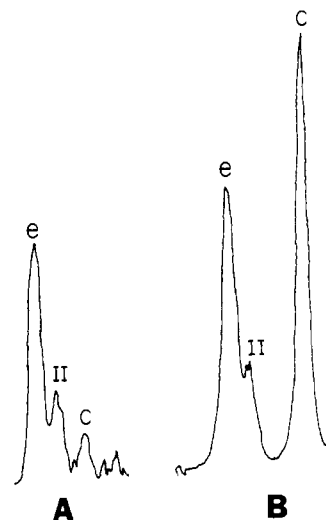
firm II at less than 10 ppb. It is our feeling, incidentally, that the interfering peaks are not from apple juice but represent as yet unidentified patulin metabolites or precursors. For example, from the proposed biosynthetic pathway for patulin (Bu'Lock and Ryan, 1958) it may be inferred that III is a precursor for patulin. Treatment of this material with BSA would lead to the formation of a trimethylsilyl derivative whose mass spectrum would also exhibit peaks at m/e 226, 211, and 183. Furthermore, bioreduction of III could result in the formation of a dihydro derivative which upon reaction with BSA would give a material with a molecular ion at m/e 228. Because of the aldehyde group, a large P − 1 contribution at m/e 227 would not be unexpected. The foregoing is, of course, only speculation, but our "feeling" is further buttressed by the fact that interferences showed up at several other known trimethylsilylpatulin fragments but not at other fragments known not to be present in the mass spectrum of this compound.

Column cleanup of the extracts on silica gel (Scott and Kennedy, 1973) failed to remove the interfering materials but did succeed in removing the carbohydrates. This, however, cut the sensitivity approximately in half because the silylated carbohydrates very effectively saturated the active sites on the glc column. It was for this reason that the calibration curve was determined by alternately injecting standards and silylated apple juice extract. The only drawback to avoiding the column cleanup was deposition of silicates on the FID detector which in this process was used only to determine the solvent front ("zero" time). On the other hand, avoiding column cleanup resulted in greater sensitivity and faster analysis time.

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Effects of Processing on the Amine Content of Pork Bellies

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The concentration of a number of amines was determined in fresh and processed pork bellies. Analyses were conducted for spermine, spermidine, putrescine, cadaverine, histamine, tyramine, tryptamine, and ethanolamine. Fresh, pickled, and fully cured and smoked bellies were obtained from two commercial sources. The amines were recovered from perchloric acid extracts of the lean meat and derivatized with dansyl chloride. The fluorescent derivatives were separated by thin-layer chromatography, extracted, and then quantitated spectrofluorometrically. The concentration per 100 g of tissue ranged from 0.03 mg for cadaverine to 8.1 mg for spermine. There were considerable variations in the levels of the individual amines within different specimens of the same sample. Processing did not significantly alter the levels of the free amines.

N-Nitrosamines are carcinogenic to various species of animals, and pose a potential health hazard to humans (Magee and Barnes, 1956). These compounds are formed through the reaction of nitrite and secondary and/or other amines. Since approximately 70% of the pork produced annually is cured with nitrite salts, and since a nitrosamine, N-nitrosopyrrolidine, has been identified in bacon after frying (Pensabene et al., 1974), information about the concentration of amines or their precursors in this product is needed.

A number of amines have been studied in physiological fluids (blood, urine, and semen) and special organs (heart, liver, and nervous tissue) (Frazen and Eysell, 1969; Guggenheim, 1940), but very little is known about amines in skeletal muscle. Putrescine, cadaverine, and tyramine have been reported in spoiled squid and octopus (Takagi et al., 1971), and there are numerous reports on the presence of methyl-, dimethyl-, and trimethylamine in fish (Gruger, 1972). The effect of storage on the concentrations of these amines has also been investigated (Keay and Hardy, 1972).

Although nitrosamines have not been reported to any extent in hams, some of these amines may be nitrosated or act as precursors for other compounds capable of forming nitrosamines. Tabor et al. (1958) showed that putrescine is a precursor of spermine and spermidine. Putrescine and cadaverine, on heating, are converted to pyrrolidine and piperidine, respectively (Lijinsky and Epstein, 1970). N-Nitrosopyrrolidine and N-nitrosopiperidine have been detected in Danish bacon after frying (Crosby et al., 1972) and nitrosopyrrolidine has been reported in bacon by other investigators in levels ranging from 10 to 108 μg/kg (Fazio et al., 1973).

This paper reports on the identification and quantitation of eight amines, spermine, spermidine, putrescine, cadaverine, tryptamine, tyramine, histamine, and ethanolamine, in pork bellies, and the effects of two curing procedures on their concentrations.

EXPERIMENTAL SECTION

Materials. Pork bellies were obtained from two commercial processors who used different techniques for curing their products. Processor A immersed fresh pork bellies (48–72 hr after slaughter) in commercial curing solution for 6–7 days. Since these cures are commercial products, the exact composition varies; usual components include sugar, sodium chloride, sodium nitrite, sodium ascorbate, and sodium tripolyphosphate. Following a 6-hr drying step at 130°F, the bellies were smoked at the same temperature for 18 hr. Processor B utilized the prevalent procedure of stitch pumping the fresh bellies to 8–10% of green weight with a commercial cure, drying for 10–12 hr at 70–90°F, and then smoking for approximately 5.5 hr at 128°F. Several bellies were obtained at three stages of operation at each processor. The first stage sampled consisted of the green bellies prior to pickling. The second set of bellies was taken after pickling but before drying, and the final set consisted of finished bacon.

Each belly was trimmed of visible fat and only the lean tissue (approximately 600 g per belly) was used for analysis. The tissue was ground, mixed, and reground four times to exclude variation in amine content among muscles.

All chemicals and solvents were Ultra Pure, spectral quality, or fluorometric grade where available commercially. Amines for standards and dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) were purchased from Sigma Chemical Co. Putrescine-$1,4$-^{14}C dihydrochloride (specific activity, 20.92 mCi/mmol) was obtained from New England Nuclear, with a purity greater than 98.5% as determined by thin-layer chromatography