partitioned between water and ether, the tentative structures assigned to these compounds by Bassir and Adekunle (1968) are presently being reevaluated in our department.

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Determination of Patulin in Apple Juice Products as the 2,4-Dinitrophenylhydrazone Derivative

A method to determine patulin, 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one, in apple juice products as the 2,4-dinitrophenylhydrazone (2,4-DNPH) derivative was developed. Patulin was derivatized by means of a micro reaction column packed with Celite–2,4-dinitrophenylhydrazine–66% H_3PO_4 and eluted with methylene chloride. The derivative was isolated by thin-layer chromatography and detected by spraying with base to produce a wine-red spot. Quantitation of the patulin derivative scraped from the thin-layer plate and extracted in solvent was carried out by measurement of the UV absorbance at 375 nm, the characteristic absorption maximum of the patulin 2,4-DNPH derivative. Satisfactory recoveries were obtained from patulin added to apple juice at levels of 50–340 ppb.

Patulin, a compound originally isolated as an antibiotic, is a toxic material that is a potential contaminant of many human foods and animal feedstuffs as a result of mold growth. Patulin administered subcutaneously produced tumors in rats at the point of injection (Dickens and Jones, 1961). Many molds are capable of producing this material and one, *Penicillium expansum*, is of particular concern as it causes storage rot in bruised or damaged apples. Patulin has been found repeatedly in commercial samples of apple juice and fresh apple cider (Stott and Bullerman, 1975). A method for analysis by thin-layer chromatography (TLC), utilizing a 3-methyl-2-benzothiazolinone hydrozone indicating spray reagent has been described (Scott and Kennedy, 1973; Scott, 1974). Many apple juice samples contain substances that interfere with TLC analysis for patulin; for example, 16 samples of pasteurized apple juice recently submitted by industry to four consulting laboratories contained interfering substances which rendered the official TLC method inapplicable to all samples (Cogley, 1975). These laboratories did not identify this material which caused widely varying results in the determination of patulin levels. Several components of apple juice, 5-(hydroxymethyl)furfural and scopoletin, have been reported to interfere with the determination of patulin by the TLC method (Ware, 1975; Scott, 1977), but it is not known whether they were the cause of the difficulty in these samples. A liquid chromatographic method that separates patulin from 5-(hydroxymethyl)furfural has been developed but has not been adopted as an official procedure (Ware, 1975).

This study was undertaken to investigate the reaction of patulin with 2,4-dinitrophenylhydrazine (2,4-DNP) to form the 2,4-dinitrophenylhydrazone (2,4-DNPH) as an alternative analytical procedure for the determination of patulin in samples that cannot be analyzed by previous methods of analysis.

EXPERIMENTAL SECTION

Materials. Pasteurized apple juice samples containing material that interfered with the official analytical method were supplied by J. R. Cogley (Knouse Foods). Fresh apple cider, without additives, was purchased locally.

Patulin was prepared in our laboratory by a fermentation procedure (Norstadt and McCalla, 1969) using freshly isolated strains of *Penicillium urticae* supplied by these investigators. A variation was made in the purification procedure that gave higher yields of patulin. This consisted of changing the solvent to $CHCl_3$ after the initial solvent extraction of the fermentation broth with ethyl acetate and chromatography on a silicic acid column. Identity and purity were determined by melting point, infrared spectra, and thin-layer chromatography.

All solvents were analytical or reagent grade. Ethyl acetate and toluene were distilled through a 10-plate Oldershaw column before use. Benzene and methylene chloride were Burdick and Jackson "distilled in glass" analytical grade.

The Celite–2,4-DNP–H_3PO_4 mixture used in this investigation was prepared by grinding 0.5 g of 2,4-DNP and 6 mL of 85% H_3PO_4 in a 6-in. mortar until dissolved. Four

milliliters of distilled water was added and the precipitated 2,4-DNP was redissolved by continued grinding. Ten grams of Celite (Johns-Manville, analytical grade) was ground with the 2,4-DNP solution until a homogeneous, damp preparation was obtained. If the material was not used immediately, it was transferred to a tightly stoppered bottle and stored at −15 °C.

The micro reaction columns were 0.7 cm i.d. × 10 cm. These were conveniently prepared from disposable "super pipets" (Curtin Matheson Scientific, Inc.) by removing most of the capillary portion below the taper. A small plug of glass wool was placed in the constriction at the bottom of the column, and the impregnated Celite was transferred in four equal portions, each portion being tamped tightly before addition of the next, until the column was filled to a depth of 5 cm. Micro cation-exchange columns containing Dowex 50W-H+, 100–200 mesh, and micro columns containing Silica Gel 60, 0.063–0.0200 mm (EM Reagents) were prepared in the same manner.

Preparative TLC separations were carried out on preabsorbent silicic acid plates (Kontes LQF plates).

Graduated chromatographic sample tubes (Kontes) were used when concentration to definite volumes was desired.

Crystalline patulin 2,4-DNPH standard was prepared by adding a solution of 2,4-DNP in 2 N HCl to a solution of patulin. The yellow precipitate that formed within 2 h was recrystallized from alcohol. At 190–200 °C, the derivative changed from yellow to red without melting. This agrees with the behavior of patulin 2,4-DNPH as described in the literature (Nauta et al., 1946). TLC separation yielded a single component that gave a red color with NaOH, a characteristic of mono-2,4-dinitrophenylhydrazones. A standard solution of this material was prepared to contain 970 ng of 2,4-DNPH derivative/1 μL of ethyl acetate.

The UV absorbance of standard and samples was determined with a Cary Model 14 spectrophotometer using 0.7-mL microcells with a 1.0-cm path length (Precision Scientific).

Extraction and Derivatization of Patulin. A 50-mL sample of apple juice was extracted with three successive 50-mL portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and concentrated, together with rinses, to 25 mL on a rotary evaporator. A micro cation-exchange column was washed with two bed volumes of ethyl acetate, which was discarded, and the sample solution was then immediately passed through the column, with application of a slight positive pressure with N_2. The column was rinsed with two bed volumes of ethyl acetate, and the combined sample solution and rinse was evaporated to 1 mL on a 50 °C bath with a gentle N_2 stream. The concentrated ethyl acetate solution was diluted with methylene chloride to 4 mL.

A Celite–2,4-DNP–H_3PO_4 micro reaction column was washed with two bed volumes of benzene, which was discarded. The sample solution was immediately added and allowed to percolate slowly through the column, followed by a rinse with two bed volumes of methylene chloride.

The combined derivative solution and rinse was immediately passed through a second prewashed micro cation-exchange column, which was then rinsed with two bed volumes of methylene chloride. The final derivative solution was concentrated under a gentle N_2 stream to 0.20 mL for TLC.

If visual observation of the solution during concentration revealed a precipitate consisting of 2,4-DNPH derivatives of other carbonyl compounds that would overload the TLC plate and interfere with the analysis of patulin, it was subjected to a silica gel column cleanup. The solution was concentrated to 0.5 mL and added to a micro silica gel column. The fast-moving components were eluted with two bed volumes of methylene chloride, and this eluate was discarded. The yellow monocarbonyl derivatives, largely patulin 2,4-DNPH, were then eluted with methylene chloride containing 20% methanol. Elution was continued until the slow-moving red derivatives were within 1 cm of the bottom of the column. The eluate containing the monocarbonyl derivatives was then concentrated to 0.20 mL in a graduated chromatographic sample tube (Kontes) for TLC.

TLC Cleanup and UV Quantitation. TLC plates were spotted with six 20-μL applications of the sample being analyzed. The last of the six spots was located adjacent to a 10-μL spot of the patulin 2,4-DNPH standard solution. The first five sample spots were used for quantitation. The migration of the sixth sample spot was compared with that of the patulin 2,4-DNPH standard spot. The TLC plates were developed with toluene–ethyl acetate (1:1, v/v) in an equilibrated tank, dried, and viewed under UV light (254 nm). If trailing occurred, the plate was developed a second time with toluene–ethyl acetate (2:1, v/v).

The first five sample spots were protected by covering, and the remainder of the plate was sprayed with 1 N NaOH. After locating the sample and standard spots representing the 2,4-DNPH derivative of patulin, the area corresponding in R_f value and containing the other five sample spots was scraped from the plate and extracted with several portions of methylene chloride containing 3% methanol. The extract was transferred to a graduated chromatographic sample tube and concentrated to 0.5 mL for determination of the UV absorbance. Methylene chloride was used for dilution when necessary. The patulin concentration in the sample was calculated from the UV absorbance at 375 nm using the molar absorptivity of 18 250.

RESULTS AND DISCUSSION

The observation that patulin readily forms the 2,4-DNPH derivative (Nauta et al., 1946) suggested that this derivative could be used for the quantitative determination of patulin. This derivative is a frequent choice for the quantitative determination of compounds having one or more carbonyl groups because the reaction is rapid and the products are stable and have excellent chromophoric properties.

The usual methods for forming the 2,4-DNPH derivatives of carbonyl compounds involve single-phase reaction with 2,4-DNP in the presence of strong acids or oxidizing agents. We investigated these methods and found that patulin gave low, variable yields of two products.

In a technique for the determination of carbonyl compounds developed by Schwartz and Parks (1961), the material to be analyzed is dissolved in a water-immiscible solvent and passed through a reaction column of Celite impregnated with a concentrated aqueous solution of 2,4-DNP and H_3PO_4. This method proved to be adaptable for obtaining reproducible yields of a single patulin derivative.

Several modifications were necessary to adapt the Schwartz and Parks procedure to the analysis of patulin. Ethyl acetate had been demonstrated to be an efficient solvent for the extraction of patulin from aqueous systems (Scott and Somers, 1968), but it was necessary to replace most of the ethyl acetate with methylene chloride prior to passage through the reaction column because ethyl

Table I. Recovery of Patulin as the 2,4-DNPH Derivative from Apple Juice Products

Added patulin, ppb	Percent recovery: Fresh apple cider	Pasteurized apple juice[a]: Clear		Opalescent	
0				(86 ppb)[b]	(103 ppb)[b]
50	74	71	78	76	104
120	73	64	71	73	92
340	85	75	79	76	80

[a] Samples containing substances that interfered with the official analytical method by TLC. [b] Levels of patulin found in the unspiked samples; recoveries of added patulin were corrected accordingly.

acetate alone eluted too much 2,4-DNP from the column. Benzene, the solvent employed by Schwartz and Parks, did not have sufficient solvent power to dissolve and carry the derivative. Direct evaporation of the ethyl acetate solution was found to result in a flocculent precipitate and loss of added patulin. Formation of a precipitate and loss of patulin were eliminated by passing the ethyl acetate concentrate through the micro cation-exchange column to remove extraneous materials extracted by ethyl acetate. A small amount of precipitate was usually formed upon addition of methylene chloride to the concentrated ethyl acetate solution, but this did not interfere with the subsequent determination.

Methylene chloride, although relatively nonpolar, eluted a small amount of 2,4-DNP from the column, and it was necessary to remove this excess reagent by passing the solution through a second micro cation-exchange column in order to avoid loss of the patulin derivative in subsequent manipulations.

The recovery of patulin from 50 mL of ethyl acetate containing 120 ppb patulin averaged 83% in six samples with a standard deviation of 14.2%.

Patulin in a standard solution was added to apple juice and cider samples at three concentration levels, and the samples were analyzed immediately by this method. The percent recovery is given in Table I. Blank analyses were conducted simultaneously on all samples to ascertain whether there was a detectable amount of patulin in the original sample. Patulin was found in two original samples of apple juice, and the percent recoveries in the table are the corrected values.

This study demonstrates the utility of the method described herein for analyzing samples that are refractory to the usual methods of patulin analysis. The patulin 2,4-DNP is readily isolated by TLC and develops a characteristic wine-red color when treated with NaOH. Most of the other materials on the TLC plate developed yellow-brown spots, and all were separated from the patulin derivative. The recovery of patulin, 64–104%, was within the range reported for the Scott method, 60–132%. The lower limit of detection for the new procedure is approximately 250 ng of patulin/spot, or 50 ppb in a 50-mL sample of apple juice or cider.

The ultraviolet absorbance of the 2,4-DNPH anions formed by adding alcoholic KOH to the derivative solution is often recommended for quantitative determination of carbonyl compounds (Parsons, 1966). This could not be used for quantitating the patulin 2,4-DNPH because the derivative was unstable in base, consistent with the instability of the original compound to base (Nauta et al., 1946). However, a characteristic absorption maximum at 480 nm was obtained by adding base to the patulin derivative, and this test could be used qualitatively as an additional means of confirming the identity of the derivative.

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Phytosterols in Some Tropical Tubers

Sterols were isolated from nine tropical tuber plants and the composition determined by TLC, GC, and GC–MS techniques. Cholesterol, campesterol, stigmasterol, and β-sitosterol were found to be the major sterols accumulated in these storage organs. Trace amounts of fucosterol were identified by GC–MS. β-Sitosterol was usually predominant although the pattern of distribution was irregular.

A number of herbaceous plants are cultivated in the tropics for food. These plants often have an enlarged rootstock, consisting mainly of starch, which acts as the storage organ (tuber) and is the part of the plant normally eaten. Although lipid constitutes less than 1% dry weight of tubers, its main constituents have a physiological role associated with the structure and function of membranes. The few reports (Lepage, 1968; Galliard, 1968; Walter et