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Note

Separation of 2,4-dinitrophenylhydrazones of α-keto dicarboxylic acids from citrus fruits

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The metabolic interconversions of various organic and amino acids in living tissues occur with some α-keto dicarboxylic acids as intermediates. These α-keto acids play key roles in respiratory and nitrogen metabolism in plants and animals. Concentrations of these keto acids indicate the metabolic state of the organisms. Various methods[1–9] have been reported for the determination of these acids. Colorimetric methods using 2,4-dinitrophenylhydrazine (DNP) to form 2,4-dinitrophenylhydrazones (DNPH) are the most widely used[3,4]. Chromatographic separation of these acids have been accomplished by thin-layer[1,5,6], paper[4], adsorption[2] and high-performance liquid chromatography (HPLC) by ion-exchange[7], ion-pairing on reversed-phase[8] and normal-phase[9] columns. Since DNP forms DNPHs with all compounds containing a keto or aldehydic carbonyl group, preliminary fractionation of the extracted DNPHs is necessary to separate the DNPH-carboxylates. This is then followed by chromatography on a Zorbax C_8 column.

METHODS

Apparatus and reagents

The apparatus used was a Waters Model 202 high-performance liquid chromatograph, equipped with a Micromeritics Model 725 automatic injector with a 20-μl loop and a Schoeffel Model 770 variable-wavelength detector, operated at 380 nm. The results were recorded with a Hewlett-Packard Model 3380A integrator.

The separation was carried out on a prepacked Zorbax C_8 column (DuPont Instruments, 25 cm × 4.6 mm I.D.), with a 3.5 cm × 4.6 mm guard column packed with 10 μm Spherisorb C_8 (Brownlee Labs.) in series.

Biochemicals were purchased from Sigma. Other reagents and solvents were obtained from various commercial sources and were used without further purification. All chromatographic solvents were the grade suitable for HPLC.

Procedure

Extracts of citrus peel were made by homogenizing 10 g of peel tissue with 100 ml 2% DNP in ethanol–10 *M* sulfuric acid (80:20) directly. The homogenate was allowed to stand for 1 h or longer and filtered through 0.3-μm pore size Gelman glass fiber filter. The filter was washed with ethyl acetate and the filtrate, including the ethyl

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acetate was diluted with an equal volume of water. The DNPHs were extracted from the filtrate with 3 × 100 ml ethyl acetate. The combined ethyl acetate extracts were washed with 3 × 100 ml portions of saturated sodium bicarbonate solution to extract the acidic compounds. The combined sodium bicarbonate extracts were acidified to pH 1 with 5 *M* sulfuric acid and were extracted with 3 × 100 ml ethyl acetate (washed previously with sodium bicarbonate solution). The final ethyl acetate extracts were combined and evaporated to dryness with a Büchi evaporator under vacuum at 40°C. The residue was dissolved in 10 ml of acetonitrile–tetrahydrofuran (1:1). Further purification of the extracts were accomplished by diluting 0.2 ml of the tetrahydrofuran–acetonitrile solution of the extract to 5 ml with 1 *M* ammonium hydroxide and this solution filtered through a C_{18} Sep-Pak cartridge (Waters Assoc.). The Sep-Pak was washed with three additional 5-ml portions of 1 *M* ammonium hydroxide, and the ammonium hydroxide solutions combined. The combined fractions were evaporated to dryness at 50°C under vacuum. The dry material was redissolved in 0.2 ml of mobile phase. A dilution of this material (1:10) was injected for chromatography.

Standard DNPHs of the α-keto dicarboxylates were prepared from commercially available acids or their sodium salts.

The chromatographic solvent system consisted of 0.015 *M* phosphoric acid in acetonitrile–water (40:60), used isocratically at a flow-rate of 1.5 ml/min. Samples injected were at 20 μl/injection, usually dissolved in the mobile phase.

RESULTS AND DISCUSSION

All compounds containing a keto or aldehyde carbonyl group give DNPH derivatives with DNP. However, the extraction procedure, modeled after Isherwood and Niavis[4] was designed to selectively remove all DNPHs and unreacted DNP, leaving in the final extract only derivatives containing the carboxyl group, *i.e.*, keto or aldehyde acid DNPHs. These derivatives are considerably more acidic than the parent keto acids[7], and require suppression of their ionization for chromatography in a reversed-phase system. Phosphoric acid serves in the mobile phase as the ionic suppressant. However, these DNPHs are still too polar after suppression of ionization to permit good separation on the widely used C_{18} columns. Attempts of separation by ion-pair chromatography with hexadecyl–trimethyl ammonium counter-ion with methanol–phosphate buffer as eluent yielded adequate separation, but some of the compounds gave overlapping peaks with keto-monocarboxylate DNPHs. The availability of column packings with shorter hydrocarbon chain coating permitted testing on these type columns. Zorbax C_8 was chosen as the column, as this material seems to have favorable mass transfer characteristics combined with greater affinity for more polar compounds. Later it was found that a 5-μm Spherisorb column (25 cm × 4.6 mm I.D., Chromanetics Corp.) produced nearly identical separations for the five α-ketodicarboxylate DNPHs to that of the Zorbax column, but operated at a significantly higher column pressure. The typical separation of the five standard DNPHs is shown in Fig. 1. Under these conditions, good separation of the standards was achieved in less than 10 min with a detection of 50–100 pmoles of DNPH/injection.

Various reports indicated that isomer formation of the DNPHs produced is common[1,3,5–9]. Nevertheless, under the conditions employed with the dicarboxylate parent acids this was not evident. However, decarboxylation products, particularly of oxaloacetate, such as pyruvate, can be observed (Fig. 1A). This compound, as well as

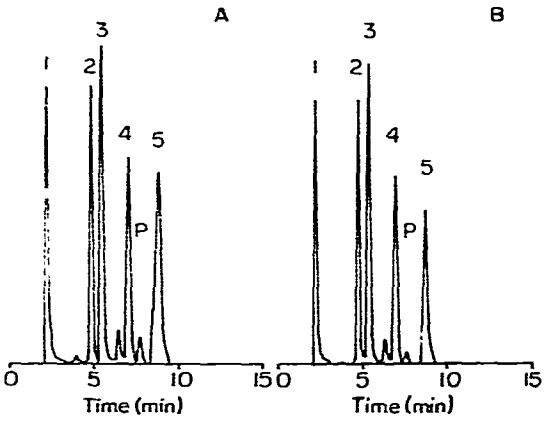

Fig. 1. Chromatogram of a standard mixture of α-keto dicarboxylate 2,4-dinitrophenylhydrazones. 20-μl injection. Isocratic separation for 15 min. Peaks: 1 = mesoxalic; 2 = oxaloacetic; 3 = α-ketoglutaric; 4 = α-ketoadipic; 5 = α-ketopimelic; P = pyruvic acid. Detector at 380 nm; 0.16 a.u.f.s. Flow-rate: 1.5 ml/min; Solvent: *0.015 M* phosphoric acid in acetonitrile–water (40:60). A, Before clean-up procedure; B, after C_{18} Sep-Pak clean-up.

other monocarboxylates do show formation of isomeric DNPHs. These isomers usually exhibit widely separated retention times in this solvent system, causing excessively long chromatographic runs. Attempts to remove these compounds in preference with the dicarboxylate DNPHs were generally unsuccessful, since they too are acidic. It was also evident upon examination of the chromatogram of the standards, that during the extraction process a small amount of DNP also remains with the extracted acidic material. When the mixture of the five dicarboxylate standards was passed through the C_{18} Sep-Pak in 1 *M* ammonium hydroxide, the resulting chromatogram showed the removal of the shoulder from the α-ketopimelate-DNPH peak. This shoulder was found to correspond to DNP (Fig. 1B). In addition, some reduction of the peak identified as pyruvate-DNPH can be observed. The recovery of the dicarboxylate peaks was in excess of 91 %.

Examination of the α-keto acids in citrus fruits may be used to establish the metabolic state of these fruits under varying conditions of growth and storage. The primary difficulty encountered, especially in preparation of peel extracts involves the presence of large quantities of pectic materials, which frequently cause difficulty in solvent extraction procedure due to formation of persistent emulsions. Acidic conditions in aqueous extracting solutions aggravate this problem. To alleviate these difficulties, alcoholic extracts were tried, maintaining strongly acidic conditions necessary for the formation of the derivatives, as well as for the reduction of instability with some of the keto acids. Ethanol–10 *M* sulfuric acid (80:20) seemed to produce the most consistent results. A modification, to include 2 % DNP in the extracting medium was tried to ascertain the total conversion of keto acids to their DNPHs, as the cells were broken during homogenization, thus releasing their content in intimate and immediate contact with the derivatizing agent. Fig. 2 illustrates results obtained from orange peel using the procedure described. The early eluting peaks in this chromatogram effectively masked components eluting at retention times corresponding to mesoxalic, oxaloacetic, α-ketoglutaric and α-ketoadipic acid DNPHs. Since the extracts contained other than dicarboxylic keto acid DNPHs, such as keto monocarboxylate DNPHs, excessive time was required to complete elution. In order to obtain chroma-

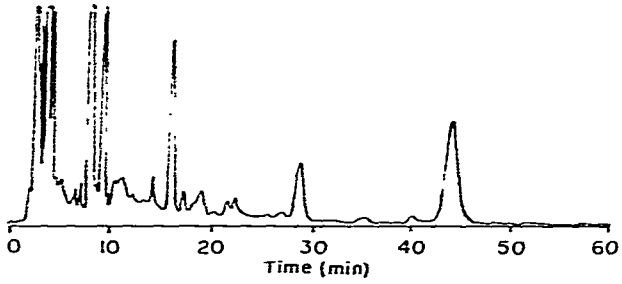

Fig. 2. Chromatogram of orange peel extract. Conditions as in Fig. 1.

tograms of the keto dicarboxylate-DNPHs in a reasonably short time, it is necessary either to increase the solvent strength after elution of the dicarboxylates to elute rapidly the remaining compounds, or to remove these to enable to perform chromatography under isocratic conditions. Application of the Sep-Pak clean-up procedure to the citrus peel extracts yielded unexpectedly simplified chromatograms, essentially free of all compounds eluting beyond 20 min (Fig. 3). This obviated the need for a gradient and the subsequent reequilibration, prior to introduction of the next sample. Compounds indicated on the chromatograms were identified by enrichment with known standards.

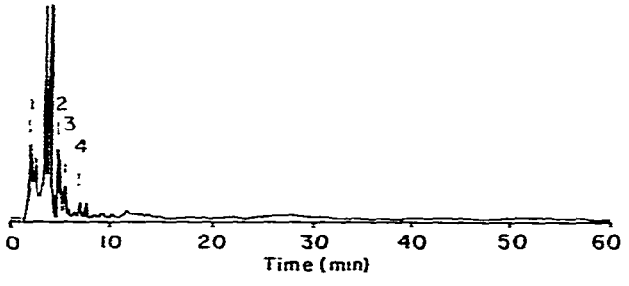

Fig. 3. Chromatogram of orange peel extract after C_{18} Sep-Pak clean-up. Conditions and peaks as in Fig. 1.

As the results indicate, direct derivatization during extraction, combined with precipitation of the interfering pectinaceous material from citrus peel is a feasible way of obtaining α-keto acids for analysis. The subsequent clean-up procedure and chromatographic system is capable of resolving the first five α-keto dicarboxylate-DNPHs, without recourse to excessively long chromatographic runs or the need for gradient operation. The separation of the keto monocarboxylates present in the extracts, as well as the isomeric derivatives that form during derivatization requires further study.

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