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FREE STEROLS OF CITRUS ROOTS

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Key Word Index—*Citrus kharna*; *Citrus medica*; *Citrus reticulata*; *Poncirus trifoliata*; Rutaceae; citrus; root sterols; 4-desmethylsterols; campesterol; sitosterol; stigmasterol; cholesterol.

Abstract—Free 4-desmethylsterols from fibrous roots of 6 citrus rootstocks were identified by combined gas chromatography and mass spectrometry as campesterol, stigmasterol, sitosterol and cholesterol (minor component). No isofucosterol was present.

INTRODUCTION

Investigations involving the quantitation of sterols from citrus (and other woody plants) are few and have been limited to tentative identification of individual sterols by co-chromatography with authentic standards on GC [1, 2]. Individual sterols affect membrane permeability in plants to different extents [3, 4] and changes in sterol composition appear to play a significant role in the response of roots to salinity [1], mycorrhyzal infection [2] and other environmental factors such as light intensity [5], hormone treatment [6] and temperature [7]. Therefore, it seemed appropriate to formally identify the free 4-desmethylsterol components of citrus roots to verify observed differences in composition in response to such environmental factors. In citrus this necessity is accentuated by the fact that isofucosterol has been tentatively identified in one investigation [2], but appears to be absent in another investigation [1] on roots of the same citrus rootstocks.

RESULTS AND DISCUSSION

GC of free 4-desmethylsterols from fibrous roots of all 6 citrus rootstocks examined showed the presence of only 4 main compounds. These corresponded in relative retention time to the authentic sterols cholest-5-en-3β-ol (cholesterol), 24ξ-methylcholest-5-en-3β-ol (campesterol), 24ξ-ethylcholest-5-en-3β-ol (sitosterol), and 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol) with the latter three compounds accounting for greater than 96% of the total sterols (Table 1). An additional peak with a relative (to 5α-cholestane) retention time greater than that of sitosterol and of similar order to that calculated for isofucosterol (24-ethylidenecholest-5-en-3β-ol) was also present in very small amounts (usually less than 1%). GC-MS of trifluoroacetate derivatives of the citrus sterols, scanned over the range m/z 50–500, gave a total ion chromatogram with peaks at the same relative retention times as the sterol standards. Cholesterol ($RR_t = 1.27$), campesterol ($RR_t = 1.51$) and sitosterol ($RR_t = 1.75$) trifluoroacetates showed characteristic ions of $[M-114]^+$ (as base peak) due to the loss of CF_3COOH and $[M-129]^+$ due to the further loss of the methyl at C-21. The abundance of the $[M-114]^+$ ion of stigmasterol ($RR_t = 1.60$) was reduced due to the production of minor peaks for $[M-142]^+$ due to the elimination of the 2C unit at C-24 (C_2H_4) and $[M-157]^+$ (the additional loss of the C-21 methyl). Ions for $[M-112]^+$ and $[M-141]^+$ indicated fragmentation of the side chain promoted by the double bond at C-22. In addition, all sterols showed ions m/z 255 and 213 due to the loss of side-chain and cleavage of ring D, indicative of the perhydrocyclopentanophenanthrene ring structure.

The peak observed running after sitosterol did not show an $[M-114]^+$ ion at m/z 394 that might be expected for isofucosterol nor other ions characteristic of the 4-desmethylsterols and is thus not isofucosterol. Serial

Table 1. Percentage distribution of 4-desmethylsterols from fibrous roots of citrus rootstocks from analytical capillary GLC* and GC-MS

Citrus rootstock	% Composition				
	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Unknown
Rangpur lime	2.1	41.0	19.9	37.0	0.9
Kharna khatta	0.6	36.4	26.2	36.9	0.6
Etrog citron	0.7	38.1	29.4	31.8	0.9
Cleopatra mandarin	3.1	24.2	18.1	34.4	1.3
Carrizo citrange	2.7	38.5	15.7	43.1	1.0
Trifoliata	2.9	40.9	16.7	39.5	1.1
Mean	2.0	36.5	21.0	37.1	1.0
GC-MS of combined sterols	2.6	37.7	20.7	39.0	0

*Analytical capillary GLC data are means ($n = 5$).

mass spectra taken during the elution of the sterols showed the peaks to be homogeneous. When scanned over the limited range of m/z 350–450, integration of the abundances gave percentage distribution figures for the 4 sterols very similar to those obtained by GC on fused silica columns (Table 1).

As relative retention indices are the same on two different phases, either as the free sterol or derivatized, and the mass spectra are the same as those of authentic compounds, the data are consistent with citrus roots containing three main 4-desmethylsterols viz. campesterol, sitosterol and stigmasterol with cholesterol (but not isofucosterol) as a minor component. The marked homogeneity of the mass peaks from mass spectral analysis of the citrus root sterols combined with the close agreement between the percentage distribution data obtained by capillary GC and GC-MS are strongly supportive of the use of capillary GC for the quantitation of these four sterols in citrus roots.

Finally, the percentage distribution data for sterols analysed by combined GC-MS are in close agreement with those published earlier [1] supporting the possible regulatory role of specific sterols in the chloride exclusion mechanism in citrus roots.

EXPERIMENTAL

Extraction and purification of sterols. The free 4-desmethylsterols were extracted from powdered, freeze-dried, fibrous root samples from citrus seedlings and separated from other lipids by TLC exactly as previously described [1]. The TLC band corresponding to 4-desmethylsterols was eluted from the silica gel with EtOAc and the sterols re-run on silica gel G TLC plates developed with $CHCl_3$ (× 2). The sterols were analysed by GLC using a fused silica capillary column (12 m × 0.2 mm) coated with SE-54 fitted to a Packard 438 GC with a Chrompack, all-glass, solid injector (Packard Instruments). Operating conditions were: isothermal (240°) operation, He carrier gas (5 ml/min), with detection by FID. Quantitation of sterol peaks from GC was performed by a Hewlett–Packard 3390 A Integrator interfaced to the GC. GC-MS of the sterols was carried out in electron impact mode on a Hewlett–Packard 5995 GC-MS at 70 eV fitted with a packed glass column (1.2 m × 2 mm i.d.) containing 1.5% (w/w) OV-101 on Gaschrom Q (100–120 mesh). The column oven was programmed from 220 to 240° at 8°/min and He carrier gas flow rate was maintained at 20 ml/min. Sterols were analysed on GC-MS as their trifluoroacetate derivatives prepared by adding trifluoroacetic anhydride (100 μl) to the sterol sample dissolved in $CHCl_3$ (100 μl) and heating the mixture (60°, 30 min). 5α-Cholestane was used as internal standard for all GC and GC-MS operations.

Plant material. White, fibrous roots sampled from 6–8 months old seedlings of Rangpur lime (*Citrus reticulata* var. *austera* hyb.?), Kharna khatta (*Citrus kharna* Raf.), Etrog citron (*Citrus medica* L.), Cleopatra mandarin (*Citrus reticulata* Blanco), Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) and Trifoliata (*Poncirus trifoliata* (L) Raf.) were used for analytical GC and GC-MS of the citrus sterols. Five replicate samples were analysed from each rootstock.

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