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Journal of Chromatography A, 874 (2000) 13–19

JOURNAL OF CHROMATOGRAPHY A

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Screening for limonoid glucosides in *Citrus tangerina (Tanaka) Tseng* by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Received 27 July 1999; received in revised form 14 December 1999; accepted 21 December 1999

Abstract

A screening method for limonoid glucosides in the peel of *Citrus tangerina (Tanaka) Tseng*, which utilizes high-performance liquid chromatography (HPLC) with diode-array detection and interfaced to electrospray ionization mass spectrometry, has been developed. In this way, the UV–Vis spectra and the mass spectra indicate the presence of limonoid glucosides without the necessity of isolating the individual compounds. Two major limonoid glucosides – obacunone glucoside (OG) and nomilin glucoside (NG) – were identified in the methanol extract of the peel. The two limonoid glucosides were taken as the target and isolated by means of preparative HPLC on a C_{18} reversed-phase column with an acidic acetonitrile–water mobile phase. The structures of OG and NG were further confirmed by nuclear magnetic resonance spectrometry.

Keywords: *Citrus tangerina*; Limonoid glucosides; Glucosides; Obacunone glucoside; Nomilin glucoside; Triterpenes; Carbohydrates

1. Introduction

Limonoids represent a group of chemically related triterpenes found in the Rutaceae and Meliaceae families. Thirty-six limonoid aglycones and 17 limonoid glucosides have been isolated from citrus and its hybrids [1]. Limonoid aglycones and their glucosides have been found to have anti-carcinogenic activity in laboratory animals and anti-feedant activity against insects [2–7]. Limonoid glucosides are non bitter in taste and water-soluble, so they could be excellent food additives as preventives against carcinogenesis [8]. The objective of this study was to develop a fast and reliable method to determine the presence and distribution of limonoid glucosides in the peel of *Citrus tangerina (Tanaka) Tseng* from China. High-performance liquid chromatography (HPLC) with diode-array detection (DAD) and electrospray ionization mass spectrometry (ESI-MS) were used to identify two limonoid glucosides. In this way, the UV–Vis spectra and the mass spectra indicate the presence of limonoid glucosides without the necessity of isolating individual compounds. The methodology includes a series of liquid–liquid extraction and column separation and LC–ESI-MS analysis of the extract. Finally, two limonoid glucosides – obacunone glucoside (OG) and nomilin glucoside (NG) – were purified by preparative

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)01337-0

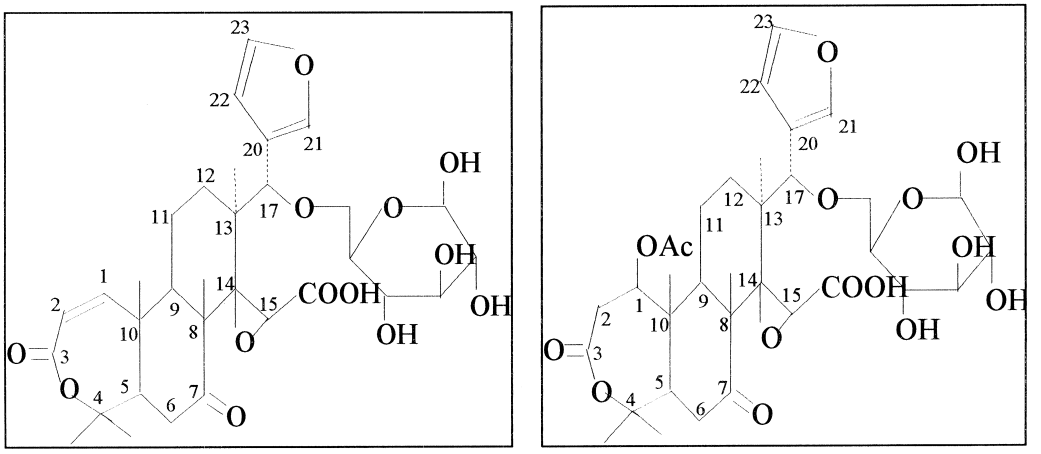


Fig. 1. Structures of the two limonoid glucosides.

HPLC and their structures were further confirmed by nuclear magnetic resonance (NMR) spectrometry. The structures of the two limonoid glucosides are shown in Fig. 1.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile (Merck, Darmstadt, Germany) was passed through a 0.45-μm filter, trifluoroacetic acid (TFA) was also from Merck. HPLC-grade water passed through 0.5-μm filter (Millipore, MA, USA). XAD-2 resin was purchased from Rohm and Haass, USA and WA-30 resin from Nihon Rensui (Tokyo, Japan).

2.2. Sample preparation

Peel of *Citrus tangerina Tseng* was ground with a Retsch mill (Brinkmann, Westbury, NY, USA) after drying at 55°C for 2 days. A 500-g amount of dried peel was placed in a Soxhlet extractor and washed overnight with 1 l hexane to remove oil. Limonoid aglycones were extracted with 1 l acetone, which was followed by 1 l 70% (v/v) aqueous methanol to extract limonoid glucosides. The methanol extract was evaporated to nearly dryness under vacuum and dissolved in 300 ml of water, the water solution was adjusted to pH 4.5 with 0.5 *M* HCl and then clarified by centrifugation at 1000 *g* for 20 min, which was applied to an XAD-2 (Rohm and Haass) column (600×60 mm), the column was washed thoroughly with 3 l water and then eluted with 2 l of methanol. The WA-30 (600×60 mm) column was conditioned with 2 l of 0.5 *M* NaOH followed by 5 l of deionized water, then the column was washed with 2 l of 0.5 *M* HCl followed by 5 l of deionized water. The 2 l methanol eluate from XAD-2 column was evaporated and dissolved in 200 ml of water, the solution was adjusted to pH 6.5 with 0.5 *M* NaOH and then applied to the WA-30 column to remove phenolic compounds [9]. The WA-30 column was washed thoroughly with 3 l deionized water and eluted with 1.5 l of 0.5 *M* NaCl to wash the limonoid glucosides, the eluate was re-applied to the previous XAD-2 column which was thoroughly washed with 3 l water to remove salts and subsequently eluted with 2 l methanol to wash limonoid glucosides. The flow-rate for the two columns was 10 ml/min. The final 2 l methanol eluate was evaporated and 7.215 g of dry substance was obtained. A solution of 0.01 g of dry

substance in 20 ml of water was made for HPLC–DAD and ESI-MS.

2.3. HPLC–DAD–MS analysis

Separation and analysis were performed on a 150 mm×3.9 mm I.D., 5 μm particle size, Symmetry C_{18} reversed-phase column (Waters, MA, USA). The LC–MS system is a Platform LCZ-2000 (Waters). The mobile phase for HPLC was acetonitrile–water (15:85, v/v), 0.05% TFA resulting in a pH of 3.2 was added to both solvents to eliminate the tailing of limonoid glucosides. The injected volume was 20 μl and the flow-rate was 1 ml/min. The effluent was scanned between 190 and 400 nm by the diode-array detector. The eluate from the HPLC column was split using a stainless steel tee-piece to deliver 10 μl/min into the electrospray ionization source. The mass spectrometer operated in the positive-ion and negative-ion electrospray ionization mode. Optimization was based on the stability of the spray and intensity of the $[M-H]^-$ and $[M+Na]^+$ signal.

2.4. Preparative HPLC conditions

A Waters 600 preparative HPLC system was used to purify limonoid glucosides. The column used for preparative HPLC was of 250×20 mm I.D., 10 μm particle size from Waters. The mobile phase was acetonitrile–water (25:75, v/v) (containing 0.05% TFA) and the injection volume was 200 μl, the column was eluted at 6 ml/min in 60 min with detection at 210 nm.

3. Results and discussion

Reversed-phase HPLC on a C_{18} column with a mobile phase of acidic acetonitrile–water enabled baseline separation of the limonoid glucosides in the methanol extract. The ion source used contains a stainless steel capillary for desolvation, which was set to 280°C for desolvation without thermal fragmentation. An ESI voltage which was set at 3.5 kV and −3.0 kV, respectively for detection of the positive ions and the negative ions gave a stable

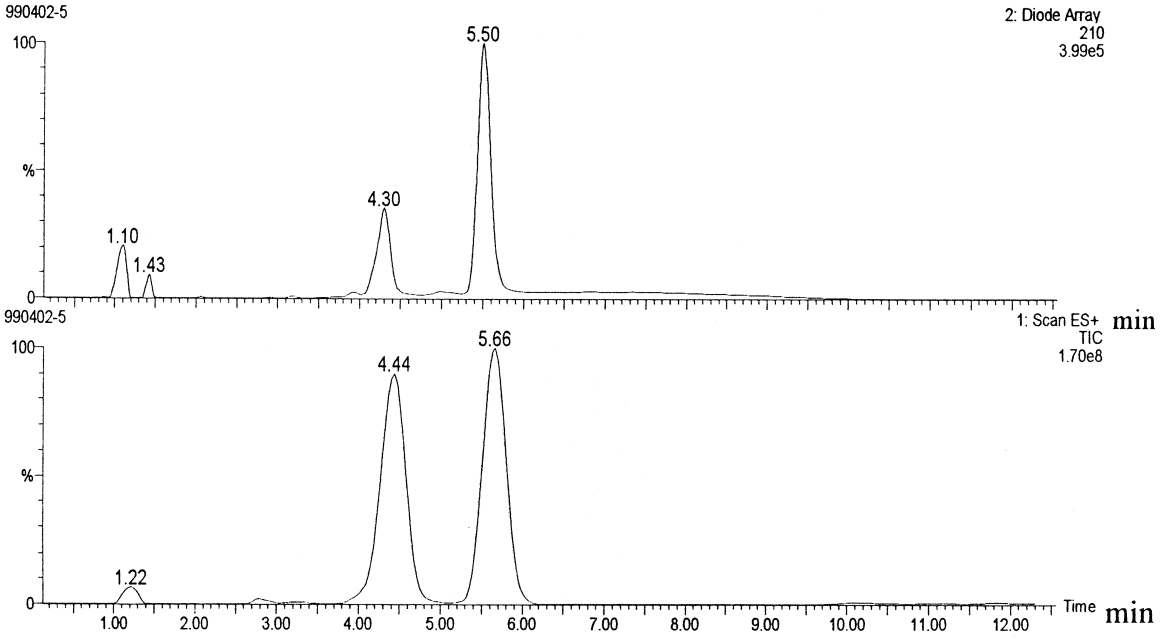


Fig. 2. HPLC–UV (210 nm) and HPLC-reconstructed TIC chromatogram.

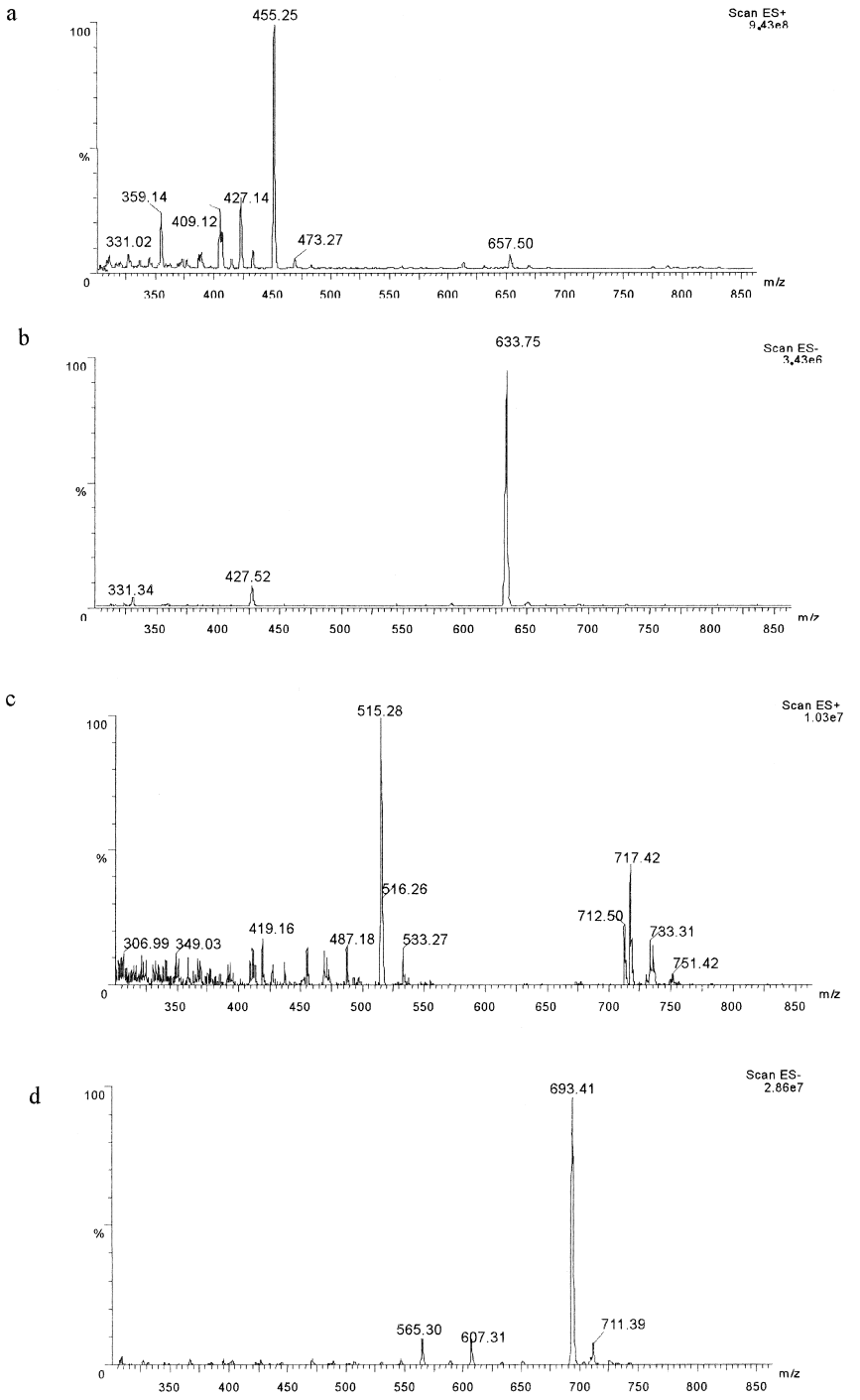
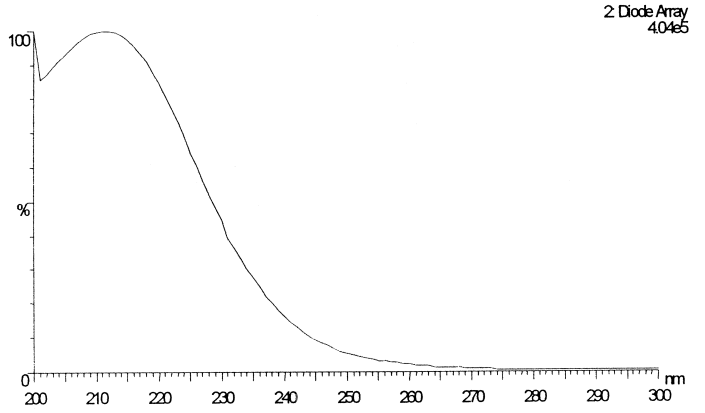
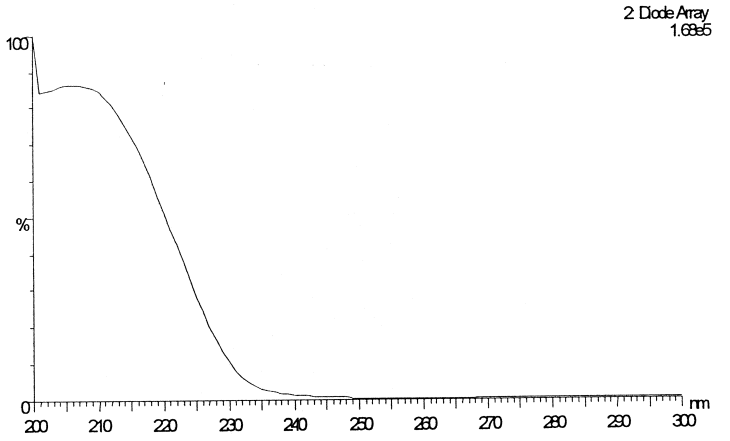


Fig. 3. LC–ESI-MS chromatograms of NG and OG. (a) Shows the positive ESI and (b) shows the negative ESI mass spectrum of OG. (c) Shows the positive ESI and (d) shows the negative ESI mass spectrum of NG. The figure shows an expansion of the region between scans 300 and 900.

spray and yielded the most abundant $[M-H]^-$ or $[M+Na]^+$ signals. In the negative-ion mode, $[M-H]^-$ ions were often detected as base peaks without any further fragment and sometimes a dimer $[2M-H]^-$ was observed. But in the positive-ion mode, the intensity of $[M+Na]^+$ ions was always very low. During analysis of the methanol extract of the peel, all peaks recorded in the UV trace (210 nm) gave a mass response in the total ion current (TIC) trace. Fig. 2 shows a representative chromatogram acquired using HPLC–ESI-MS. Both DAD and MS spectra were obtained for the two major limonoids in the methanol extract which was pretreated by absorption and ion-exchange column separation. The positive ESI mass spectrum (Fig. 3a) of compound 1 (peak at 5.66 min in the TIC spectrum of Fig. 2) gave an $[M+Na]^+$ ion at m/z 657.50 corresponding to a molecular mass (M_r) of 634. Other masses observed were at m/z 359.14, 455.25. In the negative-ion mode, the intense $[M-H]^-$ ion at m/z 633.75 and a weak ion at 427.52 was observed (Fig. 3b). The UV–Vis spectrum of this compound gave the maximum absorption at 213 nm (Fig. 4). The positive-ion ESI mass spectrum (Fig. 3c) of compound 2 (peak at 4.44 min in TIC spectrum of Fig. 2) gave an $[M+Na]^+$ ion at m/z 717.42 corresponding to an M_r of 694 and an ion of m/z at 515.28 was also observed. The intense signal in the negative-ion ESI mode at



Compound 1



Compound 2

Fig. 4. UV–Vis spectra of limonoid glucosides.

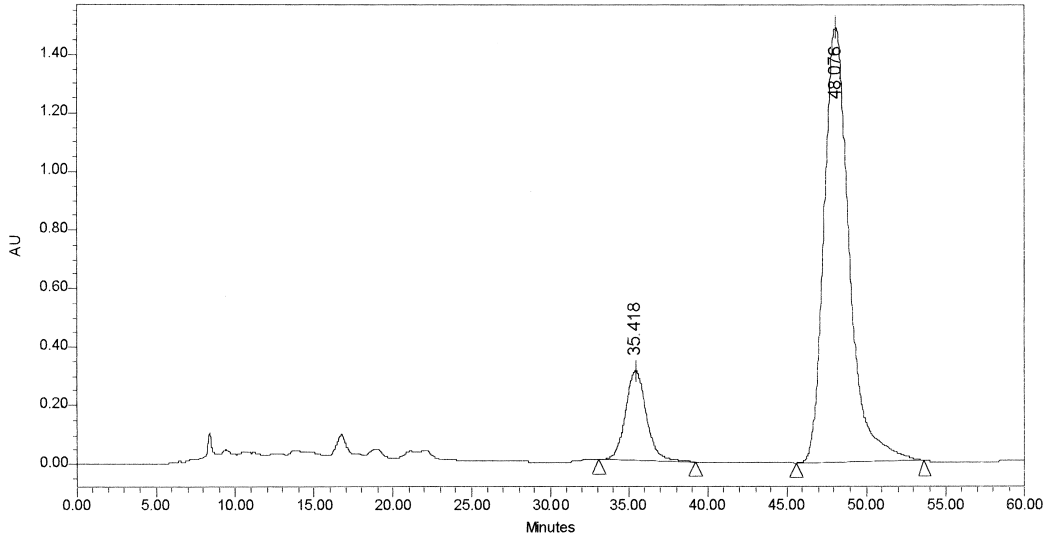


Fig. 5. Preparative HPLC chromatograms of limonoid glucosides. See text for HPLC conditions. The retention times for NG and OG were 35.418 and 48.076 min, respectively.

m/*z* 693.41 represents the $[M-H]^-$ ion of NG was observed, another ion at *m*/*z* 711.39 representing $[M+H_2O-H]^-$ was also observed (Fig. 3d). The UV–Vis spectrum (Fig. 4) shows the maximum absorption of this compound was at 208 nm. The UV–Vis and mass spectra suggested that compounds 1 and 2 were mostly like obacunone glucoside and nomilin glucoside.

In order to verify the correctness of the detection, the proposed OG and NG were taken as a target and isolated and purified by preparative HPLC on a C_{18} reversed-phase column. Fig. 5 is a typical preparative HPLC chromatogram of limonoid glucosides. Repeatedly the isolation and purification were monitored by HPLC–ESI-MS. Fractions containing OG and NG were freeze–dried, 2.785 g of OG and 0.121 g of NG were obtained and they were characterized by NMR.

Obacunone glucoside: ^{1}H-NMR (270 MHz, [$^2H_6$]dimethyl sulfoxide, 90°C): δ0.80, 1.06, 1.29, 1.34, 1.38(15H, 5*s*, C-Me), 3.05 (1H, *s*, H-15), 4.10 (1H, *d*, *J*=7.8, Glc H-1), 5.16 (1H, *s*, H-17), 5.88(1H, *d*, *J*=12.3, H-2), 6.39(1H, *d*, *J*=12.6, H-1), 6.54(1H, *s*, β-furan), 7.51(1H, *s*, α-furan), 7.42(1H, *s*, α-furan).

^{13}C-NMR (67.8 MHz, [$^2H_6$]dimethyl sulfoxide, 90°C): δ208.0(C-7), 168.7(C-16), 164.9(C-3), 149.9(C-1), 141.2(C-21), 140.4(C-23), 125.1(C-20), 119.1(C-2), 112.2(C-22), 104.1(Glc C-1), 82.8(C-4), 77.6(C-17), 76.6(Glc C-3), 75.8(Glc C-5), 73.8(Glc C-2), 70.5(C-14), 70.3(Glc C-4), 61.3(Glc C-6), 57.0(C-15), 51.0(C-8), 51.0(C-5), 46.3(C-9), 44.0(C-13), 44.0(C-10), 39.6(C-6), 29.6(C-Me), 26.4(C-12), 25.0(C-Me), 22.8(C-Me), 19.0(C-Me), 17.0(C-11), 14.8(C-Me).

Nomilin glucoside: ^{1}H-NMR (270 MHz, [$^2H_6$]dimethyl sulfoxide, 90°C): δ0.77, 0.98, 1.32, 1.35, 1.43(15H, 5*s*, C-Me), 2.02(3H, *s*, acetate methyl), 3.02(1H, *s*, H-15), 4.10(1H, *d*, *J*=7.2, Glc H-1), 4.68(1H, *d*, *J*=6.6, H-1), 5.23(1H, *s*, H-17), 6.52(1H, *s*, β-furan), 7.50(1H, *s*, α-furan), 7.51(1H, *s*, α-furan).

These data are consistent with those in the literature [10].

4. Conclusion

The HPLC–ESI-MS approach described in this paper using detection of positive and negative ions has been used successfully to rapidly screen complex mixtures in refined methanol extracts of citrus peel. The aim of this study was to investigate on-line LC–ESI-MS for the detection of limonoid gluco-

sides. The use of two detectors (diode array detector and mass spectrometer) allowed us to obtain both the UV–Vis spectrum and mass spectrum of each compound. The peel of *Citrus tangerina (Tanaka) Tseng* mainly contains two kinds of limonoid glucosides – nomilin glucoside and obacunone glucoside – the latter is the most abundant. This experiment is a good way to screen for target compounds.

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