Journal of Chromatography, 639 (1993) 295–302
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 970

Thin-layer and high-performance liquid chromatographic analyses of limonoids and limonoid glucosides in *Citrus* seeds

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(First received August 31st, 1992; revised manuscript received February 9th, 1993)

ABSTRACT

A routine method for the analysis of limonoids and limonoid glucosides in citrus seeds, which utilizes thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), is described. Seed meals were washed with *n*-hexane to remove oily materials, after which limonoids and limonoid glucosides were extracted with acetone and then methanol. The methanol extract contains the remainder of the limonoid aglycones and all limonoid glucosides. After the methanol was evaporated off, the residue was extracted with methylene chloride–water (1:1). The limonoids of both the acetone and methylene chloride fractions were separated and quantified by HPLC. The total limonoid glucoside content of the water fraction was determined by silica gel TLC. Each of the limonoid glucosides was then eluted from the reversed-phase HPLC column by a linear gradient system starting at 15% acetonitrile in 3 m*M* phosphoric acid and ending with 26% acetonitrile after 35 min, and quantitated by spectrophotometric detection at 210 nm. The limonoid aglycones and limonoid glucosides in two kinds of citrus seeds, Shiikuwasha (*Citrus depressa*) and Iyo (*Citrus iyo*), were determined. The content of limonoid glucosides in Shiikuwasha was found to be approximately two-fold higher than that of Iyo.

INTRODUCTION

The limonoids are a group of chemically related triterpenoid derivatives present in the Rutaceae and Meliaceae families. Limonoids are one of two major bitter principles in citrus juices. Among the 37 limonoids isolated from *Citrus* and its hybrids, limonin is the major cause of the bitterness in a variety of citrus juices. Recently, limonoids have been shown to be also present as glucoside derivatives in *Citrus* [1]. Nineteen limonoid glucosides have been isolated from *Citrus* and, significantly, they are all non-bitter in taste.

Limonoids possess important biological activities such as the inhibition of the growth of cancerous tumours in laboratory animals [2–4] and antifeedant activity against insects and termites [5,6]. The demand for limonoids has increased significantly in recent years. Citrus seeds are major sources of both limonoid aglycones and their glucosides and could be utilized as a source of these important compounds.

Methods for the determination of limonoid aglycones, such as limonin and nomilin, using

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0021-9673/93/$06.00

thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been reported [7–14]. In particular, Van Beek and Blaakmeer [14] performed a detailed analytical investigation of limonin in citrus juice. In this HPLC method the combination of reversed-phase columns and spectrometric detection was successfully applied to limonoid aglycone assays. Recently, limonoid glucosides in citrus juices have been also analysed using TLC [15] and HPLC [16].

The objective of this study was to establish a routine procedure for the extraction, isolation and characterization of limonoid aglycones and limonoid glucosides in citrus seeds by TLC and HPLC. For this study, the seeds of Shiikuwasha (*Citrus depressa* Hayata) and Iyo (*Citrus iyo* hort. Tanaka) were examined. The structures of major limonoids and limonoid glucosides are shown in Fig. 1.

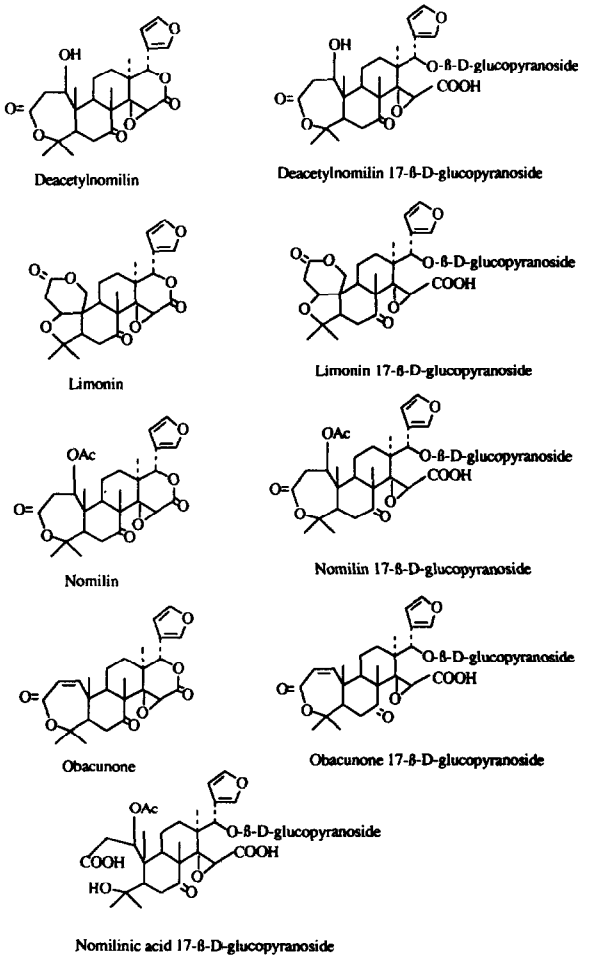

Fig. 1. Structures of major limonoids and limonoid glucosides.

EXPERIMENTAL

Materials

Shiikuwasha (*Citrus depressa* Hayata) was harvested in November, 1991, from trees grown at the Agricultural Product Processing Factory, Agricultural Cooperative Association of Okinawa, Japan. Iyo (*Citrus iyo* hort. ex Tanaka) was sampled in October, 1991, at the Tree Research Station of Saga Prefecture, Japan. The seeds were ground with a Retsch mill (Brinkmann, Westbury, NY, USA) after drying at 60°C for 3 days.

Chemicals

Silica gel HLF plates were purchased from Analtech (Newark, DE, USA). A preparative HPLC column, C_{18} reversed-phase, Partisil ODS-3, 25 cm × 2.2 cm, particle size 10 μm (Whatman, Hillsboro, OR, USA) was used. DEAE-Sephacel, Amberlite XAD-2 resin (20–60 mesh), hesperidinase and naringinase were obtained from Sigma (St. Louis, MO, USA). Sep-Pak silica cartridges, used for sample clean-up, were obtained from Waters (Milford, MA, USA).

Standards of limonoids and limonoid glucosides

Limonoid aglycones such as limonin, nomilin, deacetylnomilin and obacunone, and 17-β-D-glucopyranosides of limonin, deacetylnomilin, nomilin, nomilinic acid and obacunone, which were isolated and characterized by NMR at the Fruit and Vegetable Chemistry Laboratory, US Department of Agriculture, Agricultural Research Service, Pasadena, CA, USA, were used as standards in this study.

Apparatus for HPLC

The system consisted of two Waters 510 LC pumps, a Waters Automated Gradient Control-

ler, a C_{18} reversed-phase analytical HPLC column (250 mm × 4.6 mm, 5 μm particle size, Alltech Associates, Deerfield, IL, USA), a Perkin Elmer LC-75 spectrophotometric detector and a Shimadzu C-R3A integrator (Shimadzu, Kyoto, Japan).

Extraction of limonoid aglycones and glucosides

Aliquots of 100 g of each seed meal obtained from the dried seeds were placed in a Soxhlet extractor and washed thoroughly with *n*-hexane to remove oily materials, then extracted sequentially with acetone and methanol. The acetone extract (fraction 1) contained approximately 50% of total limonoid aglycones. The methanol extract (fraction 2) contained the remainder of limonoid aglycones and all the limonoid glucosides. This fraction was evaporated to dryness and the residue was re-extracted with methylene chloride–water (1:1). The methylene chloride fraction (fraction 3) contained aglycones, and the water fraction (fraction 4) contained glucosides.

TLC identification and HPLC analysis of limonoid aglycones

Both the acetone (fraction 1) and methylene chloride (fraction 3) fractions were combined and evaporated to analyse the limonoid aglycones. The dried material was resuspended in methanol and a portion was spotted onto silica gel TLC plates, which were developed in one dimension. The following three solvent systems were used in saturated chambers: cyclohexane–ethyl acetate (2:3), methylene chloride–methanol (49:1) and ethyl acetate–methylene chloride (2:3). The developed plate was sprayed with Ehrlich's reagent and colour was developed in a hydrochloric acid chamber [9]. R_F values were compared with those of standards for identification purposes. Another portion of the resuspended sample was injected into a C_{18} reversed-phase HPLC column, the column being eluted isocratically with acetonitrile–methanol–water (10:41:49). The limonoid aglycones were separated and identified by monitoring UV absorption at 210 nm.

HPLC and TLC analyses of the limonoid glucosides

The content of limonoid glucosides in the seeds was determined by both TLC and HPLC methods.

For the TLC, the water extract (fraction 4) was evaporated, dissolved in a measured volume of methanol and spotted on a plate, which was developed in one dimension with an ethyl acetate–methyl ethyl ketone–formic acid–water (5:3:3:1) system. Limonoid glucosides were visualized by spraying with Ehrlich's reagent followed by exposure to hydrogen chloride gas [17]. The spots were compared with those of a standard compound, and the total limonoid glucosides were determined by the relative intensity of the colour. This analysis was done in duplicate and average values were reported. Limonin glucose standards ranging from 1 to 5 μg at increments of 0.2 μg were used [15]. A preliminary analysis was needed to determine the approximate concentration of the sample to be used on TLC.

For the HPLC, a portion of the water extract (fraction 4) was treated with hesperidinase [0.010 units mg^{-1} solid: one unit was defined as 1.0 μmol of reducing sugar (as glucose) from hesperidin per min at pH 3.8 at 40°C] and naringinase [365 units g^{-1} solid: one unit was defined as 1.0 μmol of reducing sugar (as glucose) from naringin per min at pH 4.0 at 40°C] in a 0.1 *M* sodium formate buffer at pH 3.8 for 20 h at room temperature. This treatment was necessary to obtain good peak resolution by cleaving the sugars from interfering flavonoids. The sample was then loaded onto a Sep-Pak column, washed with water and eluted with methanol. This methanol fraction was evaporated in a test tube; 250 μl of 15% acetonitrile in 3 m*M* phosphoric acid were added and analysed by HPLC. For each sample extracted, duplicate 100-μl injections were made on a C_{18} reversed-phase analytical HPLC column. The flow-rate was 1 ml min^{-1} with a linear gradient system, starting with 15% acetonitrile in 3 m*M* phosphoric acid and ending with 26% at 35 min. The elution was monitored by UV absorption at 210 nm and a standard curve was run for each of the limonoid glucosides.

Isolation and characterization of limonoid glucosides

The water fraction of a seed meal extraction (fraction 4) (pH 6.5) was transferred on to a DEAE-Sephacel column (25 cm × 2.5 cm I.D.), followed by a thorough washing with water. The limonoid glucosides were eluted by an increasing linear gradient of sodium chloride in water. Fractions containing glucosides were combined, desalted by passing through a Dowex 50 column (H^+ form, 20 cm × 1.5 cm I.D.), and refractionated on an XAD-2 column (75 cm × 2.5 cm I.D.). The column was eluted with methanol. After the solvent was evaporated, the residue was dissolved in water and fractionated on a C_{18} reversed-phase preparative HPLC column. The column was eluted at 6 ml min^{-1} with a linear gradient starting with 15% and ending with 55% methanol in water over 75 min [18]. Fractions containing each limonoid glucoside were characterized by NMR [1,19] and analytical HPLC. NMR spectra were recorded on a JEOL GSX-270 NMR spectrometer in [$^2H_6$] dimethyl sulphoxide at 90°C, at 270 MHz for ^1H and 67.8 MHz for ^{13}C. NMR spectral assignments were made on the basis of ^1H-^1H COSY (correlation spectroscopy) and NOESY (nuclear Overhauser enhancement and exchange spectroscopy), DEPT (distortionless enhancement by polarization transfer) and ^{13}C-^1H COSY spectra [1,19].

RESULTS AND DISCUSSION

Determination of limonoid aglycones

Identification of limonoid aglycones by silica gel TLC. Limonoids in citrus tissue and juice have been customarily detected by TLC [7]. For identification purposes, we first examined R_F values of the major standard limonoids by TLC, using the three solvent systems described above. Table I lists the R_F values of major limonoids, including limonin, nomilin, deacetylnomilin and obacunone. Although these R_F values are a useful indicator with which to identify each of the limonoids, it is recommended that standard compounds be co-chromatographed on the same TLC plate.

Qualitative and quantitative analyses of limonoid aglycones by HPLC. The individual limonoid aglycones in the acetone and methylene chloride extracts were separated and quantified by HPLC. We used a C_{18} reversed-phase column and the column was eluted isocratically with acetonitrile–methanol–water (10:41:49). A typical chromatogram of standard limonoids is shown in Fig. 2, the retention times for limonin, deacetylnomilin, nomilin and obacunone being 15.1, 18.8, 26.3 and 43.2 min, respectively.

TABLE I

R_F VALUES OF LIMONOID AGLYCONES ANALYSED BY TLC

Limonoid aglycone	R_F value		
	Solvent system		
	1[a]	2[b]	3[c]
Limonin	0.32	0.53	0.55
Deacetylnomilin	0.12	0.33	0.36
Nomilin	0.23	0.60	0.58
Obacunone	0.57	0.74	0.81

[a] Cyclohexane–ethyl acetate (2:3).
[b] Methylene chloride–methanol (49:1).
[c] Ethyl acetate–methylene chloride (2:3).

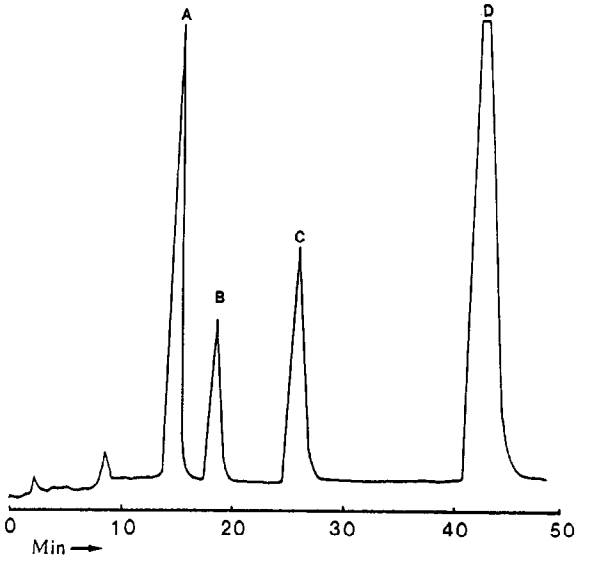

Fig. 2. High-performance liquid chromatogram of limonoid aglycones. Peaks: A = limonin (5 μg); B = deacetylnomilin (2.5 μg); C = nomilin (5 μg); D = obacunone (5 μg). See text for the HPLC conditions.

These retention times are also useful indicators for the identification of each limonoid.

In order to check the linearity of the relationship between the limonoid levels and peak area using the above separation and detection system, various amounts of dissolved limonoid standards were injected into the HPLC column in the concentration range of 0–4 μg. Except for obacunone, all the graphs exhibited good linearity and obeyed Beer's law. For the regression equation $y = ax + b$, where x is the amount of limonoid (μg) and y is the peak area, the correlation coefficients (r) of the limonoids were as follows: for limonin, $y = (4.814 \cdot 10^5)x - 2.174 \cdot 10^4$ ($r = 0.999$); for deacetylnomilin, $y = (4.216 \cdot 10^5)x - 1.221 \cdot 10^4$ ($r = 0.999$); for nomilin, $y = (4.209 \cdot 10^5)x - 4570$ ($r = 1.000$); for obacunone, $y = (1.372 \cdot 10^6)x + 1.661 \cdot 10^4$ ($r = 0.999$). These results suggest that these HPLC conditions are sufficiently sensitive to detect the levels of the major limonoids.

Determination of limonoid glucosides

Estimation of total limonoid glucosides by TLC. A portion of the water extract (fraction 4) described above was spotted on TLC plates and developed with ethyl acetate–methyl ethyl ketone–formic acid (88%)–water (5:3:3:1). The plate was then developed in the same manner mentioned above. The R_F values were compared with standards run in most cases on the same plate.

R_F values of the major 17-β-D-glucopyranosides of limonin, deacetylnomilin, nomilin and obacunone were 0.34, 0.26, 0.37 and 0.54, respectively. In the determination of the total limonoid glucosides, two judges provided estimates of the glucosides by comparing the size and colour intensity of spots with those of the standards as described above.

Qualitative and quantitative analyses of limonoid glucosides by HPLC. The standard limonoid glucosides were separated by a C_{18} reversed-phase HPLC column. The column was eluted at 1 ml min^{-1} using a linear gradient starting with 15% acetonitrile in 3 m*M* phosphoric acid and concluding with 26% after 35 min. A typical HPLC chromatogram is shown in Fig. 3, retention times for the 17-β-D-glucopyranosides of limonin, deacetylnomilin, nomilin, nomilinic acid and obacunone being 16.2, 23.4, 29.6, 30.5 and 33.8 min, respectively.

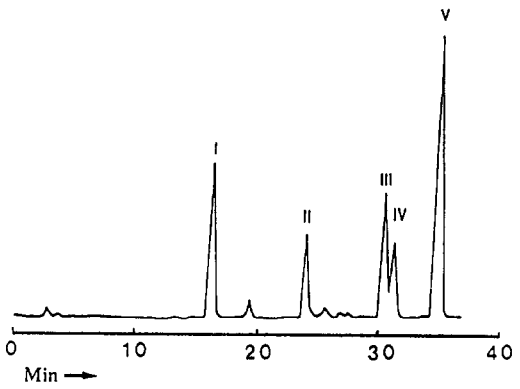

Fig. 3. High-performance liquid chromatogram of limonoid glucosides. 17-β-D-Glucopyranosides of limonin (I, 2.4 μg), deacetylnomilin (II, 1.6 μg), nomilin (III, 1.6 μg), nomilinic acid (IV, 1.6 μg) and obacunone (V, 1.6 μg).

The calibration curves for the 17-β-D-glucopyranosides of limonin, deacetylnomilin, nomilinic acid and obacunone showed good linearity in the concentrations range 0–4 μg, except for obacunone glucosides. For the regression equation $y = ax + b$, where x is the amount of limonoid glucosides (μg) and y is the peak area, the correlation coefficients (r) of the limonoid glucosides were as follows: for limonin glucoside, $y = (1.675 \cdot 10^5)x - 289.2$ ($r = 0.999$); for deacetylnomilin glucoside, $y = (1.378 \cdot 10^5)x - 7794$ ($r = 0.999$); for nomilin glucoside, $y = (1.963 \cdot 10^5)x - 1121$ ($r = 0.999$); for nomilinic acid glucoside, $y = (1.515 \cdot 10^5)x + 7795$ ($r = 0.999$); for obacunone glucoside, $y = (6.042 \cdot 10^5)x - 1677$ ($r = 0.999$). These results indicated that this HPLC condition was sufficiently sensitive to detect the limonoid glucosides.

Recovery of limonoid glucosides. Five limonoid glucosides, limonin glucoside, deacetylnomilin glucoside, nomilin glucoside, nomilinic acid glucoside and obacunone glucoside, were added at 100 mg l^{-1} to a sample water fraction (fraction 4) of Iyo containing known levels of the compounds (see Table II), followed by analysis by the HPLC method described in the Experimental section after Sep-Pak clean-up. The recoveries were 97% for limonin glucoside, 98% for deacetyl nomilin glucoside, 97% for nomilin

glucoside, 95% for nomilinic acid glucoside and 98% for obacunone glucoside. Several experiments gave recoveries in the range 93–100%. These recoveries were certainly within acceptable limits.

Application

Seeds of Shiikuwasha (Citrus depressa Hayata). TLC analysis with the three solvent systems described in Experimental section showed that Shiikuwasha contains three limonoid aglycones. They were identified as limonin, nomilin and obacunone. These are the major limonoid constituents in *Citrus* and its hybrids, and are widely spread in many species [20]. Like many other species, limonin is the predominant limonoid in the seeds of Shiikuwasha, followed by nomilin and obacunone. Limonin, which is the major cause of limonoid bitterness in a variety of citrus juices, has been shown to be biosynthesized from nomilin via obacunone, obacunoate and ichangin [21,22].

NMR analysis of the isolates showed that Shiikuwasha contains the 17-β-D-glucopyranosides of limonin, deacetylnomilin, nomilin, nomilinic acid and obacunone. Fig. 4 shows a chromatogram of limonoid glucosides in the seeds of Shiikuwasha. Table II gives the results expressed as mg g^{-1} (dried seeds). As expected, nomilin 17-β-D-glucopyranoside was the predominant limonoid glucoside, followed by obacunone 17-β-D-glucopyranoside, nomilinic acid 17-β-D-glucopyranoside, limonin 17-β-D-glucopyranoside and deacetylnomilin 17-β-D-glucopyranoside in order of decreasing concentration. This order is very common in many *Citrus* species [20].

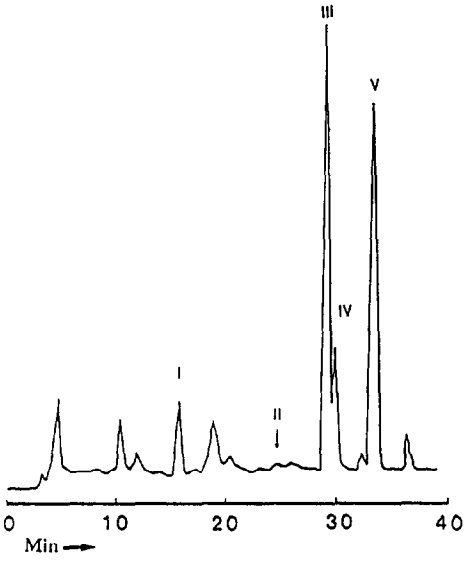

Fig. 4. High-performance liquid chromatogram of water extract from Shiikuwasha (*Citrus depressa*) seeds. 17-β-D-Glucopyranosides of limonin (I), deacetylnomilin (II), nomilin (III), nomilinic acid (IV) and obacunone (V).

TABLE II

LIMONOID AND LIMONOID GLUCOSIDES IN SEEDS OF SHIIKUWASHA (*CITRUS DEPRESSA* HAYATA) AND IYO (*CITRUS IYO* HORT. EX TANAKA)

Limonids	Content[a] (mg per g dried seed)	
	Shiikuwasha	Iyo
Limonin	1.87	4.57
Deacetylnomilin	–	0.72
Nomilin	0.96	2.53
Obacunone	0.45	0.91
Total	3.28	8.73
17-β-D-glucopyranoside of		
Limonin	1.16	0.53
Deacetylnomilin	0.18	0.48
Nomilin	7.59	2.24
Nomilinic acid	1.85	0.34
Obacunone	1.96	0.87
Total[b]	12.74	4.46
Total[c]	11.82	4.41

[a] Average values of duplicate measurements.
[b] Determined by HPLC.
[c] Determined by TLC.

Table II also shows the limonoid glucoside content as analysed by TLC. The values for total amount of limonoid glucosides determined by TLC and HPLC correlated very closely. A paired-sample *t*-test gave no significant variance at the $p = 0.05$ confidence level. Quantitative analyses by TLC and HPLC showed that the contents of limonoid glucosides in the seeds of Shiikuwasha are approximately twofold higher than that of other citrus seeds [20], whereas the content of limonoid aglycones analysed by HPLC is lower than that of others. The conversion of limonoid aglycones to their corre-

sponding glucosides occurs in *Citrus* during late stages of fruit growth and maturation [23]. This conversion may continue until fruit is harvested. The Shiikuwasha fruits used in this study were harvested at the early season of November. Thus, fruit harvested in the late season should have higher concentrations of limonoid glucosides.

Citrus limonoids have been shown to possess biological activities such as anticancer activity in laboratory animals [2–4] and antifeedant activity against insects and termites [5,6]. Thus the demand for seeds as a source of limonoids has increased significantly in recent years. Shiikuwasha is mainly grown in Okinawa, the most southern part of Japan, and is used mainly as a flavour enhancer. This work shows that its seeds are also excellent sources of limonoids, particularly their glucoside derivatives.

Seeds of Iyo (Citrus iyo hort. ex Tanaka). Four limonoid aglycones were identified. They were limonin, nomilin, obacunone and deacetylnomilin in order of decreasing concentration (Table II). As in many other *Citrus* species, limonin was the predominant limonoid aglycone in the seed of Iyo.

Analyses of the isolates by NMR spectra resulted in the identification of five limonoid glucosides from the seeds of Iyo. They are the 17-β-D-glucopyranosides of nomilin, obacunone, limonin, deacetylnomilin and nomilinic acid. Fig. 5 presents a typical chromatogram of limonoid glucosides in the seeds of Iyo. As shown in Table II, data on the total limonoid glucoside contents obtained from the two methods, TLC and HPLC, also agreed very well.

The composition and relative concentrations of limonoid aglycones and their glucosides are very similar to those of the common *Citrus* species [20]. Like the others, the ratio of aglycones to glucosides was about 2. The 17-β-D-glucopyranoside of nomilin is the predominant limonoid glucoside. This supports the previous suggestion [24] that limonoids and limonoid glucosides in seeds are biosynthesized there, independent from the biosynthesis occurring the fruit tissue.

From these results, we recommend this TLC and HPLC procedure as a convenient method for the analysis of the limonoid aglycones and limonoid glucosides in citrus seeds. We also expect that this method will be a useful in any chemotaxonomic analysis of Rutaceae and Meliaceae families and as a method for the purification of limonoids and limonoid glucosides from citrus seeds.

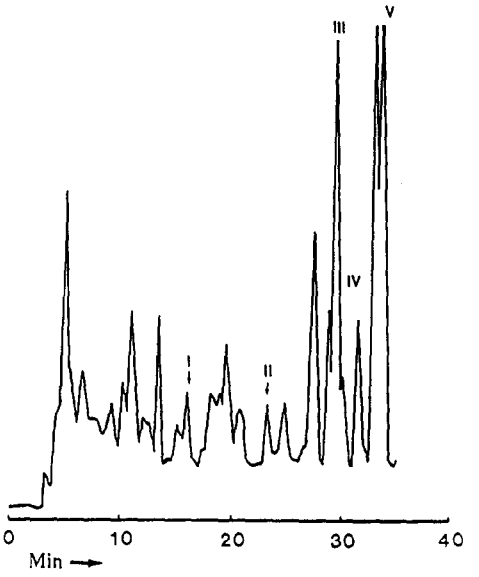

Fig. 5. High-performance liquid chromatogram of water extract from Iyo (*Citrus iyo*) seeds. 17-β-D-Glucopyranosides of limonin (I), deacetylnomilin (II), nomilin (III), nomilinic acid (IV) and obacunone (V).

ACKNOWLEDGEMENTS

This research was supported in part by a Grant-in-Aid from the Science and Technology Agency of Japan. S.H. was a recipient of Research Awards for specialists from Japanese Government.

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