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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF OLEANANE-TYPE TRITERPENES

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SUMMARY

Seven oleanane-type triterpenes and sitosterol, which may be present together in natural mixtures, were successfully resolved by normal phase high-performance liquid chromatography on a silica gel column. A rapid isocratic separation was achieved using a ternary solvent system of hexane–isopropanol–methanol (96:3.5:0.5). Derivatization was not required for compounds that were detected by UV absorption at 210 nm. This method, applied to qualitative and quantitative analysis of triterpenes extracted from seeds and callus tissue culture of *Chenopodium quinoa*, has proved to be efficient, highly reproducible and sensitive.

INTRODUCTION

Oleanane-type triterpenes are natural products that are widely distributed in the plant kingdom. They are of great interest because of their diverse pharmacological properties, for instance, anti-inflammatory, antibiotic, contraceptive, and cholesterol-lowering effects[1].

Thin-layer chromatography[2] (TLC) and gas–liquid chromatography[3] (GLC) have been useful in studying these compounds in our laboratory. However, they are time-consuming and may require sample derivatization. These inconveniences can be avoided through the use of high-performance liquid chromatography (HPLC), but few reports exist on HPLC separation of pentacyclic triterpenes: a single triterpene has been separated from a cardiac glycoside and a sesquiterpene by normal-phase HPLC (NP-HPLC)[4]; cactus triterpenes have been analyzed by reversed-phase HPLC (RP-HPLC)[5], but derivatization was required. More recently, a method for HPLC analysis of underivatized triterpenes was published[6], but the separation of compounds having a wide range of polarities was achieved only by combining normal- and reversed-phase methods.

We describe a new NP-HPLC procedure that separates, in one chromatographic run, seven underivatized and closely related mono- and dihydroxy-oleanane triterpenes, which are found in many plant families (Araliaceae, Caryophyllaceae, Umbelliferae, Ranunculaceae, Mimosaceae, Sapindaceae)[7,8]. Sitosterol, a common com-

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ponent of plant and tissue cultures, was also included in the triterpene mixture and was successfully resolved. Reproducibility and sensitivity of this method were excellent for qualitative and quantitative analyses of triterpene sapogenins. This method was applied to the analysis of seed and *in vitro* tissue culture extracts from *Chenopodium quinoa*, a South American grain crop[9], notable for its high nutritional value.

EXPERIMENTAL*

Standards and plant material

Commercial compounds used were β-amyrin (Pierce), sitosterol (Calbiochem), oleanolic acid (Chem. Procurement Lab. Inc.), and erythrodiol and echinocystic acid (Sarsyntex). Gypsogenin, hederagenin, and queretaroic acid were kindly provided by R. G. Powell (USDA, Peoria). Plant triterpenes were extracted from seeds of *Chenopodium quinoa* Willd., var. Real de Puno, a variety known to be high in saponins[2], and from its root-derived callus cultures. *Quinoa* seeds were kindly donated by Dr. J. Alvarez (University C. Heredia, Lima). Growth of callus cultures was initiated and maintained on a Murashige-Skoog medium[10] containing 3% sucrose and 1 mg/l 2,4-dichlorophenoxyacetic acid (a plant growth regulator). Callus tissues were cultivated at 24°C under white fluorescent light for 12 h per day. One-year-old cultures were harvested and dried in an oven (60°C, 24 h). Seeds and callus tissues were ground and defatted with diethyl ether, then extracted three times with 1-butanol–ethanol–water (27:53:20, 40:40:20 and 53:27:20, respectively) in a Soxhlet apparatus. Saponins were precipitated with diethyl ether and hydrolyzed in 2 *M* hydrochloric acid for 5 h. The water-insoluble hydrolyzates, recovered after filtration in a Buchner funnel, were extracted with methanol for 3 h to obtain the triterpenes.

Instrumentation

HPLC apparatus consisted of a solvent delivery system (Spectra Physics, SP 8700), an automatic sample injector (Waters Assoc., WISP 710B), a 250 × 4.6 mm I.D. column pre-packed with 5 μm silica gel (Alltech) protected by a 25 × 3.9 mm I.D. Corasil II guard column, and a variable-wavelength U.V. detector (Schoeffel, GM 770). Peak areas were quantified by a Hewlett-Packard 3390A integrator; raw analog data were also stored on magnetic tape on a ModComp computer to permit subsequent replotting with variable x and y scales.

Chromatographic conditions

Analyses were carried out under isocratic conditions with a filtered and degassed solvent mixture of hexane–isopropanol–methanol (96:3.5:0.5) delivered to the column at a flow-rate of 2 ml/min. Triterpenes were detected at 210 nm and a sensitivity of 0.4 absorbance unit full-scale (a.u.f.s.). Before injection, standard compounds and plant extract samples, dissolved in isopropanol, were centrifuged (5 min at 2500 *g*) to remove particulate material. All samples were soluble in the mobile phase.

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* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Quantitation procedure

Calibration curves (weight injected *vs.* peak area) were plotted for oleanolic acid and hederagenin, the major quinoa sapogenins[3]. These two triterpenes gave a linear response over a range of 0.5–30 μg and 1–30 μg, respectively. Peak areas showed excellent reproducibility (average relative standard deviation of 1.2%).

RESULTS AND DISCUSSION

Separation of triterpenes

A mixture of triterpenes was used to test the NP-HPLC method. Oleanolic acid, hederagenin, and echinocystic acid were chosen since they are saponin aglycones found in the Chenopodiaceae[7] and are metabolically related to β-amyrin and erythrodiol. Sitosterol is commonly produced by plants and cultured cells, and it represents an analytical problem if present as a glycoside[11–13] or if plant tissues are hydrolyzed and extracted without prior isolation and purification of saponins. Gypsogenin was added to the standard mixture to determine the effect of an aldehyde group on retention time, and queretaroic acid was included because it is an isomeric form of echinocystic acid.

Several attempts were made to resolve completely a mixture of these triterpenes and sitosterol. Separation of triterpenes could be achieved with hexane–isopropanol (96:4) under isocratic conditions at 2 ml/min flow-rate, but sitosterol was incompletely separated from oleanolic acid. However, base-line resolution of all compounds tested was finally obtained under the same conditions by the addition of a small amount of methanol, a more polar compound than isopropanol (hexane–isopropanol–methanol, 96:3.5:0.5) (Fig. 1). Queretaroic acid, the most polar triterpene, eluted 28.5 min after injection. Retention times (Table I) were highly reproducible, with an average relative standard deviation of 0.4%. Table II illustrates the chemical structures of the triterpenes studied. The chemical nature of the polar group plays a significant role in the adsorption of the molecule: expected, oleanolic acid (3)

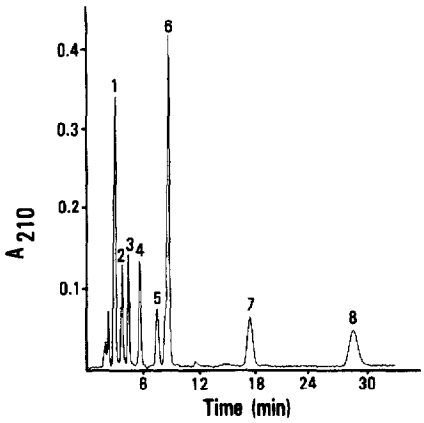


Fig. 1. HPLC separation of oleanane-type triterpene and sitosterol standards on 5 μm silica gel column. Solvent system: hexane isopropanol–methanol (96:3.5:0.5). Flow-rate: 2 ml/min. Sensitivity: 0.4 a.u.f.s. Peaks: 1 = β-amyrin, 2 = sitosterol, 3 = oleanolic acid, 4 = erythrodiol, 5 = gypsogenin, 6 = echinocystic acid, 7 = hederagenin, 8 = queretaroic acid.

TABLE I

RETENTION TIMES FOR OLEANANE-TYPE TRITERPENES AND SITOSTEROL ON NP-HPLC

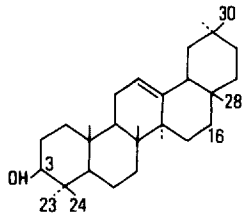
Hexane–isopropanol–methanol (96:3.5:0.5); flow-rate, 2 ml/min; t_R = retention time of compounds, t_R^* = retention time of non-sorbed solvent (= 1.93 min).

Peak number	*Compound*	*Retention times (min)*	
		t_R	$t_R - t_R^*$
1	β-Amyrin	2.90	0.97
2	Sitosterol	3.68	1.75
3	Oleanolic acid	4.39	2.46
4	Erythrodiol	5.61	3.68
5	Gypsogenin	7.50	5.57
6	Echinocystic acid	8.65	6.72
7	Hederagenin	17.45	15.52
8	Queretaroic acid	28.53	26.60

was less strongly adsorbed by the silica gel than the corresponding dihydroxy triterpene, erythrodiol (4), and the C-24 aldehydic compound, gypsogenin (5), had a shorter retention time than the similar C-23 hydroxyl compound, hederagenin (7) although part of the difference can be explained by the steric effect of the C-25 methyl group. The position of polar groups, as well as steric factors, also play an important role. We have examined both parameters within the limitations of the small number of compounds analyzed. Comparison of the retention times of β-amyrin (1) and of sitosterol (2) in Table I suggests that the two methyl groups on C-4 of β-amyrin interfere with the adsorption of the 3β-hydroxyl group (*ca.* 50% decrease in retention

TABLE II

CHEMICAL STRUCTURES OF OLEANANE-TYPE TRITERPENES



Name	*Substituent*				
	16	*23*	*24*	*28*	*30*
β-Amyrin	H	CH_3	CH_3	CH_3	CH_3
Oleanolic acid	H	CH_3	CH_3	COOH	CH_3
Erythrodiol	H	CH_3	CH_3	CH_2OH	CH_3
Gypsogenin	H	CH_3	CHO	COOH	CH_3
Echinocystic acid	OH	CH_3	CH_3	COOH	CH_3
Hederagenin	H	CH_2OH	CH_3	COOH	CH_3
Queretaroic acid	H	CH_3	CH_3	COOH	CH_2OH

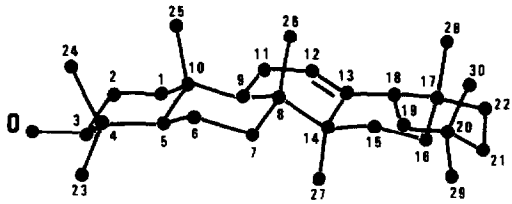


Fig. 2. Molecular structure of oleanane-type triterpenes.

time). The idea that steric factors are involved in this case, rather than polar differences of the molecules, is supported by the finding that lanosterol, a 4,4-dimethyl sterol, co-eluted with β-amyrin in our system. It is also interesting to compare the retention times of echinocystic acid (6), hederagenin (7) and queretaroic acid (8) to their parent molecule, oleanolic acid (3), as well as that of erythrodiol (4) to its parent molecule, β-amyrin (1). The presence of a second hydroxyl group at either end of the parent molecule (3) increased its retention time; for instance, a hydroxyl group at C-30 gave a 10.8-fold increase in retention time (8 *vs.* 3) and at C-23, 6.3-fold (7 *vs.* 3). The effect on retention is, however, considerably smaller when the hydroxyl group is in the middle of the molecule, as at C-16 of echinocystic acid, which shows only a 2.7-fold increase in retention time (6 *vs.* 3), and at C-28 of erythrodiol with only a 3.8-fold increase (4 *vs.* 1).

X-ray crystallographic studies[14–16] indicate a relatively planar conformation of the triterpene ring system with the methyl carbon atoms extending up and down from the plane (Fig. 2). The disposition of these methyl groups seems to interfere with the adsorption of polar groups located at C-16 or C-28. The hydroxyl group at C-30 of queretaroic acid is relatively free of such effects. Thus, it increases the retention time of the parent compound (3) almost 11-fold. The hydroxyl group of hederagenin is similarly situated, but its effect is apparently reduced by the presence of the adjacent 3β-hydroxyl group.

Quantitation of triterpenes in plant extracts

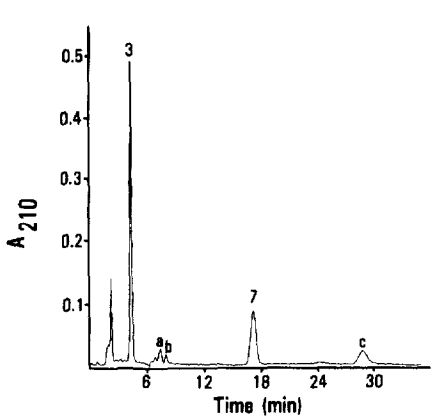
One of the advantages of this NP-HPLC method is the use of a 100% organic solvent system that ensures the solubility of triterpenes necessary for accurate quantitation. RP-HPLC methods have also been developed for analysis of oleanane-type triterpenes[5,6]. However, these compounds tend to precipitate in the aqueous mobile phases often employed in reversed-phase, thus preventing reproducible quantitation.

TABLE III

NP-HPLC QUANTITATION OF TRITERPENES FROM PLANT EXTRACTS OF *C. QUINOA* (VAR. REAL DE PUNO)

Extract	*Oleanolic acid*		*Hederagenin*	
	*μg/g**	*%*	*μg/g**	*%*
Seeds	3000.0	0.30	1433.3	0.14
Callus tissues	478.2	0.04	236.5	0.02

* Values represent the amount of triterpenes (μg) per gram of dry material.



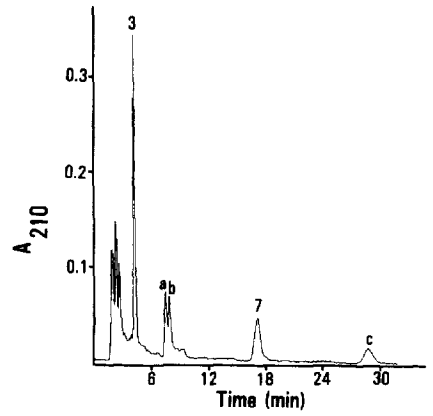


Fig. 3. NP-HPLC analysis of triterpenes extracted from *Chenopodium quinoa* (var. Real de Puno) seeds. Chromatographic conditions as in Fig. 1. Peaks: 3 = oleanolic acid, 7 = hederagenin; a, b and c = unidentified compounds.

Fig. 4. NP-HPLC analysis of triterpenes extracted from root-derived callus tissues of *C. quinoa* (var. Real de Puno). Chromatographic conditions as in Fig. 1. Peaks: 3 = oleanolic acid, 7 = hederagenin; a, b and c = unidentified compounds.

Detection of plant triterpenes was done at 210 nm rather than at their maximum absorbance close to 200 nm because of the limitations imposed by the use of chromatographic solvents with a UV cutoff at 205 nm. At a wavelength of 210 nm and a sensitivity setting of 0.4 a.u.f.s., we detected quantities as low as 400 ng of oleanolic acid and 500 ng of hederagenin. According to Halsall[17], UV absorption of triterpenes is largely due to the position and degree of substitution of the double bond.

Oleanolic acid and hederagenin are two of the most common triterpenes in many plant species[7,8], and their identities in *C. quinoa* were confirmed by mass spectrometry[3]. Quantitatively, our NP-HPLC results reveal that these triterpenes represent the major aglycones of quinoa saponins. The seed extract contained approximately six times more triterpenes than the callus tissue extract; however, the ratio of oleanolic acid to hederagenin was the same in both materials (Table III). The chromatograms of seed and callus extracts (Figs. 3 and 4) showed, in addition to the above aglycones (peaks 3 and 7, respectively), minor components which have not been identified. One of these, peak a, had the same retention time as gypsogenin by HPLC (Table I), but the two could be separated by GLC. Peak c eluted close to queretaroic acid, but these were resolved by HPLC when the standard triterpene was added to the plant extract. It should be noted that neither β-amyrin nor erythrodiol were present in the saponin fraction.

These results demonstrate that NP-HPLC is a powerful procedure for the separation of triterpenes from natural sources. Indeed, compounds can be separated without derivatization and are not subjected to high temperatures as is the case in GLC; therefore, they can be quantitatively recovered in preparative systems for further studies. Another advantage of NP-HPLC is the rapidity when compared to TLC or GLC methods; we have shown that triterpenes of differing polarity can be resolved in 30 min or less under isocratic conditions, which avoids reequilibration of the col-

umn between sample injections. This efficiency is enhanced by automatic sample application that allows analysis of a large number of samples per day. In addition, NP-HPLC presents high reproducibility of both retention time and peak area. TLC and GLC methods show different selectivities and thus can complement HPLC procedures, particularly in the preliminary characterization of triterpenes. The present NP-HPLC method is proving to be very valuable in our current biosynthetic studies on quinoa triterpenes.

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