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## Note

# Extraction and high-performance liquid chromatographic determination of gypsogenin 3,O-glucuronide

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The genus *Gypsophila* (Caryophyllaceae) is well known to contain saponins of industrial interest with various applications, *e.g.*, in the compositions of photosensitive surfaces in photography and of shampoos[1]. More recently, these saponins have been described as apparently being responsible for hypocholesterolemic effects[2]. Gypsogenin is the main pentacyclic triterpenoidal aglycone of these saponins[3]. Substituted on the 3-OH and on the 28-COOH by two chains of different glycosides, these saponins, then called bidesmosidic, are among the most glycosylated. Gypsoside, for example, extracted from *Gypsophila pacifica*, contains nine glycosides[4]. A commercial product well known as Saponin pure white (Merck), used in the past as a standard for haemolytic tests in most saponin determinations, was previously reported to be extracted from roots and rhizomes of *Gypsophila paniculata*[5]. These large saponins easily break during their extraction and purification, giving shorter saponins. Higuchi *et al.*[6] have demonstrated in this commercial product the presence of a gypsogenin 3-O-glycoside, [28-hydroxy-23,28-dioxoolean-12-en-3$\beta$-yl O-$\beta$-D-galactopyranosyl (1→2)-$\alpha$-L-arabinopyranosyl-(1→3)-$\beta$-D-glucopyranosid]uronic acid, and the roots and rhizomes of *G. paniculata* have been reported[7] to contain a main saponin, the gypsoside, the same as described in *G. pacifica*. However, high-performance liquid chromatography (HPLC) of Saponin pure white shows about nine saponins, none of them more abundant than the others (unpublished work; identification of some of them is in progress) and the routine quantitative determination of these saponins is difficult and time consuming. Tagiev and Ismailov[8] described a thin-layer chromatographic method for the determination of a *Gypsophila* saponin in the roots of *G. bicolor* after extraction, purification and measurement of the spot surface revealed by phosphotungstic acid. Although this method is interesting for a rapid evaluation, it seems very imprecise. We report here an efficient method for the determination of *Gypsophila* sp. saponins by the means of their prosapogenin, gypsogenin 3,O-glucuronide (I) (Fig. 1), which is much more stable than gypsogenin. This greater stability may be explained by the reversible formation of a hemiacetal group by the glucuronic acid carboxyl group and the genin CHO group[4]. I is present in most of the saponins isolated and identified from the genus *Gypsophila*[9,10]. We chose to test this method of quantification on Saponin pure white by hydrolysing this mixture of saponins to their prosapogenin, I, purifying it by liquid–liquid extraction with a polar solvent nonmiscible with water, in one step, and measuring it by HPLC.

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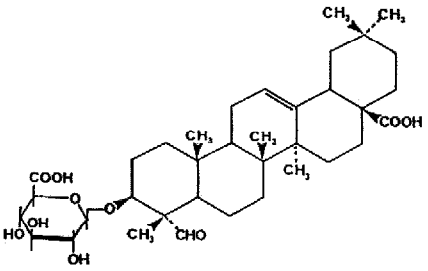

Fig. 1. Structure of gypsogenin 3,O-glucuronide.

## EXPERIMENTAL

Commercial Saponin pure white (No. 7695) was purchased from Merck (Darmstadt, F.R.G.). Sep-Pak $C_{18}$ reversed-phase cartridges were purchased from Millipore–Waters (Milford, MA, U.S.A.).

The standard hydrolysis conditions for quantitative experiments involved boiling with refluxing at 95–100°C of a maximum of 20 mg/ml of the Merck saponin mixture in 0.57 *M* sulphuric acid for at least 1 h. Under these conditions, aglycone (gypsogenin) or by-products (gypsogenin lactone) were not detected.

The extraction conditions were as follows. The carboxylic groups of the glucuronic acid-containing saponins undergo two types of dissociation. Glucuronic acid dissociates near $pK_a$ 3.6 and the carboxylic group of triterpenoidal aglycones dissociates near $pK_a$ 4.9[11]. Hence at pH 1 all acid moieties of I are in the proton-associated form and can be easily extracted with a solvent immiscible with water such as *n*-butanol or ethyl acetate with a very good yield (95%) in one step with 2 ml of solvent and 7 ml of hydrolysing mixture. The second step of the extraction gives only 5% of remaining product. *n*-Butanol and ethyl acetate show a different behaviour in neutral media owing to the difference in their polarities. I precipitates at pH 7 in ethyl acetate whereas it remains soluble in *n*-butanol and therefore it can even be purified in ethyl acetate and water by changing the pH near 7 and liquid–liquid extraction gives a yield of nearly 100% with I in the aqueous phase. The pH must be below 9 in order to avoid any degradation, even below 30°C. Further purification of the aqueous extract is effected with a single and rapid purification step on a Sep-Pak $C_{18}$ reversed-phase cartridge. The aqueous extract, adjusted to pH 2–3 with acetic acid, was passed through the cartridge, which was then rinsed with methanol–water (60:40, v/v) containing 0.1% of acetic acid. I was then eluted with 2 ml of methanol and was then ready for injection into the HPLC system.

The HPLC equipment consisted of a Waters Assoc. Model 510 pump with a Lambda Max 481 spectrophotometer and three stainless-steel columns, the first (250 × 4 mm I.D.) packed with LiChrospher 100 RP-18e (end-capped) (Merck, 5 μm), the second (300 × 3.9 mm I.D.) with LiChrospher RP-8 (Merck, 10 μm) and the third (300 × 3.9 mm I.D.) with μBondapak Phenyl (end-capped) (Waters, 10 μm). The analysis was carried out at 25°C at a flow-rate of 1.0 ml/min. The mobile phase consisted of a mixture of methanol and water in different proportions containing 5

m*M* TBA and the pH was adjusted with 0.5 *M* phosphoric acid. I (2 mg/ml, volume injected 20 $\mu$l) was detected at 206 nm.

I had m.p. 270–272°C; $[\alpha]_D = +44$ ($c$ = 10 mg/ml in methanol); IR (KBr, 1%) 3400 $cm^{-1}$ (OH), 1700 $cm^{-1}$ (CO acid); FAB-MS, with NaI, $m/z$ 669 $[M + Na]^+$, 453 [gypsogenin $- H_2O - H^+]^+$ and with KI, $m/z$ 723 $[M + K]^+$, 453 [gypsogenin $- H_2O - H^+]^+$ [M = $C_{36}H_{53}O_{10}$ (646)]; $^{13}C$ NMR, see Table I (chemical shifts consistent with those of Higuchi *et al.*[6] for gypsogenin methyl ester).

## RESULTS

### *Ion-pair HPLC for the analysis of gypsogenin 3,O-glucuronide*

Few HPLC studies of triterponoidal saponins have been reported. Most have involved ginsenosides[12,13], and diene-transformed saikosaponins[13]. Generally, HPLC is employed to separate and identify mixtures of saponins or to purify them by a preparative method (*e.g.*, ref. 14). Saponins are studied in the underivatized form[15–18] although they have no capacity to absorb UV light (except a few of them, such as glycyrrhizic acid). One of these studies[19] was carried out on I obtained from Sarsynthex (Merignac, France), who purchased it from our laboratory. Hence the purity of this product was confirmed. Saponins may show large differences in polarity. In the olean-12-ene group, a mixture of eight saponins of *Lonicera nigra* L. (Caprifoliceae) was separated on an RP-8 column using gradient elution with acetonitrile–water[17]. Among these saponins, two 3,O-glucuronides of hederagenin and oleanolic acid eluted with relatively sharp peaks, between more polar bidesmosidic saponins (with three or four sugars attached to the 3-OH and the 28-COOH of the aglycones, hederagenin and oleanolic acid) and monodesmosidic saponins (with one or two sugars attached to the 3-OH 3 of the same aglycones). Hence the efficiency of the RP-8 column was good enough to perform the HPLC separation of these glucuronides under the correct conditions. In spite of this, I shows a different behaviour, as previously observed[19] and as we also found; under the above classical conditions, I showed extensive band broadening with loss of product remaining attached to the

TABLE I

$^{13}C$ NMR CHEMICAL SHIFTS ($\delta$ VALUES) OF GYPSOGENIN 3,O-GLUCURONIDE IN $C^2H_3O^2H$

| *Carbon* | $\delta$ *(ppm)* | *Carbon* | $\delta$ *(ppm)* | *Carbon* | $\delta$ *(ppm)* | *Carbon* | $\delta$ *(ppm)* |
|---|---|---|---|---|---|---|---|
| $C_1$ | 39.4 | $C_{11}$ | 24.3 | $C_{21}$ | 35.2 | $C_1'$ | 105.4 |
| $C_2$ | 26.0 | $C_{12}$ | 123.6 | $C_{22}$ | 33.6 | $C_2'$ | 76.7 |
| $C_3$ | 73.3 | $C_{13}$ | 145.5 | $C_{23}$ | 209.3 | $C_3'$ | 83.9 |
| $C_4$ | 56.4 | $C_{14}$ | 43.2 | $C_{24}$ | 10.8 | $C_4'$ | 75.1 |
| $C_5$ | 49.7 | $C_{15}$ | 29.1 | $C_{25}$ | 16.4 | $C_5'$ | 77.6 |
| $C_6$ | 21.6 | $C_{16}$ | 24.3 | $C_{26}$ | 18.0 | $C_6'$ | 172.7 |
| $C_7$ | 34.0 | $C_{17}$ | 48.6 | $C_{27}$ | 26.8 | | |
| $C_8$ | 41.1 | $C_{18}$ | 43.0 | $C_{28}$ | 182.0 | | |
| $C_9$ | 49.1 | $C_{19}$ | 47.9 | $C_{29}$ | 33.9 | | |
| $C_{10}$ | 37.3 | $C_{20}$ | 31.9 | $C_{30}$ | 24.8 | | |

reversed stationary phase in the column. The sole chemical difference between I and the two glucuronides of hederagenin and oleanolic acid is the presence of a CHO group in the 23-position instead of the $CH_2OH$ and $CH_3$ groups in the latter two compounds. Hence a detailed study of the efficiency of HPLC under various conditions of the mobile and stationary phases combined with changes in the polarity of the molecule of I was necessary.

Recently, reversed-phase ion-pair chromatography has been increasingly used for the analysis of ionic compounds[20]. A rapid and precise method for the determination of glycyrrhizin in *Glycyrrhizae radix* utilizing an ion-pair technique was described by Sagara *et al.*[20]. Following their recommendations for glycyrrhizin, also a saponin with glucuronic acid (two glucuronic acid groups by molecule of saponin), we selected tetra-*n*-butylammonium (TBA) as the counter ion. The mixing ratio of methanol to water and the pH of the mobile phase were varied to find the optimum conditions for determination of I with different HPLC columns (Table II). With methanol-water (75:25) containing 5 m*M* TBA and adjusted to pH 5.0 with 0.5 *M* phosphoric acid, I (2 mg/ml) was eluted at an appropriate time (13 min with a capacity factor of 5.6) and with a relatively sharp peak and good effiency (22 400 theoretical

TABLE II

EFFICIENCY OF DIFFERENT COLUMNS IN THE HPLC DETERMINATION OF I (1 mg/ml) WITH DIFFERENT CONDITIONS OF THE MOBILE PHASE

Mobile phase, methanol–water in various proportions containing 5 m*M* TBA, pH adjusted with 0.5 *M* phosphoric acid; flow-rate, 1 ml/min; injection volume, 20 µl; detection, 206 nm. All assays were performed at least in triplicate. *N*/m = theoretical plates per metre; *k'* = capacity factor.

| *Methanol–water* | *pH* | *RP-18 (end-capped)* | | *RP-8* | | *µBondapak Phenyl (end-capped)* | |
|---|---|---|---|---|---|---|---|
| | | *N/m* | *k'* | *N/m* | *k'* | *N/m* | *k'* |
| 78:22 | 7.3–7.6[a] | 5000 | 3.0 | | | | |
| | 6 | 7440 | 3.0 | | | | |
| | 5 | 7200 | 3.9 | | | | |
| 75:25 | 7.3–7.6[a] | 13 600 | 4.0 | | | | |
| | 6 | 15 200 | 5.0 | | | | |
| | 5 | 22 400 | 5.6 | | | | |
| 72:28 | 7.3–7.6[a] | 12 400 | 6.5 | 1280 | 2.3 | | |
| | 6 | 13 200 | 7.7 | 2370 | 3.2 | | |
| | 5 | 20 600 | 10.0 | ND[b] | ND | | |
| 70:30 | 7.3–7.6[a] | | | 1280 | 2.8 | 1220 | 1.3 |
| | 6 | | | 1750 | 3.8 | 1330 | 1.4 |
| | 5 | | | 3236 | 5.5 | 2230 | 2.0 |
| 68:32 | 6 | | | 833 | 4.2 | | |
| 65:35 | 7.3–7.6[a] | | | | | 1530 | 2.8 |
| | | | | | | 1570 | 3.2 |
| | | | | | | 1770 | 3.5 |

[a] pH not adjusted.
[b] ND, not determined.

plates per meter) with an RP-18 end-capped column (5 $\mu$m). The HPLC trace for a typical determination is illustrated in Fig. 2.

Generally, the influence of the alkyl chain on retention and efficiency is secondary to the much greater influence of the mobile phase composition[21]. Although this assertion is in part verified here, the influence of the alkyl chain is greater than for other solutes. Under the optimum conditions of mobile phase composition, RP-8 and Phenyl reversed phases give similar efficiencies as measured by the theoretical plate number. However, the use of RP-18 increases the efficiency by a factor of about 7, especially when the remaining silanol groups are well end-capped. Tests with another $C_{18}$ column, probably with the silanol groups less protected, (results not shown) showed an intermediate efficiency. However, end-capping was not sufficient to give the best efficiency because the $\mu$Bondapak Phenyl column was end-capped. The polarity of the solute is emphasized by the use of TBA with adjustment of the pH with phosphoric acid. With all other conditions identical, the efficiency could be doubled when the pH was adjusted to 5, I giving the pairing reagent. An ion-suppression method has been described[19] for the analysis of I to overcome this difficulty, apparently giving a good efficiency, but no value for the theoretical plate number was given. In that study[19] a $C_{18}$ column was used with a 25-min water–acetonitrile gradient in

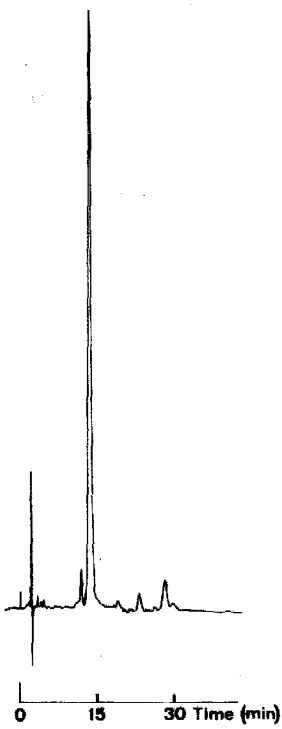


Fig. 2. Reversed-phase HPLC trace for a typical determination of I (2 mg/ml) with UV detection at 206 nm. Sample size, 20 $\mu$l; column, $C_{18}$ (Merck, 5-$\mu$m LiChrospher 100 RP-18e, 250 mm × 4 mm I.D.). Isocratic elution with methanol–water (75:25) containing 5 m*M* TBA, pH adjusted with 0.5 *M* phosphoric acid, at a flow-rate of 1 ml/min.

TABLE III

HPLC DETERMINATIONS OF I IN INCREASING CONCENTRATIONS OF SAPONIN PURE WHITE (MERCK) AFTER HYDROLYSIS AND EXTRACTION AS DESCRIBED UNDER EXPERIMENTAL AND CONTROL OF THE METHOD BY THE YIELD OF I CONTAINED IN EACH CONCENTRATION OF SAPONIN PURE WHITE TESTED

Reproducibility of yields (1.0–15 mg/ml): $X_s$ = 14.3%, S.D. = 2.15%.

| | *Saponin pure white (mg/ml)* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *0.5* | *1.0* | *1.5* | *3.0* | *4.5* | *6.0* | *7.5* | *11.25* | *15.0* | *22.5* | *30.0* |
| I (mg/ml) | 0.04 | 0.14 | 0.27 | 0.37 | 0.55 | 0.89 | 0.90 | 1.88 | 2.10 | 2.02 | 1.74 |
| Yield of I as a % of Saponin pure white | 8.7 | 14.0 | 18.0 | 12.5 | 12.3 | 14.8 | 12.0 | 16.7 | 14.0 | 9.0 | 5.8 |

the presence of trifluoroacetic acid (TFA). As TFA has a low UV absorbance, detection of I could be monitored at 210 nm. This ion-suppression method completes the results presented here with the ion-pair method. Comparison between these two methods is in progress, and the results will be reported in the near future.

Our results clearly show that, in contrast to other glucuronic acid-containing saponins, I requires a very apolar reversed-phase support with good end-capping and with an apolar protection of the molecule by a pairing reagent or transformation by acidification of the molecule to be eluted under HPLC conditions without band broadening and with a symmetrical peak.

*Determination of gypsogenin 3,O-glucuronide in Saponin pure white (Merck)*

The concentration of I in Saponin pure white and the reproducibility of the method were measured using the above HPLC conditions, with increasing concentrations of the analyte from 0.5 to 30 mg/ml submitted to the hydrolysis and extraction conditions described under Experimental. The results, given in Table III, show good correspondence between I concentration and Saponin pure white concentration in the range 1.5–15 mg/ml, with an average of 14.3% of I and a coefficient variation of 2.15%. In comparison, roots and rhizomes of *G. paniculata* contain 0.74 to 3.92% dry weight of I (unpublished results).

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