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Stability of Saponins in Alcoholic Solutions: Ester Formation as Artifacts

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Saponins containing a free carboxylic group in the molecule give the corresponding esters as artifacts when stored for a long time in alcoholic solutions. Two saponins from *Medicago sativa* L., chosen on the basis of their different positions of the carboxylic group in the molecule, were refluxed with methanol and ethanol under neutral conditions. 3,28-di-*O*-glu medicagenic acid possesses a carboxylic group on the triterpenic moiety, whereas soyasaponin I, a glycoside of soyasapogenol B, has a glucuronic acid unit as the first sugar linked to the triterpene structure. Artifacts were quantified by HPLC. The peaks identified as the corresponding esters were examined during boiling from 1 h to 5 days. Quantitative results indicated that the carboxylic group on the sugar moiety, as for soyasaponin I, is more reactive than that on the triterpenic structure, as for 3,28-di-*O*-glu medicagenic acid. Saponins having the free carboxylic groups create enough acidity in their alcoholic solutions to catalyze the formation of the corresponding esters.

KEYWORDS: ***Medicago sativa*** **L.; saponins; artifact formation; identification; HPLC quantification; ester formation**

INTRODUCTION

Saponins are glycosidic compounds present in many edible and inedible plants. Structurally they are composed of a lipid-soluble aglycon, consisting of either a sterol or, more commonly, a triterpenoid, and water-soluble sugar residues. Their biological activity is closely related to the chemical structures, which determine the polarity, hydrophobicity, and acidity of compounds (*1*−*3*), and changes in the chemical structure, for example, different sugar chains or substitution of a functional group, can strongly influence the biological activity of saponins. It has been reported that methylation of the free COOH group of some oleanolic acid glycosides increases both hemolytical and fungicidal activities (*4*).

Saponins are soluble in water/alcohol mixtures that are used for their extraction and purification. Alcohols were also used to store saponins as standard solutions for the successive steps. Not all saponins are stable in alcoholic media, especially compounds possessing a free carboxylic group in the molecule. These findings were evidenced after a long storage of saponin solutions, when artifact formation was revealed. A few years ago, we isolated 24 saponins from alfalfa roots. The methanol solutions of these saponins were chromatographically homogeneous (*5*). After 2 years at room temperature, we found by TLC that some saponin solutions had become nonhomogeneous, giving an additional spot on chromatograms. Two of these saponins (**Figure 1**) were chosen on the basis of their different positions of the carboxylic group in the molecule and treated with methanol and ethanol under neutral conditions: namely, 3,28-di-*O*-glu medicagenic acid (**1**) with the carboxylic group on the triterpenic moiety and soyasaponin I (**4**) with glucuronic acid as the first sugar linked to the triterpene structure. The aims of this study were to ascertain whether these additional spots detected by TLC were artifacts arising during the reaction of saponins with alcohols, to establish their structures, and to elucidate the mechanism of their formation.

MATERIALS AND METHODS

Isolation of Glycosides. Pure saponins were obtained from *Medicago sativa* according to the method of ref *5*. Their structures were confirmed by ^{1}H, ^{13}C NMR, and MS analyses, and data were superimposed to those reported in ref *6*.

Artifact Preparation. 1 and **4** (∼20 mg each) were treated with 50 mL of methanol and ethanol under reflux conditions. After the boiling treatment, the solvent was removed in vacuo and saponins were used for the successive determination without any other treatment. Artifact formation was monitored by TLC and HPLC analyses between 1 h and 5 days of boiling. Treatments were performed in triplicate and solutions used separately for the successive determination. Standard methyl esters **2** and **5** were prepared by treating a methanolic solution of **1** and **4** with diazomethane. Standard ethyl esters **3** and **6** were purified from the reaction mixtures by HPLC.

TLC Analyses. Five microliters of a 0.1% saponin solution was spotted on silica gel 60 precoated Merck plates and developed in ethyl

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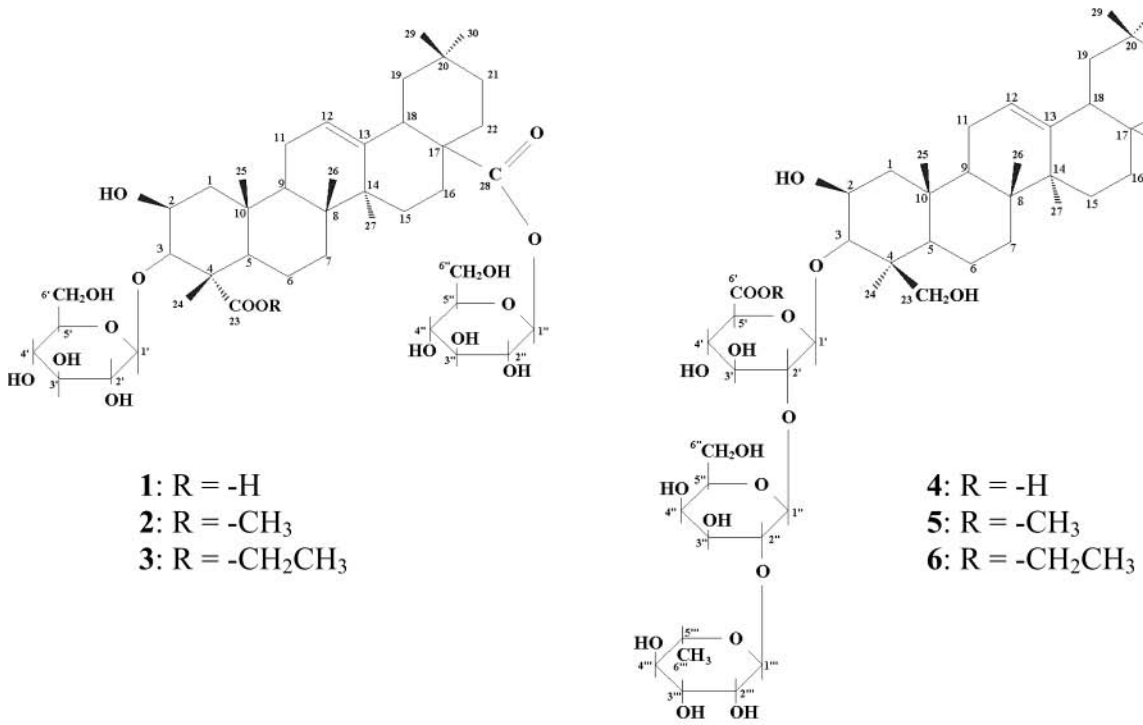

Figure 1. Structures of compounds **1**–**6**.

acetate/acetic acid/water (7:2:2). The chromatograms were spread with methanol/sulfuric acid (5:1) and heated for a few minutes at 120 °C. Saponin **1** (R_f = 0.52) heated with methanol gave an additional spot (R_f = 0.68). Saponin **4** (R_f = 0.39) after heating with methanol gave an additional spot (R_f = 0.50). Treatment with ethanol gave additional spots at R_f = 0.71 for saponin **1** and at R_f = 0.52 for saponin **4**.

HPLC Analyses. HPLC analyses were performed using a Perkin-Elmer chromatograph equipped with an LC250 binary pump and diode array 235 detector using an XTerra column (RP18, 5 μm, 4.6 × 250 mm). Solvent A was CH_3CN/0.05% CH_3COOH, and solvent B was H_2O/0.05% CH_3COOH. Chromatographic runs were carried out under gradient elution from 20 to 100% of solvent A in 30 min. Five microliters of methanolic solutions (1 mg/mL) of pure saponins **1** and **4**, their ester derivatives **2**, **3**, **5**, and **6**, and saponin mixtures after treatment with alcohols was injected. Saponins were eluted at 1.0 mL/min, and detection was by UV monitoring at 215 nm. Quantitative data were calculated as a percentage amount on the bases of the peak area from the chromatograms.

NMR Analyses. All spectra were recorded on a Bruker AV 300 spectrometer at 25 °C observing ^{1}H and ^{13}C at 300.13 and 75.47 MHz, respectively. The NMR samples contained 5−10 mg in 0.5 mL of pyridine-d_5 in 5 mm tubes. ^{1}H and ^{13}C chemical shifts were expressed in parts per million relative to the pyridine signal at 7.2 and 123.5 ppm, respectively. Structures were deduced from ^{1}H, ^{13}C, ^{13}C-DEPT135, H,H-COSY, and H,C-HSQC experiments.

RESULTS AND DISCUSSION

Artifact formation in all of the alcoholic treatments was monitored by TLC and HPLC, and both analyses revealed the presence of an additional compound in each treatment. These additional compounds showed longer retention times when analyzed in HPLC reverse phase conditions, as shown in **Figure 2**, and higher R_f value (see Materials and Methods) in silica gel TLC compared to those of the starting saponins. This behavior indicated a decrease of polarity in these new substances. The HPLC quantification, performed in triplicate between 1 h and 5 days of boiling, showed a linear increase of artifacts up to 30−45 h of boiling for all treatments and then a slow decrease in their percentage amounts (see **Figure 3**). Saponin **4** gave the maximum artifact amount after 35 h of boiling in methanol (artifact quantified as 9.8 ± 0.5%) and after 45 h of boiling in ethanol (artifact quantified as 10.9 ± 0.6%). The observed decrease of artifact amount on increasing treatment time is probably due to decomposition. Saponin **1** showed the same trend as for compound **4**, although the artifact was quantified in lower amount (2.1 ± 0.4% after 30 h in methanol and 2.3 ± 0.2% after 45 h in ethanol).

With regard to the methanol treatment, the NMR results obtained with the saponin mixture after 45 h of boiling allowed the detection of some additional signals in both ^{1}H and ^{13}C spectra well separated from those of the starting saponins, unambiguously attributable to artifacts. From these data, artifacts were identified as methyl ester derivatives **2** and **5** of the two starting saponins. Spectral data of the saponin mixture after boiling treatment allowed the detection of the unchanged triterpenic structure and the same sugar chain configuration as for the untreated compounds. This demonstrates that no hydrolysis or other breakdown reactions took place during the methanol treatment. Only some changes in the spectra were evident in the region between 3.25 and 3.75 ppm in the proton spectra and in the 50−55 ppm and 170−180 ppm regions of the ^{13}C spectra. The differences in the ^{1}H spectra were solely due to the presence of an additional singlet signal at 3.42 and 3.58 ppm for treated saponins **1** and **4**, respectively. This region of the spectra does not contain other signals, and it is diagnostic for the presence of methyl ester groups. The presence of two additional signals in the ^{13}C spectra at 54.68 and 179.88 ppm for the treated saponin **1** and at 51.82 and 170.04 ppm for the treated saponin **4** confirmed the presence of the $-COOCH_3$ group. In the two-dimensional H,C-HSQC experiments, the following new correlations were obtained: 51.82 ppm with 3.42 ppm and 54.68 ppm with 3.58 ppm, in accordance with the presence of a methyl ester group as for saponins **2** and **5**. The methyl ester caused the upfield shift of the C23 carboxylic group of saponin **1** from 180.41 to 179.88 ppm of the corresponding methyl ester (saponin **2**) and from 172.08 ppm of the 6′-

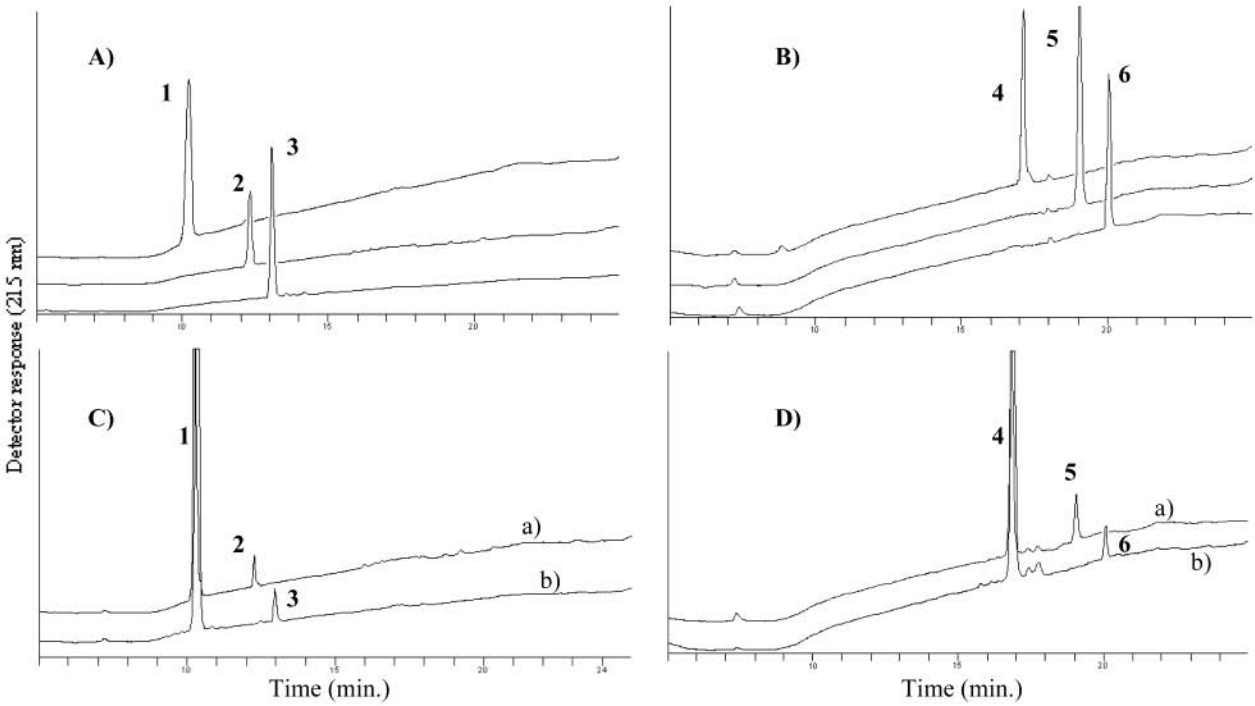

Figure 2. HPLC profile of (A) 3,28-di-*O*-glu medicagenic acid (**1**) and its methyl ester (**2**) and ethyl ester (**3**) standards; (B) soyasaponin I (**4**) and its methyl ester (**5**) and ethyl ester (**6**) standards; (C, D) reaction mixtures of saponins **1** and **4** in (a) methanol and (b) ethanol. For chromatographic conditions see Materials and Methods.

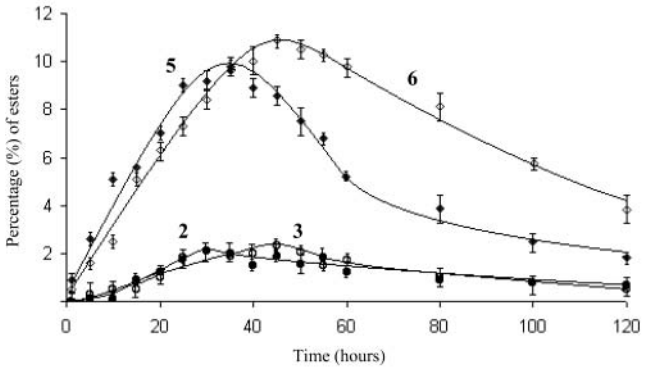

Figure 3. Formation of methyl esters **2** and **5** and ethyl esters **3** and **6** from saponins **1** and **4**, respectively, during the 5-day reaction.

carboxylic group of glucuronic acid of saponin **4** to 170.04 ppm (saponin **5**). Moreover, these signals are superimposed to those of standard methyl esters prepared with diazomethane. These compounds have also the same retention time and the same R_f values as for the previously prepared methyl esters.

The artifacts originated during the ethanol treatment were purified by HPLC. The NMR results clearly indicated these compounds are ethyl esters **3** and **6** of the two starting saponins. As for the methanol treatment, ethanol did not change the triterpenic structure and did not affect the sugar configuration. In the ^{1}H NMR the signals at 4.10 ppm (2H, q, $J = 7.0$ Hz) and 1.15 ppm (3H, t, $J = 7.0$ Hz) for compound **3** and at 4.12 ppm (2H, q, $J = 7.0$ Hz) and 1.16 ppm (3H, t, $J = 7.0$ Hz) for compound **6** indicated the presence of a $-CH_2CH_3$ group. The corresponding signals in the ^{13}C spectra were 14.30 ppm (CH_3) and 60.15 ppm (CH_2). As for the methyl ester, the same upfield shift was observed for the 23-COOH group (from 181.05 to 179.56 ppm) and for the 6′-COOH of glucuronic acid (from 172.67 to 170.11 ppm).

These data show that during the treatment with alcohols an esterification reaction occurs, giving the corresponding esters. The quantitative results indicate that the −COOH group on the sugar moiety, as for saponin **4**, is more reactive than that on the triterpenic structure, as for saponin **1**. Therefore, we can conclude that saponins give enough acidity in their alcoholic solutions to catalyze the formation of ester derivatives.

Artifacts of saponins (*7*) or, more often, their aglycons (*8*−*10*) have been reported. Formation of artifacts usually takes place during hydrolysis of saponins under acidic conditions. We did not find in the literature any report on esterification of saponins by alcohols under neutral pH conditions. The obtained results show that it is not advisable to store saponin mixtures in alcohols at room temperature for a long time. It needs to be noted that methyl esters of saponins obtained by extraction with methanol are not necessarily genuine compounds as they may be artifacts, as reported for alfalfa (*5*) or clover (*11*).

LITERATURE CITED

(1) Fenwick, G. R.; Price, K. R.; Tsukamoto, C.; Okubo, K. Saponins. In *Toxic Substances in Crop Plants*; D'Mello, J. P. F., Duffus, C. M., Duffus, J. H., Eds.; Royal Society of Chemistry, Cambridge, U.K., 1991; pp 285−327.

(2) Cheeke, P. R. Biological effects of feed and forage saponins and their impact on animal nutrition. In *Advances in Experimental Medicine and Biology. Vol. 405. Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 377−385.

(3) Hostettmann, K.; Marston, A. Triterpene saponins−pharmacological and biological properties. In *Chemistry and Pharmacology of Natural Products. Saponins*; Phillipson, J. D., Ayres, D. C., Baxter, H., Eds.; Cambridge University Press: Cambridge, U.K., 1995; pp 232−286.

(4) Tacheki, M.; Tanaka, Y. Structure−activity relationship of synthetic methyl oleanate glycosides. *Phytochemistry* **1992**, *11*, 3789−3791.

(5) Bialy, Z.; Jurzysta, M.; Oleszek, W.; Piacente, S.; Pizza, C. Saponins in alfalfa (*Medicago sativa* L.) root and their structural elucidation. *J. Agric. Food Chem.* **1999**, *47*, 3185−3192.

(6) Oleszek, W.; Price, K. R.; Colquhoun, I. J.; Jurzysta, M.; Ploszynski, M.; Fenwick, G. R. Isolation and identification of alfalfa (*Medicago sativa* L.) root saponins: their activity in relation to a fungal bioassay. *J. Agric. Food Chem.* **1990**, *38*, 1810−1817.

(7) Massiot, G.; Dijoux, M. G.; Lavaud, C. Saponins and artifacts. In *Advances in Experimental Medicine and Biology. Vol. 405. Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 183−192.

(8) Jurzysta, M. Investigations on saponins of native alfalfa (*Medicago media* Pers.) populations (in Polish). Habilitation Thesis, Ser. R (170), IUNG Pulawy 1982; pp 1−64.

(9) Mahato, S. B. Triterpnoid saponins from *Medicago hispida*. *Phytochemistry* **1991**, *30*, 3389−3393.

(10) Price, K. R.; Fenwick, G. R.; Jurzysta, M. Soyasapogenols—Separation, analysis and interconversions. *J. Sci. Food Agric.* **1986**, *37*, 1027−1034.

(11) Sakamoto, S.; Kofuji, S.; Kuroyanagi, M.; Ueno, A.; Sekita, S. Saponions from *Trifolium repens*. *Phytochemistry* **1992**, *31*, 1773−1777.

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