Saponins from Sugar Beet and the Floc Problem

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Extraction of sugar beet (*Beta vulgaris* L.) molasses resulted in the isolation of the known 3-*O*-β-D-glucuronopyranosyl-3β-hydroxy-olean-12-en-28-oic acid (**1**) and a novel compound which was shown from mass and NMR spectroscopy to be 3-*O*-[β-D-glucopyranosyl-(1–2)-(β-D-xylopyranosyl-(1–3))-β-D-glucuronopyranosyl-3β-hydroxyolean-12-en-28-oic acid (**2**). These compounds are not present in the root of the fresh plant but are found in the leaves along with another new saponin: 3-*O*-[β-D-glucopyranosyl-(1–2)-(β-D-xylopyranosyl-(1–3))-β-D-glucuronopyranosyl]-28-*O*-β-D-glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid (**3**).

INTRODUCTION

The presence of saponins in sugar beets (*Beta vulgaris*) has been known for over 100 years [cited by Van der Haar (1927)], when the major saponin was reported to be the glucuronic acid glycoside of oleanolic acid. This compound was isolated from fodder beets and also from the spent cake of the liming process produced during the sugar refining process (Marsh and Levvy, 1956). Later, two more related compounds were reported to be present in beet juice; these were identified as 3-*O*-β-D-glucopyranosyl-3β-hydroxy-olean-12-en-28-oic acid and the methyl ester of oleanolic acid (Bauserman and Hanzas, 1957).

Sugar beet saponins are thought to be the cause of difficulties, and therefore increased cost, in the process of sugar refining which includes various steps to decrease their amount in the final product. However, due to their active surface properties, saponins have been implicated in foaming problems during sugar production and also in the formation of turbidity, called floc, in acidic sugar solutions. Loss of clarity in these solutions is not acceptable for their use in the food and drink industry. Since both physiological and physical properties of saponins (Price et al., 1987) are dependent on their chemical structure, these structures need to be determined before decisions can be made in relation to their reduction or removal from food.

This paper reports the confirmation of the structure of one of the most frequently reported "saponins" in beets, together with that of a previously unreported saponin, a trisaccharide monodesmoside of oleanolic acid. These saponins were not detected in the root of the fresh plant but were isolated from molasses and aerial parts. A novel tetrasaccharide bidesmoside of oleanolic acid was also isolated from the leaves. Work on floc was done in the United Kingdom and work on the fresh plant in France with the structural elucidation done in common.

EXPERIMENTAL PROCEDURES

General Procedures. All solvents were of AnalaR grade and were redistilled before use. Methanolic solutions (5 μL) of the saponins were applied to a drop of glycerol on the copper probe tip in the fast atom bombardment attachment to a Kratos MS9/SOTC mass spectrometer. The sample was bombarded with a fast atom beam of xenon produced by an Ion-tech NF gun operating at 9 kV (nominal). FAB mass spectra were recorded in both negative and positive modes, using a UV galvanometer recorder. Spectra of sapogenins were recorded on a Kratos MS80 instrument, source temperature 250 °C, nominal ionizing voltage 70 eV. Combined GC–MS were recorded on a VS Trio IS operating under similar conditions. ^1H and ^{13}C NMR spectra were recorded in CD_3OD at 300 and 75 MHz, respectively, on a Bruker AC 300 instrument, modified to allow inverse detection. The HOHAHA experiment was done using the decoupling channel to generate the spinlock (MLEV-17; 238 ms) and ROESY experiment in the same mode with a single long pulse (200 ms).

Extraction and Purification of Saponins from Beet Molasses. Beet molasses, 3.5 L of 75 °Brix; (approximately 1.38 sg) was diluted to 5 L with demineralized water and mixed well. The solution was adjusted to pH 1.5 with HCl and then heated to 85 °C for 15 min. After cooling overnight, the mixture was filtered through a large Büchner funnel using muslin cloth coated with a Perlite filter aid as the filtering medium. The filtrate was returned through the filter two more times, and then the filter was washed with warm (60 °C) 1 N HCl; all of the filtrates were discarded. The filter was then washed with warm (60 °C) 2 N NaOH solution until the filtrate was clear. This filtrate was then transferred to a large beaker and HCl added to reduce the pH to 1.5. The precipitate obtained was filtered through a muslin cloth coated with filter aid as before and washed with warm 1 N HCl, and the filtrate was discarded. The filter was then washed with warm 2 N NaOH until the filtrate was clear. Again, this filtrate was acidified to pH 1.5 with HCl and finally filtered through Whatman No. 542 filter paper. The filter was washed with demineralized water and then transferred to a clean dry flask and washed with ca. 0.5 L of warm ethanol, until no further dissolution occurred. The filtrate was then evaporated to dryness leaving a crude floc extract. The floc material (1 g) was dissolved in 20 mL of the lower phase of a mixture of $CHCl_3$–MeOH–H_2O (65:35:10) and applied to a column of silica gel 60 (100 g; 70–230 mesh ASTM, BDH-Merck, Poole, U.K.) preconditioned with the same solvent. Column size was 25-mm diameter and 150-mm length. The column was eluted with this same solvent; the flow rate was 3 mL/min with 700 drops collected per fraction. Fractions were monitored by thin-layer chromatography (TLC) on silica gel 60 plates (BDH-Merck), again using the same solvent as for elution of the column fractions. The plates were visualized by spraying with a solution of *p*-anisaldehyde (0.5 mL) and concentrated sulfuric acid (0.5 mL) in glacial acetic acid (100 mL) and heating to 100 °C until the background color was uniformly pink. Two major components were isolated, namely saponin **1** (79 mg) and saponin **2** (162 mg) with R_f values of 0.57

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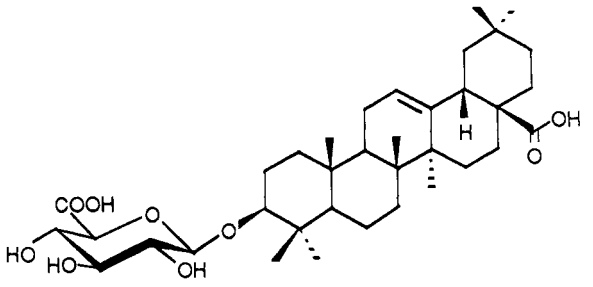

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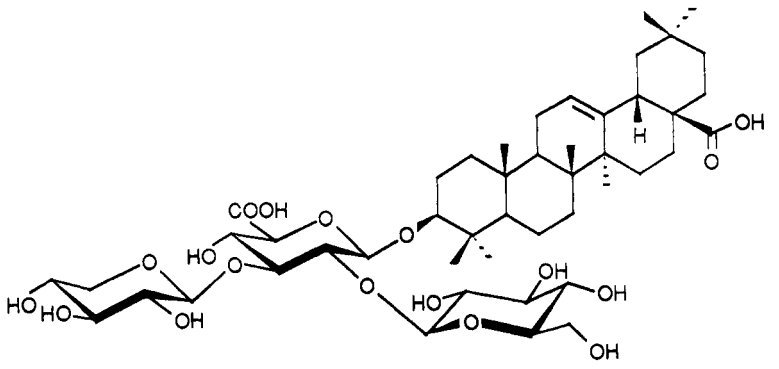

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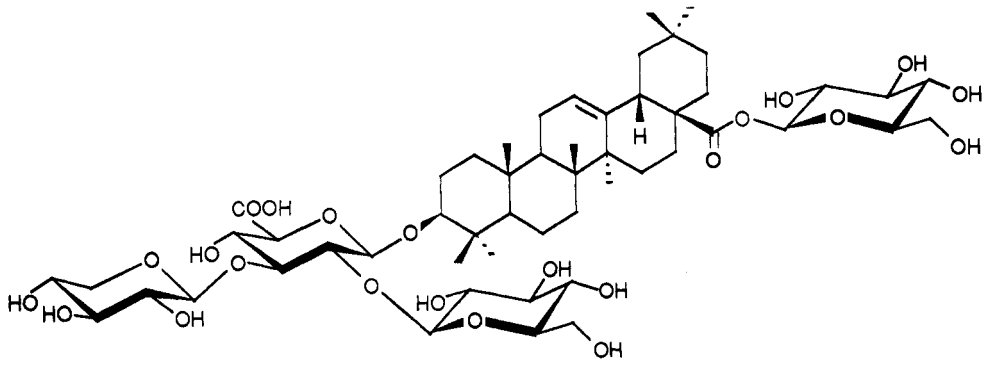

(3)

and 0.39, respectively. Saponin **1** was eluted in fractions 34–40 and saponin **2** in fractions 110–140. Saponin **2** was passed through an ion-exchange resin (Amberlite IRN 77) before its NMR analysis.

Extraction and Purification of Saponins from Beet Leaves. Dried leaves (1.4 kg) were boiled under reflux in MeOH (14 L) for 3 h. After cooling and filtration, MeOH was removed in vacuo. The residue was partitioned between H_2O and petroleum ether. Then, the aqueous layer was extracted with 1-BuOH (3 × 1.5 L). The BuOH layer was evaporated to dryness to provide a brown residue, which was dissolved in H_2O and dialyzed against water. After 72 h, the content of the tube was frozen and lyophilized to give 3 g of a brown powder. TLC of the saponin mixture on a silica gel 60 plate in $CHCl_3$–MeOH–H_2O (60:40:8) showed three spots with R_f values of 0.26, 0.39, and 0.57, corresponding to saponins **3**, **2**, and **1**, respectively. A part of this mixture (900 mg) was subjected to silica gel column chromatography (Merck 70–230 mesh; 40 g) using a gradient of $CHCl_3$–MeOH–H_2O as solvent (70:30:0, 1 L; 60:40:0, 3 L; 60:40:1, 1 L; 50:50:0, 2.5 L). Saponin **3** (50 mg) was eluted in fractions using $CHCl_3$–MeOH–H_2O (60:40:1). Saponins **1** and **2** were present in a mixture with saponin **3** in fractions eluting with $CHCl_3$–MeOH (60:40) and not further purified. Saponin **2** was purified from the saponin mixture (1 g) by exclusion chromatography (Sephadex LH 20; 70 g) using MeOH as solvent. Fractions containing compound **2** were purified by thick-layer chromatography on reversed phase (Whatman $PLKC_{18}F$) in MeOH–aqueous 0.5 M NaCl (70:30) to yield 25 mg of pure saponin **2**.

Extraction of Saponins from Beet Roots. Fresh roots were cut off collars and then sliced. The slices were frozen and lyophilized. After powdering, 1 kg of the white powder was boiled under reflux in MeOH (10 L) for 3 h. After cooling, the mixture was filtered and the filtrate was evaporated to dryness. The residue was dissolved in water (300 mL), and the solution was extracted with 1-BuOH (2 × 300 mL, 1 × 150 mL). The BuOH layer was subjected to the process used for the leaves to yield 1.95 g of saponin mixture. TLC of this mixture on silica gel plate ($CHCl_3$–MeOH–H_2O 60:40:8) showed three spots of weak intensity corresponding to saponins **3**, **2**, and **1** and more polar pink spots (R_f < 0.1).

Hydrolysis of Saponins. Each of saponins **1** and **2** (1 mg) was hydrolyzed with 5% dry hydrochloric acid in methanol (5 mL) for 3 h at reflux. The reaction mix was neutralized with ammonium hydroxide (2 M) and evaporated under reduced pressure to dryness. The hydrolysates were redissolved in water (5 mL), and the sapogenin was extracted into ethyl acetate (3 ×

Table 1. ^{13}C Assignments of 1–3, Recorded in CD_3OD

C	1	2	3	C	1	2	3
1	39.7	37.9	37.9	β-D-glucopyranose			
2	28.7	25	25	1		103.3	103
3	91	92	92	2		76.2	75.2
4	40.1	40.5	40.5	3		78.3	78.2
5	56.7	57.1	57	4		72.7	72
6	19.2	19.4	19.3	5		78.2	78.2
7	33.7	34.1	33.9	6		63.7	63.6
8	40.4	40.7	40.7	β-D-xylopyranose			
9	49	49	49	1		104.9	105.6
10	39.8	39.9	39.9	2		75.3	73.9
11	24.4	24.6	25	3		78.1	78.2
12	123.6	123.7	123.9	4		71.1	72.6
13	145	145.2	144.9	5		67.2	67.1
14	42.8	43	42.9	β-D-glucuronopyranose			
15	28.7	28.9	28.9	1	106.5	105.6	104.8
16	26.8	24.2	27.2	2	75.3	78.8	78.7
17	46.7	47.7	30.7	3	77.7	86.9	86.8
18	42.6	42.8	42.6	4	73.5	72.1	72
19	47.2	47.3	47.2	5	76.6	77.3	78.2
20	31.5	31.5	31.5	6	176.5	172	176.3
21	34.8	34.8	35	β-D-glucopyranose (ester)			
22	33.9	33.9	33.9	1			95.7
23	28.5	28.4	28.3	2			76.1
24	17	16.9	16.9	3			78.2
25	15.9	15.9	16	4			71.1
26	17.7	17.7	17.7	5			78.2
27	26.4	26.4	26.3	6			62.4
28	182.1	182.1	178.1				
29	33.6	33.5	33.5				
30	24	24	23				

5 mL). The combined extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure.

Derivatization of Sapogenins. The sapogenin from each saponin was derivatized in pyridine (200 μL) with BSTFA (200 μL) at 50 °C for 20 min in sealed vials.

Saponin 1: $[\alpha]_D$ +5.13° (MeOH, *c* 0.02); ^1H NMR (300 MHz, CD_3OD) δ 0.78 (d, *J* = 13.5 Hz, H-5), 0.80 (s, Me-26), 0.84 (s, Me-24), 0.87 (s, Me-29), 0.92 (s, Me-30), 0.93 (s, Me-25), 1.04 (s, Me-23), 1.14 (s, Me-27), 1.24 (br d, *J* = 13.5 Hz, H-19), 1.35 (t, *J* = 13.5 Hz, H-6), 1.64 (t, *J* = 13.5 Hz, H-19), 2.83 (dd, *J* = 13.5, 3 Hz), 3.19 (dd, *J* = 12, 4.5 Hz, H-3), 3.26 (t, *J* = 9 Hz, gluA-2), 3.40 (dd, *J* = 9, 8.8 Hz, gluA-3), 3.47 (t, *J* = 8.9 Hz, gluA-4), 3.59 (d, *J* = 9 Hz, gluA-5), 4.36 (d, *J* = 9 Hz, gluA-1), 5.22 (br t, *J* = 4.5 Hz, H-12); ^{13}C NMR, see Table 1.

Saponin 2: $[\alpha]_D$ +15.4° (MeOH, *c* 0.104); ^1H NMR (300 MHz, CD_3OD) δ 0.77 (br d, *J* = 12 Hz, H-5), 0.81 (s, Me-26), 0.86 (s, Me-24), 0.90 (s, Me-29), 0.93 (s, Me-30), 0.94 (s, Me-25), 1.06 (s, Me-23), 1.12 (br d, *J* = 13.5 Hz, H-19), 1.15 (s, Me-27), 1.38 (td, *J* = 13.5, 3 Hz, H-21), 1.68 (t, *J* = 13.5 Hz, H-19), 1.77 (br dd, *J* = 15, 5 Hz, H-15), 2.00 (td, *J* = 13.5, 4 Hz, H-16), 2.85 (dd, *J* = 13.5, 4 Hz, H-18), 3.13 (t, *J* = 9 Hz, glc-4), 3.16 (t, *J* = 8 Hz, glc-2), 3.20 (m, H-3), and 3.24 (br t, *J* = 11 Hz, xyl-5), 3.26 (t, *J* = 7.5 Hz, xyl-2), 3.30 (t, *J* = 8 Hz, xyl-3), 3.31 (m, glc-5), 3.35 (t, *J* = 9 Hz, glc-3), 3.51 (m, xyl-4), 3.58 (dd, *J* = 12, 6.5 Hz, glc-6), 3.59 (br t, *J* = 9 Hz, gluA-4), 3.66 (d, *J* = 9.5 Hz, gluA-5), 3.73 (t, *J* = 9 Hz, gluA-3), 3.79 (br t, *J* = 8 Hz, gluA-2), 3.82 (dd, *J* = 12, 2.5 Hz, glc-6), 3.94 (dd, *J* = 11, 5.5 Hz, xyl-5), 4.48 (d, *J* = 7.5 Hz, gluA-1), 4.63 (d, *J* = 7.3 Hz, xyl-1), 4.94 (d, *J* = 8 Hz, glc-1), 5.22 (t, *J* = 5 Hz, H-12); ^{13}C NMR, see Table 1.

Saponin 3: $[\alpha]_D$ +4.7° (MeOH, *c* 0.085); ^1H NMR (300 MHz, CD_3OD) δ 0.79 (s, Me-26), 0.85 (s, Me-24), 0.9 (s, Me-29), 0.92 (s, Me-30), 0.93 (s, Me-25), 1.05 (s, Me-23), 1.14 (s, Me-27), 2.85 (dd, *J* = 10, 6 Hz, H-18), 3.08 (t, *J* = 9.3 Hz, glc-4), 3.15 (dd, *J* = 9.5, 7.8 Hz, glc-2), 3.17 (dd, *J* = 10 , 6 Hz, H-3), 3.23 (dd, *J* = 11, 5.5 Hz, xyl-5), 3.25 (t, *J* = 7.4 Hz, xyl-2), 3.28 (dd, *J* = 10, 7.4 Hz, glc-5), 3.31 (t, *J* = 8.5 Hz, *glc*-2), 3.32 (dd, *J* = 8.5, 7.4 Hz, xyl-3), 3.35 (t, *J* = 9.4 Hz glc-3), 3.35 (m, 2H, *glc*-3 and *glc*-4), 3.37 (m, 1H, *glc*-5), 3.52 (td, *J* = 8.5, 5.7 Hz, xyl-4), 3.55 (dd, *J* = 12.2, 7 Hz, glc-6), 3.59 (m, 2H, gluA-4 and gluA-5), 3.66 (dd, *J* = 12.5, 5 Hz, *glc*-6), 3.75 (t, *J* = 8 Hz, gluA-3), 3.79 (t, *J* = 8 Hz, gluA-2), 3.81 (dd, *J* = 12.2, 2 Hz, glc-6 and *glc*-6), 3.91 (dd, *J* = 11, 8.5 Hz, xyl-5), 4.46 (d, *J* = 7.3 Hz, gluA-1), 4.63 (d, *J* = 7.4 Hz, xyl-1), 4.95 (d, *J* = 7.8 Hz, glc-1), 5.24 (t, *J* = 3 Hz, H-12), 5.37 (d, *J* = 8 Hz, *glc*-1); ^{13}C NMR, see Table 1.

RESULTS AND DISCUSSION

The three isolated saponins 1–3 were identified from a combination of mass and NMR spectrometry. FAB mass spectra were used to determine the molecular weight and the type of sugar chain present. EI mass spectra of the acid-released aglycon together with GC–mass spectra of the derivatized material enabled its identity to be determined as oleanolic acid. This was confirmed by direct comparison with an authentic sample (Extrasynthèse).

The FAB mass spectra for compound 1, negative mode (relative abundance in parentheses, u = uronic acid, p = pentose, h = hexose and w = water) gave ions of mass *m/z* 631 (100) M − H^-, 455 (12) M − H − u^-, and in the positive mode *m/z* 439 (100) M + H − u − w^+. For compound 2, negative mode gave ions of mass *m/z* 925 (100) M − H^-, 793 (27) M − H − p^-, 763 (18) M − H − h^-, 631 (12) M − H − h − p^-, 455 (18) M − H − h − p − u^- and positive mode *m/z* 949 (5) M + Na^+, 927 (6) M + H^+, 795 (5) M + H − p^+, 765 (4) M + H − h^+, 439 (100) M + H − p − h − u − w^+. For saponin 3 positive mode gave ions of mass *m/z* 1127 (4) M + K^+, 1111 (2) M + Na^+, 979 (1) M + Na − p^+, 965 (2) M + K − h^+, 833 (1) M + K − h − p^+, 817 (1) M + Na − p − h^+, 787 (1) M + Na − $2h^+$, 657 (1) M + K − h − p − u^+, 655 (1) M + Na − p − $2h^+$, 641 (1) M + Na − p − h − u^+, 439 (10) M + H − 2h − p − u − w^+.

The *m/z* fragments 455 in the negative mode and 439 in the positive mode were indicative of the presence of oleanolic acid in saponins. Saponin 1 contained a single uronic acid, while saponin 2 contained three sugars comprising uronic acid with both a pentose and a hexose indicated as being in terminal positions. Saponin 3 contained also a single uronic acid but two hexoses and a pentose in a terminal position.

EI mass spectra of the aglycon, oleanolic acid, gave ions at *m/z* 456 $M^{\bullet+}$ and 248 (retro-Diels–Alder fragmentation). GC–mass spectra of the derivatized sapogenin gave ions at *m/z* 600 $M^{\bullet+}$, 585 M − 15, 482 M − 118, 320 RDA, and 203 RDA − 117.

Confirmation of oleanolic acid as genin in these three saponins was achieved by the analysis of their HMQC and HMBC spectra and allowed assignment of the ^{13}C NMR spectra according to a previous publication (Tori et al., 1974).

The presence of a hexose in saponin 1 was confirmed by observation of an anomeric carbon at δ 106.5 bonded in the HMQC experiment to an anomeric proton at δ 4.36 (d, *J* = 9 Hz). The spin system to which this proton was attached was analyzed by means of a COSY H–H experiment and was assigned to a β-D-glucuronic acid. The large coupling constants corresponded to vicinal axial protons. A ROESY experiment showed a cross-peak between H-3 of the genin and H-1 of glucuronic acid in saponin 1, which therefore corresponds to the known 3-*O*-β-D-glucuronopyranosyloleanolic acid (Nie et al., 1984).

The ^{13}C NMR spectrum showed that saponin 2 contained three sugar residues whose anomeric carbons resonated at δ 103.3, 104.9, and 105.6. The HMQC experiment allowed location of the bonded anomeric protons at δ 4.94 (d, *J* = 7.8 Hz), 4.61 (d, *J* = 7.3 Hz), and 4.54 (d, *J* = 7.3 Hz). Identification of the sugars from these protons by means of a COSY spectrum was hampered by severe overlap in the δ 3.8–3 area. This analysis was guided by a HOHAHA experiment which allowed the link between the anomeric doublets to the seven-, six-, and five-spin systems of D-glucose, D-xylose, and D-glucuronic acid, respectively. The measurement of large coupling constants between H-1, H-2, H-3, H-4, and H-5 in each sugar confirmed their nature and β-D configuration.

Analysis of HMQC and HMBC spectra allowed the assignment of C-2 and C-3 of glucuronic acid at δ 78.8 and 86.9, suggesting that these positions were substituted by the others sugars. ROEs were observed between glucuronic acid H-1 and H-3 of oleanolic acid, between glucose H-1 and glucuronic acid H-2, and between xylose H-1 and glucuronic acid H-3. The sequencing was confirmed by the HMBC experiment which showed heteronuclear correlations between the anomeric protons and deshielded carbons C-3 of genin at δ 92, C-2, and C-3 of glucuronic acid. Thus, saponin **2** was identified as 3-*O*-[β-D-glucopyranosyl(1–2)-(β-D-xylopyranosyl(1–3))-β-D-glucuronopyranosyl]oleanolic acid. To the best of our knowledge, this carbohydrate moiety has not been described before.

The ^{1}H and ^{13}C NMR spectra of saponin **3** were not much different from those of compound **2**. Major differences were supplementary signals for a fourth sugar unit, a hexose in **3**. The deshielding of the corresponding anomeric proton at δ 5.37 (d, J = 8 Hz) linked to a shielded anomeric carbon at δ 95.7 in the HMQC spectrum suggested that the sugar esterified an acid function. The presence of the same disubstituted glucuronic acid side chain of saponin **2** in compound **3** was confirmed by the analysis of COSY, HOHAHA, ROESY, HMQC, and HMBC set of experiments. The fourth sugar was identified with a terminal β-D-glucose attached to either glucuronic acid C-6 or to oleanolic acid C-28. The observation of cross-peaks in the HMBC spectrum between H-5 and C-6 of glucuronic acid allowed assignment of the latter with the δ 176.3 carbonyl, thus leaving the δ 178.1 signal to C-28 of the aglycon. A correlation between H-1 of the glucose ester and this signal permitted the structure of saponin **3** to be identified as 3-*O*-[β-D-glucopyranosyl(1–2)-(β-D-xylopyranosyl(1–3))-β-D-glucuronopyranosyl]28-*O*-β-D-glucopyranosyloleanolic acid.

CONCLUSION

Studies of saponins in a wide range of foods such as legumes, medicinal plants, and root crops have demonstrated a large complexity both in chemical structures and biological activities. In the case of sugar beet the existence of yet two other novel structures has been shown. An initial investigation into the chemistry of fresh root saponins showed the presence of saponins **1** and **2** in minute quantities. The fundamental structures of these compounds are part of the more complex and labile structures of the major saponins from the roots of the beet plant (M. G. Dijoux et al., unpublished work). Work is in progress toward their elucidation, although they are not of direct relevance to the floc phenomenon because of their instability during chemical isolation and especially in response to the harsh conditions encountered in the sugar refining process. In the same way, saponin **1** was not detected in the fresh-leaf saponins. This compound can be considered as one product of a partial hydrolysis of the other saponins **2** and **3** or from these similar more complex structures also present in the extract of fresh material.

The problems caused by the presence of bioactive saponins in food and the turbidity cannot be solved until the chemical nature of the saponins is understood and sufficiently well-defined material is available for further study into these problems. The method described here for the isolation of the two main saponins present in sugar beet molasses and the structure elucidation of these is the first step toward understanding how to overcome these types of problems in the food industry.

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