Thin Layer Chromatography of Sugar Beet Saponins

A. J. VAN DUUREN[1]

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Introduction

Van der Haar (3,4)[2] first recognized oleanolic acid and glucuronic acid as components of sugar beet saponin. He isolated three saponins with different solubility properties. In as much as saponins easily form emulsions and addition compounds with many substances (fats, fatty acids, phosphatids, e.g.) they are very difficult to purify and have not yet been obtained in crystallized form. The sapogenins on the other hand crystallize readily and can be obtained in a relatively pure state. It is, however, very difficult to determine the purity of a sapogenin preparation as the molecular weight is high (oleanolic acid = 456), the melting-point, accordingly, is very high and there are several substances in the same group with only slight differences in composition and structure.

Paper chromatography seemed to offer a good way to analyze these substances. Walker and Owens (9) investigated floc components of beet sugar by this method and by paper electrophoresis. They did not get a satisfactory separation; with several solvents the spots either failed to move or moved with the solvent front. Finally a solvent was found that separated oleanolic acid and saponin with R_F values of 0.2 and 0.8 respectively. The resolution of the spots, however, depended on the concentration of the mixture. Paper electrophoresis was not satisfactory either, because of poor resolution of different sapogenins. There was an indication that saponin from floc consisted of two substances.

Bauserman and Hanzas (1) found that on paperchromatograms, purified beet saponin behaved differently from saponins in beet juice. They ascribed the differences in behavior to saponins in the beet juice being in the salt form; with purified saponin salts they too obtained as R_F values either 0.0 or 1.0 and in some cases variable R_F values; R_F of oleanolic acid was 1.0 or 0.0. Mg, Ba and Ca were found associated with the saponin spots. With water as solvent, two spots were found for saponin.

Thin layer chromatography was first developed by Kirchner, Miller and Keller (5); they used glass strips, covered with a thin layer of absorbent (chromatostrips). Silica gel appeared to be the best absorbent for terpenes. Other workers used glass plates (chromatoplates) covered with absorbent (6,7,8,10).

[1] Head Research Chemist, Instituut voor Rationele Suikerproductie, Bergen op Zoom, Netherlands.

[2] Numbers in parentheses refer to literature cited.

This new method, in contrast to paper partition chromatography, is essentially absorption chromatography, and as such is suitable for substances insoluble in water. Therefore, it seemed a good means of separating sapogenins and possible also saponins.

Methods

The chromatograms were made on glass plates (15x15 cm) covered with a thin layer (~ 0.3 mm) of silicagel (Kieselsaüregel G, Merck, a special preparation of silicagel, mixed with a small amount of gypsum). The plates were dried in the air at room temperature. The spots were placed 2 cm from one side. The plates stood vertically in glass tanks (18x18x12.5 cm), the bottom of which was covered with 100 ml solvent. The time required for development is very short, about 20 to 40 minutes.

A saturated solution of antimony trichloride in chloroform was used as a spray reagent. After heating for 15 to 30 minutes

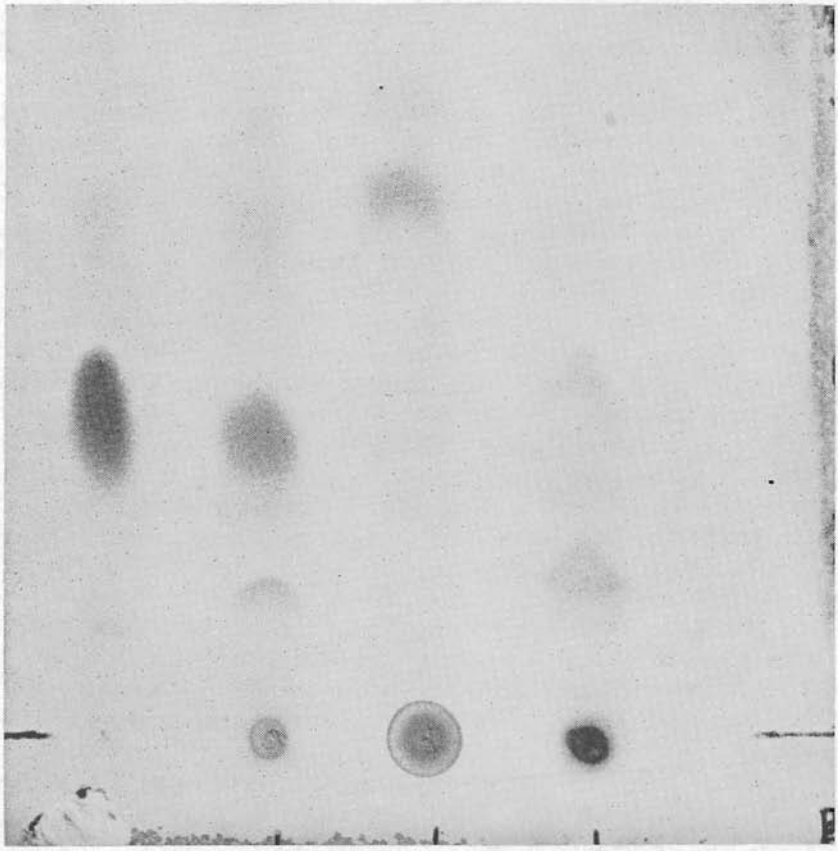

Figure 1.—1 = beet sapogenin b, 2 = beet sapogenin a, 3 = quillaic acid, 4 = glycyrrhetic acid. Solvent: (benzene 90 ml, ethanol 96% 10 ml).

at 100°C, blue-violet and sometimes yellow spots are visible. The blue-violet spots have a light orange fluorescence in ultraviolet light.

The procedure used for the isolation of saponin from sugar beet was as follows:

Acidify raw juice to pH 1 and heat at 90°C for 1 hour, let cool overnight and collect the precipitate by decantation and centrifugation and wash with slightly acidified water. Extract the wet precipitate with 96% ethanol. Evaporate the ethanol carefully in a vacuum desiccator at room temperature.

The saponin preparation can be separated in two fractions as follows: to obtain fraction a, extract the dry matter with warm acetone and evaporate the acetone at room temperature in a vacuum. The residue, fraction b, is soluble in dilute ammonia and may be precipitated by acidification.

The preparation of sapogenin was the following: hydrolyze by boiling the saponin for 6 to 7 hours in a solution, containing, 45 to 50% ethanol and 5% HCl. After cooling, dilute with water and collect the precipitate. For comparison with beet sugar saponins we isolated some closely related saponins from the same chemical group (β- amyrin group, Figure 2).

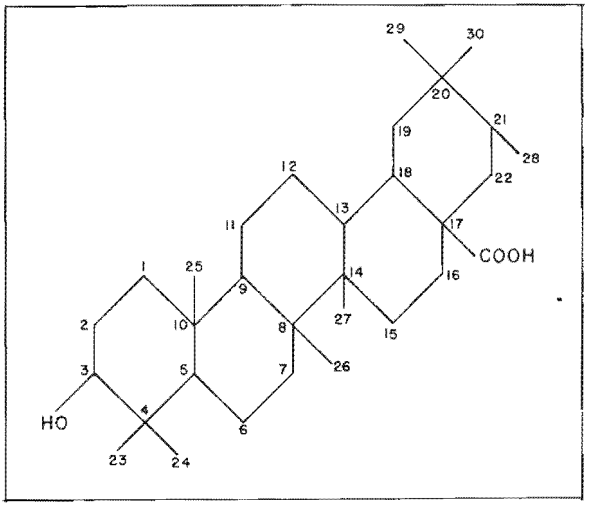

Figure 2.—Sapogenin formula.

β- amyrin group

oleanolic acid	: 23,24,25,26,27,29 and 30 CH_3 - groups; 28 = - OH group
hederagenin	: 28 = OH - group 24,25,26,27,29 CH_3 - groups; 30 : - CH_2OH group
quillaic acid	: 23 = CHO - group 24,25,26,27,29 and 30 CH_3 - groups to C- atom 16 is an OH group attached
Glycyrrhetic acid	: To C 11 is an = O group attached; between C 12 and C 13 is double binding; 24,25,26,27,29 and 30 : CH_3- groups.

The saponin from soapbark *(Quillaja saponaria)* was isolated in the same manner. Glycyrrhizic acid was extracted from licorice *(Radix liquiritiae)* with ethanol and precipitated with ether. Hederagenin was also extracted with ethanol, from soapnuts (*Sapindus rarak* from Indonesia).

The unpurified preparations were used for chromatography.

Discussion and Results

After some experimentation we found the following solvents suitable for the separation of beet and closely related sapogenins:

benzene- ethanol 90:10 (see Table 1 and Figure 2)

A mixture of hexane-ethylacetate was also found to be suitable for the separation of the sapogenins (see Table 2).

With the benzene-ethanol solvent the saponins remained practically at the starting point, while with the hexane-ethylacetate solvent a good separation was obtained (see Table 3).

A fairly good separation of saponins was also obtained, using one of the solvents in use in paper chromatography (butanol-acetic acid- water 4:1:1, see Table 4).

It appears that the major components of beet sapogenin fractions a and b are probably the same; they have about the same R_F value in two solvents. The major components of the other sapogenins are distinctly different from the major component of beet sapogenin.

It is of course usual in chromatographic research to compare the unknowns with the pure substances that are supposed to be in it. However, in this case we did not succeed in obtaining the pure substances for comparison of the components of the sapogenins tested. The sapogenins of the β-amyrin group (see Figure 2), oleanolic acid, hederagenin, quillaic acid and glycyrrhetic acids, are known to be the main components of the saponins of respectively sugar beet (3,4), sapindus varieties (2), soap bark and licorice.

From the chromatograms of the sapogenins it is clearly seen which are the major components. So it is highly probable that these substances are indeed the above mentioned sapogenins. This has to be confirmed of course by experiments with the pure substances.

Figure 2 gives the probable structual formula of the four sapogenins. The differences are relatively small. The difference is slightest between oleanolic acid and hederagenin, as is also the case on the chromatograms.

Table 1.—Chromatographic behavior of sugar beet sapogenins and sapogenins from other sources. Solvent: benzene-ethanol 90:10.

Beet sapogenin fraction a			Beet sapogenin fraction b			Sapogenin from *Sapindus rarak*			Quillaic acid sapogenin			Glycyrrhetic acid sapogenin		
$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color
21.5	w	g				19	w	g	9	vw	bg	17	+	y
26	+	rv				37	+++	bp	18	vw	bg	21	++	y
47	+++	bp	48	+++	bp	61	+	b	27	vw	bg	44	+	b
64	w	b	64	+	b									
73.5	w	b	75	+	b	71	w	p	65	+	bg	65	vw	b
92	+	p	91.5	+	p	92	w	bp	85	w	bg	74	vw	b

Legend for table 1,2,3 and 4: b = blue, p = purple, g = green, y = yellow, r = red, w = weak, vw = very weak, + = moderate, ++ = strong, +++ = very strong.

Table 2.—Chromatographic behavior of sugar beet sapogenins and sapogenins from other sources. Solvent: hexane-ethylacetate 50:50.

Beet sapogenin fraction a			Beet sapogenin fraction b			Sapogenin from *Sapindus rarak*			Quillaic acid sapogenin		
$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color
15	vw	p	18	+	b	8	+	bg	24	vw	b
22	+	b							28	vw	b
34	+++	b	32	++	b	32	+++	b	35	vw	b
60	+	b	60	+	b						
68	vw	bp	68	+	bp	68	+	b			
75	+	b	76	+	b	77	+	bp	73	+++	b
91	++	p	89	+	p	92	+	b	82	+	b

Table 3.—Chromatographic behavior of sugar beet saponin and saponin from *Sapindus rarak*. Solvent: hexane-ethylacetate 50:50.

Beet saponin fraction a			Beet saponin fraction b			Saponin from *Sapindus rarak*		
$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color
22	w	b	18	vw	b	0	+++	bp
35	w	b	34	vw	b			
64	++	b	63	–	b			
81	w	b						
91	+++	b	89	–	b			

Table 4.—Chromatographic behavior of sugar beet saponin and saponin from *Sapindus rarak*. Solvent: butanol-acetic acid-water 4:1:1.

Beet saponin fraction a			Beet saponin fraction b			Saponin from *Sapindus rarak*		
$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color	$R_F \times 100$	Intensity of spot	Color
61.5	w	p	61.5	w	p	28.5	+++	p
75	++	b	75[1]	+	b			
78.5	++	b	79[1]	++	b			
81	+	g						
90.5	++	p	93	w	p			

[1] The spots R_F 75 and 79 were horizontally elongated and very clearly separated.

It appears from the chromatograms that there are probably at least six different sapogenins in sugar beet saponin, probably also closely related substances. Possibly quillaic acid is one of them.

There seems to be little difference between fraction a and b sapogenin, only in relative amounts.

It may be thought that the six sapogenin spots represent partial hydrolysis products of the sugar beet saponin.

As however oleanolic acid and related substances only contain one hydroxyl group to attach a carbohydrate moiety, it is extremely unlikely that one molecule of oleanolic acid binds more than one carbohydrate molecule. Van der Haar (3) indeed found from molecular weight determinations one molecule glucuronic acid in one molecule of beet saponine. So the presence of partial hydrolysis-products is unlikely.

The saponins, fraction a and b, too are much the same, except in relative amounts. The difference in solubility, therefore, has to be ascribed to the presence of complexes or absorption compounds.

It is remarkable that *Sapindus* saponin gives only one spot, but contains as many sapogenins as the beet saponin. Probably the carbohydrate moiety determines this property to a high degree.

It is possible that in beet saponins other carbohydrates occur apart from glucuronic acid. This has not yet been investigated by modern methods as far as we know. Even the presence of glucuronic acid is not without doubt, as this has been confirmed only by color reactions.

If other carbohydrates also occur in beet saponins the number of possible compounds is high.

Summary

With thin-layer chromatography it is possible to separate closely related sapogenins and saponins. From chromatographic evidence it seems likely that beet saponin contains at least six different sapogenins, four of which are as yet unknown. Fractions of saponins obtained by solubility differences contain the same sapogenins.

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