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Review

Chromatographic determination of plant saponins

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Abstract

The methods used for saponin determination in plant materials are presented. It is emphasised that the biological and spectrophotomeric methods still being used for saponin determination provide, to some extent, valuable results on saponin concentrations in plant material. However, since they are sensitive to the structural variation of individual saponins they should be standardized with saponin mixtures isolated from the plant species in which the concentration is measured. However, one plant species may contain some saponins which can be determined with a biological test and others which cannot. That is why biological and colorimetric determinations do not provide accurate data and have to be recognized as approximate. Thin-layer chromatography on normal and reversed-phases (TLC, HPTLC, 2D-TLC) provides excellent qualitative information and in combination with on-line coupling of a computer with dual-wavelength flying-spot scanner and two-dimensional analytical software can be used for routine determination of saponins in plant material. The densitometry of saponins has been very sensitive, however, to plate quality, spraying technique and the heating time and therefore appropriate saponin standards have to be run in parallel with the sample. Gas–liquid chromatography has limited application for determination since saponins are quite big molecules and are not volatile compounds. Thus, there are only few applications of GC for determination of intact saponins. The method has been used for determination of TMS, acetyl or methyl derivatives of an aglycones released during saponin hydrolysis. However, structurally different saponins show different rates of hydrolysis and precise optimisation of hydrolysis conditions is essential. Besides, during hydrolysis a number of artefacts can be formed which can influence the final results. High performance liquid chromatography on reversed-phase columns remains the best technique for saponin determination and is the most-widely used method for this group of compounds. However, the lack of chromophores allowing detection in UV, limits the choice of gradient and detection method. The pre-column derivatisation with benzoyl chloride, coumarin or 4-bromophenacyl bromide has been used successfully in some cases allowing UV detection of separation. Standardisation and identification of the peaks in HPLC chromatograms has been based on comparison of the retention times with those observed for authentic standards. But new hyphenated techniques, combining HPLC with mass spectrometry and nuclear magnetic resonance are developing rapidly and allow on-line identification of separated saponins. Capillary electrophoresis has been applied for saponin determination only in a limited number of cases and this method is still being developed.

Keywords: Reviews; Saponins; Triterpene glycosides; Steroidal glycosides

Contents

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1. Introduction

Saponins are widely distributed in plant species, being reported in nearly 100 families. They are naturally occurring glycosides which are found mainly, but not exclusively (lower marine animals) in the plant kingdom. They consist of non-sugar aglycone coupled to sugar chain units. These sugars can be attached as one, two or three sugar chains and the terms monodesmoside, bidesmoside and tridesmoside has been given to these saponins, respectively (Greek *desmos*=chain) [1]. According to the nature of the aglycone they can be classified into steroidal or triterpene groups. Some authors also include within the saponins steroidal glycoalkaloids of solanidans and spirosolan classes. All classes of aglycones may have a number of functional groups (–OH, –COOH, $–CH_3$) causing big natural diversity only because of aglycone structure. Over 100 steroidal and probably even larger numbers of triterpene sapogenins have been identified [2]. This diversity can be further multiplied by the composition of sugar chains, sugar numbers, branching patterns and type of substitution. It is well recognised that even one plant species may possess a number of individual saponins, e.g., alfalfa roots contain at least 25 medicagenic acid, hederagenin, zanhic acid, soyasapogenol and bayogenin glycosides, with the attached number of sugars ranging from one to seven [3]. These structures and their amounts may differ depending on the plant part studied. This structural diversity and resulting wide range of polarities makes determination of individual saponins very difficult.

The early determination of saponins in plant material was based predominantly on gravimetry [4] or on methods taking advantage of some of their chemical or biological features. The foaming property, which is a well-known feature of most saponins, was used to search for plant saponin content. Froth formation after shaking in water solution is specific to most saponins, but some of them, especially those with two or three branched sugar chains do not form stable froth, and conversely some plant extracts not containing saponins may produce froth, providing misleading information.

Some saponins show haemolytic activity which has been used for the development of semiquantitative tests for their determination. In the simplest haemolytic method, saponin-containing material or its water extract is mixed with blood or with washed erythrocytes in isotonic-buffered solution (0.9% NaCl). After 20–24 h samples are centrifuged and haemolysis is indicated by the presence of haemoglobin in the supernatant. For the evaluation of haemolytic activity, European Pharmacopoeia uses as a unit the haemolytic index (HI), which is defined as the number of millilitres of ox blood (2%, v/v) that can be haemolysed by 1 g of crude saponins or plant material. The saponin mixture of *Gypsophila paniculata* L. (HI=30 000) or Saponin white (Merck, HI=15 000) are usually used as reference. Haemolytic indices of saponins are calculated according to the following equation: $HI=HI_{std}\times a/b$; where HI_{std} is the haemolytic index of standard saponin, and a and b are the lowest concentrations of test and standard saponin, respectively, at which full haemolysis occurred. Mackie et al. [5] measured the absorbency at 545 nm of the supernatant after haemolysis and defined one unit of activity as the quantity of haemolytic material that caused 50% haemolysis.

Another modification of the haemolytic method is the haemolytic micromethod [6] in which cow's

blood stabilised with sodium citrate (3.65%, w/v) is mixed with gelatine solution. For this, gelatine (4.5 g) is dissolved in 100 ml of isotonic-buffered solution and 75 ml of this are mixed with 20 ml of blood. The gelatine–blood mixture is spread on a glass plate (10×20 cm) to a thickness of 0.5 mm and, after coagulation, the plates are used for tests. Saponin samples (10 μl) or mashed plant material is placed in localised areas on the gelatine–blood-covered plates and after 20 h the widths of the resulting haemolytic rings are measured. A ring of standard saponin is measured in parallel on each plate. The detailed description of HI and microhaemolytic methods and their limitations in relation to alfalfa (*Medicago sativa*) saponins can be found in a separate publication [7]. It has been clearly documented that saponins differ in their haemolytic activities depending on their structures and also on the haemolytic method used. Monodesmosidic saponins are usually more active than their bidesmosidic analogues [2,7]. The HI method has been used successfully for determination of oleane type saponins in *Aerva lanata* and *A. javanica* [8] and in *Phytolacca dodecandra* [9].

Haemolytic effects can also be used successfully for spotting haemolytic saponins on TLC plates [10,11]. For this, developed plates need to be carefully dried from the solvent residue and then covered with a layer of gelatine–blood solution. After few hours, whitening spots can be seen on plates indicating the presence of saponins. Care has to be taken, as some hydrophobic compounds prevent plates from wetting properly and in fact these areas can be mistakenly identified as haemolytic spots.

Quantitation of saponins can be also performed with other biological methods including growth of sensitive fungus colony *Trichoderma viride* [12] or with *Tribolium castaneum* growth [13] or germination of lettuce seeds [14].

The biological methods, in spite of their simplicity, are at best only approximate and do not distinguish between different saponins. They can be used successfully in work in which the main goal is the comparison of total saponin concentrations, e.g., for breeding purposes where seedlings can be compared quickly without destroying the plants and one mashed leaf is enough for the test. To semiquantify saponins, the test must be standardised with saponin mixtures isolated from the species studied. However, the biological methods fail when saponin preparations are used for biological activity tests. Because the biological activity of saponin has been closely correlated with the chemical structure of individual saponins and because their concentration may change with different factors, including plant age, growth phase, environmental stresses, etc., the exact composition of saponin mixture is crucial. The poor characterisation of saponin preparations is the main cause of difficulties in interpreting findings on their biological activity and differences in the data obtained on the same preparation by different laboratories.

Non-biological methods of total saponin determination include spectrophotometry [15–17], TLC-densitometry, gas chromatography (GC), high-performance liquid chromatography (HPLC), hyphenated techniques such as LC–MS, LC–NMR and capillary electrophoresis (CE). While first three methods were used for quantitation of sapogenins and/or saponins in plant material, recent hyphenated techniques enable rapid initial screening of crude plant extracts, providing preliminary information on the content and the nature of constituents in the matrix. They provide a good method for identification of new compounds with potential biological activity and assure avoidance of unnecessary isolation of common compounds of minor interest [18].

2. Thin-layer chromatographic determination of saponins

2.1. TLC–densitometry

Thin-layer chromatography in one- and two-dimensional (1/2D) modes is a powerful technique which has been used successfully in the separation and determination of a large number of saponins in plant extracts. A major problem with these techniques is first of all the parallel running of the appropriate standards minimising the variation between different plates and colour reactions with spraying agents. The second difficulty is spot detection by means of sophisticated instrumentation for data acquisition and handling to scan the whole plate surface at high speed. This can be achieved, how-

ever, by on-line coupling of a computer with a dual-wavelength flying-spot scanner and two-dimensional analytical software. A great number of saponins are being determined by these techniques and some of them are listed in Table 1.

Most frequently, silica gel plates are used and developing systems consist of choloroform–methanol–water or butanol–acetic acid–water mixtures for saponins and benzene–acetone for aglycones. The most frequently used visualisation sprayers include Carr–Price reagent [30], Liebermann–Burchard reagent [31], phosphotungstic acid [32], 1% $CeSO_4$ in 10 H_2SO_4 [33], 10% H_2SO_4 in EtOH [34], phenol–H_2SO_4 [35], 0.5% *p*-anisaldehyde 1% H_2SO_4 in OHAc [34]. Linear relationships between the peak area and the amount of standard saponins can be found in the range of 1–5 μg per spot with recovery being at the level of 98% and standard deviation of around 3–5%. Comparison of the data with those obtained by HPLC shows that the method is sufficiently accurate for quality control monitoring and is particularly suitable for assays in series [36]. With 2D-TLC it was possible to analyse 35 saponins extracted from stems and leaves of ginseng [37], and *Calendula officinalis* saponins [38]. Fifteen ecotypes of quinoa (*Chenopodium quinoa*) used in breeding programs in the UK were successfully analysed for the concentrations of three groups of saponins, including oleanolic acid, hederagenin and phytolaccagenic acid glycosides [39]. The glycyrrhizic acid in liquorice extracts [40], panaxadiol and panaxatriol [41,42] in ginseng and *Avena sativa* saponins [35] were determined with this technique. The triterpene saponin, escin, in horsechestnut extracts and phytopharmaceutical preparations was measured with high-performance TLC (HP-TLC) with post-chromatographic derivatisation and in situ reflectance measurements. This method enabled determination of large numbers of samples and did not require any tedious clean-up steps prior to analysis, and was highly recommended in pharmaceutical quality control practice.

2.2. TLC–colorimetry

In a large number of investigations, the saponins have been analysed qualitatively/quantitatively with TLC–colorimetry. The saponins can be determined colorimetrically in the crude extract and TLC is just a means of confirmation of their presence in the sample, or the TLC-separated bands are scraped, extracted with alcohol and the extract is treated with a specific reagent. Most frequently used colorants include Ehrlich or vanillin reagents and measurements are made at λ=515–560 nm. The major disadvantage of this procedure is the fact that some other components of the extract such as sterols and bile acids with hydroxyl group at C_3, may give a colour reaction with the reagent, providing misleading information. An anisaldehyde–sulphuric acid–

Table 1
Determination of some saponins by TLC–densitometry

Saponin	Plates	Solvent system	Spray reagent	Refs.
Cucurbitacin B, D,E,I	Silica gel	MeOH–H_2O (55:45)	UV 254 nm	[19]
Cucurbitacin C	Silica gel	EtOAc–C_6H_6 (75:25)	Vaniline–orthophosphate in EtOH	[20]
Ginsenosides	Silica gel G,H	$CHCl_3$–MeOH–H_2O (65:35:10) BuOH–EtOAc–H_2O (4:1:2)	NH_4HSO_4 in in 15% H_2SO_4	[21]
	Silica gel	1,2-Dichloroethanol–BuOH–MeOH–H_2O (30:40:15:25)	NH_4HSO_4 in EtOH	[22]
	Silica gel H	$CHCl_3$–MeOH–H_2O (65:35:10)	10% H_2SO_4	[23]
	Silica gel H	$CHCl_3$–MeOH–H_2O (21:11:4) I dir. BuOH–EtOAc–H_2O (4:1:1) II dir.	Vaniline in H_2SO_4	[24]
Glycyrrhizin	Silica gel 60	BuOH–OHAc–H_2O (5:1:4)	H_2SO_4	[25]
Gypsosides	Silica gel LS	BuOH–OHAc–H_2O (4:1:5)	Phosphotungstic acid	[26]
Oleanolic acid	Silica gel G	$CHCl_3$–Et_2O–MeOH (30:10:1)	Anisaldehyde–H_2SO_4	[27]
	Silica gel G	C_6H_6–Me_2CO (36:13)	10% H_2SO_4	[28]
Triterpene	Silufol	C_6H_6–Me_2CO (8:2)	Phosphotungstic acid	[29]

Table 2
The TLC-colorimetric determination of saponins

Sample	Reagent	Wavelength (nm)	Refs.
Cyclamiretin A	10% vaniline–OHAc, $HClO_4$	560	[45]
Ginsenosides	8% vaniline–EtOH, H_2SO_4	544	[46]
	8% vaniline–EtOH, H_2SO_4	544	[47]
	vaniline–OHAc	560	[48]
	vaniline–$HClO_4$	560	[49]
	HCl–H_2SO4	520	[50]
Protodioscin (I)	Ehrlich	515	[51]
Soyasapogenols	OHAc–H_2SO_4	530	[17]
Steroidal aglycones	Anisaldehyde–H_2SO_4–EtOAc	430	[16]

ethyl acetate reagent gives with steroidal sapogenins a colour reaction which is in general free of any influence from interfering compounds [16]. A good improvement of the colorimetric procedure can be the application of a reversed-phase clean-up stage (SPE) prior to the determination. Depending on the nature of saponins, their retention on reversed-phase support is different and appropriate selection of the solvents allows their considerable purification. This was successfully applied for soybean [43] and alfalfa [44] saponins. This clean-up procedure is a very useful technique also for purification of extracts prior to HPLC determination.

When silica gel TLC is used prior to colorimetric determination, a considerable portion of the compound can be adsorbed irreversibly in the stationary phase and the quantities present may be underestimated. To use this procedure for routine analysis, it first has to be calibrated against a more sophisticated technique, e.g., HPLC. Examples of the TLC–colorimetric determinations are presented in Table 2.

3. Gas–liquid chromatography

A basic requirement for a compound to be analysed by GC is its volatilisation. Saponins as polar and quite large molecules are not easily volatile compounds and they have to be volatilised by derivatisation to acetyl, methyl or trimethylsilyl ethers. This method has been used almost exclusively for the separation of aglycone moiety (Table 3). However, a few attempts have been made to use gas chromatography for the determination of intact saponins and these showed that analysis of trimethylsililated glycosides possessing four or less sugar units was satisfactory [63]. However, these sugars have to be attached not through an ester linkage, otherwise they undergo deglycosidation reaction in the injection port of the gas chromatograph [64].

The first step in GC analysis of sapogenins is hydrolysis of intact saponins to their aglycones. This is one of the crucial points of analysis since hydrolysis products are not always natural genins but depending on the saponin structure, time and conditions of hydrolysis they may form number of artefacts. For instance, hydrolysis of soyasaponins present in soybean, alfalfa and a number of other legumes leads to the formation of several aglycones (Table 4). Their relative quantities change significantly depending on the time of hydrolysis and also on the type of hydrolysing agent. Soyasapogenols A and E seem not to be to sensitive to these parameters, while the concentration of soyasapogenol B decreases with gradual increase in soyasapogenols C, D and F, the artefacts of soyasapogenol B degradation. Also the solvent used in the hydrolysis mixture seems to influence the final results, and creation of artefacts is possible in aqueous hydrolysis. Similar results were obtained by Ireland and Dziedzic [66], where in aqueous hydrolysis soyasapogenol B_1, C, D and E were registered while genuine aglycones soyasapogenol A and B were obtained when soysaponins were hydrolysed with sulphuric or hydrochloric acid in anhydrous methanol [67].

The situation becomes even more complicated

Table 3
The gas–liquid chromatography analysis of saponins

Sample	Saponins	Column	Refs.
Alfalfa	Medicagenic acid, hederagenin soyasap. B,C,D,E,F, oleanolic acid (methylated-acetylated)	OV-1	[52]
	Medicagenic acid dimethyl ester, di(trimethylsilyl)	OV-17 on Chromosorb or 3% SP-2250 on Supelcoport	[53]
	Soyasapogenols A-E, medicagenic acid TMS	OV-17 on Chromosorb W HP	[54]
	Medicagenic acid TMS	SP-2250 on Supelcoport	[55]
Ginseng	Ginsenosides TMS	OV-101 on Chromosorb WHP	[32]
Legumes	Soyasapogenol B TMS	Technochrom SPB-1	[56]
	Soyasapogenol A, B TMS	OV-1 on Diatomit CQ AW DMCS	[57]
Lentil	Soyasapogenol B TMS	DB-1, 0.25 micron film	[58]
Lupin	Soyasaponin I, VI TMS	DB-1, 0.25 micron film	[59]
Quinoa	oleanolic acid TMS	SE-30 on Chromosorb W AW DMCS	[60]
Soybeans	Soyasap. I, II, III, A1, A2 TMS	FFAP on Chromosorb GAW DMCS	[33]
	Soyasapogenol A, B TMS	OV-1 on Chromosorb	[33]
Triterpene	Oleanane, ursane type TMS	OV-101	[61]
	Oleanolic and ursolic acids	OV-17	[62]

when saponins possessing different aglycones are hydrolysed. The complete hydrolysis of all saponins into sapogenins seems to be a key step for their quantitation. Tava et al. [52] showed that the optimal conditions for hydrolysis of alfalfa root saponins was obtained upon refluxing for 8 h with 2 *M* HCl in 50% aqueous methanol. Prolonged times of hydrolysis gave reduced medicagenic acid yields, probably due to decomposition. Under these conditions, soyasapogenol B was almost completely decomposed to yield the artefacts (Fig. 1). Similar changes in medicagenic acid concentration versus time of hydrolysis were reported [55]. The best results were obtained when the reflux time was 12 h and the sulphuric acid concentration was 0.5 *M* in 1,4-dioxane–water (1:3, v/v).

A number of saponins generate, during hydrolysis, not the genuine aglycone but a mixture of it and the lactone. This can happen in case of the glycosides of hederagenin and oleanolic acid [68,69], jujubogenin→ebelin [70], cochalic acid→echinocystic acid [71], zanhic acid→lucernic acid [72], and some others. Acid-catalysed double-bound migration [73], epimerisation [71], and dehydration [74] are often observed during hydrolysis. The same is true with steroidal saponins where, for instance, furostanol saponins in the presence of methanol can be converted to their 22-OCH_3 derivatives. This great number of possibilities for artefact formation makes GC analysis complicated.

Identification of derivatised aglycones can be performed by comparison of retention times of peaks in the sample GC spectrum with the retention times of appropriate standards. Best resolutions with relatively short retention times are obtained for trimethylsilyl derivatives. However, these derivatives are rather difficult to use in GC–MS since identification of the MS peaks is complicated. Rao and Bories

Table 4
Influence of the conditions and the time of hydrolysis of alfalfa saponins on soyasapogenol concentration [65]

Hydrolysing agent, time	Soyasapogenol concentration (% of total)					
	A	B	C	D	E	F
2 N H_2SO_4 in 50% MeOH, 1 h	21.9	55.2	6.9	7.6	1.9	6.3
2 N H_2SO_4 in 50% MeOH, 30 h	23.2	12.5	22.4	21.6	1.7	18.6
1 N H_2SO_4 in dioxan–water (1:3), 8 h	24.5	48.3	11.3	–	2.2	13.7

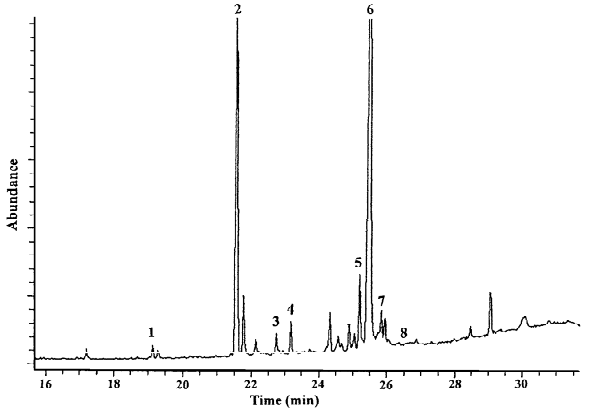


Fig. 1. GC analysis of derivatized sapogenins from alfalfa roots: (**1**) soyasapogenol C; (**2**) betulinol (internal standard); (**3**) hederagenin; (**4**) soyasapogenol D; (**5**) soyasapogenol E; (**6**) medicagenic acid; (**7**) soyasapogenol F; (**9**) soyasapogenol B. Column: DB5 (30 m×0.32 mm I.D.; 0.25 μm film thickness). Chromatographic conditions: injector, 310 °C; detector, 340 °C; column temperature, 90–300 °C; carrier gas, He.

[55] compared GC separation of di-*o*-acetyldimethyl ester of medicagenic acid with its trimethylsilyl derivative on an OV-17 column and reported that much higher temperatures and longer times were necessary to achieve the elution of acetylated and/or methylated sapogenins. In spite of these difficulties, a mass spectral study performed on medicagenic acid present in a mixture of acetylated and methylated sapogenins using GC–MS displayed a base peak at *m*/*e* 203 due to the expected retro-Diels-Alder fragmentation, together with other characteristic ions at *m*/*e* 262, 494, 554 and 614 (M^+).

4. High-performance liquid chromatography

High-performance liquid chromatography is the most powerful and the most frequently used technique for saponin determination due to the fact that it can deal effectively with non-volatile, highly polar compounds. It has been used extensively for determination of both aglycones and intact saponins. The separations are performed usually on normal (silica gel) and reversed-phase (C_8, C_{18}) columns, of which C_{18} has been definitely preferred, but other modified silica gel supports including NH_2 and DIOL are occasionally used (Table 5). In some instances when resolution of saponins on reversed-phase is insufficient, the selected carbohydrate, borate anion-exchange and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] supports have been used successfully. Carbohydrate and NH_2-modified columns have been shown to be very effective in the separation of glycoalkaloids [102] but some steroidal saponins can also be successfully analysed [135] The chaconines and solanine were nicely separated with Bondapack NH_2 column in the reversed-phase mode in less than 7 min (detection limit, 5–15 ppm) [100] The borate anion-exchange chromatography depends on the formation of borate complexes with *cis*-diols in the saccharide moiety. The formation of these complexes in some cases improves significantly the resolution and separation of isomeric glycosides which are not separated on reversed-phase support [98]. After separation, genuine saponin can be recovered by removing borate as volatile methyl borate by repeated co-distillation of the eluate with methanol. Resolution of closely related compounds can be also improved by the application of hydroxyapatite support. The hydroxyapatite is more hydrophilic than silica gel support and allows separation of two glycosides differing only in a terminal pentose [99].

The main problem in HPLC analysis of saponins is detection. Since only a few saponins, for instance glycyrrhetinic acid and its glycosides and cucurbitacins, have absorption maxima in UV range, they are the only compounds that can be easily detected at 254 nm. The majority of saponins do not possess chromophores necessary for UV detection, and the separation of intact glycosides or their aglycones has to be traced at lower UV wavelengths ranging from 200 to 210 nm. The sensitivity with this detection mode has been satisfactory and depending on the nature of the saponin ranges from 50 ng for avenacoside B [81] to 300 ng for ginseng saponins [92]. The detection at lower wavelengths, however, limits the selection of solvents and the gradients that can be used. Since acetonitrile gives much lower absorption at lower wavelengths than methanol, the selection of acetonitrile–water gradients is the mode of choice. Similarly, the gradient cannot cover a wide range of concentrations due to the baseline drift, which creates an additional problem with saponin analysis. The bidesmosidic saponins elute in

Table 5
The high-performance liquid chromatographic determination of saponins

Sample	Column	Solvent system	Detection (nm)	Refs.
Aescin	C_{18}	MeCN–H_2O	206	[75]
Aesculus sap.	C_{18}	MeCN–H_2O–H_3PO_4	210	[76]
	C_{18}	MeCN–H_2O–H_3PO_4	205	[77]
Agave sapogen.	C_8	MeCN–H_2O (benzoate esters)	254	[78]
Amaranthus sap.	C_{18}	MeCN–H_2O (Br derivatives)	260	[79]
Aster sap.	C_{18} Nova-Pak	MeCN–H_2O	210, TIC[a]	[80]
Avenacosides	RP8	MeCN–H_2O	200	[81]
Calendula sap.	C_{18}	Me–OH–H_3PO_4	210	[82]
Cucurbitacins	C_{18}	MeOH–H_2O (45:55 or 70:30)	254	[20]
Diosgegnin acet.	Silica gel	Hexane-*iso*-PrOH	219	[83]
Ginsenoside Rb1	Lichrosorb-NH_2	MeCN–H_2O–BuOH (8:2:1)	210	[84]
Ginsenosides	Silica gel LS-310	*n*-C_6H_{14}–CH_2Cl_2–MeCN (15:3:2) Benzoyl	240	[85]
	Silica gel	*n*-Heptan–BuOH–MeCN–H_2O	207	[86]
	Silica gel	CH_3Cl–MeOH–H_2O (30:17:2)	RI[a]	[87]
	Spherical silica gel	Hexane–Et_2O–EtOAc	206	[88]
	Carbohydrate	MeCN–H_2O (80:20–94:6)	210	[89]
		MeCN–H_2O–BuOH (86:14:10)		
	Bondapack/carbohydr.	MeCN–H_2O–BuOH (80:20:15)	RI	[90]
	Bondapack C_{18}	MeCN–H_2O (1:1–9:1)	220	[91]
	C_{18}	MeOH–H_2O	203	[86]
	C_{18}	MeCN–H_2O	203	[92]
	C_{18}	MeOH–H_2O	203	[93]
	C_{18}	MeCN–50 n*M* KH_2PO_4	202	[94]
		MeCN–H_2O–H_3PO_4		
	C_{18}	MeCN–H2O–H3PO4	205	[95]
	C_{18}	MeCN–H_2O	203	[96]
	C_{18}	MeCN–H_2O	203	[97]
	Anion-exchange	MeCN–0.25 *M* H_3BO_3 (12.5:87.5)	UV	[98]
	Hydroxyapatite	MeCN–H_2O (80:20)	210	[99]
		MeCN–H_2O (90:10 →70:30)		
Glycoalkaloids	Bondapack NH_2	THF–H_2O–MeCN (56:14:30)	208	[100,101]
	NH_2	MeCN–KH_2PO_4–H_2O	208	[102]
	Carbohydrate	MeCN–THF–H_2O	215	[103]
	C_{18}	MeOH–H_2O–H_3PO_4	206	[104]
	C_{18}	MOH–0.01 *M* Tris, MeCN–0.01 *M* Tris	205	[105]
	C_{18}	MeCN–H_2O–ethanolamine (45:55:0.1)	210	[106]
	YMC-Pack SiO2	$CHCl_3$–MeOH–NH_4OH (10:13:1; 7:13:2)	230	[107]
Glycyrrhizin	C_{18}	MeOH–H_2O–TBA–H_3PO_4	254	[108]
	C_{18}	MeOH–H_2O–OHAc	254	[109]
	C_{18}	MeOH–H_2O–$HClO_4$	254	[110]
	C_{18}	MeCN–MeOH–H_2O + ammonium perchlor.	254	[111]
Gypenosides	C_{18}	MeOH-H_2O	210	[112]
Gyspogenin	glcAC$_{18}$	MeOH-H_2O-TBA-H_3PO_4	206	[113]
H. helix sap.	C_{18}	MeCN–H_2O	205	[114]
Medicagenic acid	C_{18} Hypersil	MeOH–H_2O–HCOOH	210	[115]
Medicagenic acid glc	Eurospher C_{18}	H_2O–MeCN (10–90%) Br-derivatives	260	[116,117]
Nerium sap.	C_{18} Nova Pak	MeCN–H_2O–TFA (20:80→45:55)	210, TIC	[80]
Oleanolic acid	C_{18}	MeCN–H_2O	210	[118]
Oleane glc.	Bondapak C_{18}	H_2O–MeCN (70:30→ 50:50)	206	[119]
		H_2O–MeCN (40–70%) Br-derivatives	254	[119]
	RP-8, DIOL	H_2O–MeCN (85:15	206	[120]

Table 5. Continued
The high-performance liquid chromatographic determination of saponins

Sample	Column	Solvent system	Detection (nm)	Refs.
Primula sap.	C_{18}	MeCN–H_2O–H_3PO_4	195	[121]
Ruscin	C_{18}	MeCN–H_2O	202	[122]
Saikosaponin	Aquasil, hydroxyapatite	H_2O–MeCN, $CHCl_3$–MeOH–H_2O	205	[123]
	Develosil-ODS	MeOH–H_2O	210	[124]
	Nucleosil 50-5	$CHCl_3$–MeOH–EtOH–H_2O (62:16:16:6)	210	[87]
	Silica gel	$CHCl_3$–MeOH–H_2O (30:10:1)	RI	[87]
	C_{18}	MeCN–H_2O	210	[124]
	C_{18}	MeOH–H_2O–OHAc-trimethylamine	254	[125]
Soyasapogenols	Silica gel	Petrol–EtOH	MD[a]	[126]
Soyasaponins	Lichrosorb RP18	MeOH–PrOH–H_2O–OHAc (32:4:63:0.1)	RI	[127]
	Silica gel	$CHCl_3$–MeOH–H_2O–OHAc	MD	[128]
	C_{18}	MeCN–H_2O (coumarin derivatives)	Fluor.[a]	[129,130]
	C_{18}	MeCN–*n*-PrOH–H2O–OHAc (32:4:63:0.1)	205	[131]
Soyasaponin VI	Ultrasphere C_{18}	MeCN–OHAc (1000:0.3) (S1) H_2O–OHAc–EDTA (1000:0.3:0.15) (S2)	205	[132–134]
Steroid saponin	Silica gel	Hexane–EtOH–H_2O	208	[135]
	NH_2	MeCN–H_2O	208	[135]
Steroidal sapog.	Silica gel	Hexane–Me_2CO, hexane–EtOH	206	[136,137]
	C_{18}	MeCN–hexane–THF, MeOH–H2O	206	[136,137]
Swartzia sap.	C_{18}	MeCN–H_2O–TFA (30:70→50:50)	210, TIC	[80]
Triterpene sap.	Alltech C_{18}	H_2O–MeCN (20–80%)	210	[138]
	Hydroxyapatite	MeCN–H_2O (87:13; 80:20)	210	[99]
Zanhic acid glc.	Spherisorb C_{18}	H_2O–MeCN (10→90%) Br-derivatives	260	[139]

[a] TIC, total ion current; RI, refractive index detector; MD, mass detector; Fluor., fluorescence detector.

water–acetonitrile gradient at relatively low concentrations of MeCN while monodesmosides elute later and are consequently much more difficult to quantify. Some plant extracts may contain large numbers of glycosides differing in polarities due to the number of sugars attached. Analysis of such a complicated mixture would need wide range of solvent concentration. Application of a gradient completely excludes detection with a refractive index detector (RI) and this is why this type of detection has been rarely used.

The alternative to low wavelength UV or RI detection has been pre-column derivatisation of saponins in order to attach a chromophore that facilitates UV detection at higher wavelength (254 nm). Several attempts has been made to introduce chromophores. Kitagawa et al. [129] employed the derivatisation of saponins from soybean with coumarin and the registration of HPLC profiles with a UV-fluorescent detector. Similar attempts have been made by others to introduce benzoyl chromophore through the pre-column reaction of saponins with benzoyl chloride in pyridine and detection at 254 nm [76,85]. These derivatisation procedures, however, create some practical problems due to the steric shape of the saponin molecule and differentiated rate of substitution of functional –OH groups. Some of these groups are not readily derivatised, and thus the reaction mixture of a single saponin may contain a number of its derivatives. During the derivatisation of complex saponin mixtures many peaks are observed and their interpretation and quantification are difficult.

Promising results has been obtained by derivatisation of saponins with 4-bromophenacyl bromide in the presence of crown ether. Originally this derivatisation mode was employed for the analysis of fatty acids and prostaglandins. Slacanin et al. [119] applied this procedure for the determination of oleane saponins in *Phytolacca dodecandra*. To be derivatised with 4-bromophenacyl bromide, a saponin molecule has to possess at least one carboxyl group, either at the aglycone or sugar part. Therefore, this method can be applied only to some groups of saponins. The oleanolic acid monodesmosides of *P. dodecandra* could be easily derivatised, while

bidesmosides had to be determined in a separate HPLC run with 206 nm detection. This method was excellent for saponin determination in the roots and aerial parts of alfalfa [116,117]. The medicagenic acid glycosides, both in monodesmosidic and bidesmosidic forms can be chromatographed after derivatisation in a single run on a C_{18} column (Fig. 2). Soyasaponin I and monodesmosides of hederagenin can also be determined. Zanhic acid tridesmoside in the aerial part of alfalfa had both –COOH groups glycosylated and could not be readily determined. Due to the very high polarity of this compound, determination at 210 was also very difficult. This problem can be avoided by alkaline hydrolysis of zanhic acid tridesmoside prior to the derivatisation with 4-bromophenacyl bromide. The resulting prosapogenin derivatises readily and can be chromatographed at 260 nm [139]. To apply 4-bromophenacyl bromide derivatisation to the analysis of mixtures of mono-, bi- and tridesmosidic saponins, the method can be modified to allow chromatography of all three groups. For this, two HPLC runs are necessary. In the first run, only monodesmosides or

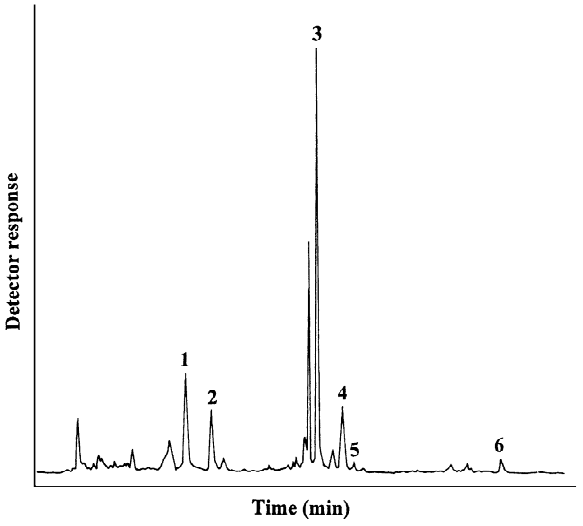


Fig. 2. HPLC separation of 4-bromophenacyl bromide derivatives of saponins from alfalfa roots: (**1**) 3Glc,28AraRhaXyl medicagenic acid; (**2**) 3Glc,28Glc medicagenic acid; (**3**) 3GlcA,28AraRhaXyl medicagenic acid; (**4**) 3AraGlcAra hederagenin; (**5**) soyasaponin I; (**6**) 3Glc medicagenic acid (for structures see Fig. 3). Column, Eurospher C_{18}, 5 μm (4.6×250 mm); eluent, linear gradient 20–80% acetonitrile in water; flow-rate, 1 ml/min; detection, 260 nm.

bidesmosides with one free –COOH are determined. For the saponins not having free –COOH group, the plant extract is treated with saturated lead acetate solution in H_2O and the resulting precipitate is centrifuged at 3000 *g*. The precipitate contains all carboxyl-free saponins, and the supernatant is passed through a C_{18} Sep-Pak cartridge which retains saponins. They are then washed with methanol, alkaline hydrolysed and derivatised for chromatography [117].

No matter which method of detection is used for HPLC determination of saponins, a solid-phase clean-up step is highly desirable prior to analysis. The C_{18} [43,44,76,95,96,128,139,140] for saponins and NH_2 [102] Sep-Pak cartridges for glycoalkaloids have been used successfully, with recovery of glycosides higher then 95%. All carbohydrates and most of the phenolic compounds can be removed from the matrix by the careful selection of eluent, usually methanol–water mixture. The carbohydrates pass readily C_{18} Sep-Pak with water, and most of the phenolic acids and flavonoids can be removed with 40% methanol at which concentration the majority of saponins are retained on the solid-phase [44]. The saponins removed with methanol are highly purified and HPLC profiles are much easier to interpret. The solid-phase clean-up procedures by simplifying extraction and purification ensure mild conditions for these processes and protect saponins from multiple artefact formation [141].

5. Hyphenated techniques in HPLC

The HPLC methods for the determination of saponins do not ensure identification of individual peaks because the absorption spectra are not specific. This is not like in the case with the determination of phenolics where absorption spectra registered with a photodiode array detector (DAD) provide useful information about the type of chemical and its oxidation pattern. Identification of saponins must rely exclusively on the comparison of retention times of unknown peaks with retention times of an appropriate saponin standards, for example alfalfa saponins [116]. None of the detection modes permits the registration of all the saponins encountered in plant extracts within a single analysis. To overcome

this problem, some authors have used off-line peak identification with high-performance thin-layer chromatography (HPTLC), multiple internal reflection infrared spectroscopy (MIR-IR) and field desorption mass spectrometry (FD-MS) [92,96,142].

More recently, hyphenated techniques coupling HPLC with different spectroscopic detection methods have been developed. The use of on-line detection/identification systems allowing chemical screening of plant extracts not only for saponins but for a number of phytochemicals is a promising breakthrough in the determination and structural analyses of natural products. This detection/identification can be achieved by coupling LC-UV to mass spectrometry (LC-UV–MS) [80,143–145] or with nuclear magnetic resonance (LC-UV–NMR) [18,146,147].

In the LC-UV–MS mode three different interfaces are used most frequently. These include thermospray (LC–TSP-MS) [148], continuous flow FAB (LC–CF-FAB) [149], and electrospray (LC–ES-MS) technique [150]. The most suitable of these for phytochemical analysis is TSP, since this allows introduction of aqueous phase into MS system at a flow-rate compatible with that usually used in phytochemical analysis (1–2 ml/min). However, this interface allows satisfactory ionisation (NH_4OAc buffer, positive ion mode) of molecules within the mass range 200–800 mu, which means that only saponins having up to three sugars can be analysed satisfactorily [145]. For larger molecules with MW$>$ 800 mu, CF-FAB or ES are the methods of choice [151]. Oleanolic acid glycosides from *Swartzia madagascarensis* Devaux in TSP mode showed clear MS molecular peaks only when they possessed up to three sugars. Much clearer spectra for three and four sugar-bearing saponins were obtained with CF-FAB and ES, but even though, the LC–MS–MS mode was necessary for full identification [18].

The LC–NMR method is not yet a widely accepted technique and many technical problems have still to be overcome including low sensitivity and the conditions for using non-deuterated solvents (MeCN, MeOH, H_2O) [18].

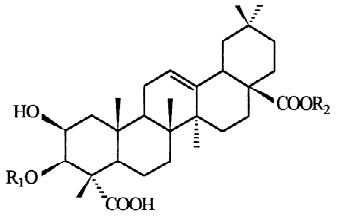
These methods, however, are still in the early stages of development and are generally regarded as pure research tools, without any major routine uses. However, for laboratories which can afford to purchase this extremely expensive equipment, the hyphenated HPLC techniques can be a powerful tool for efficient targeted isolation of new types of saponins with potential biological activities, replacing bioassay-guided search.

6. Capillary electrophoresis (CE) of saponins

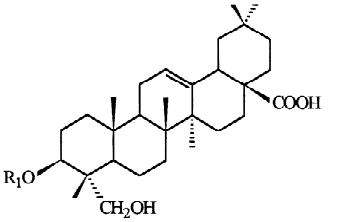
Capillary electrophoresis is an excellent analytical technique for the separation and quantitation of a broad range of phytochemicals. Millions of theoretical plates that can be obtained, rapid separation (1–30 min), minimal sample requirement (nl) and extremely low reagent consumption makes EC a very promising tool [152]. However, the minimal sample requirements for separation are in contrast with the high concentrations required for detection and this drastically limits the use of CE for saponin analysis [153].

The results of only a few trials on CE separation and determination of saponins have been published. Iwagami et al. [154] analysed ginseng saponins using the micellar electrokinetic capillary chromatography (MECC) mode with borate buffer at pH 7 with 25% ACN and 75 m*M* cholate as modifiers. The column 102 cm$\times$50 μm (95 cm to detector) at 30 °C and 30 kV ($+$ve to $-$ve) were used. Detection was performed at 200 nm. Separation of triterpenoid in *Silene onites* was done by MECC with phosphate–borate buffer (pH 9.4), SDS and 5% MeOH. The 72 cm$\times$50 μm (50 cm to detector) column heated to 50 °C and 20 kV ($+$ve to $-$ve) voltage were used for separation. Detection was performed at 240 nm. By this technique the more hydrophilic compounds had shorter migration times than the lipophilic ones and the elution pattern was very similar to that observed with reversed-phase separation [155]. The saponin concentration of herb mix I-tzu-teng was determined both with HPLC and CE [156]. In MECC technique with SDS and sodium cholate buffer, the complete separation of four anthraquinoids, five flavones, two carboxylic acids and saponins was obtained within 14 min, while for HPLC analysis 50 min were necessary.

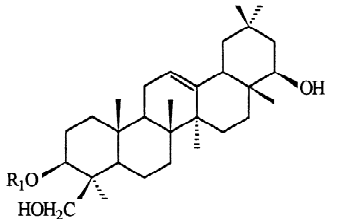
A number of medicagenic acid glycosides from alfalfa roots (Fig. 3) have been used for optimisation of CE separation [157]. After a number of experi-



1 R_1=Glcp R_2=[Xylp(1-4)Rhap(1-2)Arap]
2 R_1=Glcp R_2=Glcp
3 R_1=GlcAp R_2=[Xylp(1-4)Rhap(1-2)Arap]
6 R_1=Glcp R_2=H
7 R_1=[Glcp(1-2)Glcp] R_2=[Xylp(1-4)Rhap(1-2)Arap]



4 R_1=[Arap(1-2)Glcp(1-2)Arap]



5 R_1=[Rhap(1-2)Glcp(1-2)GlcAp]

Fig. 3. Chemical structures of alfalfa saponin standards used in HPLC and CE separation. Glcp, glucopyranosyl; GlcAp, glucuronopyranosyl; Arap, arabinopyranosyl; Xylp, xylopyranosyl; Rhap, rhamnopyranosyl.

ments with different temperatures (20–30 °C) and borate buffer concentrations (100–200 m*M*), the optimal conditions for separation of standard saponins were found to be 150 m*M* buffer concentration and room temperature. Under these conditions, complete separation was achieved within 7 min (Fig. 4). It was evident that glycosides having two free COOH groups eluted much later (one sugar saponin **6** and four sugars **3**) than the compounds with only one group free. Borate complexation at the OH groups of sugars and the mass effect due to different numbers of sugar units allowed separation of groups containing the same number of free COOH groups. The pattern of separation is substantially different from that obtained on reversed-phase HPLC [117]. Zanhic acid tridesmoside (not shown in the figures), which does not have free COOH eluted shortly after EOF, before any other compounds. There were also problems with registration of soyasaponin I, in spite of the fact it possesses COOH on the glucuronic acid molecule. Separation of the Sep-Pak C_{18} purified saponin mixture from alfalfa roots (Fig. 5) produced electrophoregraphs with a drift of baseline, indicating the need for further purification of the mixture prior to CE analysis.

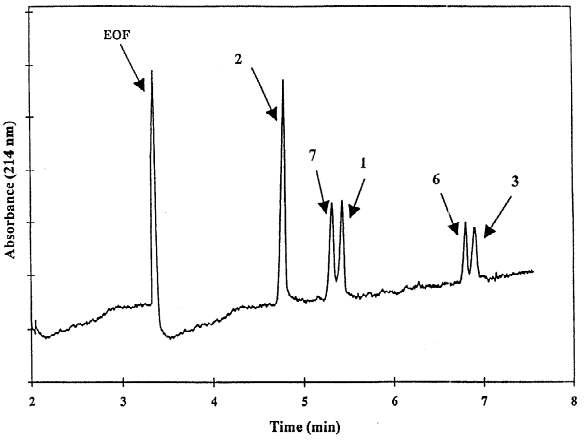


Fig. 4. CE separation of medicagenic acid (Ma) glycoside standards: EOF, internal standard; (**6**) 3 Glc Ma; (**2**) 3Glc,28Glc Ma; (**1**) 3Glc,28AraRhaXyl Ma; (**3**) 3GlcA,28AraRhaXyl Ma; (**7**) 3GlcGlc,28AraRhaXyl Ma.

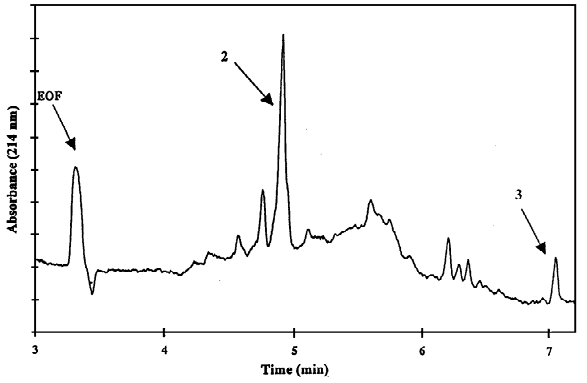


Fig. 5. CE separation of saponins from alfalfa roots: (**2**) 3Glc,28Glc Ma; (**3**) 3GlcA,28AraRhaXyl Ma.

The CE methods for saponin determination are also in the early stages of development but the data published so far give some hopes for future use of this technique in routine saponin analysis.

7. Conclusion

Chromatographic determination of saponins in plant material is still a challenge to the phytochemist. There is no one single method that can be recommended as routine procedure for analysis of complex saponin mixtures. None of these methods provides a fingerprint, which allows the quality to be monitored during the production of batches and to control properly the stability, which is required for registration of herbal medicinal products. Also none of them provides an adequate tool for the separation or preparative isolation of saponin components; a combination of several techniques is required to obtain single standard compounds.

All methods presented have advantages but also multiple limitations. Simple chromatographic methods (TLC, TLC–densitometry), if used properly by a well-trained person, can be of the same value as more sophisticated procedures including hyphenated techniques, which are not available in every laboratory due to the high cost. A good example is escin, the saponin mixture obtained from the seeds of *Aesculus hippocastanum* L, for which German Pharmacopoeia DAB 1997 recommends a colorimetric method.

High-performance liquid chromatography finds widest application. But since saponins possess a wide range of polarities and are lacking chromophores which allow UV detection, precise pre-column clean-up of the saponin fraction from the other components of the matrix is essential for this procedure. New types of more selective columns, allowing separation of saponins differing by just one terminal sugar are needed. Again work on escin can be a good example to follow; use of a Waters symmetry shield C_8 column can provide fingerprinting in just one chromatographic run [158].

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