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High-performance liquid chromatography of alfalfa root saponins

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ABSTRACT

A method for the separation of individual saponins present in alfalfa roots using high-performance liquid chromatography is described. The derivatized saponins are monitored using UV detection and the potential of the method for the quantitation of intact saponins in plant extracts is discussed.

INTRODUCTION

The presence of saponins in alfalfa has been known for a long time and their harmful effect on ruminants, poultry, fungi, mice and germinating seeds has been reviewed [1–4]. These detrimental effects stimulated an interest in monitoring the saponin content in alfalfa, and a number of analytical methods have been developed, usually involving hydrolysis of the plant extract, followed by titrimetric [5], gas [6] or high-performance liquid chromatographic (HPLC) [7] determination of the released aglycones. Although these methods may be a useful source of structural information they are less useful for quantitative analysis due to the potential loss of material during hydrolysis and derivatization.

Other analytical methods are based on the biological activities of the various saponins. The most commonly used tests are based on the sensitivity of *Trichoderma viride* [8–11] and on haemolytic activity [12–14]. These methods, in spite of their simplicity, are at best only approximate and do not distinguish between different saponins. Also they do not show changes in the individual components of the saponin mixture whose biological activity may vary according to their chemical structure [15,16].

The development of new, more dependable analytical techniques for saponin determination, *e.g.* HPLC has been limited. This has been due firstly to the difficulties with the detection of triterpene saponins which do not contain a UV-chromophore and secondly to the lack of appropriate standards. There have been several attempts to overcome the detection problems, which include detection of the underivatized saponins at 190–210 nm [17,18] and monitoring with a light-scattering detector [19].

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These mode of detection, however, have some limitations as to the solvents and gradients that can be used [20].

One solution is to derivatize the saponins with a chromophore which facilitates UV detection [20–22]. The method of Slacanin *et al.* [20] included the derivatization of saponin with 4-bromophenacyl bromide which can be completed with any saponin containing a free carboxylic acid group. All of the alfalfa root saponins fulfil these requirements as they are composed of: (i) medicagenic acid glycosides which in both mono- and bisdesmosidic form contain at least one free carboxyl group; (ii) soyasaponin I which possesses a COOH group on the glucuronic acid moiety; and (iii) hederagenin monodesmosides substituted only at the 3-O position [16]. All these compounds have recently been isolated and identified [16] and we report here the HPLC separation of these derivatized alfalfa root saponins. The potential of using this method for both quantitative and qualitative determination is also discussed.

EXPERIMENTAL

Materials

All chemicals used were of AnalaR grade (BDH, Poole, U.K.). Saponin standards were isolated from alfalfa (*Medicago sativa* L.) roots [16] and the mixtures of crude saponins from *Medicago sativa* and *Medicago lupulina* roots were prepared as previously described [16,23].

Preparation of saponin derivatives

The method previously described [20] was applied with some modifications. Saponin (2.5 mg) was dissolved in 0.25 ml of methanol and 2 ml of water containing 1 mg of potassium hydrogencarbonate was added dropwise with occasional sonication. The samples were lyophilized and treated with 1 ml of a mixture of 4-bromophenacyl bromide (Sigma, Poole, U.K.) (3.5 g) and 18-crown-6 (Sigma) (680 mg) in acetonitrile (100 ml). This was then refluxed at 100°C for 90 min and purified on a Silica gel Sep-pak cartridge (Waters Assoc, Milford, MA, U.S.A.) by eluting with dichloromethane (10 ml) followed by chloroform–methanol (1:1) (10 ml). After evaporation of the latter solvent the sample was redissolved in 2 ml of methanol and used for HPLC analysis.

For the root material, 10 mg of *M. sativa* and *M. lupulina* crude saponins were derivatized in a similar manner to the saponin standards.

Instrumentation

HPLC analyses were performed on a Perkin-Elmer Series 3B liquid chromatograph equipped with Perkin-Elmer LC-75 spectrophotometric detector and Trilab 2000 data collection system. Separations were performed on Spherisorb S 5-μm ODS-2 column (25 cm × 4.6 mm I.D.) (Phase Separation, Deeside, UK.).

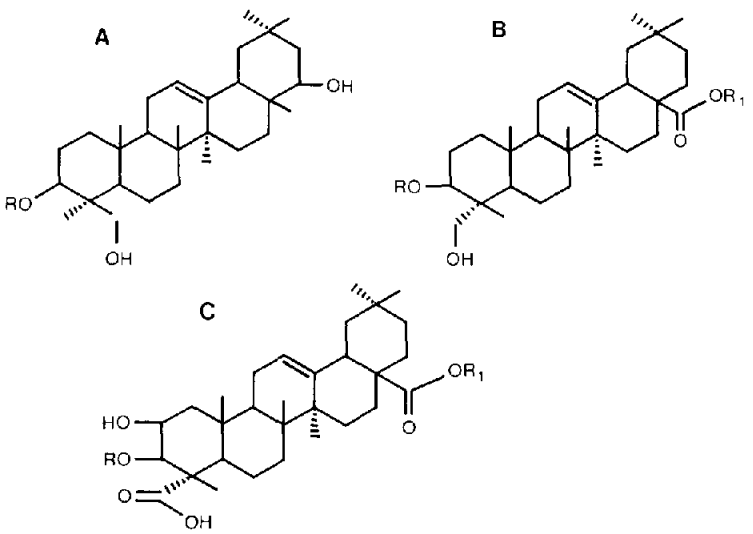
Chromatographic conditions

Chromatographic runs were carried out with an acetonitrile–water gradient elution system. Solvents were acetonitrile–water (10:90, v/v) (solvent A) and acetonitrile–water (90:10, v/v) (solvent B). Solvents were filtered through a Millipore filter (0.45 μm) and degassed by sonication prior to use. Gradients used were not linear and

were run according to the following programme: a concave gradient from 60% A to 25% A over 15 min, followed by the same gradient from 25% A to 0% A over 10 min and continued with 100% B over the next 15 min. A reequilibration period of 10 min was used between individual runs. Derivatized saponins were eluted at 1 ml/min and detection was by monitoring at 260 nm.

RESULTS AND DISCUSSION

The six individual alfalfa root saponins (Fig. 1) that included four medicagenic acid glycosides, Soyasaponin I and the glycoside of hederagenin were derivatized with 4-bromophenacyl bromide according to Slacanin *et al.* [20] with some minor modifications. Conversion of the saponin into its potassium salt can be easily carried out when it is readily soluble in water, whereas it is difficult for saponins such as soyasaponin I which possess low solubility in water. This problem can be overcome, however, by first dissolving the saponin in a small volume of methanol followed by dropwise addition of potassium hydrogencarbonate solution. Other steps of deri-



Compounds	Aglycone	R	R_1
I	C	Glc	Ara-Rha-Xyl
II	C	Glc	Glc
III	C	GlcA	Ara-Rha-Xyl
IV	A	GlcA-Gal-Rha	—
V	B	Ara-Glc-Ara	H
VII	C	Glc	H

Fig. 1. Chemical structures of the saponins.

TABLE I

SEPARATION OF SAPONINS PRESENT IN ALFALFA ROOTS: RETENTION TIMES, t_R, RESPONSE CURVE EQUATIONS AND STANDARD ERRORS, R^2, FOR THE INDIVIDUAL SAPONINS AND PHENANTHRENE AS AN INTERNAL STANDARD

Compound	t_R (min)	Equation	Error	R^2
I	12.7	$y = 45.4x + 3.2$	8.3	0.997
II	14.8	$y = 78.4x + 19.7$	18.5	0.995
III	21.6	$y = 112.0x - 10.6$	21.6	0.998
IV	23.6	$y = 60.0x + 1.6$	19.3	0.991
V	24.5	$y = 65.5x + 18.1$	16.2	0.994
VI	28.4	$y = 588.7x + 16.3$	27.7	0.989
VII	35.5	$y = 123.4x - 7.3$	29.0	0.997

vatization can then be followed since the potassium salt of the saponin is readily soluble in water.

All the saponins present in alfalfa roots could be derivatized via their free carboxylic acid groups. Those saponins containing only one free carboxylic acid (I, II, IV and V) had lower responses than those containing two free acid groups (III and VII) (see Table I). In the former group the location of the free acid moiety within the molecule is different, *i.e.* IV possesses a free carboxylic acid group in the carbohydrate chain while V has a free C-28 acid group and I, II both have a free C-23 acid group. The different spatial arrangements and resultant steric hindrances involved probably account for the differences in responses found within this group (Fig. 2).

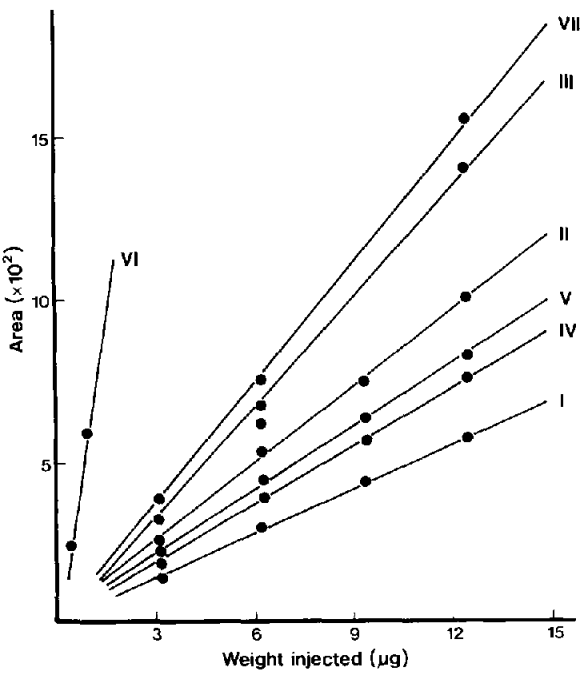


Fig. 2. Calibration graphs for derivatized saponins I–VII.

The stability of the 4-bromophenacyl derivatives varied strongly and depended on the structure of the saponin. The most stable, at room temperature, were derivatives of compound II and VII whereas other compounds had decomposed after 48 h. Their stability, however, was satisfactory when samples were stored at −10°C. Thus, for routine analysis it is recommended that either the HPLC determination is carried out just after derivatization or the samples are stored in methanol at low temperature. Dry samples appear to be stable for periods in excess of one month.

Separation of a mixture of the derivatized standard saponins is shown in Fig. 3. The saponins showed sharp and symmetrical peaks and no peak broadening caused by possible ionization of the carboxylic groups was observed. No ion-suppression reagent was therefore added to the mobile phase. The separation was satisfactory using just a water–acetonitrile gradient and a complete separation could be completed in 30–35 min. However, when a linear gradient from 60% A to 100% B solvent was used, saponins I/II and IV/V were poorly separated. This was overcome by using a non-linear gradient, with optimal separation achieved using the following gradient programme: a concave gradient from 60% A to 25% A over 15 min followed by the same gradient from 25% A to 100% B over 10 min and finally 100% B for a further

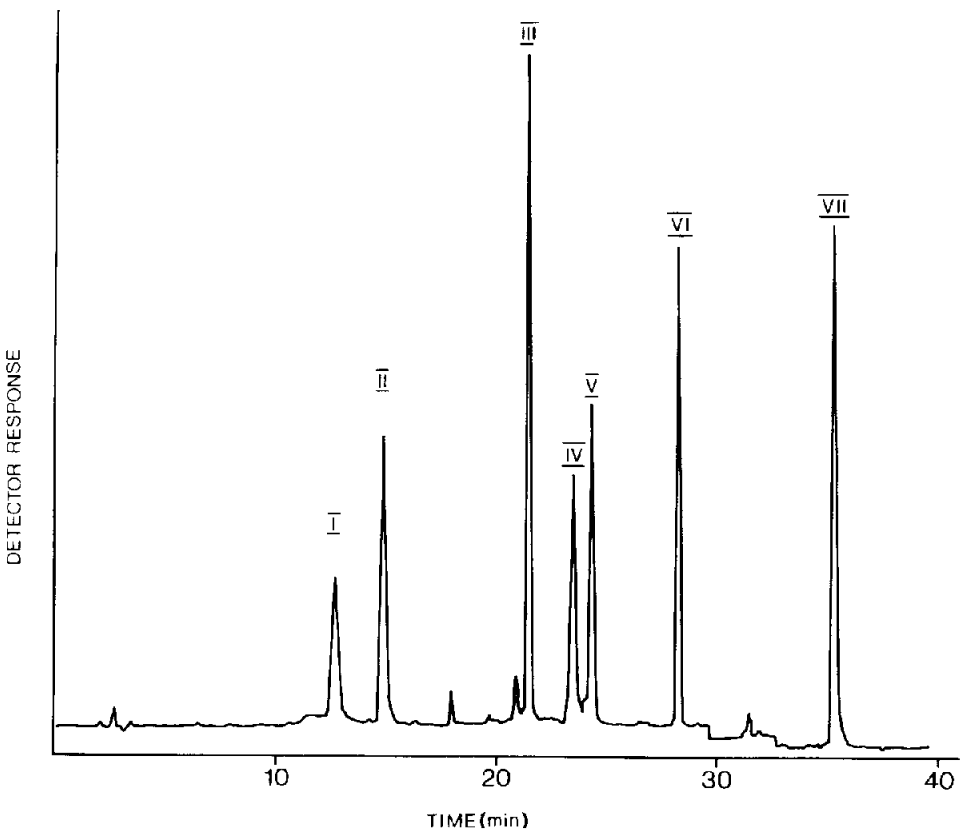


Fig. 3. Separation of derivatized standard saponins I–VII.

15 min. Under these conditions all standards were satisfactory separated and the last peak eluted after 35.5 min. A mixture of standards was spiked with 50 μl of methanolic solution (1 mg/1 ml) of phenanthrene; with a retention time of 28.4 min, this component was well separated from the saponin standards tested and proved satisfactory for use as an internal standard.

Fig. 4 shows the analysis of saponins present in alfalfa (*Medicago sativa*) root extracts. A comparison of retention times of particular peaks of the mixture with those of standards readily allowed the identification of several peaks. The root mixture contained the highest amount of bisdesmoside III followed by compounds I, II, IV and VII respectively. The relative amounts found in the mixture by HPLC were in good agreement with the quantities of each component found during preparative isolation [16], and also confirms our previous findings which indicate that alfalfa root saponins consist mainly of medicagenic acid glycosides [24].

Similarly, the analysis of saponins present in *Medicago lupulina* root extract (Fig. 5) shows the presence of three main compounds namely IV, VII and II. The sapogenols derived from the saponins in this mixture contained medicagenic acid (46%) and soyasapogenol B (45%) [23,25] which is in close agreement with the

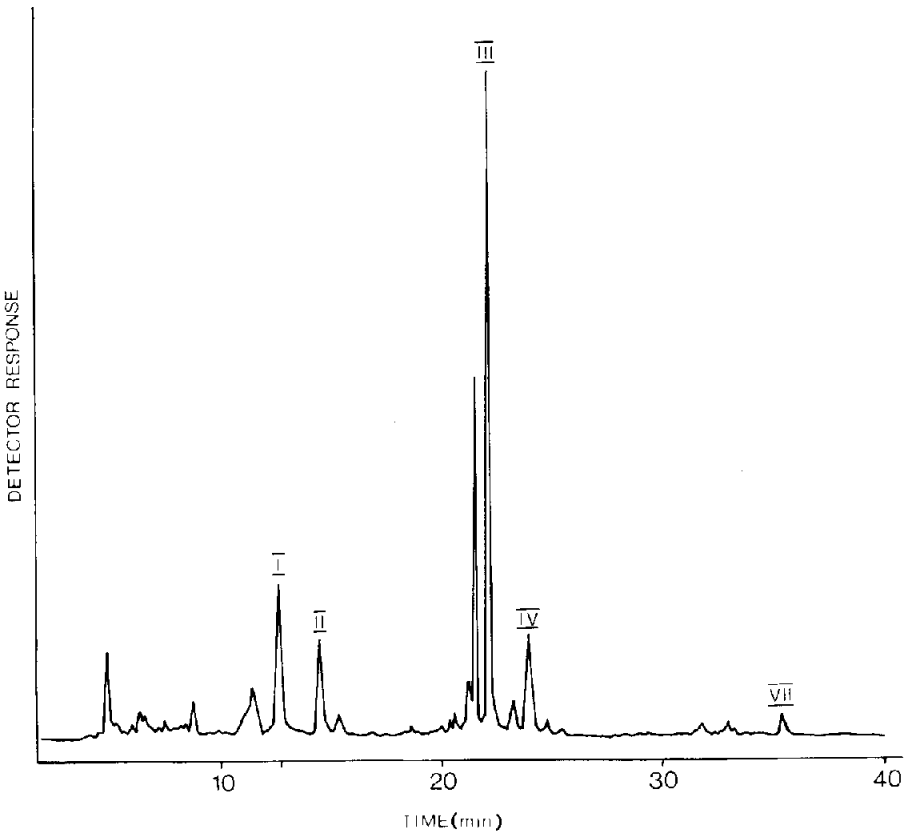


Fig. 4. Separation of derivatized *Medicago sativa* root saponins.

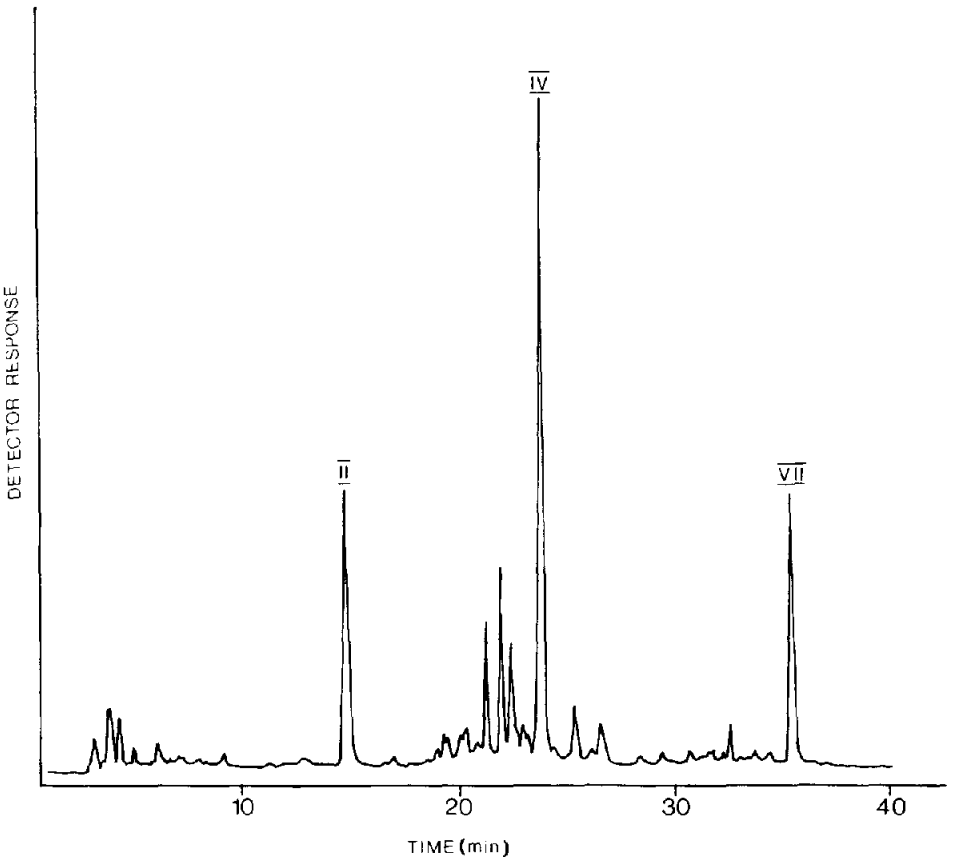


Fig. 5. Separation of derivatized *Medicago lupulina* root saponins.

amounts derived from IV, VII and II found in the present study. The methods used previously, however, were not able to distinguish between the various saponins present and whose biological activities varied depending on their structure [15,16]. The HPLC procedure proposed in this paper allows the quantitative and qualitative determination of individual alfalfa root saponins.

CONCLUSIONS

Mono- and bisdesmosidic olean-12-ene saponins from alfalfa roots can be easily derivatized with 4-bromophenacyl bromide to produce UV absorbing compounds. The derivatized saponins can be chromatographed in a single run on a silica C_{18} column. The method described here could find an application in the study of saponins in alfalfa roots. This may be applied both for the quantification of individual glycosides and also for qualitative screening to find species with the highest content of the most biologically active compounds for pharmaceutical purposes such as their fungitoxic activity against the medically important yeasts *Candida*, *Terulopsis*, etc.

This technique may also be used for the study of alfalfa top saponins for plant breeding and nutritional purposes as soon as appropriate standards are available.

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