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Separation of a triterpenoid saponin mixture from *Maesa lanceolata*: semipreparative reversed-phase wide pore high performance liquid chromatography with temperature control

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Abstract

A mixture of triterpenoid saponins derived from the dried leaves of *Maesa lanceolata* was separated, without structure deterioration, in its components. Seven fractions (I–VII) of high molecular weight (1234–1358) saponins were obtained on a semipreparative scale using wide pore reversed-phase high performance liquid chromatography with an acetonitrile–trifluoroacetic acid (500:0.3 w/w)–water–trifluoroacetic acid (391:0.3, w/w) gradient from 35 to 56% in 30 min. The mobile phase was cooled in an ice bath (0°C) during chromatography in order to prevent bubble formation and to improve the quality of the separation. Freeze-dried fractions IV, V, VI and VII were further separated using solvent systems developed for each of the fractions. Fourteen pure triterpenoid saponins were isolated in this way and their molar weight determined.

Keywords: *Maesa lanceolata*; Triterpenoid saponins; Semipreparative chromatography; Reversed-phase HPLC; Wide pore; Temperature control

1. Introduction

Maesa lanceolata Forsskal var. *gonlungensis* (Myrsinaceae) is a shrub or small tree growing in many African countries used in traditional medicine, e.g. in Rwanda. Bioassay guided fractionation of the methanol extract of dried leaves resulted in the isolation of a triterpenoid saponin mixture [1]. This mixture exhibited a moderate virucidal and high haemolytic activity and showed a severe toxic effect on *Biomphalaria glabrata* snails (molluscicidal activity) [2]. Screening of the *Maesa lanceolata* saponins for antiangiogenic activity in the chick embryo chorioallantoic membrane (CAM) assay gave promissing results [3].

In order to evaluate the biological activity in more detail and to establish a structure–activity relationship, it was necessary to obtain pure saponins. Therefore, a semipreparative high perfor-

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mance liquid chromatography (HPLC) method was developed to separate this mixture in its components without structure deterioration. Fourteen closely related oleanane-type triterpenoid saponins were obtained and their mass spectra were recorded.

2. Experimental

2.1. Solvents, chemicals

Acetonitrile (HPLC grade) was obtained from Lab-scan; trifluoroacetic acid (Uvasol®, for spectroscopy) was from Merck. Deionised water was used (milli-Q quality, Millipore).

2.2. HPLC equipment

A liquid chromatograph (Shimadzu) with two LC-10AD pumps, a CBM-10A communications Bus Module, a manual Rheodyne injector, a guard and an analytical column was used for the separation on analytical scale. The compounds were detected using a SPD-M10A Shimadzu diode-array detector (DAD) (λ_{max}, 210 nm).

The semipreparative separation was performed on an apparatus constructed with the following parts: two Varian 2010 HPLC pumps, a Varian 2020 gradient programmer, a Waters U6K manual injector, a guard and a semipreparative column, a Pye Unicam PU 4020 UV detector and a Waters Data Module type 730.The columns used were:

- guard column: Waters refillable guard column; Delta-Pak™ C_{18}, 15 μm, 300 Å
- analytical column: VYDAC™ RP C_{18}, 5 μm, 300 Å (250 × 4.6 mm)
- semipreparative column: VYDAC™ RP C_{18}, 5 μm, 300 Å (250 × 10 mm).

2.3. Liquid chromatographic conditions

2.3.1. Mobile phase

The mobile phase was prepared gravimetrically. When preparing H_2O containing 0.06% trifluoroacetic acid (TFA)(solvent A), ~0.3000 g TFA was filled up to 500 g (pH = 2.24 at ± 5°C) with water in a 500 ml bottle. Acetonitrile (ACN) containing 0.06% TFA (solvent B) was prepared in the same way by filling the 500 ml flask up to 391.0 g with ACN. To degas the solvents, the bottles were put in an ultrasonic bath for at least 15 min. The mobile phase was cooled down during chromatography in an ice bath.

2.3.2. Injection volume–solvent system-flow rate

Analytical:

- Injection volume: 10 μl.
- Flow rate: 1 ml min^{-1}
- Solvent system:

Mixture: a solution of the saponin mixture was injected and analysed using a gradient from 35 to 56% solvent B in 30 min.

Fractions IV, V and VI: these fractions were further separated using a mobile phase containing 34% of solvent B in solvent A.

Fraction VII: Fraction VII was separated into its components isocratically by a mobile phase consisted of solvent A and B (62:38, v/v).

Preparative:

- For the mixture, the injection volume was scaled up to 1 ml. The load of fraction IV was ~125 μl. Approximately 200–250 μl of fractions V and VI and ~300 μl of fraction VII was injected.
- Flow rate: 5 ml min^{-1}; flow rate programme for fraction IV: 0–14 min: 5 ml min^{-1}, 14–20 min^{-1}: decreasing from 5 to 2 ml min^{-1}.
- The solvent systems were the same as for the analytical scale.

2.4. Mass spectroscopy

Negative–ion fast atom bombardement (FAB) mass spectra using glycerol as liquid matrix were recorded on a Fisons VG70SEQ mass spectrometer [1].

2.5. Extraction and isolation

Dried and powdered leaves of *Maesa lanceolata* were exhaustively extracted with aqueous

methanol (90% v/v). The methanol extract was partitioned between *n*-hexane, *n*-butanol and water. Using herpes simplex virus type 1 as target, a bioassy guided fractionation of the *n*-butanol extract by column chromatography on Silicagel 60 (Merck) and Sephadex® LH-20 (Pharmacia), led to the isolation of the saponin mixture [1].

2.6. Sample preparation

Analytical: ~1 mg of each sample to be separated was disolved in 1 ml H_2O/ACN (80:20, v/v) containing 0.06% TFA.

Semipreparative:

Mixture: ~7 mg was disolved in 1 ml H_2O/ACN (80:20, v/v) containing 0.06% TFA

Fraction IV: ~7.5 mg was disolved in 1 ml H_2O/ACN (80:20, v/v) containing 0.06% TFA

Fraction V: ~7.5 mg was disolved in 1 ml H_2O/ACN (66:34, v/v) containing 0.06% TFA

Fraction VI: ~3 mg was disolved in 1 ml H_2O/ACN (66:34, v/v) containing 0.06% TFA

Fraction VII: ~2.5 mg was disolved in 1 ml H_2O/ACN (62:38, v/v) containing 0.06% TFA

All samples were kept in a ultrasonic bath for 1 min. Fraction VI had to be gently heated in a water bath until a clear solution was obtained.

3. Results and discussion

In order to establish the best conditions for HPLC, the mixture of saponin was first analysed using both normal and reversed phase (C_{18}) thin layer chromatography. With reversed phase TLC a better separation was achieved, so the mixture was analysed on the analytical column using a gradient from 0 to 100% of solvent B in 60 min. This chromatogram (not shown) contained peaks due to saponins as well as peaks due to other minor components which were not further investigated. Peaks due to saponins were characterised by their UV spectra from DAD. This is illustrated in Fig. 1, which shows some examples of spectra of saponins (a) and of spectra of some other minor components (b). On this basis a gradient system, increasing concentration from 33 to 56% of solvent B in 30 min was chosen to separate the saponins. A representative chromatogram of saponins was given in Fig. 2.

There are several reasons for using a column with pore size 300 Å. Firstly, there is a high degree of structural similarity between the saponins of *Aesculus hippocastanum* and the saponins of *Maesa lanceolata* [4]. For the development of an HPLC method to determine β-escin in *Aesculus hippocastanum* extracts and preparations, four colums have been evaluated before [5]:

- Hibar Lichrospher® 100CH-18/2, 5 μm, 100 Å (125 × 4 mm) (Merck).
- Ultrasphere® ODS RP-18, 5 μm, 100 Å (250 × 4.6 mm) (Altex).
- Supelcosil® LC-318, 5 μm, 300 Å (50 × 4.6 mm) (Supelco).
- Vydac® 218TP54, 5 μm, 300 Å (250 × 4.6 mm) (Sigma).

Results suggested that columns with a pore size of 300 Å gave the best separation for saponins due to the larger intrinsic load capacity of column and a stronger interaction between the molecules and the stationary phase. The C_{18} coated Vydac, which shows an unique selectivity inherent to the siliceous matrix, achieved the best resolution. The unique process by which Vydac TP silica beads are manufactured results in a long column lifetime and no measurable phase leaching. Another advantage of this manufacturing process is the stability of the column in mobile phases containing up to 0.5% TFA [5–7]. In addition, Beutler proved in his study on the separation of high molecular weight saponins of *Archidendron ellipticum* that the pore size of the stationary phase played a key role in the separation and the wide pore 300 Å column appeared to yield the best resolution [8]. Finally, it has been proved that better separation was achieved for macromolecules such as nucleotides, peptides and proteins with wide pore columns [9,10].

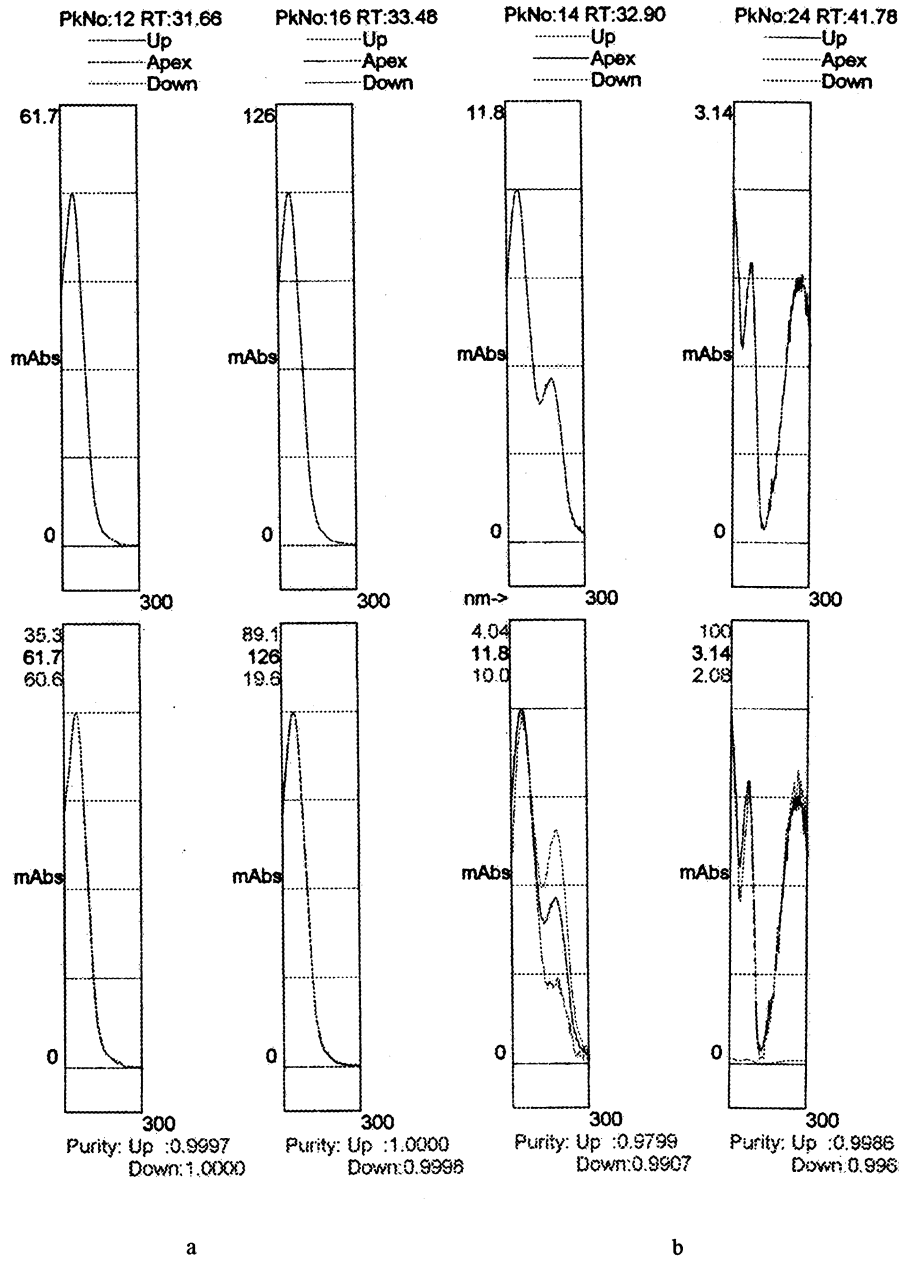

Fig. 1. (a) UV spectra of saponins; (b) UV spectra of other minor compounds which were not further investigated.

Various practical priviledges favoured the use of TFA above any other acid (e.g. phosphoric acid). It is an ion-pairing agent, volatile, suppresses the ionization of silanol groups, enhances the stability of the matrix and has a low UV absorbance [5].

A rarely investigated but important factor in the performance of HPLC colums is temperature which influences the thermodynamic and hydrodynamic properties of the column [11]. Welsch et al. stated that a decrease of resolution is due to viscous heating in the column [12]. Another con-

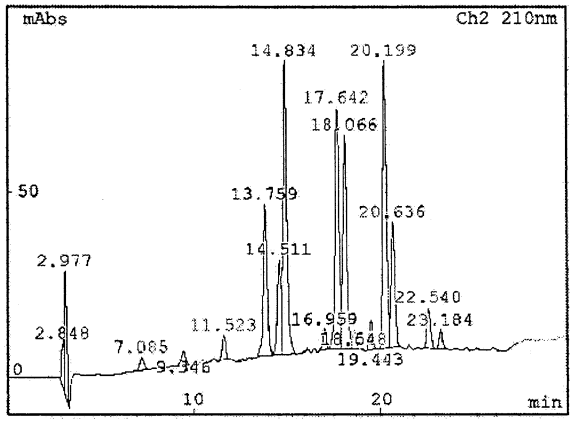

Fig. 2. Analytical separation of the triterpenoid saponin mixture.

siderable bandspreading effect in preparative columns is the distribution in inlet and outlet frits. By sub-cooling the eluent it is possible to compensate viscous heating as well as distribution problems in the column. Brandt et al. considered temperature control to be an interesting tool for the preparative separation of substances [11]. Bubble formation was also suppressed in this way.

To scale-up the separation, the flow rate and the load had to be adjusted. To maintain the same retention times when scaling, the flow rate needed to be scaled proportionally. The preparative flow rate was determined by the following equation [13]:

$$F_P = F_A \times \frac{L_P}{L_A} \times \frac{d_P^2}{d_A^2}$$

where F is the volumetric flow rate, L an d are the respective column lengths and diameters of the analytical (A) and preparative (P) columns. For our columns, this gave the following:

$$F_P = 1 \text{ ml min}^{-1} \times \frac{250}{250} \times \frac{10^2}{4.6^2} = 4.7 \text{ ml min}^{-1}$$

Thus, a flow rate of 5 ml min^{-1} was used. The amount of saponin molecules to be separated was increased to determine the maximum load that retained adequate resolution and purity [13]. Using the same gradient as on the analytical scale, 1 ml of the saponin mixture solution was analysed.

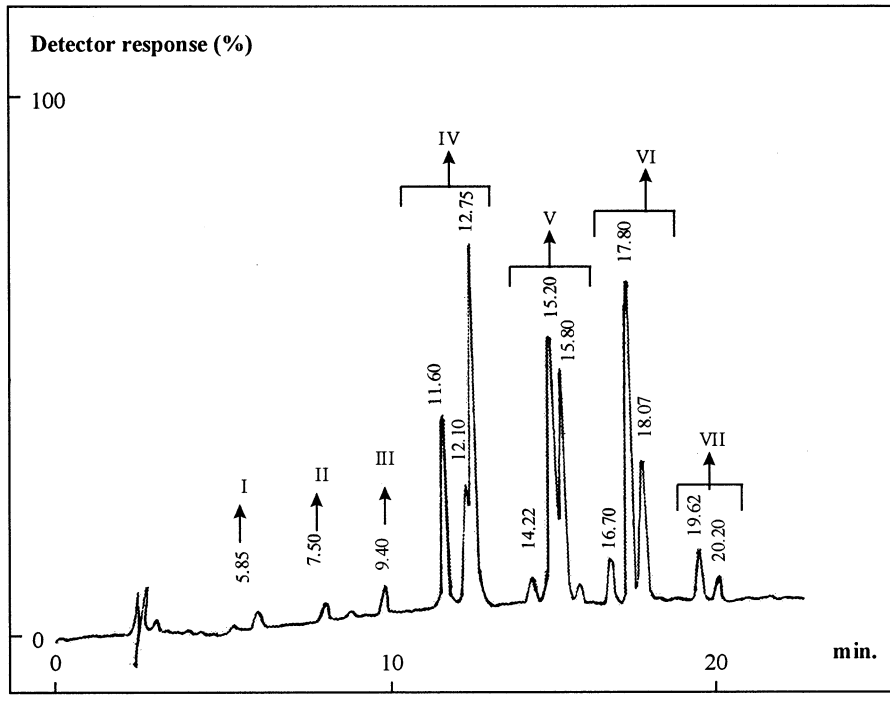

Fig. 3. Separation of the triterpenoid saponin mixture on the semi-preparative Vydac column (load 25 μl of a 5 mg ml^{-1} solution in 20% of solvent B in solvent A, flow rate 5 ml min^{-1}): assignment of the fraction numbers I–VII.

The mixture (760 mg) was treated in this way; seven fractions (I–VII) were obtained and freeze-dried. In Fig. 3, the assignment of the fraction numbers (I–VII) is shown. The triterpenoid saponin mixture obtained from the leaves of *Maesa lanceolata* was separated through the procedure given in Chart 1. Fractions I (8 mg), II (10 mg) and III (18 mg) had a M_r of 1234, 1276 and 1306, respectively. Fraction IV (226 mg) was further separated in its three components using an isocratic system of 34% of solvent B. To obtain a good separation, it was necessary to decrease the flow rate during the analysis to 2 ml min^{-1}. Out of a total of 100 mg fraction IV, 28.3 mg of subfraction IV_1 (M_r 1248), 14.7 mg of subfraction IV_2 (M_r 1318) and 42.4 mg of IV_3 (M_r 1290) were obtained after freeze-drying. 80 mg of fraction V (143.1 mg) was further separated in three subfractions: 1.8 mg of V_1 (M_r 1260), 27.8 mg of V_2 (M_r 1332) and 22.3 mg of V_3 (M_r 1304). From 45 mg of fraction VI (88.9 mg), 2.2 mg of VI_1 with M_r 1338, 16.2 mg of VI_2 with M_r 1316 and 14.5 mg of VI_3 with M_r 1346 was obtained after separation and freeze-drying. Fraction VII (16.2 mg) was separated into its two components using a 38% B isocratic system. 14 mg of fraction VII delivered 7.0 mg of VII_1 (M_r 1358) and 2.9 mg of VII_2 (M_r 1314).

After freeze-drying, the purity of all of the obtained fractions and subfractions was checked by means of analytical HPLC.

Complete structure elucidation will be reported elsewhere in due course.

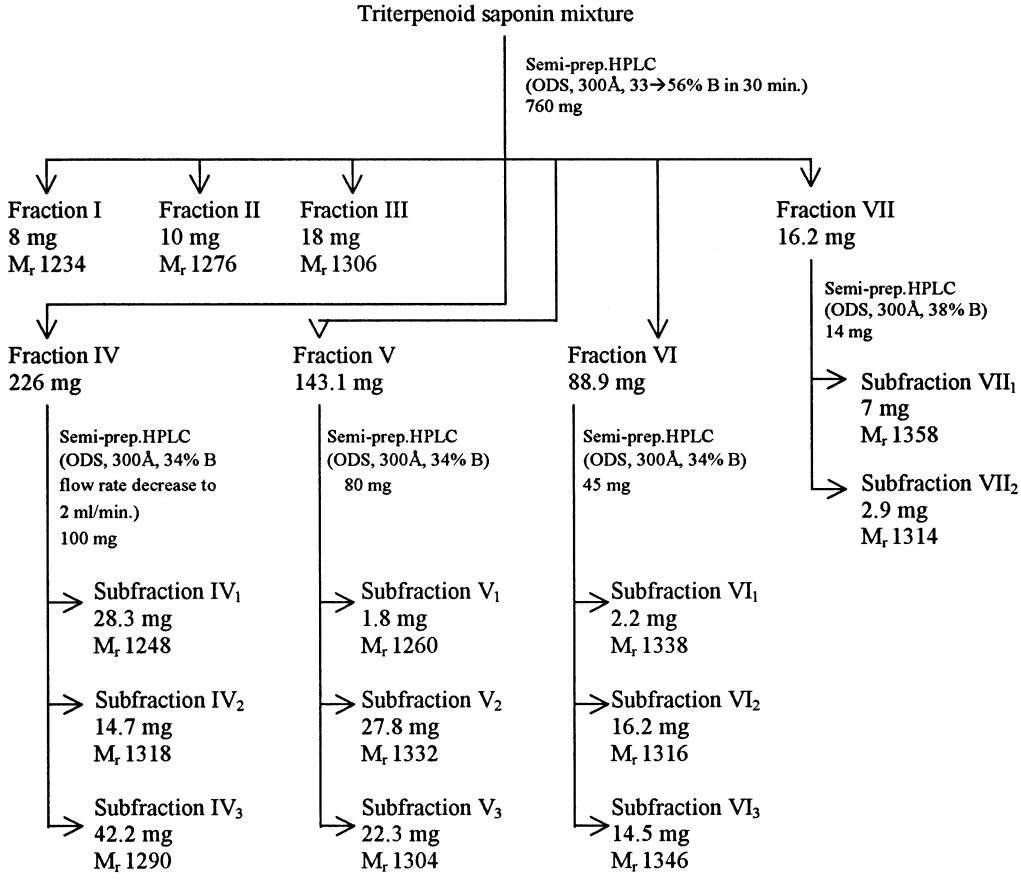

Chart 1: Separation procedure of the triterpenoid soponin mixture obtained from the leaves of *Maesa lanceolata*

4. Conclusions

The triterpenoid saponin mixture from *Maesa lanceolata* was successfully separated into its 14 components, whereas originally only seven were reported [1]. Two chromatographic factors, pore size and temperature of the mobile phase, are shown to be important in the separation of closely related saponins. Alteration of the flow rate during chromatography also improved the separation of components which were difficult to separate.

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