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Determination of saponins in *Maesa lanceolata* by LC-UV: Development and validation

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Abstract

Triterpene saponins are a class of plant natural products with a wide range of bioactivities, which makes them an interesting research subject. The small tree *Maesa lanceolata*, growing in African countries, is used in traditional medicine against various diseases. In previous work a triterpenoid saponin mixture was isolated from the leaves of *M. lanceolata* and the compounds were identified as closely related oleanane type triterpenes [Apers, S., Foriers, A., Sindambiwe, J.B., Vlietinck, A., Pieters, L., 1998. Separation of a triterpenoid saponin mixture from *Maesa lanceolata*: semi preparative reversed-phase wide pore high performance liquid chromatography with temperature control. J. Pharm. Biomed. Anal. 18, 737; Apers, S., De Bruyne, T.E., Claeys, M., Vlietinck, A.J., Pieters, L.A.C., 1999. New acylated triterpenoid saponins from *Maesa lanceolata*. Phytochemistry 52, 1121]. The compounds showed virucidal, haemolytic, molluscicidal and antiangiogenic activity [Apers, S., Baronikova, S., Sindambiwe, J.B., Witvrouw, M., De Clercq, E., Vanden Berghe, D., Van Marck, E., Vlietinck, A., Pieters, L., 2001. Antiviral, haemolytic and molluscicidal activities of triterpenoid saponins from *Maesa lanceolata*: establishment of structure–activity relationships. Planta Med. 67, 528; Apers, S., Bürgermeister, J., Baronikova, S., Vermeulen, P., Paper, D., Van Marck, E., Vlietinck, A.J., Pieters, L.A.C., 2002. Antiangiogenic activity of natural products: *in vivo* and *in vitro* test models. J. Pharm. Belg. 57 (Hors-série 1), 47]. Here we report the development of an extraction and quantification method to analyse saponin compounds in roots and leaves of *M. lanceolata*. After a purification step using C_{18} solid phase extraction (SPE) cartridges, the samples were analysed on a LC-UV/MS system. The identification of the peaks from the different saponins was confirmed based on the retention time and mass spectrum. The quantification was performed using the UV signals. The standard oleanolic acid curve was linear over a concentration range of 2.8–140.0 μg/mL. The recovery from the leaves was 94.5%. The precision of the method with respect to time and concentration was acceptable, with relative standard deviation (RSD%) values of 4.9 and 4.3, respectively.

Keywords: *Maesa lanceolata*; Myrsinaceae; Method development; Method validation; HPLC; Mass spectrometry; Saponin

1. Introduction

Maesa lanceoalata is a shrub or small tree growing in many African countries. The plant is used in Rwandan traditional medicine against various diseases including infectious hepatitis, bacillary dysentery, impetigo, some types of dermatoses and neuropathies. Bioassay guided fractionation of the methanol extract of the dried leaves resulted in the isolation of a triterpenoid saponin mixture. All saponins identified contained the same glycan part in position 3 of the aglycon, and the same oleane-derived triterpenoid (C30) skeleton. The saponins showed a different esterification pattern at the C16, C21 and C22 hydroxyl groups (Fig. 1) (Apers et al., 1998, 1999). The saponin mixture

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	R1	R2	R3	m/z [M-H]⁻	RT (min)
Maesasaponin I	H	angeloyl	H	1233	13.3
Maesasaponin II	acetyl	angeloyl	H	1275	16.0
Maesasaponin III$_1$*				1304	18.5
Maesasaponin III$_2$	H	angeloyl	acetyl	1275	18.5
Maesasaponin IV$_1$*				1247	21.0
Maesasaponin IV$_2$	acetyl	angeloyl	acetyl	1317	21.6
Maesasaponin IV$_3$	H	angeloyl	propanoyl	1289	21.9
Maesasaponin V$_1$*				1259	24.0
Maesasaponin V$_2$	acetyl	angeloyl	propanoyl	1331	24.7
Maesasaponin V$_3$	H	angeloyl	butanoyl	1303	25.0
Maesasaponin VI$_1$*				1337	26.4
Maesasaponin VI$_2$	H	angeloyl	angeloyl	1315	27.1
Maesasaponin VI$_3$	acetyl	angeloyl	butanoyl	1345	27.5
Maesasaponin VII$_1$*	acetyl	angeloyl	angeloyl	1357	29.4
Maesasaponin VII$_2$				1313	29.9

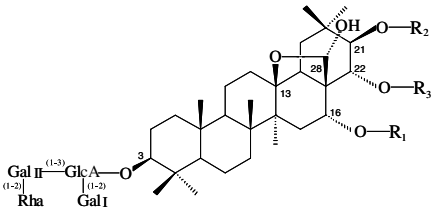

Fig. 1. Structures, m/z $[M-H]^-$ values and retention times (RT) of the known saponins of *M. lanceolata*. *Structures unknown, but compounds characterized based on their molecular mass.

exhibited a moderate virucidal and pronounced haemolytic activity and showed a severe toxic effect on *Biomphalaria glabrata* snails (molluscicidal activity). Testing of the *Maesa lanceolata* saponin mixture for antiangiogenic activity in the chick embryo chorioallantoic membrane (CAM)-assay also gave promising results. Structure–activity relationships for the virucidal, haemolytic, molluscicidal and antiangiogenesis activities were established (Apers et al., 2001, 2002; Sindambiwe et al., 1998). Unfortunately, maesasaponin II, the best angiogenesis inhibitor showing only a low membrane irritating effect and no haemolytical activity, was only a minor compound of the mixture.

In view of creating transgenic plants and cells with a higher production in maesasaponins and in maesasaponin II in particular, a method to quantify all known saponins in *M. lanceolata* needed to be developed. Furthermore, because the method is compatible with a LC-UV/MS system, newly formed saponins could be discovered based on their molecular mass and MS/MS spectra.

2. Results and discussion

The major challenges in developing a new quantitative method for assaying medicinal plants are: (1) the unknown content of the molecule of interest in the sample, which urges the completeness of extraction, (2) the high variability of content due to the influences of growth circumstances, time of harvest of the plant material or the conditions used in the *in vitro* cell cultures, what demands methods that cover a big range, and (3) the lack of commercial available reference substances.

Creating a new method includes two steps: the development and the validation. Both are described below for the quantification method of saponins in *M. lanceolata*.

2.1. Method development

The extraction and separation of the triterpenoid saponin mixture from *M. lanceolata* dried leaves were already described in previous articles (Sindambiwe et al., 1996; Apers et al., 1998). Tests on the composition of the extraction solvent, the time of extraction, the number of repetitions and the extraction method, were evaluated to develop a quantitative method by which extraction till total exhaustion is guaranteed. Due to the complexity of the extract obtained and in order to remove interfering compounds, the use of a SPE column appeared necessary as a sample clean up step, before bringing the sample on the HPLC-UV/MS system. The volume and the MeOH concentration of the washing steps were tested. It was proven, based on TLC profiles, that washing steps with MeOH 30% (v/v) removed interfering compounds, while the saponins of *M. lanceolata* remained on the cartridge. The saponins were recovered from the cartridge by using MeOH as eluens, leaving lipophilic compounds on the cartridge. The HPLC gradient was optimised, using a Vydac column with a smaller diameter (3.2 mm) than described in Apers et al. (1998). This smaller diameter resulted in a lower flow, making the method compatible with LC–MS conditions, and in a better separation of the saponin mixture (Fig. 2). Another adaptation of the method was the acid used in the mobile phase, i.e. TFA was replaced by HCO_2H since TFA is known to suppress ionisation, necessary for MS.

In short, in the final method the saponins were extracted from the plant material by boiling the powdered plant

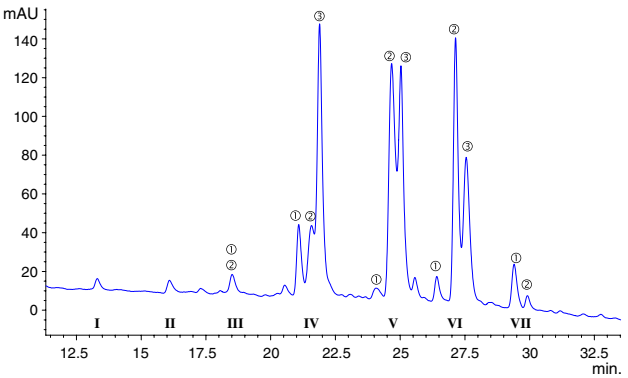

Fig. 2. UV chromatogram of the mixture MC3B1. The compounds are numbered as in Fig. 1.

material under reflux in MeOH 50% (v/v). This procedure was repeated using the same volume of fresh solvent. Both extracts were combined and the solvent was removed in vacuum. The residue was redissolved in H_2O and cleaned up using a SPE C_{18} cartridge. The resulting solution was injected on the HPLC-UV/MS system. Compounds were identified based on their retention time and MS spectra and quantified using UV-detection.

2.2. Method validation study

In order to prove that the final method was suitable for its intended use, the method was validated on the dried (wild) leaves of *M. lanceolata* according to the ICH guidelines on the validation of analytical methods (ICH guidelines, 1994, 1996).

2.2.1. Calibration model – response function

Because the saponins of *M. lanceolata* are not commercially available, a compound with similar properties and structure, namely oleanolic acid was selected as standard. This compound is less polar than the maesasaponins and elutes later from the C18-RP column (retention time: 49.7 min). In the samples currently under investigation, oleanolic acid is not present and could be added as an internal standard (IS) to the sample. Since in the transgenic plants and cells that will be constructed, we are not sure though that oleanolic acid will not be present, it is used as an external standard.

The linearity was examined by analysing oleanolic acid reference solutions at eight levels (2.8–140.0 μg/mL, in duplicate). For assessing the linearity the least square line and the correlation coefficient were calculated. The calibration curves (area versus concentration) obtained were tested on slope ($a \neq 0$) and intercept ($b = 0$) by means of the Student's *t*-test. In order to evaluate the lack of fit of the linear model a lack-of-fit (LOF) test was performed and the residuals graphically examined (Fig. 3).

The results are shown in Table 1. The LOF test did not accept the linearity of oleanolic acid in the range tested, however graphical examination of the residuals and the correlation coefficient proved the method to be linear. The slope of the curve was significantly different from 0. The *t*-test revealed that point (0,0) fell within the calibration curve, therefore a single-point calibration was justified.

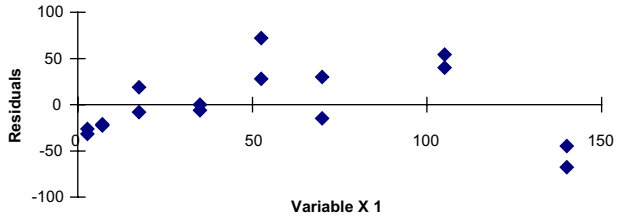

Fig. 3. Residual plot of the oleanolic acid reference solutions, injected in duplicate in eight different concentrations. The residuals are randomly scattered.

Table 1
Overview of the linearity data of oleanolic acid

	Oleanolic acid
Correlation coefficient	0.9994
Slope ± standard error	110.7 ± 0.21
Intercept ± standard error	18.2 ± 15.0
Confidence interval (95%)	−14.1 to 50.5
F_{LOF} ($F_{crit} = 4.3$)	9.0
Range (μg/mL)	2.8–140
Number of standards (duplo)	8

2.2.2. Accuracy

Accuracy is often calculated as percent recovery by the assay of known, added amount of analyte to the sample. Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels.

The accuracy of the method was investigated by means of a recovery experiment, adding the saponin mixture, isolated from the leaves in previous work (Sindambiwe et al., 1996), to the samples at the start of the extraction. A mean recovery % ($n = 9$) of 92.0% (RSD% = 7.3%) (Fig. 4) was obtained. This was still acceptable, considering the complexity of the method, i.e. all the different steps during extraction and purification.

2.2.3. Precision

Here, the precision was investigated at two levels: the repeatability (precision under the same conditions over a short interval of time) and the intermediate precision (investigating the effect of performing the analysis on different days).

The repeatability and interday intermediate precision were determined by analysing six independently prepared samples (100%) following the below-described method on three different days. The standard deviation and relative standard deviation (RSD%) were calculated for each day. In order to evaluate the results from the three different days, the results were analysed by means of an ANOVA single factor. Within and between day RSD%'s were

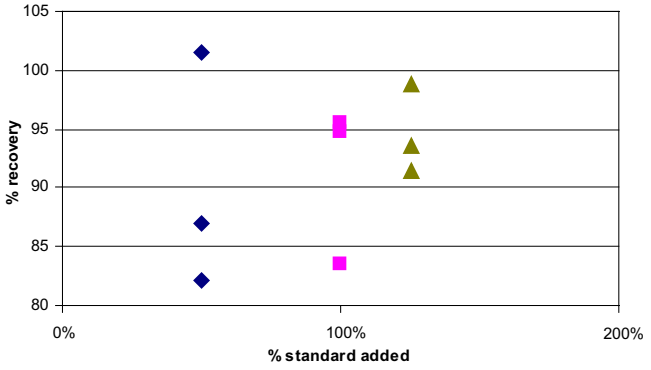

Fig. 4. Recovery percentage of samples with 50%, 100% and 125% of MC3B1 mixture. A mean recovery of 92.0% (RSD% = 7.3%) was obtained.

Table 2
Validation data: precision

Parameter	Maesasaponins		
Precision on different days (n = 3)			
Repeatability			
Number of replicates	6		
Mean content (%)	1.16	1.17	1.12
RSD% (day1/day2/day3)	4.61	3.86	5.47
Intermediate precision			
Number of days	3		
Number of replicates	6		
$RSD\%_{between\ groups}$/Horwitz[a]	4.92	2.50	
F_{calc} ($F_{crit} = 3.68$)	1.69		
Precision on concentration levels			
Repeatability			
Number of replicates	6		
Mean content (%) (50%/200%)	1.14	1.15	
RSD% (50%/200%)	3.04	4.03	
Intermediate precision			
Number of days	5		
Number of replicates	6		
$RSD\%_{between\ groups}$/Horwitz[a]	4.30	2.50	
F_{calc} ($F_{crit} = 2.76$)	1.11		

[a] 2/3 $RSD\%_{Horwitz}$.

calculated. Obtained RSD% values on each day are summarized in Table 2. These results showed that the developed method showed acceptable repeatability. From the statistical point of view, the ANOVA proved that there was no significant difference between the results obtained on the three different days. The RSD%'s (within and between) were higher than the limit set by Horwitz (Albert and Horwitz, 1997), but still smaller than 5%, which is an acceptable limit for methods with several steps. Therefore, the method can be considered precise.

In order to evaluate the precision of the method over the whole range, six samples weighing half of the mass (50%, i.e. 0.75 g) and six samples weighing double (200%, i.e. 3.0 g) were analysed according to the method. The standard deviation and RSD% were calculated for each level. The variations at these concentrations were compared with the variation at 100% by the Cochran's test. This test revealed that the variation is equal over the whole range of the method. Furthermore, the results were analysed by an ANOVA single factor to evaluate the results obtained on the three levels. This ANOVA showed that the results on the three levels were not significantly different. Within and between level RSD%'s were calculated. Results are shown in Table 2. The RSD%'s (within and between) were higher than the limit set by Horwitz (Albert and Horwitz, 1997), but still smaller than 5%. Because we were working with plant material and a lot of extraction and purification steps were necessary, we still consider these results as repeatable in the range of 50–200%.

To summarize, the dried leaves contained on average 1.2% of the known saponins. The precision of the determination was acceptable with a $RSD_{between\ days}$ of 4.9% and a $RSD_{between\ levels}$ of 4.3%.

Table 3
Percentage content of saponins in *M. lanceolata* leaves and roots

	Leaves wild type	Leaves greenhouse	Roots greenhouse
MSI			
MSII	0.005		
MSIII	0.012		
$MSIV_1$	0.250		0.453
$MSIV_2$		0.019	
$MSIV_3$	0.238	0.086	0.034
MSV_1	0.030		0.091
MSV_2	0.066		
MSV_3	0.145	0.819	0.396
$MSVI_1$	0.023		
$MSVI_2$	0.285	4.030	0.295
$MSVI_3$	0.064		
$MSVII_1$	0.059	0.380	0.098
$MSVII_2$	0.066		
Total%	1.2 ± 0.1	4.9 ± 0.8	1.5 ± 0.3

2.2.4. Specificity – selectivity

In the present study, the specificity of the analytical method was proven by the technique used, i.e. LC-UV/MS. Based on the UV-chromatogram of the standard saponin mixture, the retention time of the 14 compounds (Apers et al., 1998, Fig. 1) was defined. Moreover, in the negative ion modus, the ions $[M-H]^-$ of these 14 different components could be identified by mass spectrometry in the purified saponin mixture (Fig. 1), as well as in the samples tested.

2.3. Analysis of leaves and roots of greenhouse plants

In a preliminary experiment, leaves and roots of greenhouse grown *M. lanceolata* plants were analysed for their saponin content (Table 3). In the leaves of *M. lanceolata,* maesasaponin IV_2, IV_3, V_3, VI_2 and VII_1 were observed. Clearly the amount of total saponins in young leaves of greenhouse grown plants ($4.9\% \pm 0.8$; $n = 3$) was much higher than the amount in leaves of the in-nature-grown, older tree ($1.2\% \pm 0.1$; $n = 18$). As the saponin content depends on several factors such as the cultivar, the age, the physiological state, etc. (Hostettmann and Marston, 1995), here the different growth conditions and the difference in age of the plants could explain the difference in saponin content. Furthermore an infection of the greenhouse plants with woolly aphid was reported. Since it was previously described that saponins have insecticidal activity (for review see Hostettmann and Marston, 1995), this could also be a reason for the higher concentration of saponins in the greenhouse grown plants.

In the roots of the greenhouse plants, maesasaponin IV_1, IV_3, V_1, V_3, VI_2 and VII_1 were detected. The total saponin content was lower than in the leaves of the same plants, namely $1.49\% \pm 0.25$.

3. Conclusions

A LC-UV method was developed to determine the saponin content in *M. lanceolata*. A sample preparation step using a C18 SPE cartridge was necessary to eliminate interfering compounds of the total extract. The method was validated according to the ICH guidelines and shown to be linear within the established ranges. Furthermore, the repeatability and accuracy were acceptable, considering all the different sample preparation steps. Therefore this method can be used for quantitative purposes to asses transgenic plants. Since this method is compatible with LC-UV/MS, it can also be used for the discovery of new compounds produced by these plants.

4. Experimental

4.1. General experimental procedures

MeOH for HPLC, CH_3CN HPLC far UV and HCO_2H (p.a.) were purchased from Acros organics (NJ, USA) and distilled water (RiOs) was prepared with a Millipore water purification system (Millipore, Bedford, MA, USA).

Oleanolic acid (HPLC) (Extrasynthèse, Genay, France) was used as an external standard, in a concentration of 70.0 μg/mL, solved in MeOH 80% (v/v).

HPLC analysis was performed on an Agilent 1100 with a diode array detector using a silica-based 300 Å monomeric C18 column (Grace Vydac, Hesperia, USA) (250 × 3.2 mm, 5 μm); detector: 210 nm; flow rate: 0.5 mL/min; solvent A: 0.05% HCO_2H; solvent B: CH_3CN + 0.05% HCO_2H; gradient: 10 min 25%B – from 25%B to 60%B in 30 min – from 60%B to 90%B in 7 min – from 90%B to 25%B in 3 min – 5 min 25%B; injection volume: 20 μL. This HPLC system was coupled to a Bruker esquire 3000 plus ion trap MS (Bruker Daltonics, Billerica, USA). The samples were brought into the MS via an ESI source and measured in negative ion modus, using a capillary voltage of 4500 V and a capillary exit voltage of −187 V. Nebulization was achieved using nitrogen gas at a pressure of 50 psi. The capillary temperature was set at 365 °C. Mass spectra were recorded over the range m/z 50–2200. The ion trap was operated under an ion current control of 10,000 and a maximum acquisition time of 200 ms.

4.2. Plant material

Leaves of *M. lanceolata* Forsskal var. *golungensis* Welw. were collected in Butare, Rwanda in August 1989. The plant material was identified by Dr. J. Mvukiyumwami of the botanical department of the IRST (Institut de la Recherche Scientifique et Technique), where a voucher specimen is kept.

Leaves and roots of *M. lanceolata* were collected from greenhouse grown plants. The plant material was grown at VIB-Ghent under a day/night regime (16 h–25 °C/8 h–20 °C). Seeds were kindly provided by Frank Mbago, Department of Botany, University of Dar-Es-Salaam (Tanzania).

The maesasaponin mixture isolated from *M. lanceolata* (Sindambiwe et al., 1996; Apers et al., 1998; Fig. 1) was used to develop the HPLC-UV/MS method and for the accuracy experiments.

4.3. Sample preparation

To determine the quantity of saponins in *M. lanceolata* leaves, the samples were dried and powdered. For extraction 1.5 g of plant material was refluxed in 70 mL 50% MeOH (v/v) for 1 h. After filtration the residue was redissolved in 70 mL 50% MeOH (v/v) and refluxed for another hour. This extract was combined with the first fraction and dried under vacuum. In order to clean up the resulting sample, it was dissolved in 10.0 mL H_2O under sonification. This solution was centrifuged (10 min, 3000 g) and 2 mL was brought on a Chromabond® SPE C_{18} cartridge (1000 mg) (Machery-Nagel, Germany) preconditioned with MeOH and water. After sample application the column was successively rinsed with 6 mL water and 18 mL MeOH 30% (v/v). Finally the saponins were eluted with 12.0 mL MeOH 100%. This fraction was dried under vacuum and redissolved in 2.0 mL MeOH 80% (v/v).

4.4. Preparation of the oleanolic acid standard solution

About 3.5 mg of oleanolic acid was accurately weighed in a volumetric flask of 25.0 mL and dissolved in MeOH 80% (v/v). 5.0 mL of this solution was diluted to 10.0 mL with MeOH 80% (v/v).

4.5. Validation

The method was validated according to the ICH guidelines on the validation of analytical methods (ICH guidelines, 1994, 1996). All results were expressed as percentages, where n represents the number of values. For the statistical analysis Excel 2000 (Microsoft Office) was used. A 5% level of significance was selected.

4.5.1. Linearity

Reference solutions were prepared at eight concentration levels, ranging from 2.8 to 140.0 μg/mL. Each concentration was analysed twice.

4.5.2. Accuracy

The accuracy of the method was investigated by means of a recovery experiment: to 50% of the leaves (0.75 g), the maesasaponin mixture was added on three different concentration levels (50%, 100% and 125%) at the start of the analysis. For each of the three concentrations, three samples were analysed according to the developed method.

4.5.3. Precision

The repeatability and the interday intermediate precision were determined by analysing six independently prepared samples (100%; 1.5 g) according to the above described method on three different days. In order to evaluate if the precision of the method is equal over the whole range, six samples weighing half of the normal mass (0.75 g) and six samples weighing the double of it (3.0 g) were analysed according to the method developed.

Acknowledgments

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