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Rapid HPLC Analysis for Quantitative Determination of the Two Isomeric Triterpenic Acids, Oleanolic acid and Ursolic acid, in *Plantago Major*

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Abstract

Plantago major has been used in traditional medicine for the treatment of various diseases. In order to evaluate the quality of *P. major*, a simple, rapid and accurate high-performance liquid chromatography (HPLC) method was developed for the assessment of the two bioactive triterpenic acids: oleanolic acid (OA) and ursolic acid (UA). A LiChrosorb octadecylsilyl silica (ODS) column with methanol, tetrahydrofuran and aqueous acetic acid as mobile phase and detection at 220 nm were employed for HPLC analysis. The method is precise with relative standard deviations for these two constituents that ranged between 0.5–1.1% (intraday) and 0.8–1.8% (interday). The contents of these two phytochemicals in the leaves of *P. major* growing in Friuli Venezia Giulia Region were determined in order to establish an effective and reproducible method.

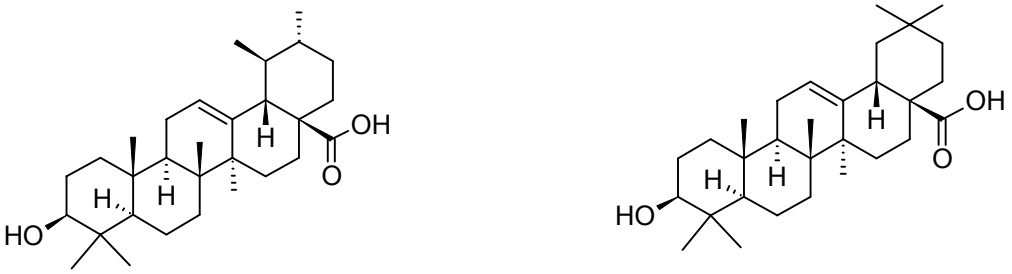
Keywords

Analysis • *Plantago major* • HPLC • Oleanolic acid • Ursolic acid

Introduction

Plantago major L. is a perennial plant that belongs to the *Plantaginaceae* family. The leaves are traditionally used as wound healing remedy and in the treatment of a number of diseases, including skin, respiratory, digestive and circulatory affections. *P. major* contains biologically active compounds such as polysaccharides, lipids, caffeic acid derivatives, flavonoids, iridoid glycosides and terpenoids. Oleanolic acid (OA) and ursolic acid (UA) (Figure 1) are the two major terpenoids of *P. major* [1]. These compounds, ubiquitous in plant kingdom, are known to possess different biological activities that could be involved in the therapeutic effects of *P. major* leaves and of other herbal preparations containing these secondary metabolites [2]. In particular, OA shows antifungal [3], anti-inflammatory [4, 5], anti-HIV [6, 7], diuretic [8], glucose-lowering [9], and anticancer [10] activities. UA also posses anti-angiogenic [11], anticancer [10, 12], and anti-inflammatory [13] activities.

Although the discovery of *P. Major* health-promoting effects has been well described, the content of OA and UA in extracts of *P. Major* leaves should be accurately determined in order to investigate the extent to which these active components are absorbed, their pharmacokinetics and bioavailabilities. Thus, the development of a rapid and reproducible analytical method for the quantitative determination of the structural isomers UA and OA in *P. Major* crude extracts is required.



ursolic acid oleanolic acid

Fig. 1. Chemical structures of UA and OA.

Although different approaches for the analysis of OA and UA in various medicinal plant extracts have been reported, the separation and quantification of these triterpenic acids in natural extracts remains still difficult.

For example, a micellar electrokinetic capillary chromatography (MECC) [14] method was used to determine OA and UA isomers content in *Ligustrum lucidum* Ait; a reliable and reproducible liquid chromatography–mass spectrometric assay [15] (LC–MS) was developed for the determination of UA in laboratory-made mixtures, in leaves and twigs extracts of *Staphylea holocarpa* Hemsl; Anandjiwala *et al.* [16] have ursolic and oleanolic acids, from the leaves of green and black varieties of *Ocimum sanctum* using high-performance thin-layer chromatography (HPTLC) with densitometry and different methods in HPLC gradient [17, 18].

According to the most relevant bibliography, HPLC is the method of choice for the analysis of these two triterpenic acids because of its versatility, precision and relatively low cost.

However, the proposed HPLC methods were gradient [17, 18] or isocratic [19] analysis and slow down the time of analysis.

In this paper a simple, rapid, and isocratic HPLC method with UV detection was developed and validated for the determination of OA and UA in crude extracts of *P. major*. This method, compared to those described in literature, shows a characteristic composition of the mobile phase due to an increase of the methanol component and to a reduction of aqueous acid component, reducing the time of analysis for OA and UA.

Results and discussion

HPLC conditions optimization

With the aim to develop a method for the quantitative determination of these structural isomers, a raw methanolic extract of *P. Major* leaves was used. To this purpose, top priority was given to the complete separation of the two analytes of interest, OA and UA, from the other components of the extract.

The mobile phase was chosen after several trials with acetonitrile, methanol, tetrahydrofuran and water in various proportions and at different pH values. After investigation, a mobile phase consisting of methanol – water – tetrahydrofuran (94:5:1 v/v), pH 5 adjusted with acetic acid was finally selected in order to achieve optimal separation, high sensitivity and good peak shape.

A typical chromatogram is reported in Figure 2 which illustrates the separation of the two acid isomers in standard solution and in a crude methanolic extract from *P. major* leaves.

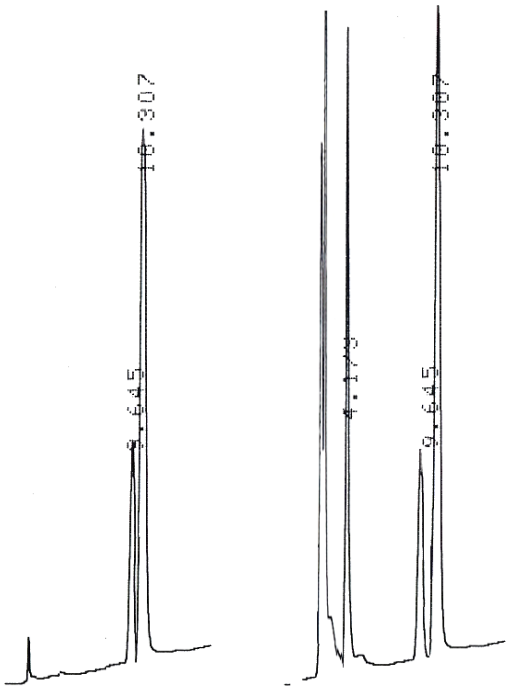
Gradient elution was usually used for the separation of triterpenes in plant tissues, containing different components, difficult to separate during a short period of the run time. Nevertheless, in the present study, OA and UA were well separated using an isocratic mobile phase. Although these different not easily removable compounds, were present in the extract, fortunately, the interference of the relevant peaks for the analytes was negligible because their retention times did not overlay with those of the two triterpene acids analysed. Under the chromatographic conditions described (see Experimental Section), OA and UA were eluted in ca. 11 minutes.

The detection wavelength was chosen at 220 nm since the OA and UA have better absorption and sensitivity at this wavelength.

This chromatographic method is more rapid compared with those reported in literature, under the conditions reported above the ritention time of OA and UA is halved [19].

Calibration and method validation

The HPLC method was validated by defining the linearity, limit of detection (LOD), lower limit of quantification (LLOQ), precision, accuracy, and stability. For qualitative purposes, the method was further evaluated by taking into account the precision of the retention times of the analytes.



a **b**

Fig. 2. a) A typical chromatogram of standard solution: oleanolic acid (RT: 9.645 min), ursolic acid (RT: 10.307 min). b) A typical chromatogram of crude methanolic extract from *P. major* leaves: oleanolic acid (RT: 9.645 min), ursolic acid (RT: 10.307 min).

Standard solutions of the two triterpene acids, in the concentration range 2.5-100 μg/mL, were prepared and analysed. The regression equations of these curves and their coefficients of determination (R^2) were calculated as follows:

$$OA, y = 4E + 06x + 21628, 0.9997$$
$$UA, y = 4E + 06x - 35396, 0.9998$$

The methods showed a linear relationship between peak areas and concentrations over this range for all two compounds.

A signal five times higher than noise was regarded as the detection limit. The LOD and LLOQ of OA and UA were 0.03 and 0.10 μg/mL, 0.1 and 0.30 μg/mL, respectively. Under the present LLOQ, the concentrations of the two triterpene acids could be determined in the extract of leaves, which was sensitive enough to investigate the content of the two isomers.

The intra- and interday precisions (expressed as RSD) and accuracy (expressed as recovery) for the two analytes were determined by spiked samples with the standard solutions of the two triterpene acids (n=6), consecutively, using the analytical method described above. The coefficient variations of intra- and interday studies were both less

than 2.0%. The results of the recovery of OA and UA ranged from 98.9 to 101.6%; the RSD of recovery of the two acids ranged between 1.2 and 2,8%. The precision as well as the reproducibility of this method was satisfactory (Table 1).

When the sample solution was analyzed in the same manner the peaks were identified by comparison of the retention time with those corresponding to authentic samples purified from *P. major*. Regarding the extraction efficiency, three time work-up was sufficient since it allowed an over 98% extraction of two constituents.

The percentage content of OA and UA in *P. major* leaves collected in Friuli Venezia Giulia Region in July 2007 is 0.2% and 0.8%, respectively.

A newly established HPLC isocratic method is validated for the quantification of the bioactive triterpenes OA and UA. The method described here is rapid, precise, reproducible, may be applied to investigations not only of plant material, cell culture lines, herbal preparations, but also of ecological factors influencing the production of active principles and for chemotaxonomical studies.

Tab. 1. Intra- and interday precision and accuracy during method validation.

		Intraday precision [a]		Interday precision [b]	
Compound	**Nominal concentration (µg/mL)**	**Detected concentration (µg/mL)**	**RSD (%)**	**Detected concentration (µg/mL)**	**RSD (%)**
OA	5.2	5.23	1.1	5.27	1.6
OA	30.0	30. 4	0.5	30.8	0.8
UA	16.0	15.95	0.7	15.89	1.8
UA	75.0	74.76	0.6	74.80	1.0

[a] Analysed on the same day (n=6). [b] Analysed on six different days (n=6)

Experimental

Plant material

The plant material (leaves, 1400 g) was harvested in July 2007 in Friuli Venezia Giulia Region (NE-Italy), where *P. major* is very common. A voucher specimen was deposited at the Herbarium of the Department of Biology (TSB-165/4) of the University of Trieste (Italy).

Reagents

The solvents used were analytical or HPLC grade (Aldrich, Milano, Italy) and were used without further purification. Only water, used as mobile phase, was deionised, distilled and filtered through 0.22 µm Millipore (Bedford, USA) before use. The reference compounds, oleanolic acid OA (97%) and ursolic acid UA (90%), were Sigma products (Milano, Italy). The solutions of OA and UA were always freshly prepared.

High-performance liquid chromatography system

The HPLC instrument consisted of a Perkin Elmer (Series 4 Liquid Chromatograph)

equipped with a septumLless injector (Rheodyne Injector 7125S) with a 20 µL sample loop, a multiple wavelength detector (Perkin Elmer LC 235 Diode Array, UV-VIS Digital Spectrophotometric Detector and LC Autocontrol) and a Shimadzu C-R3A Chromatopac integrator. Satisfactory separation was performed using a LiChrosorb RP18 column (5 µm, 250 mm x 4.6 mm i.d., Perkin-Elmer) eluted at a flow rate 1 mL/min with methanol – water – tetrahydrofuran (94:5:1 v/v), pH 5 with acetic acid as mobile phase; the detection wavelength set at 220 nm.

All the determinations were performed at room temperature (22°C) and at isocratic conditions of elution.

Preparation of calibration curves

To prepare standard solutions, OA and UA standards (10.0 mg respectively), accurately weighted, were dissolved in methanol (10 mL). Volumes of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 10 mL of the mixture of oleanolic and ursolic acids standard solution were placed in 100 mL volumetric flasks and methanol added to a final volume of 100 mL. Six samples of 20 µL of each solution were injected six times and run for calibration curves: the ratio of the peak area of the OA and UA to concentration was constant.

Preparation of sample solution

Fresh *P. major* leaves were rapidly powdered (under N_2 liquid), accurately weighted (100 g) and extracted with 300 mL methanol in Soxhlet for 8 h. The extracts were lyophilized in order to avoid chemical degradations. Before use the residue (1.2 g) was dissolved in 10 mL methanol. 1 mL of this solution was placed in a 10 mL volumetric flask and filled up to 10 mL with methanol. The afforded solution was filtered through a 0.45 µm syringe filter prior to HPLC.

Preparation of recovery studies

Tab. 2. Recovery of OA and UA in sample solution.

Standard	Amount added (mg)	Recovery (n=6) Mean	RSD (%)
OA	5.29	101.6	1.6
OA	30.96	100.9	1.2
UA	15.87	98.9	2.8
UA	70.56	99.3	1.4

To tested plant leaves (5 g), appropriate amounts of OA and UA were added to approximately the double contents of two triterpenes in treated materials. The follow-up extractions and HPLC analyses were accomplished in the same manner as detailed above. The recovery (Table 2) was determined as follows:

$$\text{Recovery}\,(\%) = (A - B)/C * 100\%$$

Where, A is the amount of detections above, B is the amount of sample without added standards, C is the added amount of the standards.

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Authors' Statement

Competing Interests

The authors declare no conflict of interest.

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