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ORIGINAL

Novel Method for HPLC Analysis of Triterpenic Acids Using 9-Anthryldiazomethane Derivatization and Fluorescence Detection

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Abstract Triterpenic acids are a group of secondary plant metabolites which are part of the cuticular waxes covering fruits, leaves, and flowers. To date, quantitative analysis of these compounds has often been conducted using high-performance liquid chromatography coupled with spectrophotometric detection or mass spectrometry; however, these methods have some major drawbacks. This paper reports a new method of analysis implementing derivatization with 9-anthryldiazomethane and fluorescence detection. The method consists of the extraction of analytes from a matrix, purification with anion exchanging SPE columns, and an optional step of the alkaline hydrolysis of triterpenic acid esters. The paper also describes a fast and easy method for the synthesis of the derivatization agent. The detection limits of the method presented are approximately 100-fold lower than in a similar method using ultraviolet spectrophotometry as the mode of detection. The recovery and repeatability of the method are at satisfactory levels.

Keywords HPLC-FD · Ursolic acid · Oleanolic acid · Betulinic acid · 9-Anthryldiazomethane

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Introduction

Triterpenic acids are a group of plant secondary metabolites occurring in cuticular waxes covering fruits, flowers, and leaves. These compounds are attributed to the secondary functions of the cuticle; prominent examples are the protection of underlying tissues against UV damage and anti-feeding properties [1]. The most abundant triterpenic acids are oleanolic and ursolic acid. However, a large number of other compounds from this group inter alia: glycyrrhetinic, gypsogenic, betulinic, maslinic, and euscaphic acid are mentioned in the scientific papers [2, 3]. The structures of molecules investigated in this study are presented in Fig. 1.

The relevance of triterpenes is associated with their health-promoting properties. Activities attributed to triterpenes and their derivatives include: the ability to inhibit tumor growth and metastasis formation, antimicrobial properties, antioxidative activity, and the ability to protect internal organs against chemically induced damage [4–6]. Terpenes, along with sterols (other constituents of the cuticle waxes), are the most widely used group of plant-derived drugs with estimated annual sales of 12.4 billion USD [7].

The most commonly used method for the quantification of triterpenic acids is high-performance liquid chromatography coupled with UV/Vis spectrophotometry or mass spectrometry (MS) detection [8–11]. The triterpene molecules lack strong chromophores; therefore, spectrophotometric detection is limited to a wavelength range with a very low specificity (200–220 nm). On the other hand, mass spectrometry equipment is very expensive and requires greater expertise from analysts. An alternative method of detection can be a derivatization of the compounds and use of fluorescence spectroscopy detection. Thus far, such strategy has been used by two research teams [12, 13]. The scientists reported the high sensitivity of the developed methods; however, both

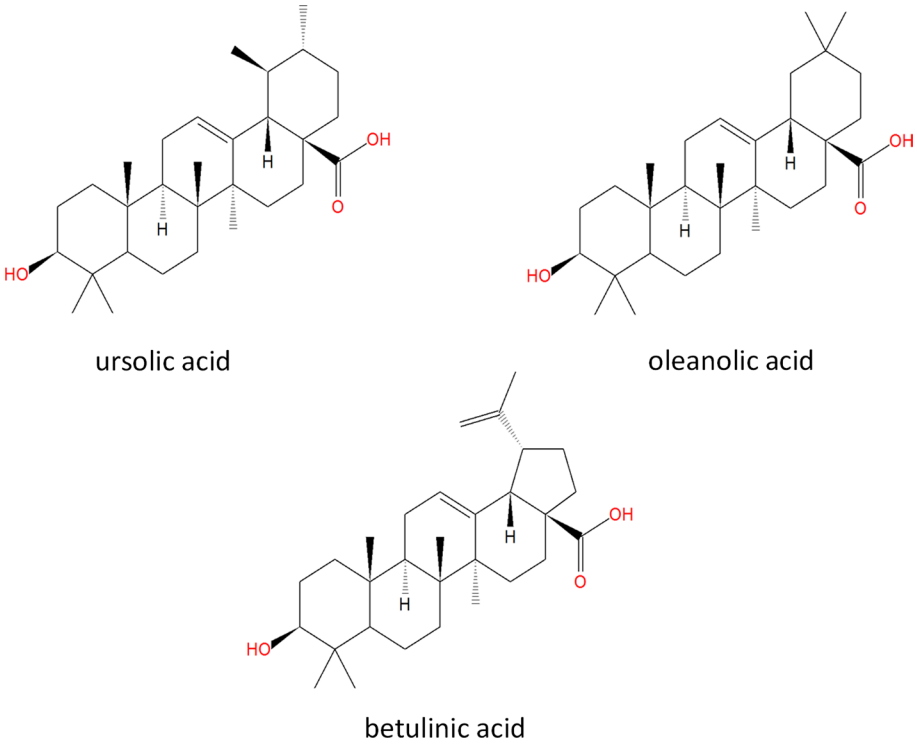

Fig. 1 Structures of selected triterpenic acids

the methods had a major disadvantage—the labeling agents were synthesized in time-consuming multi-step reactions.

ADAM (9-anthryldiazomethane) is a derivatization agent which proved to be useful for the analysis of diverse carboxylic acids with HPLC-FD systems. It has been applied successfully for the quantification of a wide range of the compounds including: fatty acids [14], diarrhetic shellfish poisoning toxins [15], jasmonic acid [16], and vitamin B_7 [17]. The greatest assets of this compound are its high selectivity towards carboxylic groups and mild derivatization conditions [15]. ADAM is available in a commercial sale or can be easily synthetized prior to analysis.

This paper reports a new method of analysis of selected triterpenic acids with the use of the high-performance liquid chromatography coupled with fluorescence spectroscopy. The optimization of parameters of chromatographic separation and the preparation of sample (extraction and purification with Solid-Phase Extraction) were performed.

Materials and Methods

Reagents and Materials

The analytical standards of ursolic, oleanolic, and betulinic acids were bought from Sigma-Aldrich (Saint Louis, MO, USA), while the standard of ursolic acid methyl ester came from Carl Roth (Karlsruhe, Germany). The analytical grade reagents: hydrazine hydrate, quinuclidine, *N*-chlorosuccinimide, 9-anthraldehyde, tetrahydrofuran, toluene, ethyl acetate, and dibasic sodium phosphate heptahydrate were purchased from Sigma-Aldrich. The analytical grade citric acid, potassium hydroxide, absolute ethanol, *n*-hexane, diethyl ether, and HPLC-grade methanol and acetonitrile were acquired from POCh (Gliwice, Poland). Dried leaves of rosemary (*Rosmarinus officinalis* L.) were acquired from a local vendor.

Instrumentation

The chromatographic analyses were conducted on Waters (Milford, MA, USA) equipment: 2695 Separation Module, 2996 Photodiode Array Detector, and 2475 Multi λ Fluorescence Detector. The Solid-Phase Extraction (SPE) of the samples was carried out with Extraction Manifold (Waters) and DOA-P504-BN vacuum pump (Gast Manufacturing, Benton Harbor, MI, USA). During the preparation of samples, ME235S analytical balance (Sartorius AG, Göttingen, Germany) and Rotavapor R-300 rotary evaporator (Büchi, Flawil, Switzerland) were used.

Sample Preparation

Extraction

Several solvents were investigated for the extraction of triterpenic acids from the samples: *n*-hexane, toluene, diethyl ether, tetrahydrofuran, ethyl acetate, acetonitrile, methanol,

and water. The yield of triterpenic acids was selected as a primary factor for selection of the solvent; whereas selectivity of the separation, miscibility with water, and safety of use were secondary.

The extraction procedure was performed with a Soxhlet apparatus. The matrix was dried leaves of rosemary (*Rosmarinus officinalis* L.)—a plant with a high content of triterpenes [18]. In each of the experiments, 5 g of the sample was extracted with 250 mL of solvent for 8 h. Afterwards, the yield of triterpenic acids was determined with HPLC–UV method and the total yield of extraction was measured as mass of a residue after evaporation of the solvent.

Alkaline Hydrolysis

The method can be modified with an additional stage of alkaline hydrolysis (saponification). In several plants, species part of the triterpenic acids is present as esters [19, 20]. The total amount of triterpenic acids can, therefore, be determined after the hydrolysis of the latter.

The hydrolysis was conducted by modifying a method presented by Jóźwiak et al. [21]. The reaction mixture was prepared by dissolving 0.75 g of potassium hydroxide in 1.0 mL of water and adding 4.0 mL of ethanol and 1 g of a sample dissolved in 5.0 mL of toluene. The hydrolysis reaction lasted 60 min and was conducted at a temperature of 90 °C. After cooling the samples to room temperature, the upper (organic) phase was collected, whereas lower (aqueous) phase was re-extracted three times with a fresh toluene. The organic fractions were combined and condensed to dryness using a vacuum evaporator.

Solid-Phase Extraction

For the purification of the obtained extracts, a solid-phase extraction (SPE) method presented by Tarvainen et al. [22] was used. The samples were evaporated under reduced pressure and dissolved in methanol. Supelclean LC-SAX 3 mL cartridges (Sigma–Aldrich) were used for separation of triterpene separation. The cartridges were conditioned with 9 mL of methanol and 9 mL of water. Then, 5 mL of water was added to the top of the cartridge and 1 mL of the sample was loaded. The cartridge was rinsed with 9 mL of water and the triterpenic acids were then diluted with 6 mL of methanol.

Derivatization with 9-Anthryldiazomethane (ADAM)

ADAM is commercially available from a few chemical companies; however, it should be kept at a temperature of −80 °C, and in some regions, access to it can be problematic. Therefore, an in situ method for synthesis of this compound using *N*-chlorosuccinimide was implemented. There are alternative methods using HgO [23] and MnO_2 [24]; however, their yields are relatively low. An outline of ADAM synthesis and its reaction with analyte is presented in Fig. 2.

The first step of the reaction was the synthesis of 9-anthraldehyde hydrazone using a method presented by Nakaya et al. [23]. A portion of 9-anthrylaldehyde (4.50 g) was dissolved in 75.0 g of absolute ethanol, and subsequently, 4.35 g of hydrazine hydrate was added dropwise. The mixture was stirred for 3 h at a room temperature. Afterwards, the precipitated product was filtered and purified by recrystallization from ethanol. The purity of the product was confirmed by the HPLC analysis of substrate leftovers in samples; the crude product contained approximately 2.4% of 9-anthrylaldehyde, while recrystallization lowered that value to 0.7%. A description of this analysis is presented in the Electronic Supplementary Material.

The second step was the in situ synthesis of diazo compound described by Quilliam et al. [15]. Prior to the analysis,

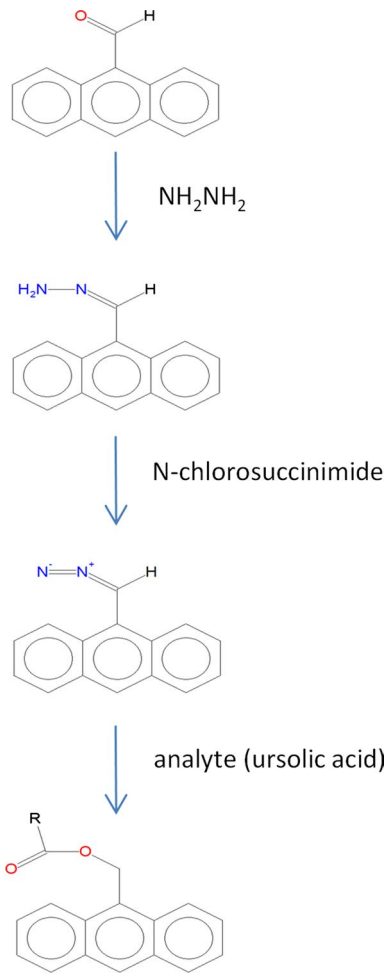

Fig. 2 Method of in situ synthesis of 9-anthryldiazomethane and its reaction with ursolic acid

solution of 9-anthrylaldehyde hydrazone (35 mmol L^{-1}) was mixed with quinuclidine (70 mmol L^{-1}) and *N*-chlorosuccinimide (35 mmol L^{-1}); all the reagents were dissolved in tetrahydrofuran. The mixture was left for 1 h in darkness to ensure the formation of ADAM.

The mixture was combined with the sample and, after incubation, injected into HPLC system. The derivatization reaction lasted 1 h and was performed at room temperature (approx. 25 °C). To ensure efficient derivatization, ADAM and analytes ratio should be at least 5:1.

Instrumental Analysis

HPLC–UV Method

The analysis was conducted using a method presented by Giménez et al. [10]. Analyses of 25 µL samples were carried out using Zorbax Eclipse PAH column (Agilent Technologies, Santa Clara, CA, USA). The column was thermostated at a temperature of 30 °C and isocratically eluted with a 0.6 mL min^{-1} flow of mixture of citrate–phosphate buffer (pH 3.0) and methanol (1:9). The detection was carried out using a wavelength of 210 nm. The separation of a mixture of the standards is shown in Fig. 3.

HPLC-FD Method

The selection of the parameters of the separation and derivatization is presented in the "Results and Discussion" section. The final method is presented below.

Analyses of 25 µL samples were carried out using Zorbax Eclipse PAH column (Agilent Technologies). The column was thermostated at a temperature of 20 °C and was isocratically eluted with a 1.0 mL min^{-1} flow of mixture of acetonitrile and water (4:1). The detection was carried on an excitation wavelength of 254 nm and a detection wavelength of 412 nm. The separation of the mixture of the standards is presented in Fig. 4.

Validation

The following parameters were established to validate the HPLC–UV and HPLC-FD methods: limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability, and linearity. The above-mentioned parameters were determined according to guidelines for pharmaceuticals presented by The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) [25].

Computer Software

The data were analyzed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and Design Expert 10 (Statsoft, Tulsa, OK, USA) software.

Results and Discussion

Extraction

The results of the Soxhlet extraction carried out with the use of various solvents are presented in Table 1.

Most of the tested solvents, with two exceptions, *n*-hexane and water, were able to extract triterpenes from the plant matrix. The yield of triterpenic acid was virtually identical—therefore, it can be assumed that the extraction process enabled full recovery of the triterpenic acids from the matrix. The properties of the solvents influenced the selectivity of the extraction; in general, water-miscible solvents were able to recover a much wider range of compounds from the leaves (the total yield of extracts was higher). The higher selectivity of the extraction is

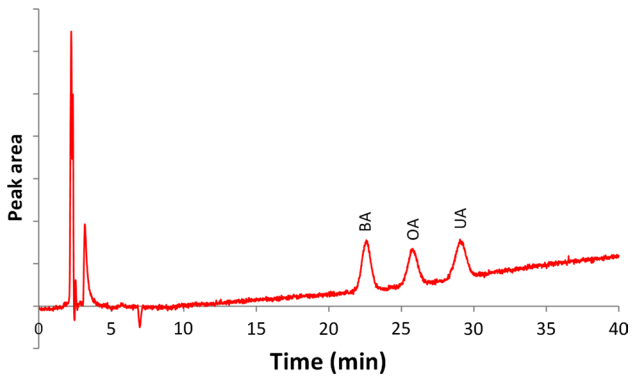

Fig. 3 Separation of the mixture of standards with the HPLC–UV method; *BA* betulinic acid, *OA* oleanolic acid, and *UA* ursolic acid

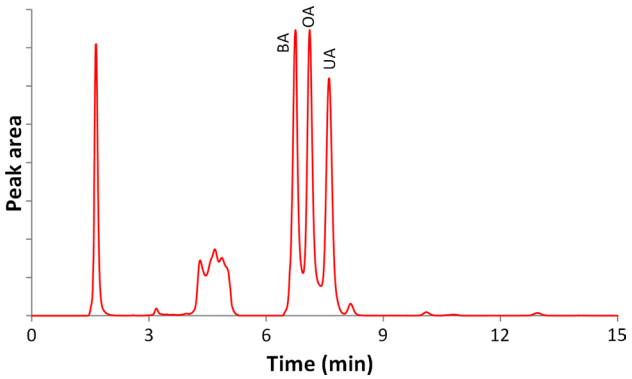

Fig. 4 Separation of the mixture of standards with the HPLC-FD method; *BA* betulinic acid, *OA* oleanolic acid, and *UA* ursolic acid

Table 1 Comparison of the yield of Soxhlet extraction of dried rosemary leaves with various solvents and selected properties of the solvents [26]

Solvent	Yield of triterpenic acids (mg g^{-1})	Total yield (mg g^{-1})	Polarity	Water solubility (g L^{-1})	Boiling point (°C)
n-Hexane	n.d.	162 ± 6	0.009	9.5×10^{-3}	69
Toluene	27.0 ± 0.3	182 ± 4	0.099	0.52	111
Diethyl ether	27.2 ± 0.1	178 ± 5	0.117	69	35
Tetrahydrofuran	27.2 ± 0.2	218 ± 9	0.207	Miscible	66
Ethyl acetate	27.3 ± 0.2	191 ± 6	0.228	83	77
Acetonitrile	27.2 ± 0.3	277 ± 6	0.460	Miscible	82
Methanol	27.3 ± 0.2	288 ± 8	0.762	Miscible	65
Water	n.d.	260 ± 14	1.000	–	100

n.d. not detected

desired as it lowers effort needed for the purification of the fractions obtained. The polarity of triterpenic acids is low compared to other compounds containing carboxylic groups; therefore, the selection of a more hydrophobic solvent reduces the amount of substances that can interfere the reaction of triterpenic acids with ADAM (e.g., Krebs cycle intermediates). Non-miscibility with water would also be advantageous when analyzing liquid samples, including metabolic fluids (e.g., blood and urine). The liquid–liquid extraction is the preferred method for such samples due to the possibility of direct extraction of the matrix and the high partition coefficients of triterpenic acids ($\log P > 6$) [27].

Among the tested solvents, the authors encourage the use of ethyl acetate. In addition to its good selectivity, it is characterized by relatively low toxicity and flammability. The use of diethyl ether is hazardous due to its volatility, while Soxhlet extraction with toluene is problematic as a result of high temperature of the process. The use of other solvents, including non-standard media such as supercritical carbon dioxide, is also possible; however, the efficiency of the extraction should be verified.

Alkaline Hydrolysis

The suitability of the hydrolysis method was verified using ursolic acid methyl ester. The solution of the standard was hydrolyzed and the amount of liberated ursolic acid was determined and compared with the theoretical yield of the reaction. The efficiency of the process was 99.8 ± 0.3%; therefore, the presented process parameters seem to be sufficient to liberate triterpenic acids from their esterified form.

The selection of toluene as a reaction medium allows the reaction to be conducted at high temperatures. The use of a more volatile solvent would limit the upper temperature of the process to its boiling point; therefore, a significantly longer time might be needed for full hydrolysis.

Solid-Phase Extraction (SPE)

The suitability of the SPE method for purification of the extracts was confirmed by measuring the recovery of the triterpenic acids in the fractions leaving the cartridge. For the experiment, 1 mL of a solution of oleanolic acid (10 g L^{-1}) was inserted into a cartridge and purified according to the method presented in Sect. "Solid-Phase Extraction"; after separation, the cartridge was washed with an additional 10 mL of methanol. The analysis showed that the recovery of the method was 98.4 ± 0.6%. No detectable amount of analyte was found in other fractions.

The purification with SPE is able to decrease an amount of substances interfering during the analysis. The use of an anion exchanging columns allows acidic compounds to be separated from neutral and basic substances. The combination of methods using different separation approaches: by polarity (extraction) and by acidic/basic properties (SPE) leads to obtaining samples with a low matrix background.

Optimization of HPLC-FD Method

An overview of the optimization results is presented in Table 2. More detailed information can be found in the Electronic Supplementary Material.

The control of the reaction of ADAM and analytes is crucial to ensure high recovery of the analysis. The experiments showed that, despite the temperature, 60 min was long enough for full derivatization. The ratio of ADAM and analytes should be estimated before the analysis; the authors suggest that it should be at least 5:1.

Among three tested liquid chromatography columns, only one was able to separate oleanolic and ursolic acid with an adequate resolution. Despite the fact that all tested columns were reversed phase, the differences in the chemistry of their stationary phase affected the outcome.

Combinations of mobile phases of various polarity and pH were tested. The best results (in terms of resolution) were obtained for a mixture of acetonitrile and water (4:1). The

Table 2 Results of the optimization of the HPLC–FD method

Parameter	Tested variants	Selected variant
Temperature of derivatization (°C)	25, 35, 45	25
Duration of derivatization (min)	30, 60, 90, 120	60
ADAM–analyte ratio (molar)	10:1, 5:1, 2:1, 1:1	5:1
HPLC column	Zorbax eclipse PAH Sunfire C18 Sunfire C8	Zorbax eclipse PAH
Temperature of analysis (°C)	20, 30, 40	20
Mobile phase composition	Combinations of acetonitrile, water and buffers	Acetonitrile–water (4:1)
Excitation wavelength (nm)	240–290	254
Detection wavelength (nm)	400–450	412

Table 3 Results of the validation of the methods

Parameter	HPLC–UV	HPLC-FD
Limit of detection (μg L^{-1})	750	8
Limit of quantification (μg L^{-1})	2500	27
Linearity (R^2)	0.9986 (2.5–100.0 mg L^{-1})	0.9722 (50–2500 μg L^{-1})
Repeatability (expressed as relative standard deviation) (%)	2.1 (at 5.0 mg L^{-1})	6.8 (at 250 μg L^{-1})
Recovery (%)	97.8 ± 0.8 (at 5.0 mg L^{-1})	93.1 ± 3.5 (at 250 μg L^{-1})

acidity of the solvent did not influence the separation, probably because the carboxylic groups of analytes were covalently bounded to ADAM. The temperature of the column during the analysis did not significantly affect the resolution and retention times.

Optimal wavelengths were established by measuring the peak area during analysis of the standard. The tested ranges of excitation and detection wavelength were selected using analyses of other compounds with ADAM [14–17].

The process of the synthesis of ADAM was not optimized during this study. The authors performed all the operations according to data in the literature. The fact that ADAM is commercially available is another advantage of the method.

Validation

The overview of the validation results of the developed HPLC–FD method, compared with HPLC–UV, is presented in Table 3.

The main aim of the work, increasing the sensitivity of the method, was achieved. The use of derivatization and subsequent fluorometry analysis enabled the limits of detection and quantification to be decreased almost 100 times. The limit of detection of the presented method is comparable to the values for the HPLC–MS methods presented in the literature; the LOD is strongly dependent on the equipment used and values of 0.5–91 μg L^{-1} were reported [28–30] The increased complexity of the method resulted in obtaining worse results of other validation parameters: linearity, repeatability, and recovery; although the results are still satisfactory.

Conclusions

The method presented in this paper is suitable for quantifying triterpenic acids in concentrations which are two orders of magnitude lower than in methods using UV–Vis spectrometry. The method included extraction, purification using solid-phase extraction, and derivatization with 9-anthrylaldehyde and HPLC–FD analysis. An additional facilitating step is alkaline hydrolysis able to liberate acids bounded in the form of esters. The presented methodology is simple, compared to other FD methods, and can be easily implemented in many research labs. The sensitivity of the method is comparable to mass spectrometry techniques, although an expensive equipment is not required for the analyses. The validation data show that the method is characterized by low limits of detection and quantification, good repeatability, and acceptable recovery.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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