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Rapid simultaneous determination of pentacyclic triterpenoids by mixed-mode liquid chromatography–tandem mass spectrometry

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ABSTRACT

Pentacyclic triterpenoids (PCTs) possess high biological activity, including antitumor, anti-inflammatory, antiviral and hepatoprotective properties and are widespread in a plant biomass. Due to significant differences in polarity and other physicochemical properties, the simultaneous determination of different classes of PCTs by the methods of reversed phase liquid chromatography is difficult. In the present study, we proposed a new approach to chromatographic separation of such compounds based on the use of a stationary phase with a mixed retention mechanism combining hydrophobic, weak anion exchange and hydrophilic interactions. The use of the Acclaim Mixed-Mode WAX-1 column and tuning the selectivity by changing the contributions of different types of analyte–stationary phase interactions allowed the separation of 10 PCTs (betulin, erythrodiol, uvaol, friedelin, lupeol, β-amyrin, α-amyrin, betulinic, oleanolic and ursolic acids) belonging to four different classes (monools, diols, ketones and triterpenic acids) during 7.5 min in isocratic elution mode. The combination of this approach with atmospheric pressure chemical ionization tandem mass spectrometric detection and pressurized liquid extraction of analytes with methanol allowed to develop a rapid, accurate and highly sensitive method for analyzing PCTs in plant tissues with a total duration of the analytical cycle (including sample preparation steps) of not more than 40 min. It provides the detection limits in plant biomass extracts of 3–12 μg L^{-1} (44 μg L^{-1} for friedelin). The developed method was validated and successfully tested in the analyses of real birch bark and lingonberry peels.

1. Introduction

Being the important class of secondary metabolites of plants, pentacyclic triterpenoids (PCTs) are widely spread in nature. Their content is greatest in the peels of fruits and berries, leaves and bark and can reach several percent and even tens of percent [1–3]. One of the most known industrial sources of triterpenoids is birch bark, containing in its outer layer betulin, lupeol, and a number of related compounds in amounts up to 35% [4,5].

The increased interest in PCTs is associated with their high biological activity, including antitumor, anti-inflammatory, antiviral and hepatoprotective properties, which, in combination with low toxicity, cause their expanding use in the pharmaceutical industry [6–11]. In this regard, the development of rapid, selective and highly sensitive methods for the determination and screening of PCTs in plant feedstock, medicinal products and biological fluids is an important task. To solve it, gas and liquid chromatography methods are mainly used [12]. The latter do not require preliminary derivatization of analytes and differ by a lower laboriousness of sample preparation and analysis [13,14].

Compared to UV absorption [15–17] and evaporative light scattering [18,19], an increase in sensitivity (by 2–3 orders of magnitude) and selectivity can be provided by using mass spectrometric detection. Comparison of various methods of atmospheric pressure ionization (chemical ionization APCI, photoionization APPI and electrospray ionization ESI) in determining PCTs of the lupane, oleanane and ursane families by HPLC–MS [20–23] demonstrated the advantage of APCI due to rather low polarity of analytes. The obtained LOD values for various compounds are in the ranges of 0.67–455 μg L^{-1}, with the highest sensitivity being achieved for triterpenic acids. The remaining triterpenoids exhibit detection limits at the level of tens and hundreds of μg L^{-1}. Tandem mass spectrometric detection allows to achieve a further increase in sensitivity and selectivity and to completely avoid any procedures for cleanup of plant tissue extracts before analysis. Thus, when using HPLC–ESI(+)–MS/MS for the direct determination of four triterpenic acids, erythrodiol and uvaol in olive leaf extracts [24], the obtained LOD values for all analytes are within 26–91 μg L^{-1}. Replacing ESI with a more suitable APPI [25] led to a decrease in detection limits to a level of 0.4–20 μg L^{-1} for a wide range of PCTs

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and plant steroids. The exception is β-amyrin, for which LOD was 158 μg L^{-1}. Kosyakov et al. [26] successfully used the HPLC–APCI–MS/MS method for highly sensitive determination of betulin, betulinic acid, lupeol, and erythrodiol in birch bark extracts, with the obtained LOD values of 0.7–1.8 μg L^{-1}.

The most important problem in existing approaches to the determination of PCTs is the chromatographic separation of analytes, which should ensure both the effective separation of the target components from the matrix and the sufficient resolution of isomeric compounds. Due to the large difference in polarity of such groups of PCTs as triterpenic acids, diols and monools, the use of reversed phase (RP) HPLC leads to an unacceptably long analysis time, broadening of chromatographic peaks and, as a result, significant loss of sensitivity. The use of gradient elution does not fully solve the problem, since the potential to reduce retention times in this case is limited by loss of resolution for structurally close isomers. In this regard, the most critical pairs of compounds are erythrodiol/uvaol, α-amyrin/β-amyrin and oleanolic/ursolic acids and some others, which structures differ only in the position of the methyl group [20].

Consequently, the works known in the literature are focused primarily on the determination of compounds from either individual groups of PCTs, for example, triterpenic acids [27–29], or a small number of analytes belonging to different groups that do not include positional isomers [26]. To overcome this problem, instead of the traditional octadecyl silica, the use of the stationary phase designed for the analysis of polycyclic aromatic hydrocarbons with polymeric C18 bonding has been proposed recently for the separation of PCTs due to its well-known resolution power towards geometric isomers [30]. This allowed achieving separation of five PCTs (including oleanolic/ursolic acids and erythrodiol/uvaol pairs) and betulinic acid as an internal standard in olive extracts and rat plasma with the analysis time 35 min [30,31].

In this study, we propose an alternative approach to PCTs separation, based on the use of multifunctional mixed-mode stationary phase with retention providing orthogonal selectivity when compared with RP phases. Taking into account the ionogenic properties of triterpenic acids, the high hydrophobicity of the PCTs hydrocarbon backbone and the presence of polar hydroxyl and carbonyl groups in their structure, great prospects are associated with the use of stationary phases combining weak anion-exchange properties, ability for hydrophobic interactions with analytes and possibility of implementing a hydrophilic interactions chromatography mode (HILIC). Among the commercially available stationary phases of this type, one of the most known is Acclaim Mixed-Mode WAX-1, developed by Dionex Corp. (Sunnyvale, USA) [32]. It possesses a unique selectivity for organic acids and has already been successfully used for separation of compounds of different classes, including steroids structurally close to PCTs [33].

The large number of available variables affecting the retention of analytes by the mixed mechanism ensures the attainment of the necessary selectivity of the analysis and its fine-tuning [34]. At the same time, this factor complicates the procedure for developing the chromatographic method, which should be based on understanding and taking into account the contributions of various interactions to the retention of analytes. Thus, the aim of the present work is to develop on this basis the method for rapid and sensitive determination of wide range of pentacyclic triterpenoids of various classes in plant tissues by mixed-mode liquid chromatography hyphenated to tandem mass spectrometry.

2. Experimental

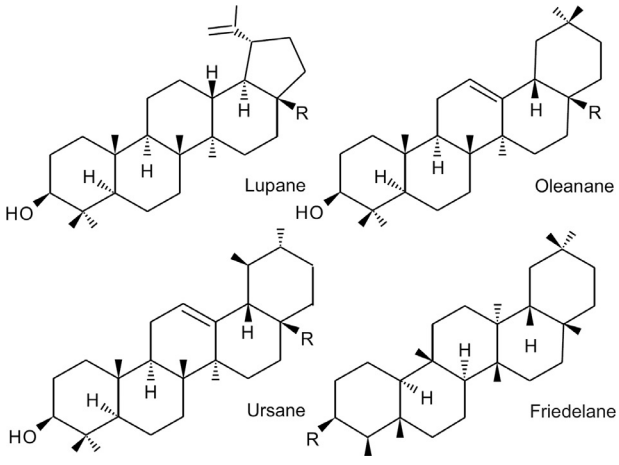
2.1. Reagents and materials

Ten commercially available (Aldrich, Steinheim, Germany) pentacyclic triterpenoids (Fig. 1, Fig. S1) belonging to the classes of triterpenic acids, monohydric and dihydric alcohols, and also ketones, of four families (with oleanane, ursane, friedelane and lupane type hydrocarbon skeleton) were selected as analytes under study: betulin, I (≥98%); erythrodiol, II (≥97%); uvaol, III (≥95%); friedelin, IV (tech. grade); lupeol, V (≥90%); β-amyrin, VI (≥98,5%); α-amyrin, VII (≥98.5%); betulinic acid, VIII (≥97%); oleanolic acid, IX (≥97%) and ursolic acid, X (≥90%).

HPLC-hypergradient grade acetonitrile (Cryochrom, S.-Petersburg, Russia), formic acid, ≥96%, "ACS Reagent" (Sigma-Aldrich, St. Louis, USA), ammonium formate, 10 M aqueous solution (Sigma-Aldrich, St. Louis, USA), and "type I" Milli-Q high-purity water were used for the preparation of mobile phase. HPLC-gradient grade methanol (Merck, Darmstadt, Germany) was used for analytes solutions preparation and extraction of plant materials.

The mobile phase was prepared by mixing an aqueous formate buffer solution of the appropriate concentration (taking into account subsequent dilution) and acetonitrile. The pH of the mobile phase was determined for an aqueous buffer solution using HI-2215 pH meter with HI-1131 glass electrode (Hanna Instruments, Smithfield, USA) and tuned by the addition of formic acid.

The stock solutions of analytes in methanol with concentration 250 mg L^{-1} were prepared from precisely weighed portions and stored in dark at 4 °C for no longer than 1 week. The working and calibration solutions with concentrations of each compound in the range 25.0–0.0125 mg L^{-1} were prepared by mixing and



№	Compounds	Family	R
I	Betulin	Lupane	-CH_2OH
II	Erythrodiol	Oleanane	-CH_2OH
III	Uvaol	Ursane	-CH_2OH
IV	Friedelin	Friedelane	=O
V	Lupeol	Lupane	-CH_3
VI	β-Amyrin	Oleanane	-CH_3
VII	α-Amyrin	Ursane	-CH_3
VIII	Betulinic acid	Lupane	-COOH
IX	Oleanolic acid	Oleanane	-COOH
X	Ursolic acid	Ursane	-COOH

Fig 1. Chemical structure of the studied pentacyclic triterpenoids.

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consecutive dilutions of stock solutions with methanol directly before experiment.

2.2. Plant materials and extraction

The silver birch (*Betula pendula*) bark and lingonberry (*Vaccinium vitis-idaea)* fruits were collected in the boreal forest in Arkhangelsk region of Russia in August 2017. The outer layer of bark and peels of berries were separated manually and dried in oven at 50 °C overnight. Dry material was grinded to the particles size 0.5–1 mm and stored in desiccator over silica gel in dark at room temperature.

Pressurized liquid extraction (PLE) was performed on ASE-350 (Dionex, Sunnyvale, USA) accelerated solvent extraction system according to the earlier developed procedure [35]. Sample of dry plant material (1 g) was mixed with 1 g of Dionex ASE Prep DE diatomaceous earth dispersant and placed into 10-mL stainless steel extraction cell. Two extraction cycles (10 min each) were performed with methanol as extractant at a temperature 100 °C and pressure 100 bar in high-purity (99.99%) nitrogen atmosphere. At the next stage, cell was rinsed with a fresh portion of extractant (6 mL). The obtained extract (~30 mL) was brought to a volume of 50 mL, and then aliquot was diluted 10-fold with methanol, passed through nylon membrane filter (0.22 µm) and subjected to chromatographic analysis.

2.3. Liquid chromatography

The LCMS-8040 (Shimadzu, Kyoto, Japan) liquid chromatography–tandem (QqQ) mass spectrometry system with LC-30 "Nexera" chromatograph was used. HPLC system included DGU-A5 vacuum degasser unit, two LC-30AD pumps, CTO-20A column oven, SIL-30AC autosampler, and CBM-20A system controller.

Chromatographic separations were carried out at 40 °C on the Acclaim Mixed-Mode WAX-1 column (Thermo Scientific, Sunnyvale, USA), 2.1 × 150 mm, particle size 3 µm, equipped with guard column (2.1 × 10 mm), with the same stationary phase. Mobile phase flow rate was 0.4 mL min^{-1}, injection volume – 10 µL. Control of the HPLC–MS/MS system, collection and processing of data were carried out using the LabSolutions 5.65 software (Shimadzu, Kyoto, Japan). Experimental determination of the void volume of the chromatographic system using various polar (DMF, methanol, acetone) and non-polar (toluene) markers gave significantly different and clearly overestimated results (500–650 µL) due to the inability to completely avoid interactions with the stationary phase of a multifunctional nature. As a consequence, we used the approximate value (416 µL), calculated on the basis of the geometric characteristics of the column, guard column (377 µL, 68% of the empty column volume for fully porous packing material) and connecting capillaries between injector and ion source of mass spectrometer (39 µL, 2940 mm × 0.13 mm i.d.) [36,37].

Other HPLC columns tested during screening of stationary phases (Section 3.1) were reversed phase Zorbax Eclipse Plus C18 and Zorbax SB-Aq, 3 × 150 mm, particle size 3.5 µm (Agilent, Santa Clara, USA); Nucleodur PolarTec 150 × 2 mm, particle size 1.8 µm (Macherey-Nagel, Duren, Germany) with embedded amide groups and Nucleodur HILIC, 150 × 3 mm, particle size 3 µm (Macherey-Nagel, Duren, Germany), with zwitterionic sulfobetaine stationary phase.

2.4. Mass spectrometry

As noted above, due to the low polarity of PCTs APCI is the preferred method of ionization of analytes, and therefore it has been used in all of our experiments. The following APCI ion source parameters, optimized during preliminary experiments, were used: ion source, heating block, and desolvation line temperatures – 350, 250 and 250 °C, respectively; corona discharge voltage – 3.5 kV; nebulizing and drying gas (nitrogen) flow rates – 4 and 15 L min^{-1}, respectively.

To ensure the necessary selectivity in the analysis of such complex objects as plant extracts, we used tandem mass spectrometric detection in the multiple reaction monitoring (MRM) mode. Considering the stability of the pentacyclic triterpene backbone to collision-induced dissociation (CID) argon was used as a collision gas, which, compared to nitrogen, provides a higher intensity of the product ion signals in the MS/MS spectra.

Since hydroxyl-containing PCTs easily undergo dehydration in the ion source under APCI conditions, the protonated dehydrated molecules $[M+H-H_2O]^+$, which peaks dominate in the mass spectra, were selected as precursor ions. The exception is friedelin, which is characterized by the formation of the $[M+H]^+$ ion.

Based on the tandem mass spectra of the precursor ions obtained at different collision energies (CE), for each analyte two product ions with the intense signals were selected, for which the CE values and bias voltages at segmented quadrupoles were optimized (Table 1). For each analyte, the ion transition giving the

Table 1
Optimized conditions of PCTs mass spectrometric detection in MRM mode.

Compounds	Monoisotopic mass, Da	Precursor ion, *m/z*	Product ion, *m/z*	Q1 bias, V	Collision energy, V	Q2 bias, V
I	442	425	95	−43.5	32	−37.1
			191*	−46.8	17	−11.3
II	442	425	191	−46.8	14	−46.8
			217*	−46.8	17	−21.0
III	442	425	191	−46.8	17	−37.1
			217*	−43.5	16	−21.0
IV	426	427	109	−46.8	27	−46.8
			95*	−40.3	34	−40.3
V	426	409	95	−43.5	33	−14.5
			137*	−40.3	20	−46.8
VI	426	409	95	−43.5	36	−43.5
			231*	−43.5	16	−14.5
VII	426	409	95	−40.3	40	−37.1
			231*	−43.5	20	−14.5
VIII	456	439	95	−46.8	40	−40.3
			137*	−43.5	21	−24.2
IX	456	439	191	−21.0	15	−17.8
			203*	−50.0	27	−43.5
X	456	439	191	−43.5	15	−17.8
			203*	−46.8	26	−40.3

* used for confirmation.

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most intense signal in MRM mode was used for quantification and another one for confirmation purposes.

2.5. Method validation

The values of the instrumental detection limit (LOD) and the lower limit of quantification (LOQ) of analytes were determined using the signal-to-noise ratio (S/N) criteria 3 and 10, respectively. A preliminary assessment of these parameters was used when analyzing standard solutions with analyte concentrations of 0.5 mg L^{-1}, followed by refinement as a result of 10 repetitive analyses of PCTs solution with concentrations close to estimated LOQ values (50 µg L^{-1}). The obtained standard deviation value multiplied by the coefficients 3 and 10 was taken as the final LOD and LOQ values, respectively.

The matrix effect and accuracy of PCTs quantification in plant extracts were estimated by the spike-recovery test at three concentration levels (500, 5000 and 12,500 µg L^{-1}). Known amounts of analytes were introduced into lingonberry PLE extracts and analyzed in three replicates.

The intra-day precision was estimated at the low concentration level, close to LOQ (50 µg L^{-1}) in a series of consecutive chromatographic analyses of standard solution ($n = 7$). The inter-day precision was determined in the same manner within 48 h ($n = 14$).

3. Results and discussion

3.1. Screening of stationary phases

As expected, the use of the "classical" reversed stationary phase (C18) did not allow separating the two critical analyte pairs mentioned above – erythrodiol/uvaol and oleanolic/ursolic acids, while for the weakly polar amyrin isomers, which differ only in the position of the methyl group in ring E, an acceptable separation is achieved (Fig. S2). And the resolution of critical pairs of more polar analytes turned out to be so small that they could not be separated by decreasing the eluting power of the mobile phase and applying a gradient elution simultaneously with solving the problem of determining less polar PCTs. Surprisingly, the separation deteriorated significantly along with a decrease in the retention of analytes when using the reversed stationary phase Zorbax SB-Aq with a slightly different selectivity for compounds with polar groups (Fig. S3). The best results in the separation of monools (V–VII) and diols (I–III) were obtained using the octadecyl phase with embedded amide groups (Fig. S4). Nevertheless, despite the good retention of triterpenic acids (VIII–X), selectivity was completely insufficient for their separation, regardless of the mobile phase composition. An attempt to solve the problem of separation of polar analytes by using HILIC on the zwitterionic stationary phase was not successful – retention of PCTs was unacceptably weak (Fig. S5).

The encouraging results were obtained only with the use of the Acclaim Mixed-Mode WAX-1 stationary phase possessing a bonded ligand with a long alkyl chain with embedded amide and terminal tertiary amino groups (Fig. S6). It provides a combination of a hydrophobic retention, HILIC and weak ion exchange and, as a result, high selectivity in the analysis of PCTs of different families. Optimization of the chromatographic separation of analytes on a given stationary phase, including tuning of the mobile phase composition as well as its pH and ionic strength to change the contributions of the three mentioned retention mechanisms, is described in the following paragraphs.

3.2. Effect of mobile phase composition

The most important feature of the separation of PCTs on the Acclaim Mixed-Mode WAX-1 stationary phase is the unusual elution order of studied classes of analytes compared to reversed phase chromatography: diols–ketone friedelin–monools–triterpenic acids. The anomalous position in this series of triterpenic acids with the highest polarity among the studied compounds is apparently due to the main contribution to their retention mechanism (along with hydrophobic interactions) of ion exchange with the trialkylamine groups of the stationary phase. This leads to unacceptably high retention factor values (k) when the content of acetonitrile in the mobile phase is less than 80% (v/v). With an increase of acetonitrile content, the retention of acids rapidly decreases (Fig. 2) due to augmentation of the mobile phase eluting power for the RP retention mechanism. Noteworthy, that the dependences of the k values of acids pass through an extremum and with a further increase in the concentration of organic solvent (>85%), their retention increases. This effect can be associated with an increase in the contribution of the HILIC to the retention of analytes containing polar groups. In this case, along with ion-exchange interactions, retention is determined by the partition of PCTs between the mobile phase and the water-enriched solvent layer retained by the amide and amine groups of the stationary phase. This assumption is supported by numerous literature data demonstrating, with examples of various classes of analytes, the possibility of implementing HILIC or mixed HILIC/WAX retention mechanisms on the Acclaim Mixed-Mode WAX-1 and similar stationary phases at high acetonitrile concentrations in the mobile phase [38–42]. Naturally, a similar (but less pronounced due to the lower polarity compared with the acids) pattern is observed for diols. The behavior of the four less polar PCTs (monools and friedelin) is typical for the

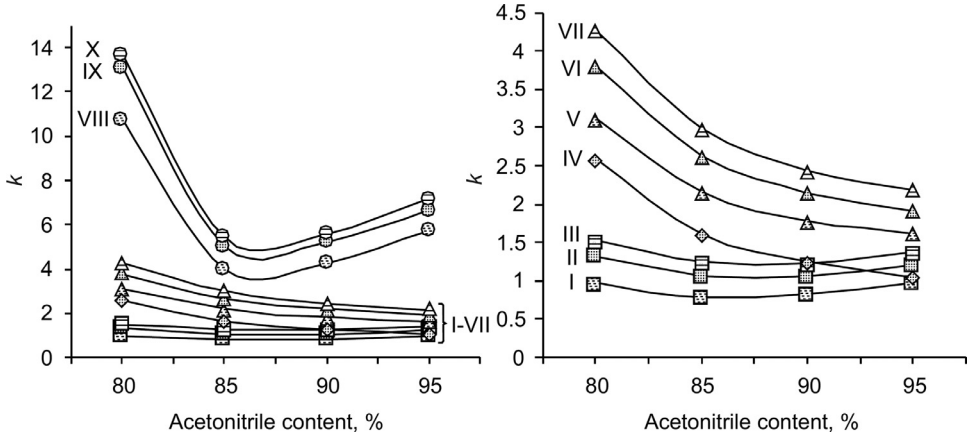

Fig 2. Effect of acetonitrile content on the retention factors of PCTs on Acclaim Mixed-Mode WAX-1 stationary phase (ammonium formate concentration 5 mM, pH 4).

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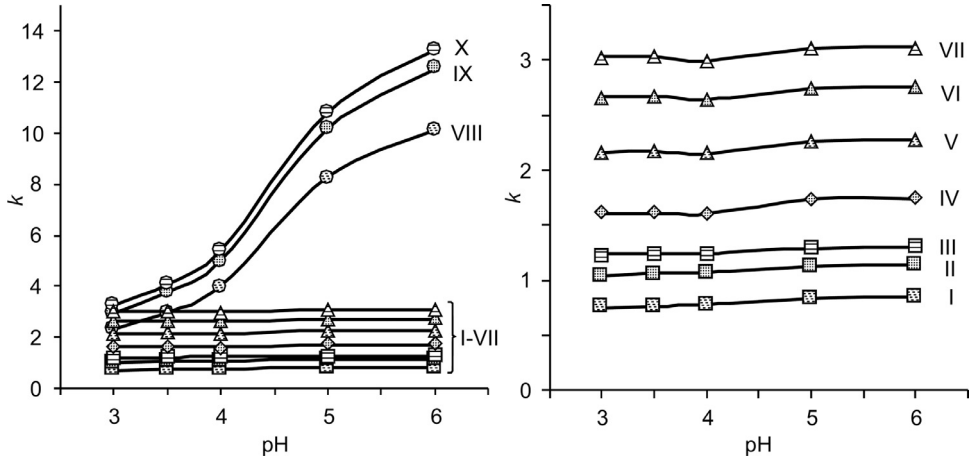

Fig. 3. Effect of pH on the retention factors of PCTs on Acclaim Mixed-Mode WAX-1 stationary phase (ammonium formate concentration 5 mM, acetonitrile content 85%).

reversed-phase retention, which is decisive for them when using the selected stationary phase. However, the dependences of log k for these analytes on the volume fraction of acetonitrile in the mobile phase are not strictly linear (Fig. S7) [34] due to some contribution of hydrophilic interactions. The consequence of a significant change in the contributions of hydrophobic and HILIC interactions to the retention of diols is the inversion of the elution order of these compounds and friedelin observed at the acetonitrile contents of 90–95%.

An additional confirmation of the significant role of the hydrophilic interactions in the retention of acids and diols is the behavior of analytes when acetonitrile in the mobile phase is replaced by methanol [41]. The latter is not suitable for the implementation of HILIC due to its high affinity for the polar groups of the stationary phase and, as a result, competition with water. In this case, all families of analytes exhibit a similar behavior, while the log k values decrease almost linearly with an increase in the volume fraction of methanol in the mobile phase (Fig. S8). The absence of a noticeable contribution of hydrophilic interactions does not allow the use of a mobile phase based on aqueous methanol for the separation of PCTs due to the loss of chromatographic resolution both between the groups of analytes and the compounds within each group.

3.3. Effect of mobile phase pH and ionic strength

Since the pK_a values of the terminal tertiary amine groups of the grafted stationary phase lie far beyond the recommended working pH range (2.8–6.5) of the chromatographic column, the effect of this parameter on the separation of analytes can be caused only by changing the degree of dissociation of triterpenic acids, which pK_a in aqueous solution is close to 5. This is confirmed by the obtained dependences of the retention factors of PCTs on pH (Fig. 3), from which it can be seen that a significant change in k values is observed for betulinic, oleanolic, and ursolic acids (VIII–X). The increase in the retention of these analytes with an increase in the pH of the mobile phase is due to the conversion of acids into anionic form, along with the above-mentioned decisive contribution of ion-exchange interactions to the mechanism of their retention on the stationary phase. It is noteworthy that the dependencies are S-shaped with an abrupt change in k near the pK_a value of the carboxyl groups, where the effect of pH on the concentrations of the molecular and anionic forms is most pronounced. The insignificant increase (within ~0.1 unit) in retention factors with increasing pH for other analytes is associated apparently with a slight change in the composition and ionic strength of the mobile phase due to the varying content of formic acid in the formate buffer solution used.

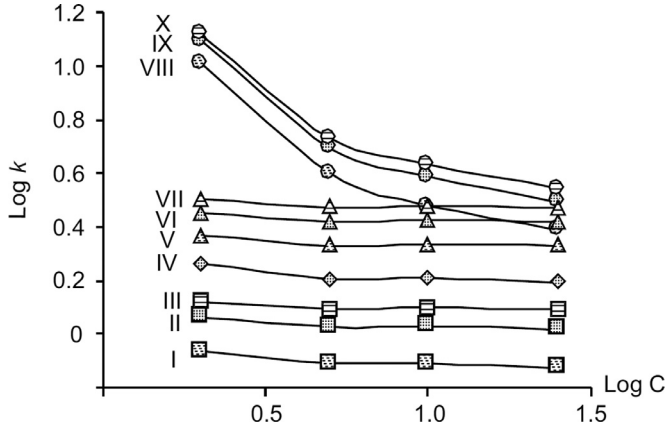

Fig. 4. Effect of ammonium formate concentration on the retention factors of PCTs on Acclaim Mixed-Mode WAX-1 stationary phase (pH 4, acetonitrile content 85%).

The ionic strength of the mobile phase determines the intensity of electrostatic interactions of analytes with charged groups of the stationary phase and is an important factor affecting the retention of triterpenic acids (VIII–X, Fig. 4). It is expected that the values of k for them sharply decrease with increasing concentration (C) of ammonium formate in the mobile phase, while the nonlinearity of the dependences in the coordinates log k–log C [43] confirms the conclusion that a significant contribution to the retention mechanism of triterpenic acids belongs to interactions which are not related to ion exchange. The k values of the remaining compounds slightly increase with a decrease in the concentration of the buffer salt lower 5 mM. This is probably due to the suppression of ion–dipole and dipole–dipole interactions of the stationary phase with analytes by ammonium formate.

3.4. Optimal conditions of chromatographic separation

Taking into account the need to ensure maximum rapidity of analysis, sufficiently complete separation of analytes and high robustness of the method, based on the dependencies obtained (Fig. 2–4) it is easy to conclude that optimal conditions for chromatographic separation are achieved when the acetonitrile content in the mobile phase is 85–90%, at ammonium formate concentrations of 5–10 mM and pH 3.5–4. The fine tuning of the parameters in the indicated ranges while taking into account the

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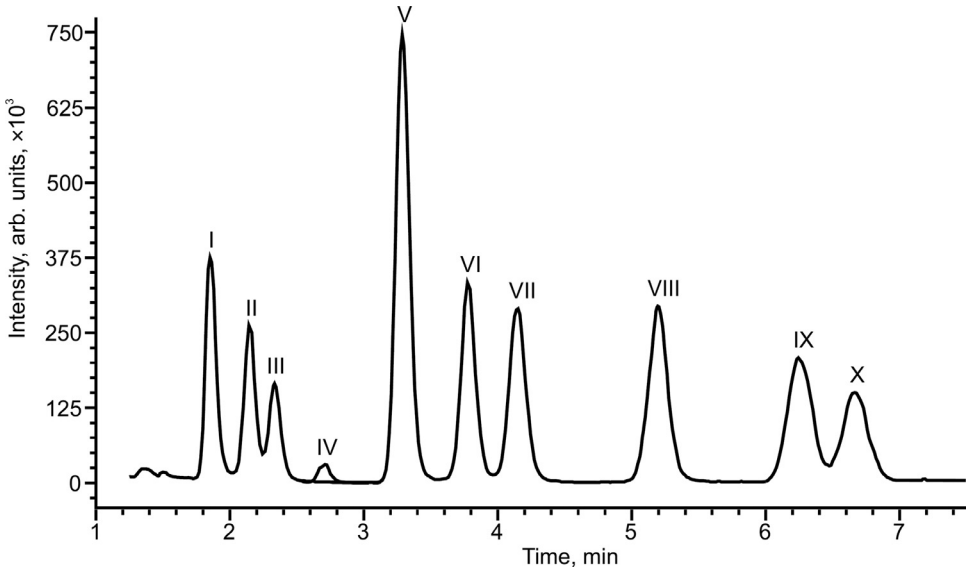

Fig. 5. HPLC-MS/MS chromatogram of analytes model mixture (12.5 mg L^{-1} of each compound) obtained on Acclaim Mixed-Mode WAX-1 stationary phase in optimized conditions.

desirability of using the lowest possible salt concentration to ensure high sensitivity of mass spectrometric detection (due to suppression of analytes ionization in the ion source in the presence of background electrolyte) allowed to suggest the following composition of the mobile phase: 33.3 mM aqueous formate buffer solution with pH 4 and acetonitrile in the ratio of 15:85 (v/v), which corresponds to 5 mM buffer salt content in the final solution. At the maximum mobile phase flow rate recommended by the manufacturer for this type of chromatographic column (0.4 mL min^{-1}), the selected conditions allowed to achieve separation of all 10 analytes within 7 min (Fig. 5, Table S1), with a close to optimal range of PCTs retention factors from 0.78 (betulin) to 5.41 (ursolic acid).

The attained analysis time is 2–5 times shorter compared to the PCTs chromatographic separation methods proposed previously in the literature [20–26,30,31] and mentioned in Introduction. It should be noted that the developed approach uses the isocratic elution mode, which gives an additional gain both in the equilibration time between the chromatographic runs and in the robustness of the method.

Despite the short analysis time, the baseline separation of the studied PCTs was achieved. The exceptions are the erythrodiol/uvaol and oleanolic/ursolic acids pairs, for both of which the chromatographic resolution (R_s) was 1.25 and selectivity factor (α) close to 1.1 (Table S1). However, this does not create significant problems for their simultaneous quantification. It should be noted, that the R_s value for the third critical pair of analytes (α/β-amyrins) reaches the close to optimal value of 1.70.

3.5. Quantification and method validation

The obtained LOD and LOQ values lie in relatively narrow ranges of 3–12 and 10–40 µg L^{-1}, respectively (Table 2). The exception is friedelin (LOD = 44 µg L^{-1}), the only analyte that forms a protonated molecule $[M+H]^+$ during ionization and differs from other PCTs under study by a lower precursor ion generation efficiency. A similar observation was made in Ref. [25] when determining PCTs by HPLC–APPI–MS/MS. The adequacy of the obtained values is clearly confirmed by the chromatogram of a mixture of analytes with concentrations of components close to LOQ (Fig. S9). Comparison of the obtained LOD values with those given in the literature shows that the developed method is distinguished by high sensitivity, which is comparable or substantially higher than that achieved in various works (see Introduction section) for determining PCTs by HPLC–MS/MS (0.5–157.9 µg L^{-1}).

The obtained calibration dependences are linear ($r^2 > 0.999$) for all studied components in the concentration range from LOQ to the highest value we used (25.0 mg L^{-1}), covering three orders of magnitude (Table 2). It is noteworthy, that in optimized detection conditions the response factors s (slopes of linear calibration plots) for most analytes lie in a relatively narrow range of 13×10^4–19×10^4, this can be used for the purposes of semi-quantitative screening using the minimum set of PCT standards.

The results of the evaluation of intraday and inter-day reproducibility of the analysis on standard analytes solutions with concentrations of each component 50 µg L^{-1} (150 µg L^{-1} for friedelin)

Table 2
Calibration dependences ($y = sx+a$) for the area of chromatographic peak versus analyte concentration, limits of detection and quantification of analytes by the mixed mode HPLC–MS/MS.

Analyte	Linear concentration range, µg L^{-1}	s	a	R^2	LOD, µg L^{-1}	LOQ, µg L^{-1}
I	33–25,000	107,568	887	0.999	10	33
II	20–25,000	60,673	528	0.999	6.0	20
III	30–25,000	55,960	260	0.999	8.8	30
IV	147–25,000	15,551	336	0.999	44	147
V	10–25,000	355,678	275	0.999	2.5	10
VI	23–25,000	169,985	332	0.999	6.5	23
VII	20–25,000	150,489	249	0.999	5.7	20
VIII	40–25,000	188,872	721	0.999	12	40
IX	13–25,000	179,116	280	0.999	6.3	20
X	20–25,000	130,986	210	0.999	4.1	13

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showed that the standard deviation in both cases did not exceed 10%, and the accuracy was close to 100% (Table S2).

A more adequate assessment of the method accuracy, taking into account possible matrix effects, was obtained by the spike-recovery test using the lingonberry PLE extract as a real matrix. Since the latter already contained significant concentrations of some analytes (see Section 3.6), the lower limit of the available range of the spiked concentrations for them was relatively high. The obtained results (Table S3) prove the absence of noticeable interferences from the matrix for all detectable compounds – the obtained recovery values were in the range of 96–104%. Effective elimination of matrix effects is attained due to the good chromatographic separation of analytes from the matrix components, the use of APCI, as well as the significant dilution of the extract.

The robustness of the developed method is based on the application of isocratic elution, as well as the selection of optimal conditions for chromatographic separation (see Section 3.4) with the minimum possible sensitivity of the retention times with respect to small changes in the mobile phase composition. Our tests showed that variations of pH within 0.05 units, ionic strength within 0.5 mM and chromatographic column temperatures in the range of 38–42 °C did not lead to a significant (>2%) shift in t_R values, loss of chromatographic resolution, as well as noticeable change in the results of analytes quantification. Analysis of more than 100 samples of plant extracts on a single chromatographic column did not lead to a significant deterioration of the chromatographic separation.

3.6. Plant biomass analyses

For approbation of the developed method, the birch bark and the peels of berries are chosen as real objects, which are characterized with a complex chemical composition and considered as an important industrial feedstock for the production of triterpenoids and dietary source of a number of biologically active PCTs, respectively. PLE extraction with methanol was used as a sample preparation method, providing quantitative and rapid extraction of analytes from plant tissues [35].

The obtained chromatograms (Fig. 6) demonstrate the presence of the majority of analytes in both samples studied in very different (up to four orders of magnitude) concentrations (Table 3).

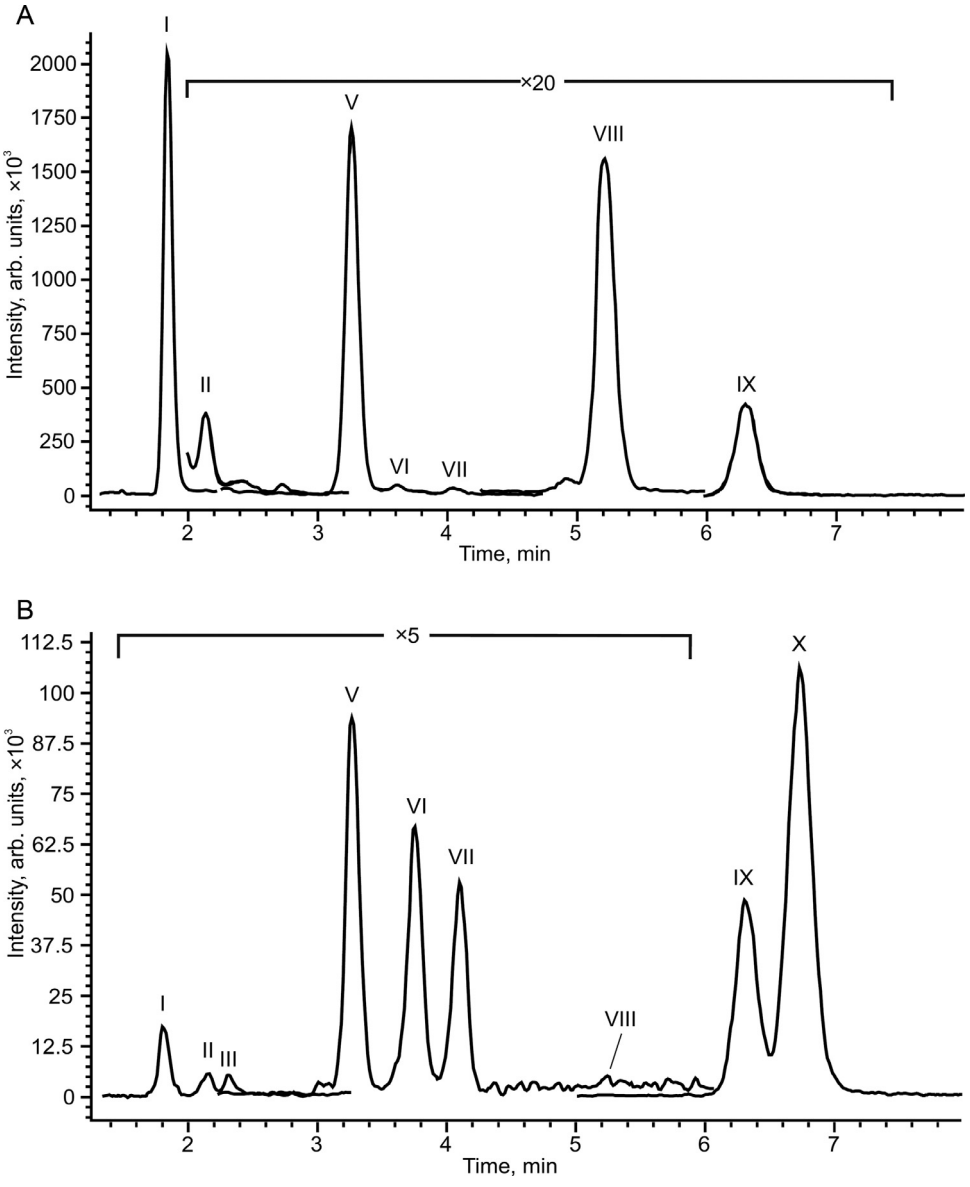

Fig. 6. HPLC–MS/MS chromatograms of birch bark (a) and lingonberry peels (b) PLE extracts.

Table 3
The content of PCTs in plant tissues ($n = 3$, $P = 0.95$) determined by pressurized liquid extraction and mixed mode HPLC–MS/MS.

Compounds	Birch bark, mg g^{-1}	Lingonberry peels, mg g^{-1}
I	280 ± 10	0.38 ± 0.02
II	4.6 ± 0.2	0.059 ± 0.003
III	–	0.15 ± 0.07
IV	–	–
V	6.4 ± 0.2	0.59 ± 0.02
VI	0.29 ± 0.01	0.71 ± 0.03
VII	0.39 ± 0.02	0.99 ± 0.04
VIII	15 ± 1	0.016 ± 0.001
IX	5.1 ± 0.2	4.6 ± 0.2
X	–	15 ± 1

Betulin (I), erythrodiol (II), uvaol (III), betulinic (VIII) and oleanolic (IX) acids predominate in the outer layer of birch bark, which is consistent with the literature data [5]. Among the minor components, β- and α-amyrins (VI, VII) were detected in amounts of 0.29 and 0.39 mg g^{-1}. It is curious that while the presence of the β-isomer is known in the literature, the detection of α-amyrin in birch bark has not been previously reported. One reason for this may be the difficulty of chromatographic separation of these analytes.

Lingonberry extract differs from birch bark by a much more complex chemical composition, which, however, does not interfere with the determination of PCTs. In it, we found all the analyzed compounds with the exception of friedelin. The predominant components are ursolic (X) and oleanolic (IX) acids (15.74 and 4.59 mg g^{-1}, respectively), the remaining analytes are present in amounts from 0.016 to 0.99 mg g^{-1}. The wide linear range of PCTs calibration curves allows simultaneous determination of both major and minor analytes in plant extract without additional dilution or concentration of samples, which further decreases the overall analysis time.

4. Conclusions

The use of a stationary phase with a mixed retention mechanism, comprising hydrophobic and weak anion exchange interactions, as well as HILIC, allows for rapid chromatographic separation of complex mixtures of pentacyclic triterpenoids of various classes, which differ greatly in their physicochemical properties and polarity. The great opportunities for tuning chromatographic separation selectivity due to changing the contributions of different types of analyte–stationary phase interactions by varying the mobile phase composition and ionic strength ensured the separation of 10 major PCTs belonging to four different classes (including three critical analytes pairs – erythrodiol/uvaol, α-amyrin/β-amyrin and oleanolic/ursolic acids), during 7.5 min in isocratic elution mode. Based on a combination of this approach with atmospheric pressure chemical ionization tandem mass spectrometric detection and pressurized liquid extraction of analytes with methanol, a rapid, accurate and highly sensitive method for analyzing PCTs in plant tissues has been developed and validated. With a total duration of the analytical cycle (including sample preparation steps) of not more than 40 min, it provides the detection limits in plant biomass extracts of 3–12 µg L^{-1} (44 µg L^{-1} for friedelin).

Declaration of Competing Interest

The authors declare no conflicts of interest in relation to this research.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2019.460458.

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