Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-022-03723-3

REVIEW ARTICLE

Adsorbents, mobile phases, and strategies for the chromatographic separation of betulinic, oleanolic, and ursolic acids

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Received: 13 September 2022 / Revised: 6 December 2022 / Accepted: 27 December 2022

Abstract
A critical overview of the separation of three relevant triterpenic acids, namely betulinic, oleanolic, and ursolic acids, by liquid and supercritical fluid chromatography is presented in this review. These triterpenic acids are commonly found in different biomass residues and have raised great research attention in recent years due to their broad and valuable therapeutic properties. Accordingly, fundamental aspects such as solubilities in single and mixed solvents, commercial stationary phases (octadecylsilyl and other bonded phases like triacontylsilyl, as well as porous graphitic columns), custom adsorbents (molecularly imprinted polymers and other polymeric adsorbents), mobile phases (conventional HPLC solvents and super/subcritical fluids), mobile-phase modifiers (acidic, basic, and cyclodextrins), pre-column derivatization strategies, and influence of temperature have been compiled, analyzed and thoroughly discussed in terms of (calculated) separation selectivities, peaks resolution, orders of elution, systems descriptors, etc. The most relevant achievements and gaps in this area of research are highlighted, together with a final comparison of the separation performance of the chromatographic systems reported in the literature.

Keywords Chromatography · Natural products · Purification · Stationary phases · Triterpenoids · Triterpenic acids

1 Introduction

There is a growing interest in revisiting natural products (NPs) for drug discovery as they provide unique and large structural diversity with a wide variety of pharmacophores and a high degree of stereochemistry [1]. Parallel to the revival of natural products, there is the current concept of biorefinery in which biomass is integrated and converted into energy and an array of marketable and high-value products [2, 3], fomenting resource efficiency, waste prevention, as well as recycling and circularity [4–6] while simultaneously complementing NPs research interest.

Among the various NPs, pentacyclic triterpenoids, and in particular, betulinic, oleanolic, and ursolic acids, have raised considerable research attention due to their various pharmaceutical and nutraceutical activities [7–11]. Triterpenoids are structurally diverse NPs that are synthesized in plants from squalene or oxidosqualene by a series of intramolecular condensation reactions [12, 13]. According to their backbone structures, they are classified as lupane, oleanane, and ursane, respectively, as shown in Fig. 1. Structurally, they contain five- and six-membered rings (A, B, C, D, and E in Fig. 1), five methyl groups linked to C-4, C-4, C-8, C-10, and C-14, and in the case of oleanolic acid, two other methyl groups are linked to C-20, while in the case of ursolic acid, these two other methyl groups are linked to separate carbons, C-19 and C-20, in the ring E and in an equatorial position. As result, ursolic acid has higher degree of planarity than oleanolic acid [14, 15]. In the case of betulinic acid, a prop-2-enyl group is located at carbon C-19, making this molecule stand apart in terms of its structure from oleanolic and ursolic acids. The double bonds in C-20–C-30 in betulinic acid structure, and in C-12–C-13 in oleanolic and ursolic acid structures, the hydroxyl groups at C-3, and the carboxyl groups at C-28 are three "active" positions generally used

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Published online: 23 January 2023

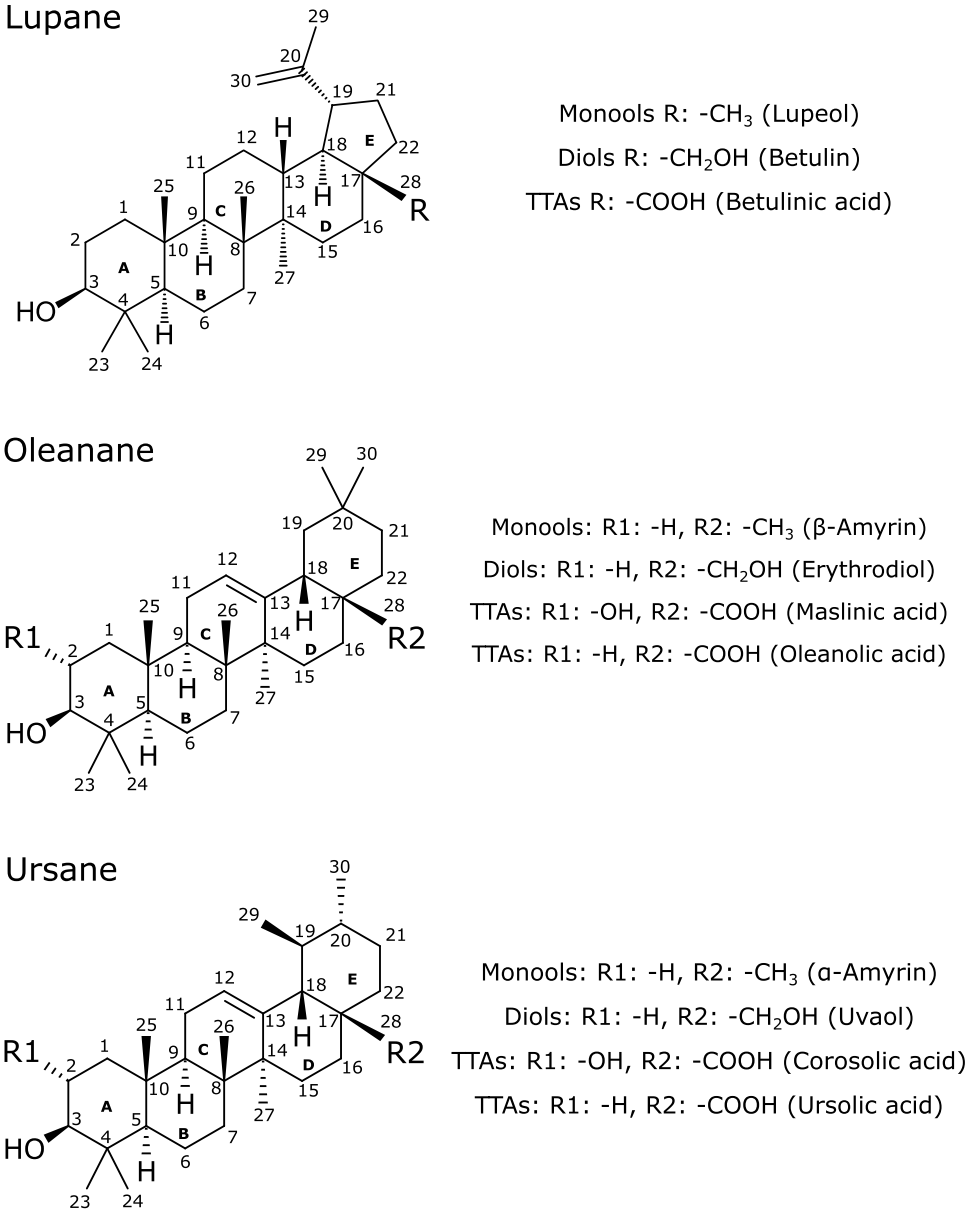

Fig. 1 Chemical structures of some lupane (lupeol, betulin, and betulinic acid), oleanane (β-amyrin, erythrodiol, and maslinic and oleanolic acids), and ursane (α-amyrin, uvaol, and corosolic and ursolic acids) pentacyclic triterpenoids commonly found in natural matrices. The letters A, B, C, D, and E identify carbon rings

to perform chemical modifications to tune or enhance their potency [16, 17].

These three triterpenic acids (TTAs) are ubiquitously distributed in nature and commonly found along with some other structurally related analogues such as lupeol, betulin, β-amyrin, erythrodiol, maslinic acid, α-amyrin, uvaol, and corosolic acid, among others (structures shown in Fig. 1) [18–21].

Betulinic acid is widespread in the Betulaceae family, particularly in the outer barks of multiple *Betula* spp. in varying concentrations along with its lupane analogue betulin [20]. Other known sources of betulinic acid include, for example, *Diospyros* spp. [22], *Ziziphus* spp. [23, 24], and *Quercus suber* L. [25, 26]. Betulinic acid has been shown to exhibit multiple and varied biological activities, namely anti-HIV (its derivative *bevirimat* reached phase II clinical trials as an anti-HIV drug), antimicrobial, antimalarial, antidiabetic, anti-inflammatory, anthelmintic, and cytotoxic activity against multiple cancer cell lines [27–30].

Oleanolic and ursolic acids possess similar structures and occur together in plants of many families [21]. Oleanolic acid, for instance, has been isolated from over 1600 species [31]. One of the most abundant sources of oleanolic acid is the Oleaceae family of plants, namely olive trees [32–34]. Oleanolic acid has shown low toxicity and is known for its antitumoral, hepatoprotective, anti-inflammatory, and cytotoxic activities [16, 35]. Ursolic acid was first isolated in cranberry fruit (Ericaceae) [36, 37], is ubiquitous in plant tissues belonging to Lamiaceae [38, 39] and Rosaceae [40, 41] families, and also abundant in plant tissues of *Eucalyptus*

spp. including leaves, fruits, and barks [21, 42, 43]. Similarly to oleanolic acid, ursolic acid has shown low toxicity and is known for its antimicrobial, hepatoprotective, anti-inflammatory, anti-hyperlipidemic, and cytotoxic activities [35, 44, 45]. It has also demonstrated potential for the prevention and treatment of obesity- and muscle mass-mediated metabolic consequences [46].

In the Portuguese perspective, *Eucalyptus globulus* is a potential abundant source of TTAs *as* this species is dominant in the Portuguese forest [47] and the most used source of fiber for pulp and paper production. These industries create substantial amounts of byproducts such as leaves, bark, and branches that may be further exploited with additional extraction and purification steps. As an example, an average *E. globulus* kraft pulp mill with a production of 500,000 tons/year of bleached pulp could generate 100,000 tons/year of bark, which could be roughly translated into *ca.* 134 tons/year of ursolic acid, 46 tons/year of oleanolic acid, and 29 tons/year of betulinic acid [21]. It is thus clear that this biomass residue is an important source of biologically active TTAs that can add significant value to these industrial residues.

The subsequent valorization of TTAs after extraction requires, however, efficient and reliable separation procedures. Due to their structural similarity and simultaneous occurrence, their complete and efficient separation is challenging. For the analysis and separation of pentacyclic triterpenoids, a review article was recently published covering multiple methods [48] and, among these, liquid chromatography coupled to different detection systems is one of the most used methods [21]. Liquid chromatography is an attractive and versatile technique but its successful implementation is critically dependent on the correct selection of adsorbents and eluents as well as operating conditions.

Accordingly, this article is intended to review and discuss critically the analytical separation of betulinic, oleanolic and ursolic acids by liquid chromatography. Overall, this appraisal will start with a review of TTAs solubility in single- and mixed-solvent systems, which is a crucial variable in the design of a chromatographic process. After that, the conditions reported in multiple works for their separation are compiled and discussed in terms of their separation selectivity and resolution results. These conditions comprehend the use of commercial stationary phases (octadecylsilyl and other bonded phases as well as porous graphitic columns) and respective mobile phases (traditional HPLC solvents and super/subcritical fluids), mobile-phase modifiers (acidic, basic, and cyclodextrins), pre-column derivatization strategies, influence of temperature, and custom adsorbents (molecularly imprinted polymers and other polymeric adsorbents). A general comparison between the separation selectivities for different conditions is also provided, foreseeing, with that, an elucidation on what adsorbents and strategies stand out for the separation of these triterpenic acids and that may be further utilized, for example, in preparative chromatography applications.

2 Solubilities of betulinic, oleanolic, and ursolic acids: solubilization strategies

The solubility is a crucial information for the proper design of any separation process. Ideally, a good eluent for a chromatographic process should promote extensive dissolution of the solutes to be separated while simultaneously impose a low-pressure drop. Several works have been dealing with the determination of the solubilities of betulinic [49–52], oleanolic [50–59], and ursolic [50, 53, 54, 56–59] acids for various unary and mixtures of solvents, as well as a function of temperature [49, 55–59]. Overall, solubilities of all triterpenic acids remain low for the vast majority of the reported solvents, with an exception for betulinic acid in tetrahydrofuran [49]. Regarding the effect of temperature, its increase significantly increments the solubility of all triterpenic acids for all reported solvents. On the other hand, due to the TTAs polycyclic hydrocarbon structure (as illustrated in Fig. 1), the addition of water has a very negative effect on their solubility, i.e., small concentrations of water induce an abrupt decrease of all TTAs solubility [52, 53]. A detailed analysis of TTAs solubility in pure and mixed conventional solvents together with temperature influence are presented in Supplementary Material.

In an attempt to improve the aqueous solubilization of TTAs and/or to avoid conventional organic solvents, other authors tried different approaches regarding the dissolution of triterpenic acids. For instance, Jin et al. [53] determined the solubility of oleanolic and ursolic acids in aqueous surfactant solutions (sodium lauryl sulfate, cetyltrimethylammonium bromide, polyoxyethylene (10) oleyl ether), and liquid polyethylene glycols (PEGs) at 298 K. The surfactant solutions showed moderate solubilizing capacities, and ionic surfactants showed a higher solvent power. Nonetheless, there was no significant difference between anionic and cationic species. Regarding the polyethylene glycol solutions, the lower the degree of polymerization the higher the solubility of oleanolic and ursolic acids, with values between *ca.* 5 and 6 mg/mL for PEG 200.

Jäger et al. [60] determined the solubility of betulinic and oleanolic acids in alkaline water at different pH values. The authors reported solubilities of 40.1 and 77.2 µg/mL for betulinic and oleanolic acids, respectively, at pH 11.4. Fan et al. [61] determined the solubilities of betulin and betulinic acid in sodium hydroxide aqueous solutions at temperatures from 283 to 323 K. All solubilities increased with increasing temperature and with increasing sodium hydroxide content, particularly above 300 K. Wang et al. [62] reported

the solubilities of betulin and betulinic acid in aqueous solutions containing different cyclodextrins (CDs) as host molecules. Non-derivatized and derivatized CDs (β-CD, (2-hydroxypropyl)-β-CD (HP-β-CD), γ-CD, HP-γ-CD) as well as three γ-CD thioethers (octakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-γ-CD, octakis-[6-deoxy-6-(2-sulfanyl ethanesulfonic acid)]-γ-CD, and octakis-[6-deoxy-6-(3-sulfanyl propanoic acid)]-γ-CD) were used at fixed concentration of 6.0 mM. All CDs imparted significant solubility enhancements for betulin and betulinic acid, with betulinic acid showing the highest solubilities of 2.06 and 1.60 mg/mL with octakis-[6-deoxy-6-(2-sulfanyl ethanesulfonic acid)]-γ-CD and octakis-[6-deoxy-6-(3-sulfanyl propanoic acid)]-γ-CD, respectively. De Faria et al. [63] determined the solubility of ursolic acid in various aqueous solutions of ionic liquids at 303 K reporting an enhancement of 8 orders of magnitude when compared with pure water. More recently, Silva et al. [64] measured the solubility of ursolic acid in several bio-based molecular solvents (limonene, menthol, thymol, γ-valerolactone, and α-pinene) as well as in menthol-based natural deep eutectic solvents (NADES) with phenyl propionic acid and thymol in different molar ratios at different temperatures. High solubilities were obtained, particularly at room temperature (*ca.* 30 mg/g(NADES)).

3 Stationary phases for triterpenic acids separation

In the next subsections, different TTA separation conditions and strategies will be discussed and presented along with two chromatographic performance indicators—selectivity and resolution. The chromatographic selectivities ($S_{i,j}$) under discussion were calculated as:

$$S_{i,j} = \frac{k_i'}{k_j'} = \frac{t_{r,i} - t_0}{t_{r,j} - t_0} \quad (1)$$

where k_i' and k_j' and $t_{r,i}$ and $t_{r,j}$ are the retention factors and retention times of species i and j, respectively, and t_0 is the column hold-up time, equal to the elution time of a non-retained species able to penetrate into the particle pores. The data used to determine t_0 was obtained from different sources and different methods, and thus, it is assumed that the same column brand (from the same manufacturer) possesses identical packing characteristics across different production batches (i.e., total porosity). Moreover, when the use of guard columns was reported, these were considered to influence the retention of analytes when dimensions were specified and the packing material of guard columns was the same as the main HPLC column. Otherwise, their effect on analyte retention was neglected. In fact, due to their small length compared to the main column, their influence on selectivities is negligible. The total porosities used to calculate the selectivities for each column discussed in the following subsections are compiled in Table SM1 along with the description of the method used to obtain them.

The peaks resolution ($R_{i,j}$) was estimated by the chromatograms provided by the different works as:

$$R_{i,j} = 1.18\frac{(t_{r,i} - t_{r,j})}{(w_{0.5H,i} + w_{0.5H,j})} \quad (2)$$

where $w_{0.5H,i}$ and $w_{0.5H,j}$ are the chromatographic peak widths at half height for species i and j, respectively. A baseline separation with a touching bands situation corresponds to a resolution of 1.5 while a resolution of 1.0 means an overlap of 3% of the peaks [65]. It is known that high resolution and preparative chromatographic applications are two antagonistic requirements as preparative chromatography is based on large sample injections and high concentrations, while analytical chromatography is based on small and diluted injections [66]. Nonetheless, peaks resolution is still provided here in this work as an additional performance indicator.

3.1 Commercial adsorbents

3.1.1 Octadecylsilyl-bonded phases

Octadecylsilyl (ODS or C18)-bonded phases have undoubtedly received the most attention thus far, with multiple C18 columns with different packing features (pore sizes, specific surface area, carbon load, and ligand densities) employed for the analytical separation/identification of betulinic, oleanolic and ursolic acids. Coupled to the various C18-bonded phases, two main detection systems have been reported: UV detection and mass spectrometry (MS). Both allow the detection, quantification, and identification of analytes in samples with UV detection being the general method of choice due its price, simplicity and versatility. On the other hand, MS offers the possibility of identification of known and unknown analytes in samples, since detection and identification are based on mass-to-charge ratio (m/z). In fact, regardless the type of mass spectrometer that is coupled to the HPLC, the use of a MS detector opens new dimensions, since MS is highly specific, sensitive and delivers structural features of the analytes. In Table SM2 a compilation of several works dealing with the analytical separation of TTAs is provided along with the used C18 columns, packing features, mobile phases, flow rate, temperature, detection conditions, and calculated separation selectivities and peaks resolution. These works and main results will be briefly discussed in the following.

Yang et al. [67] reported the use of a non-endcapped Zorbax Stable Bond (SB) C18 stationary phase (100 × 4.6 mm, 1.8 μm) for the separation of seven triterpenoids from *Chaenomeles sieneis*: pomolic acid, betulinic acid, oleanolic acid, ursolic acid, acetyl ursolic acid, betulin, and erythrodiol. A mobile phase consisting of a gradient of acetonitrile and water was used and all triterpenoids were obtained with a baseline separation within a 5-min analysis.

Olmo-García et al. [68] tested three different C18 columns for the separation of maslinic acid, betulinic acid, oleanolic acid, ursolic acid, erythrodiol, and uvaol under high pH conditions with mobile phases of methanol/acetonitrile 60/40 (%, v/v): a Zorbax Extend C18 (100 × 4.6 mm, 1.8 μm), a Zorbax Eclipse (150 × 4.6 mm, 1.8 μm), and a Gemini one (dimensions not specified). The Zorbax Extend was ultimately selected as it provided higher stability and retention time repeatability, and combined with a mobile phase of methanol/acetonitrile/water 36/54/10 (%, v/v; 1.5 mM ammonium formate, adjusted to pH 9.6 with ammonium hydroxide) the baseline separation between betulinic, oleanolic and ursolic acids was achieved. In contrast, Xia et al. [69] previously used the same column packing material with a mobile phase of acetonitrile/water 90/10 (%, v/v; 0.5% acetic acid) but no baseline separation was obtained between oleanolic and ursolic acids.

One of the earlier uses of polymeric stationary phases for the separation of oleanolic and ursolic acids was reported by Zhang et al. [70]. A polymeric Ultimate XB-PAH column (250 × 4.6 mm, 5 μm) was compared with three monomeric endcapped columns, an Ultimate XB-C18, a Luna C18, and a Shim-pack CLC-C18 column (all 250 × 4.6 mm, 5 μm) and oleanolic and ursolic acids were eluted with acetonitrile/water 85/15 (%, v/v). The distinct separation performance was clearly evident, with the Ultimate XB-PAH providing complete baseline separation and well-distanced chromatographic peaks with a selectivity of $S_{UA,OA}$ of 1.18 *versus* 1.04 for the Luna C18 and Shim-pack CLC-C18 columns and 1.03 for the Ultimate XB-C18 column. Giménez et al. [71] compared a Zorbax Eclipse PAH C18 column (150 × 4.6 mm, 3.5 μm) with a Luna C18 column using mixtures of methanol/water 83/17 (%, v/v) as mobile phase at 293 K. Selectivities $S_{OA,BA}$ and $S_{UA,OA}$ of 1.17 and 1.16, respectively, were obtained, indicating that with this polymeric stationary phase the separation between betulinic and oleanolic acids and the separation between oleanolic and ursolic acids is equally difficult to carry out. Regarding the Luna C18 column, oleanolic and ursolic acids are almost co-eluted together, even under acidic or basic conditions. Later, Giménez et al. [72] performed the separation of betulinic, oleanolic and ursolic acids at 303 K with the same mobile phase obtaining higher elution times that resulted in $S_{OA,BA} = S_{UA,OA} = 1.13$. Jang et al. [73] also reported the use of a Zorbax Eclipse PAH (250 × 4.6 mm, 5 μm) but with a gradient of acetonitrile and water obtaining a selectivity $S_{UA,OA}$ of 1.13.

Xu et al. [74] and Aniceto et al. [50] reported the use of an Apollo C18 column (250 × 4.6 mm, 5 μm) with methanol/water 95/5 (%, v/v) at 293 and 296 K, obtaining $S_{UA,OA} = 1.07$ and $S_{UA,OA} = 1.06$, respectively. In Fig. 2a–c, illustrative chromatograms of TTA separations from the works of Zhang et al. [70], Giménez et al. [72], and Aniceto et al. [50] are provided. It is evident the clear baseline separation between oleanolic and ursolic acids in the conditions reported by Zhang et al. [70].

Strzemski et al. [75] reported that using a LiChrospher 100 RP-18e column (250 × 4.6 mm, 5 μm) with acetonitrile/water 75/25 (%, v/v; 1% phosphoric acid) for the analysis of betulinic, oleanolic, and ursolic acids was adequate, but the retention times of other triterpenoids such as β- and α-amyrin and β- and α-amyrin acetate would be relatively higher even for pure acetonitrile and high flow rate values (above 2 mL/min). The best results for the separation of amyrins and amyrin derivatives were obtained with a monolithic RP column (RP18e Chromolith 100, 100 × 2 mm).

Guo et al. [76] compared the performance of a Hypersil C18 column, a Diamonsil C18 column, and a Waters SunFire C18 column (all columns 250 × 4.6 mm, 5 μm) for the separation of 11 triterpenic acids with a gradient of methanol and water as it provided better separation and resolution of target peaks (oleanolic and ursolic acids) than acetonitrile and water. The best results were found with the Hypersil C18 column and a methanol/water gradient modified with 0.3% acetic acid and 0.15% triethylamine (%, v/v) at 298 K.

Wang et al. [77] compared two Symmetry C18 columns (150, 250 × 4.6 mm, 5 μm) and an Atlantis T3 column (150 × 4.6 mm, 5 μm) for the separation of betulin, betulinic, and oleanolic acids, and the best peak shape was provided by the 250-mm symmetry column. Methanol/water and acetonitrile/water mixtures were tested, and the latter ones were selected as methanol/water mixtures produced more baseline noise. Isocratic and gradient modes of operation were tested, and while both modes of operation were able to effectively separate betulinic and oleanolic acids, only gradient elution was able to effectively separate these triterpenoids within appropriate time and improved peak shape. Zhang et al. [70] also reported that mixtures of acetonitrile and water were likely to produce less baseline noise despite the higher peak symmetry with the methanol/water mixtures.

Xing et al. [78] reported that better separations between oleanolic and ursolic acids were obtained with a Shim-pack CLC-ODS (M) column in comparison with an Inertsil ODS-SP column using methanol/water 91.7/8.3 (%, v/v; 0.05% phosphoric acid) at 294 K.

Rada et al. [79] compared an Agilent LiChroshper C18 and a Spherisorb ODS-2 C18 (both 250 × 4.6 mm, 5 μm) with an acetonitrile-based mobile phase in detriment of a

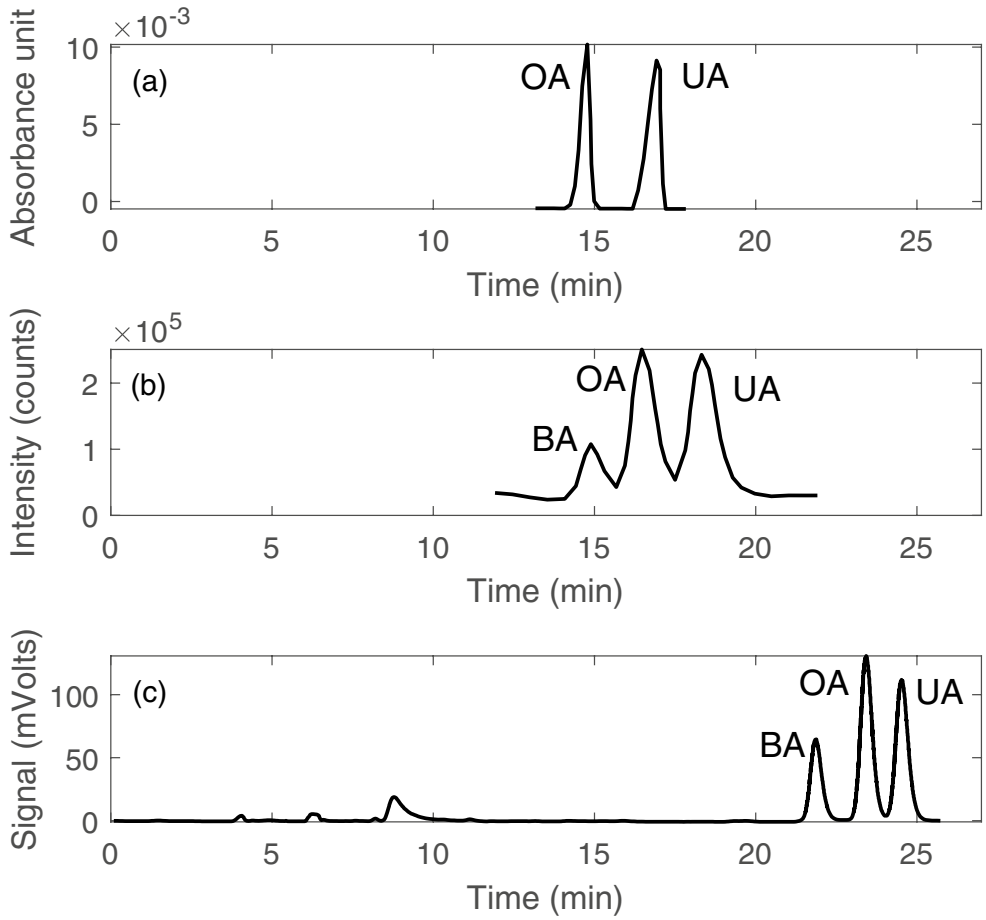

Fig. 2 HPLC chromatograms with different C18 columns. **a** Ultimate XB-PAH column (250×4.6 mm, 5 µm) with acetonitrile/water 85/15 (%, v/v) at 298 K and flow rate of 1.0 mL/min [70]. **b** Zorbax Eclipse PAH C18 column (150×4.6 mm, 3.5 µm) with methanol/water 83/17 (%, v/v) at 303 K and flow rate of 0.8 mL/min [72]. **c** Apollo C18 column (250×4.6 mm, 5 µm) at 296 K and flow rate of 0.4 mL/min [50]. Initial parts of chromatograms in (**a**) and (**b**) are omitted for simplicity. (BA, betulinic acid; OA, oleanolic acid; UA, ursolic acid). Figure 2 **b** and **c** adapted from Giménez et al. [72] and Aniceto et al. [50], respectively, with permission from Elsevier

methanol-based one to avoid blank interferences. The Spherisorb ODS-2 column ended up being selected to conduct the separation between betulinic, oleanolic, ursolic and glycyrrhetinic acids.

Guo et al. [23] separated 14 compounds (triterpenic acids, saponins, and flavonoids) from leaves of two *Ziziphus* species and concluded that despite a Hypersil C18 column (250×4.6 mm, 5 µm) provided better separations for the triterpenic acids (betulinic and oleanolic acids included) it provided poor separation performance for saponins. An Apollo C18 and a SunFire C18 column (250×4.6 mm, 5 µm) were tested and it was found that the SunFire C18 provided an overall better resolution for all compounds.

Azenha et al. [51] compared the performance of three columns, an Apollo C18 (250×4.6 mm, 5 µm), a Spherisorb ODS-2 (250×4.6 mm, 5 µm), and a Spherisorb ODS-2 (250×4.6 mm, 10 µm) with mobile phases of methanol/water mixtures and methanol/acetonitrile mixtures for the separation of betulinic and oleanolic acids. Better separations (in terms of selectivity and resolution) were obtained with the Apollo C18 column, particularly with methanol/acetonitrile mixtures, as the selectivity $S_{OA,BA}$ increased the increasing acetonitrile content. The opposite was observed in a different work for the separation of ursolic and oleanolic acids [80], as the selectivity $S_{UA,OA}$ showed a slight decrease with acetonitrile content increase. The Spherisorb ODS-2 with the larger particle diameter (250×4.6 mm, 10 µm) showed always a lower peak resolution. In Fig. 3a–c, chromatograms of betulinic and oleanolic acids are provided for the Apollo C18 column (250×4.6 mm, 5 µm and Spherisorb ODS-2 columns (250×4.6 mm, 5 and 10 µm) with methanol/acetonitrile 50/50 (%, v/v) at 296 K. It is possible to see that the Apollo C18 column provides the highest retention times with $S_{OA,BA} = 1.15$. The Spherisorb ODS-2 columns with 5 and 10 µm particles with $S_{OA,BA} = 1.16$ and $S_{OA,BA} = 1.14$, respectively, were not able to provide

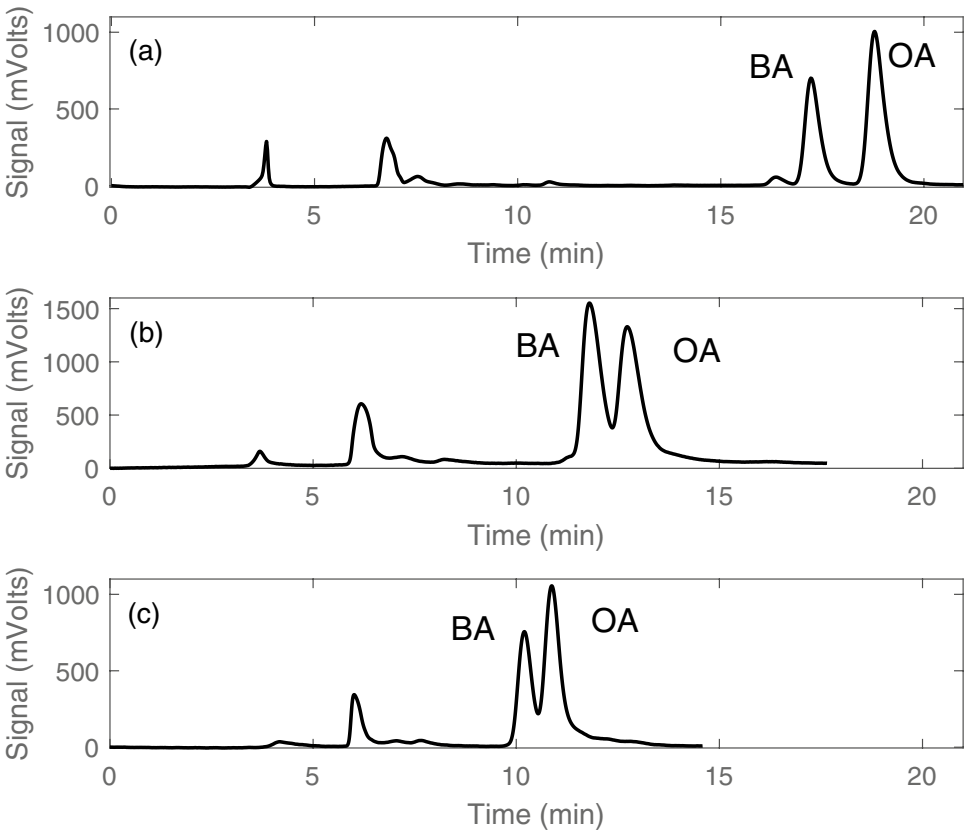

Fig. 3 HPLC chromatograms with methanol/acetonitrile 50/50 (%, v/v) at 296 K with a flow rate of 0.4 mL/min and different C18 columns [51]. **a** Apollo C18 (250×4.6 mm, 5 µm). **b** Spherisorb ODS-2 (250×4.6 mm, 5 µm). **c** Spherisorb ODS-2 (250×4.6 mm, 10 µm). (BA, betulinic acid; OA, oleanolic acid). Adapted from Azenha et al. [51] with permission from Elsevier

baseline separation between the two TTAs peaks. Moreover, with 10 µm, the retention provided by the stationary phase was inferior.

Kümmritz et al. [81] compared the performance of various columns for the separation of oleanolic and ursolic acids, namely: a Zorbax-SB C18 (250×4.6 mm, 5 µm) column, a Luna C18-2 (250×3 mm, 5 µm) column, a Nucleosil-100 C18 (250×4 mm, 5 µm) column, a Discovery HS C18 (250×4.6 mm, 5 µm) column, and an Eurospher 100 – 10 C18 (250×4 mm, 10 µm) reporting that amongst these columns only the Discovery HS C18 ensured good separation, particularly with methanol/water 92/8 (%, v/v; 0.1% formic acid) at 293 K. Li et al. [82] tested different methanol/water, acetonitrile/water, and methanol/acetonitrile/water mobile phases with an Alltima C18 column (250×4.6 mm, 3 µm) for the separation of oleanolic and ursolic acids and found that methanol/water mixtures produced better separations and lower background noise. Ammonium acetate was used in the mobile phase to improve peak selectivity and resolution. Sun et al. [83] compared two C18 columns, an Acquity UPLC BEH column and an Acquity UPLC HSS T3 (50×2.1 mm, 1.8 µm) for the separation of multiple pentacyclic triterpenes (betulinic, oleanolic, and ursolic acids included) and found out that a balanced retention performance for polar and hydrophobic molecules with shorter retention times was achieved with the HSS T3 column. A mobile phase consisting of acetonitrile/methanol 70/30 (%, v/v) was the best compromise between separation and detection conditions. It was concluded that acidic conditions provide better peak shapes and that between acetic acid and formic acid, the former was selected due to the higher signal-to-noise ratio (SNR).

Regarding the order of elution of the three triterpenic acids in the study, in the vast majority of works listed in Table SM2, one may consider it obeys: $t_{r,BA} < t_{r,OA} < t_{t,UA}$. Moreover, the order of elution of $t_{r,lupane} < t_{r,oleanane} < t_{t,ursane}$ is also observed for the different family of compounds (monools, diols) shown in Fig. 1, with diol molecules showing usually higher retention times than triterpenic acids [33, 67, 68, 71, 75, 84, 85]. Nonetheless, few exceptions regarding the order of elution are also present in literature [79, 85]. Additionally, maslinic acid (MA), with an additional hydroxyl group in C-2 (see Fig. 1) in comparison with OA, shows systematically lower retention times than the triterpenic acids (betulinic, oleanolic, and ursolic acids) [33, 68, 71, 85].

3.1.2 Bonded phases different from octadecyl

Apart from the multiple C18-bonded phases previously discussed, other alkyl-bonded phases and polar embedded phases have been mentioned in literature as alternatives for

the separation of betulinic, oleanolic and ursolic acids. Some of these works will be discussed in the following and in Table SM3 a compilation of different works dealing with the separation of betulinic, oleanolic and ursolic acids is provided along with column packing features, mobile phases, flow rate, temperature, detection conditions, and calculated selectivities and peaks resolution.

Ganbold et al. [86] reported the use of amino, phenyl, cyano, C18, and a PFP(2) (pentafluorophenyl) phases with mobile phases of water, methanol, hexane, isopropanol, and tetrahydrofuran (and their respective mixtures) using isocratic and gradient elutions. Despite the best peak resolution provided by the PFP(2) stationary phase, better selectivity between oleanolic and ursolic acids was provided by the C18 column. Zhang et al. [70] compared multiple C18 phases with cyano, phenyl, and C8 columns (all 250 × 4.6 mm, 5 µm) for the separation of oleanolic and ursolic acids with acetonitrile/water 85/15 (%, v/v) at 298 K. While all C18 stationary phases showed a separation capacity towards the two TTAs (particularly the polymeric PAH columns as previously discussed), all the three different stationary phases (cyano, phenyl, and C8) revealed to be incapable of providing any separation degree as both TTAs co-eluted in one single peak with the tested mobile phase. Using a Cosmosil πNAP (naphthalene bonded silica) column (150 × 4.6 mm, 5 µm) and methanol/water 87/13 (%, v/v), Gleńsk et al. [87] reported complete oleanolic and ursolic acids separation at 283 K with selectivity $S_{UA,OA} = 1.13$.

Mixed-mode (or multimode chromatography) is a chromatographic method in which at least two separation mechanisms contribute actively for the retention of solutes and it has become increasingly popular due to its unique selectivity and retention towards a variety of compounds [88, 89]. Recently, Falev et al. [84] compared the performance of five distinct columns to conduct the separation of 10 pentacyclic triterpenoids (betulin, erythrodiol, uvaol, friedelin, lupeol, β-amyrin, α-amyrin, betulinic acid, oleanolic acid, and ursolic acid). An Acclaim Mixed-Mode WAX-1 with embedded amide and terminal tertiary amino groups (150 × 2.1 mm, 3 µm), a Zorbax Eclipse Plus C18 and Zorbax Stable Bond Aq (150 × 3 mm, 3.5 µm), a Nucleodur PolarTec with embedded amide groups (150 × 2 mm, 1.8 µm), and a Nucleodur HILIC with a zwitterionic sulfobetaine stationary phase (150 × 3 mm, 3 µm) were tested, and, regardless of mobile phase, best separation results were obtained with the Acclaim Mixed-Mode WAX-1 which was used for more detailed assessment of mobile-phase composition influence on the separation of these 10 pentacyclic triterpenic acids. One interesting feature was the different order of elution of the various classes of triterpenoids ($t_{r,diols} < t_{r,ketone} < t_{r,monools} < t_{r,TTAs}$) in comparison with the typical reversed-phase packings. Different acetonitrile volumetric ratios were tested and the influence of mixtures of acetonitrile/water on the retention of different classes of triterpenoids was assessed. With acetonitrile/water mixtures between 80/20 and 95/5 (%, v/v) the retention times of betulinic, oleanolic, and ursolic acids undergo a minimum at *ca.* acetonitrile/water 85/15 (%, v/v) while high retention factors were found below 80 (%, v/v). This dependency on acetonitrile/water content was attributed to two retention mechanisms: a reversed-phase contribution and a hydrophilic contribution to the retention of analytes containing polar groups (a similar but much more subtle effect was also reported for the diol compounds). Overall, ion exchange and partition mechanisms were attributed to contribute actively and differently for the retention of the analytes and the observed phenomena. Additional confirmation of the relevance of hydrophilic interactions in the retention of acids and diols was given by the substitution of acetonitrile with methanol, which is not suitable for hydrophilic interaction liquid chromatography (HILIC) operation as it competes with water for polar groups of stationary phase, and, as a result, retention factors of all classes of analytes exhibited the same trend, decreasing with methanol increase. In Fig. 4a and b, the selectivity between oleanane/lupane (open symbols) and ursane/lupane (closed symbols) for the different classes of compounds, diols (full lines), monools (dashed-dotted lines), and triterpenic acids (dashed lines) are plotted as a function of acetonitrile and methanol content, respectively, in mobile phases of acetonitrile/water and methanol/water (ammonium formate concentration 5 mM, pH 4). It is possible to conclude that, with the packing material of the Acclaim Mixed-Mode WAX-1, the separation between oleanane and lupane compounds is easier to achieve and selectivities tend to be more sensitive to the organic modifier, showing a decrease as both acetonitrile and methanol increase. On the other hand, selectivities between ursane and oleanane compounds are generally more insensitive to the influence of an organic modifier and difficult to achieve particularly for methanol (selectivities very close to 1.00) with the tested mobile phases.

Since their introduction in liquid chromatography [90–92], triacontylsilyl (C30)-bonded phases have proven to be effective adsorbents in the analysis of plant extracts, food samples, biological tissues, and synthetic mixtures of carotenoids and geometric isomers [93–95]. Moreover, C30 phases are known to provide higher sample loadings and more reproducible retention behavior than C18 phases when operated in highly aqueous solvent environments [93, 96].

Recently, Azenha et al. [52, 97] studied, in different works, the separation of betulinic and oleanolic acids and the separation between ursolic and oleanolic acids with a triacontylsilyl (C30) stationary phase (250 × 4.6 mm, 5 µm). For the separation of betulinic and oleanolic acids [52], solvents such as methanol, water, acetonitrile, ethanol, isopropanol, ethyl acetate, acetone, and mixtures thereof were

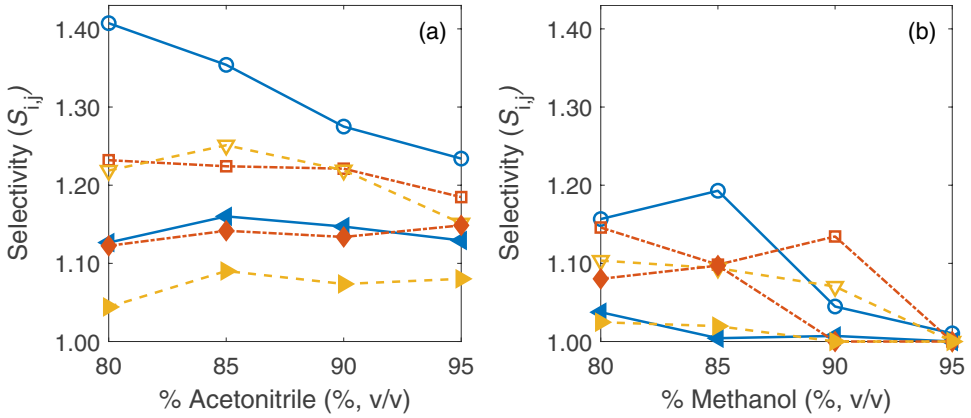

Fig. 4 Separation selectivity ($S_{i,j}$) of oleanane/lupane (open symbols) and ursane/oleanane (closed symbols) compounds as a function of **a** acetonitrile and **b** methanol content in mixtures of acetonitrile/water and methanol/water, respectively, (ammonium formate concentration 5 mM, pH 4). Symbols: ○—$S_{erythrodiol,betulin}$; □—$S_{\beta-amyrin,lupeol}$; —$S_{OA,BA}$; ◁—$S_{uvaol,erythrodiol}$; ◇—$S_{\alpha-amyrin,\beta-amyrin}$; ▷—$S_{UA,OA}$. Lines: continuous line—diol class (betulin, erythrodiol, and uvaol); dashed-dotted line—monools class (lupeol, β-amyrin, α-amyrin); dashed line—triterpenic acid class (BA, OA, and UA). Calculated results using data from Falev et al. [84]

tested. Increasing the size of the alcohol aliphatic chain, from methanol to ethanol and isopropanol, the separation of betulinic and oleanolic acids suffered a severe selectivity decrease, occurring co-elution of both acids for ethanol and isopropanol ($S_{OA,BA} = 1.00$). With the modification of methanol with water, as well as with acetonitrile, the selectivity and resolution increased with the increasing amount of modifier, reaching a selectivity $S_{OA,BA} = 1.24$ and resolution $R_{OA,BA} = 3.70$ for methanol/acetonitrile 30/70 (%, v/v). Noteworthy is also the higher sensitivity of the separation to small increments of water than to acetonitrile, since retention times of betulinic and oleanolic acids more than doubled with a 10% (%, v/v) water increase (compared with pure methanol). For the separation of ursolic and oleanolic acids [97], while the modification of methanol with water improved selectivity, the modification with acetonitrile conducted to a slight selectivity decrease. Methanol/acetone 50/50 (%, v/v) was also tested for the separation of oleanolic and betulinic acids, and a value of $S_{OA,BA} = 1.20$ was obtained but the chromatographic peaks were severely overlapped. The modification of ethanol and isopropanol with acetonitrile resulted equally in an improvement of $S_{OA,BA}$. Overall, and similarly to what was observed with C18 stationary phases [50, 51, 80], better separations of betulinic and oleanolic acids were achieved with binary methanol/acetonitrile mixtures and the separation of ursolic and oleanolic acids with methanol/water mixtures (higher selectivities and resolutions). The performance of the Acclaim C30 column was compared with the performance of two C18 columns from previous works: an Apollo C18 column (250×4.6 mm, 5 µm) [51, 80] and a Zorbax Eclipse Plus C18 column (150×4.6 mm, 1.8 µm) [68] for betulinic and oleanolic acids separation using methanol/acetonitrile mixtures with different volumetric ratios. The retention factors and selectivities as a function of acetonitrile content are represented in Fig. 5a and b, respectively. From Fig. 9a, it is possible to see that the retention factors (k') of betulinic and oleanolic acids increase with increasing acetonitrile content being this effect more pronounced for the C18 columns. On the other hand, the selectivities (Fig. 9b) increase at decreasing rate for all columns with a transition zone around 50% (v/v) of acetonitrile. The packing of the Acclaim C30 column provides the highest selectivities when compared with both C18 columns, and the ratio of selectivities between C30 and C18 columns remains approximately constant and equal to 1.08 throughout the whole acetonitrile range. Regarding the Apollo and Zorbax Eclipse C18 columns, it is interesting to note how the selectivities seem to coincide. In fact, the Apollo C18 column possesses 15% of carbon distributed over a specific surface area of 340 m^2/g and pore diameter of 100 Å [98], and the Zorbax Eclipse Plus C18 contains a carbon load of 9% distributed in *ca.* half the surface area, (160 m^2/g), and pore size of 95 Å [99], which results approximately in the same density of octadecyl bonded chains.

3.1.3 Porous graphitic columns

Porous graphitic columns (PGCs) have been highly regarded since their commercial introduction in 1988 due to their superior performance in areas where alkyl-bonded phases fail to provide satisfactory selectivity separation [100]. PGCs exhibit strongly retentive behavior for non-polar compounds, but in opposition to alkyl-bonded phases with a brush-like surface, the flat, highly crystalline, and polarizable surface of graphite results in unique retention and selectivities towards polar and structurally related compounds [100, 101], which are often difficult to resolve and retain in typical reversed-phase liquid chromatography. Overall, the

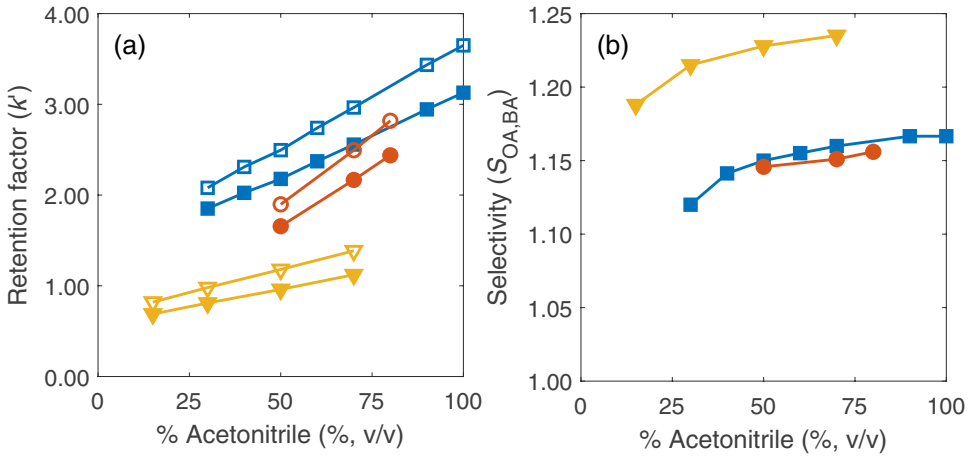

Fig. 5 **a** Retention factors (k') of betulinic (closed symbols) and oleanolic (open symbols) acids and **b** selectivity $S_{OA,BA}$ as a function of acetonitrile content (%, v/v) in methanol/acetonitrile mixtures. Triangle symbols are the results from Azenha et al. [52], with an Acclaim C30 column (250×4.6 mm, 5 µm) at 296 K, square symbols are calculated results with the data from the work of Olmo-García et al. [68], with a Zorbax Eclipse Plus C18 column (150×4.6 mm, 1.8 µm) at 298 K, and circles are the results from previous works [51, 80] with an Apollo C18 column (250×4.6 mm, 5 µm) at 296 K. Adapted from Azenha et al. [52] with permission from Elsevier

retention is determined by hydrophobicity and by the interaction of polarizable or polarized groups of analytes with the polarizable surface of graphite. The molecular shape plays a decisive role since the more planar the analyte is, the greater the interaction between the analyte and graphite surface, and thus, an increase in retention is observed [100]. PGCs' extreme chemical stability allows their use in harsh conditions of pH, salt concentration, and temperature [100, 101].

The first use of a PGC for the separation of triterpenic acids by liquid chromatography was reported by Bérangère et al. [102]. In their work, the separation of betulinic, oleanolic, ursolic, 18α-glycyrrhetinic, and 18β-glycyrrhetinic acids was performed with a Hypercarb column (100×4.6 mm, 5 µm) and the impact of different eluent mixtures of acetonitrile/chloroform, acetonitrile/methylene chloride, acetonitrile/methyl *tert*-butyl ether, and methanol/methyl *tert*-butyl ether under isocratic conditions was assessed. Regarding the separation of betulinic, oleanolic and ursolic acids, the order of elution was always the same for all binary solvent mixtures ($t_{r,BA} < t_{r,OA} < t_{r,UA}$) and the retention factors of the three acids decreased with the increasing content of the less polar solvent of all tested mobile phases. The selectivities calculated with the retention factors provided by Bérangère et al. [102] are shown in Fig. 6. Overall, the separation of oleanolic from betulinic acid (closed symbols) was easier to accomplish, especially with acetonitrile/methyl *tert*-butyl ether and methanol/methyl *tert*-butyl ether (dashed-dotted and dashed lines, respectively, in Fig. 6), than the separation of ursolic from oleanolic acid (open symbols). It is also worth of mention the different behaviors of selectivity with the increase of the less polar solvent in the mixture. While $S_{UA,OA}$ tends

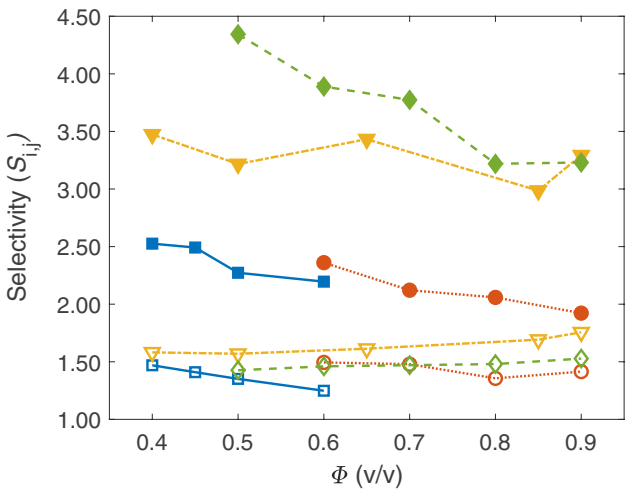

Fig. 6 Variation of the selectivities of oleanolic/betulinic acids ($S_{OA,BA}$, closed symbols) and ursolic/oleanolic acids ($S_{UA,OA}$, open symbols) with the volumetric fraction (ϕ) of the less polar solvent in mobile phases consisting of acetonitrile/chloroform (continuous lines), acetonitrile/methylene chloride (dotted lines), acetonitrile/methyl *tert*-butyl ether (dashed-dotted lines), and methanol/methyl *tert*-butyl ether (dashed lines). Selectivities calculated using the retention factors provided by Bérangère et al. [102]

to decrease monotonously with the increase of chloroform in mixtures of acetonitrile/chloroform (full lines), the separation seems to be not impaired as the fraction of methyl *tert*-butyl ether increases in mixtures of acetonitrile/methyl *tert*-butyl ether and methanol/methyl *tert*-butyl ether, dashed-dotted and dashed lines, respectively. On the opposite, $S_{OA,BA}$ decreases with increasing content of methyl *tert*-butyl ether in methanol/methyl *tert*-butyl ether mixtures.

In an attempt to elucidate the separation mechanism in this PGC, Bérangère et al. [102] determined the following five molecular descriptors: the logarithm of the octanol/water partition coefficient ($\log P_{O/W}$), the dipole moment, the van der Waals volume, the radius of gyration, and the globularity for each triterpenic acid. Volume, radius of gyration, and globularity were not able to explain the observed selectivities as these descriptors were all similar. The complexity of the separation mechanism was proven by analyzing the values of $\log P_{O/W}$ and dipole moment of each acid. Firstly, the observed anti-correlation between the order of elution of each acid and $\log P_{O/W}$ was referred to be characteristic of a normal phase mechanism. Secondly, the observed direct correlation between the dipole moment and the order of elution proved that the separation mechanism was directly related to electrostatic interactions between the stationary phase and TTAs, a mechanism described by Knox et al. [103] and termed as polar retention effect of graphite (PREG). Finally, it was also registered a decrease in the retention times of each TTA with the increase of the less polar solvent percentage in the mobile phase, which is characteristic of a reversed-phase process. Globally, these three observations constituted for the authors a proof of the dual mechanism of adsorption onto the surface of PGC (PREG effect) and a partition process between the hydrophobic TTAs and the mainly apolar mobile phase. In Table SM4 the conditions for the separation of betulinic, oleanolic and ursolic acids with acetonitrile/chloroform 40/60, 50/50, 60/40 (%, v/v) are provided along with the respective selectivities and resolutions calculated from the chromatograms reported by Bérangère et al. [102].

More recently, Rhourri-Frih et al. [104] performed the separation of 11 triterpenes with a Hypercarb column (100 × 2.1 mm, 5 µm) employing a gradient of acetonitrile/isopropanol at 298 K. The conditions and chromatographic results are listed in Table SM4. Once again, high selectivities were obtained, with $S_{OA,BA} = 3.31$ and $S_{UA,OA} = 1.54$, and peaks were completely resolved. Chromatographic conditions were previously optimized, namely the effect of acidic modifier, mobile-phase composition, and temperature on the separation of betulin from betulinic acid, lupeol from uvaol, and β-amyrin from α-amyrin. Regarding the effect of acidic modifiers, formic acid was used with mobile phases consisting of acetonitrile/isopropanol, acetonitrile/ethyl acetate, and methanol/diethyl ether and its effect was negligible up to a concentration of 100 mM at 298 K. With reference to mobile-phase composition, mixtures of methanol modified with isopropanol, ethyl acetate, and diethyl ether and mixtures of acetonitrile also modified with isopropanol, ethyl acetate, and diethyl ether were studied, and those containing acetonitrile were reported to favor selectivity, with acetonitrile/isopropanol being the best compromise between high resolution and total time of analysis. The best temperature to conduct the separation was found to be 298 K.

Grigoras et al. [105] performed the separation of betulinic acid, oleanolic acid, ursolic acid, erythrodiol, and uvaol with a Hypercarb column (50 × 4.6 mm, 5 µm) with a gradient composed of methanol/acetonitrile/isopropanol. The experimental chromatographic conditions and the results obtained from the provided chromatogram are listed in Table SM4. A high selectivity $S_{OA,BA}$ of 4.32 was obtained, the pair of oleanolic and ursolic acids was clearly isolated from the erythrodiol and uvaol pair, and the elution order was $t_{r,OA} < t_{r,UA} < t_{r,erythrodiol} < t_{r,uvaol}$. Overall, the mentioned works demonstrated the excellent capacity of PGCs to isolate structurally related isomers such as betulinic, oleanolic, and ursolic acids.

3.2 Molecularly imprinted polymers

Molecularly imprinted polymers (MIPs) are synthetic and tailor-made materials with artificially created recognition sites able to bind reversibly and very selectively to a target or target molecules in the presence of other structurally related analogues [106]. MIPs have not only the potential to be finely tuned for a specific task, and thus offer high affinity and selectivity, but also the ability to withstand harsh conditions of temperature, pressure, pH, mechanical stress, and solvent compositions combined with affordable and straightforward synthesis protocols [107–109]. As a result, MIPs have raised much research interest in the last years which is reflected, for example, by the number of review articles describing their use in a myriad of applications, including catalysis [110, 111], sensors [112, 113], solid-phase extraction [114, 115], chromatography [116, 117], sensors and membranes for enantiomeric separations [118], waste-water treatment [119, 120], food analysis [121, 122], drug delivery [123], and illicit drug analysis detection [124], among others. Globally, chromatography and solid-phase extraction (SPE) are the two major areas of application of MIPs [116].

The molecular imprinting process is based on the formation of a complex between an analyte (also referred to as a template) and functional monomers either through covalent or non-covalent approaches. In the presence of a cross-linking agent, a three-dimensional polymer network is created, and after polymerization, the removal of the template leaves an impressed polymer (MIP) with cavities and recognition sites complementary in shape, size, and chemical functionality to the template molecule (see Fig. 7). The prepared MIP can now interact with other compounds, and selectively uptake the target analyte similarly to a "lock and key" mechanism [126]. Alternatively, in applications where the template analyte may interfere in posterior quantitative analysis, a dummy template (a structurally related analog) may be used instead [116]. Simultaneously with the synthesis of a

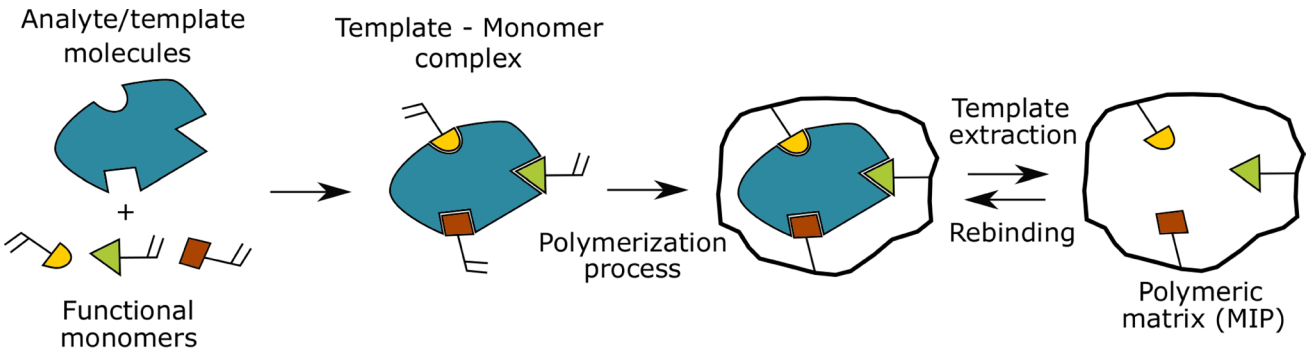

Fig. 7 Illustrative scheme of molecular imprinting for the preparation of a molecularly imprinted polymer (MIP). Adapted from Vasapollo et al. [125]

MIP, a non-imprinted polymer (NIP) is commonly prepared following the same procedure adopted for a MIP but without including the template molecule. In this way, the imprinting effect can be assessed and the resulting MIP should show a higher adsorption capacity due to selective interactions with the analyte [125].

Many of the parameters involved in the imprinting process such as the choice of reagents (functional monomers, cross-linkers, solvents/porogens) as well as their relative proportions and polymerization strategies (bulk, suspension and precipitation, emulsion, surface, in situ, etc.) can have a detrimental impact on the information associated with the recognition binding sites and overall MIP performance. Therefore, they require careful optimization to obtain a MIP with a desired performance [114, 116, 121, 125, 127, 128].

Due to the similar structures of betulinic, oleanolic, and ursolic acids (see Fig. 1) and subsequent low selectivities provided by typical octadecyl columns and other related packing materials, MIPs seem to constitute promising adsorbent alternatives for TTA separation with high selectivities. In the last few years, there have been multiple works dealing with the different MIP synthesis strategies for the separation/isolation of these three triterpenic acids and these will be discussed in the following. Table SM5 lists these works along with synthesis conditions and obtained selectivities.

To the best of our knowledge, the first application of MIPs in the analysis of betulinic, oleanolic, or ursolic acids was provided by Claude et al. [129]. A MIP was prepared by thermal polymerization using methacrylic acid as a functional monomer, ethylene glycol dimethacrylate as a cross-linking agent, chloroform as a porogenic solvent, and 18-β-glycyrrhetinic acid as a template (oleanane triterpene). MIP performance was first tested with model mixtures containing erythrodiol, oleanolic acid, echinocystic acid, and the target analyte. Excellent MIP/NIP selectivity was obtained with an average recovery for MIP of 100% towards 18-β-glycyrrhetinic acid (NIP recovery was 5%), and 50, 5, and 0% recoveries for echinocystic acid, oleanolic acid, and erythrodiol, respectively. Later, Claude et al. [130] synthesized a MIP to recover betulin and betulinic acid from a methanolic extract from plane bark. MIPs were synthesized by thermal polymerization with betulin as a template. The effect of methacrylic acid (MAA) and acrylamide (AA) as functional monomers was assessed; ethylene glycol dimethacrylate was used as a cross-linking agent, and chloroform as a porogen solvent. MAA-based polymers exhibited higher selectivities and recoveries towards betulin and related structural analogues (betulinic acid, betulinic aldehyde, acetylbetulinic aldehyde, lupeol, linoleic acid). Recoveries obtained for betulinic acid were close to those obtained for betulin reflecting their high structural similarity.

Liu et al. [131, 132] reported the preparation of β-cyclodextrin (β-CD)-based MIP microspheres (MIMs) for the extraction of ursolic acid from *Ilex kudingcha* C. J. Tseng. MIMs were prepared with bonded β-CD and acrylamide in combination with functionalized poly(glycidyl methacrylate) microspheres acting as support, and the resulting particles had an average diameter of 7.0 μm. The potential application of the synthesized MIMs as stationary phases was assessed using acetonitrile/water 90/10 (%, v/v) (0.06% acetic acid) at 298 K. A baseline separation between oleanolic and ursolic acids was obtained with a selectivity value of 1.68.

Multiwalled carbon nanotubes (MWCNTs) are ideal MIP support materials due to their strength, stability under acidic conditions, lack of swelling, and large surface areas [133], and their incorporation in the synthesis of MIPs for the analysis of triterpenic acids has received some attention. Zhang et al. [134] prepared a molecularly imprinted electrochemical sensor with specific recognition ability for oleanolic acid by modification of MWCNTs decorated with tin oxide nanoparticles (nano-SnO_2/MWCNTs) and polypyrrole-imprinted polymer on a carbon electrode. The sensor demonstrated high selectivity towards oleanolic acid with $S_{OA,UA} = 8.78$, i.e., using ursolic acid as an interfering compound. The sensor was then compared with other common

methods to determine oleanolic acid, such as liquid chromatography and capillary zone electrophoresis, showing higher selectivity, simplicity, and cost, with good stability up to 2 months at room temperature. However, this method is only suitable for the detection of oleanolic acid and not for extraction, enrichment, and separation from different matrices. Xi et al. [135] developed a composite imprinted material based on MWCNTs using ursolic acid as a template molecule and PEG-functionalized MWCNTs as the matrix. The resulting MIP showed a selectivity between ursolic and oleanolic acids of 2.88 through static adsorption experiments. The total theoretical adsorption capacity (i.e., maximum solid loading) of the MWCNT/MIPs towards UA was 100 μmol/g. More recently, Chen et al. [136] synthesized oleanolic acid-based imprinted polymers coated on MWCNT surface using 4-vinylpyridine as a functional monomer and divinylbenzene as a cross-linker. The selectivity between oleanolic and ursolic acid (batch adsorption experiments) of the resulting MIPs was 2.11 with an adsorption capacity of 30.96 mg/g for oleanolic acid.

Zhang et al. [137] prepared MIPs to induce crystallization of oleanolic acid in supercritical CO_2 (SC-CO_2) by precipitation polymerization using oleanolic acid as a template, acrylamide (AM) as functional monomer, ethylene glycol dimethacrylate (EGDMA) as a cross-linker and azobisisobutyronitrile (AIBN) as an initiator in a mixture solvent of chloroform and methanol. The influence of the ratio of the template molecule, functional monomer, and cross-linker on the MIPs performance was investigated and the best proportion was 1:4:20 (v/v), respectively. The shape of the polymer was irregular with an average size of 23 μm and through static adsorption experiments it was found $S_{OA,UA} = 1.68$. Regarding the crystallization experiments in SC-CO_2, the presence of MIPs greatly enhanced the purity of the obtained oleanolic acid, which reached 95.7%. In a more recent work, Zhang et al. [138] developed molecularly imprinted membranes for inducing the crystallization of oleanolic acid in SC-CO_2. Compared to the previous work, this time methacrylic acid (MAA) was used as a functional monomer and polysulfone (PSF) ultrafiltration membranes were used as porous supports. In this recent work, the authors were able to improve the OA purity from 95.7 to 98.3% (despite the small decrease in the production rate).

Molecular crowding is a relatively new concept to obtain MIPs with greater capacity and selectivity, originated from the molecular environments in biological cells, where high concentrations of biomacromolecules like proteins and nucleic acids often cause peculiar environments in which the stability of higher order structures of biopolymers is affected and the association of biomolecules is promoted [139, 140]. Ionic liquids, regarded as greener "designer solvents" due to their tunable characteristics, are an interesting class of solvents with unique characteristics. Their negligible vapor pressure may reduce problems of MIP bed shrinkage, act as porogen solvents, and accelerate the polymerization reaction while simultaneously improving selectivity and adsorption properties [117, 141]. Zhang et al. [142] developed a strategy to increase the affinity of MIPs by combining a porogenic solvent of an ionic liquid with a macromolecular crowding agent. A polymethylmethacrylate (PMAA) solution in a ternary mixture of chloroform, dimethyl sulfoxide, and 1-butyl-3-methylimidazolium tetrafluoroborate was used as porogen solvent. MIP monoliths (100 × 4.6 mm) were prepared using 4-vinylpyridine as a functional monomer and the influence of PMMA concentration, the type of ionic liquid and crowding agent, and their proportions were studied. The cooperative effect of ionic liquid and macromolecular crowding agent was confirmed by the higher imprinting factors, and the resulting MIP monoliths were tested as stationary phases with pure OA and UA and acetonitrile/water 70/30 (%, v/v, acetate buffer, pH 4.2) as eluent resulting in baseline separation.

Tang et al. [143] reported the preparation of monodisperse MIPs for the enrichment of oleanolic acid. The incorporation of ethanol as a cosolvent in a mixture of acetonitrile/ethanol 3:1 (v/v) as a porogenic agent revealed to be an effective way to obtain particles with uniform size distribution (polydispersity values of 1.024 and average size of 3.15 μm). The effect of functional monomer concentration, cross-linker, and initiator concentration was also evaluated. The authors concluded that a ratio of OA/methacrylic acid (MAA) 1:4 (v/v) was conducted to monodisperse particles and an additional increase led to a decrease of the specific adsorption of oleanolic acid. Divinylbenzene (DVB) and ethylene glycol dimethacrylate (EGDMA) were used as cross-linkers and it was found that increasing the proportion of DVB led to an increase in particle size. The same result was also found when the initiator concentration was increased but at the expense of a broader size distribution. The resulting MIPs showed a selectivity of 3.48 relative to ursolic acid (with batch adsorption experiments).

More recently, Lu et al. [144] developed a two-stage precipitation polymerization method for the extraction of oleanolic acid from a grape pomace extract. The process consisted of the formation of 4-vinylpyridine divinylbenzene and trimethylolpropane trimethacrylate copolymeric microspheres (first stage), which were subsequently modified/coated with a molecularly imprinting layer of oleanolic acid as a template, methacrylic acid as a functional monomer, and divinylbenzene and ethylene glycol dimethacrylate as a cross-linker. The obtained MIP microspheres had a narrow size distribution (polydispersity values of 1.011) with an average particle diameter of 4.43 μm. Batch adsorption experiments were performed revealing an adsorption

capacity of 27.4 mg/g and a selectivity towards ursolic acid of 3.82.

A very important level of MIPs characterization is related to their molecular recognition, such as the binding capacity, which is normally performed by batch adsorption experiments. A Scatchard analysis is a common way to study the binding behavior of a MIP. Typically, Scatchard plots with two straight lines are obtained, indicating binding sites with heterogeneous adsorption energies in the polymer—high- and low-affinity binding sites [125]. Regarding MIPs for TTAs analysis, several works [132, 135, 137, 142–144] present Scatchard plots with two distinctive straight lines. Alternatively, MIPs can also be packed into a chromatographic column to provide a quicker and easier analysis about their binding features. One of the main drawbacks regarding MIPs for HPLC columns is the excessive broadening and peak tailing often found in chromatograms for templates attributed to the heterogeneity of binding sites [145]. Concerning their application as stationary phases for the separation of oleanolic and ursolic acids, Liu et al. [132] and Zhang et al. [142] reported baseline separations but at the expense of a considerable amount of tailing and peak broadening.

3.3 Other polymeric adsorbents

Despite not falling in the category of MIPs due to the absence of template analyte, other works dealing with the preparation of polymeric adsorbents for the analysis/separation of triterpenic acids have been published recently and will be briefly discussed in the following paragraphs.

Sowa et al. [146] obtained a solid-phase extraction (SPE) adsorbent based on silica gel coated with polyaniline (Si-PANI) by in situ polymerization directly on carrier particles for the enrichment of triterpenic acids. Si-PANI synthesis was referred to as relatively inexpensive and the resulting adsorbent withstands aggressive solvents in wide ranges of pH. The impact of the amount of water on the adsorption of methanolic solutions of betulinic, oleanolic, and ursolic acids was also investigated, and it was found that water content did not affect the retention of TTAs. Breakthrough curves were measured revealing a lower adsorption capacity of the Si-PANI adsorbent when compared with other materials.

Pang et al. [147] prepared a metal organic framework (MOF)-polymer monolithic column (50 × 4.6 mm) using a modified MOF and *N*-methylolacrylamide (NMA) as co-monomers for the simultaneous enrichment and quantification of ursolic acid in Chinese herbal medicine samples. Nitrogen adsorption/desorption experiments were performed to assess the monolith porous structure, revealing a type III isotherm, and the MOF presence was shown to play an important role in the resulting specific surface area, with the MOF-monolith showing 18.3 m^2/g of surface area versus 8.73 m^2/g when the MOF was absent. The retention mechanism of the monolithic column was investigated using six aromatic compounds: chlorobenzene, biphenyl, p-nitro-chlorobenzene, fluorene, anthracene, and pyrocatechol. Their retention factors decreased with increasing methanol content, which was attributed to a reversed-phase retention mechanism. Moreover, the presence of the MOF increased the adsorption capacity of the monolith (37.3 mg/g for MOF-monolith *vs.* 15.14 mg/g without MOF) and was able to provide significantly lower backpressures than a C18 column, despite providing lower adsorption capacity.

Yu et al. [148] synthesized a monolithic polymer for the purification and enrichment of ursolic acid. Functionalized multi-walled carbon nanotubes (f-MWCNTs) and styrene were used as co-monomers, ethylene glycol dimethacrylate (EGDMA) and benzoyl peroxide (BPO) were used as cross-linker and initiator, respectively, and a mixture of isopropyl alcohol and butan-1-ol was used as porogen. The synthesis conditions were optimized to ensure low backpressure while maintaining high permeability. The addition of f-MWCNTs resulted in monoliths with twice the surface area when compared with monoliths without them, which translated into a higher adsorption capacity. HPLC elution experiments were performed to assess the reproducibility of the monolith after 1000 utilizations but no selectivity tests towards oleanolic and/or betulinic acids were carried out.

Yu et al. [149] prepared a monolithic column (50 × 4.6 mm) using functionalized graphene oxide (f-GO) and *N*-isopropylacrylamide (NIPAAm) as functional monomers for the SPE enrichment of ursolic acid. Synthesis conditions were optimized to assess the impact of each variable. Ethylene glycol and isopropyl alcohol 1:3 (v/v) were found to provide a good media for GO dispersion (which was a challenging task) and good monolith homogeneity and permeability. The effect of two cross-linkers [trimethylolpropane triacrylate (TMPTA) and ethylene dimethacrylate (EDMA)] was found to be similar and with their increasing concentration, the resulting monolith possessed a higher backpressure and lower permeability. Lastly, the effect of the monomer was investigated, and it was found that the presence of f-GO and NIPAAm was able to provide a monolith with higher permeability, lower backpressure and a more uniform pore size than a monolith with only NIPAAm. Nitrogen adsorption/desorption experiments showed that the composite monolith exhibited type IV hysteresis and that the presence of f-GO greatly enhanced the specific surface area (50.5 m^2/g versus 15.1 m^2/g without f-GO). The stability of the monolith was also studied and it was reported that after 1000 injections the elution performance was not affected.

More recently, Pang et al. [150] prepared a MOF polymeric monolith, by in situ radical polymerization with derivatized UiO-66-2COOH and NIPAAm as co-monomers and propan-1-ol and PEG400 as porogen, for the enrichment and

purification of ursolic acid. A more regular particle shape and uniform porous morphology were attributed to the use of the MOF, and nitrogen adsorption/desorption assays revealed a type IV isotherm and surface area of 84.16 m^2/g. The resulting monolith exhibited an adsorption capacity towards ursolic acid of 44.92 mg/g and revealed good separation ability for small molecules, demonstrated by the baseline separation of multiple acidic, basic and neutral aromatic compounds. Nonetheless, ursolic acid was the only triterpenic acid studied.

4 Strategies for TTA separation enhancement

4.1 Pre-column derivatization strategies

The accurate analysis of triterpenic acids represents multiple challenges as these compounds lack suitable chromophores, they appear in trace amounts, and the matrices from which they are extracted often contain several triterpenoids with similar structures and polarities. To solve these issues, in the last few years pre-column derivatization strategies (i.e., derivatization reactions preceding the single-column HPLC experiments) have been developed to improve both selectivity and sensitivity [151–153]. Compounds with reactive functional groups can be brought to reaction with suitable labeling reagents, and by replacing a reactive functional group with a substituent of different chemistry, its detectability is improved along with the selectivities between other compounds. It is important to refer, particularly in the case of TTAs studied here, that after derivatization the separation is no longer between the pure acids but between compounds that may retain some characteristics of the given pure triterpenic acids. This may be highly advantageous if selectivity is greatly enhanced and the pure triterpenic acid may be easily recovered from the labeling reagent after TTA separation. In the following paragraphs, different pre-column derivatization strategies adopted for TTA analysis will be discussed. These works are compiled in Table SM6 along with the column packing features, mobile phases, flow rate, temperature, detection conditions, labeling reagents, and calculated selectivities and resolutions.

Li et al. [154] proposed the use of acridone-9-ethyl-p-toluenesulfonate (AETS) as a labeling agent for the analysis of maslinic acid, betulinic acid, betulonic acid, oleanolic acid, and ursolic acid from fruit extracts. The amount of labeling reagent was optimized by response surface methodology, and the limits of detection ranged from 1.68 to 2.04 ng/mL depending on the triterpenic acid. Regarding the chromatographic separation, a Hypersil BDS C8 column (200 × 4.6 mm, 5 µm) with a gradient of acetonitrile and water at 305 K were chosen (different C18 columns were also tested). Regarding the order of elution, it is interesting to note that it is completely reversed from what is typically observed with non-labeling approaches and most columns: $t_{r,UA} < t_{r,OA} < t_{r,BA}$. Selectivities $S_{BA,OA}$ and $S_{OA,UA}$ were 1.07 and 1.05, respectively, and resolutions $R_{BA,OA}$ and $R_{OA,UA}$ were 2.19 and 1.26, respectively. Despite the different order of elution, betulinic, oleanolic and ursolic acids eluted well separated from maslinic and betulonic acids. Later, Li et al. [155] reported the use of 2-(5-benzoacridine) ethyl-p-toluenesulfonate (BAETS) as a labeling agent for the analysis of triterpenic acids from *Swertia* spp. A Hypersil BDS C8 column (200 × 4.6 mm, 5 µm) with a gradient of acetonitrile and water were used. With this method, ursolic acid was eluted first then oleanolic acid; the selectivity $S_{OA,UA}$ was improved to 1.09 and the analysis time was shortened in half. The method offered limits of detection of 1.10 and 1.30 ng/mL for ursolic and oleanolic acids, respectively. Hu et al. [156] reported a method using BAETS as pre-column derivatization reagent for the analysis of TTAs (corosolic, ursolic, oleanolic, and betulonic acids) in *Hippophae rhamnoides* L. TTAs derivatives were separated in a Hypersil BDS C8 column (200 × 4.6 mm, 5 µm) with a gradient of acetonitrile and water at 303 K. Selectivity $S_{OA,UA} = 1.06$ was obtained and the limits of detection were between 1.71 and 2.14 ng/mL. More recently, Ma et al. [157] reported the use of BAETS for the determination of five triterpenic acids (asiatic, maslinic, corosolic, oleanolic, and betulinic acids) from *Corydalis* plants. A Hypersil Gold C18 column (250 × 4.6 mm, 5 µm) and a gradient of acetonitrile and water were used to obtain a separation between oleanolic and betulinic acids with $S_{BA,OA} = 1.05$. The limit of detection was in the range 0.71 – 1.02 ng/mL.

Chen et al. [158] presented a method using 2-(2-(pyren-1-yl)-1H-benzo[d]imidazol-1-yl)-ethyl-p toluenesulfonate (PBIOTs) as labeling reagent for the detection of seven triterpenic acids (tormentic, ursolic, oleanolic, betulinic, betulonic, corosolic, and maslinic acids) in rat plasma samples. A genetic algorithm combined with an artificial neural network approach was employed to optimize the derivatization reaction. An Akasil-C18 column (250 × 4.6 mm, 5 µm) emerged as the best alternative in terms of resolution [a Spherisorb C18 column (200 × 4.6 mm, 5 µm) and a Hypersil ODS C18 column (200 × 4.6 mm, 5 µm] were also tested) and a gradient of acetonitrile and water was used at 303 K. The order of elution was $t_{r,UA} < t_{r,OA} < t_{r,BA}$ and selectivities $S_{BA,OA}$ and $S_{OA,UA}$ were the same and equal to 1.09. A limit of detection between 0.67 and 1.08 ng/mL was reported. Sun et al. [159] synthesized a label reagent, 2-(2-(anthracen-10-yl)-1H-phenanthro[9,10-d]imidazol-1-yl)ethyl 4-methylbenzenesulfonate (APIETS), for the analysis of eight fatty acids and four triterpenic acids (oleanolic, ursolic, betulinic and maslinic acids). The derivatization conditions were optimized by response surface methodology and the triterpenic

acids were separated using a Hypersil BDS C8 column (200 × 4.6 mm, 5 µm) (other C8 columns were tested) with a gradient of acetonitrile and water at 303 K. In this work, the order of elution was $t_{r,OA} < t_{r,UA} < t_{r,BA}$ and selectivities were $S_{BA,UA} = 1.04$ and $S_{UA,OA} = 1.02$. The authors reported a limit of detection of 21.06, 25.75, and 23.74 fmol for oleanolic, ursolic, and betulinic acids, respectively.

Zhang et al. [160] reported the development of a sensitive method for the determination of triterpenic acids (maslinic, corosolic, betulinic, oleanolic, and ursolic acids) using 1-(9H-carbazol-9-yl) propan-2-yl-methanesulfonate (CPMS) as a derivatization reagent. The separation of the derivatized TTAs was performed in a Zorbax SB C18 column (50 × 2.1 mm, 1.8 µm) using a gradient of acetonitrile and water at 303 K. By using this specific marker, an interesting phenomenon emerged: after derivatization, each triterpenic acid produced two distinctive chromatographic peaks with different retention times and similar areas. The relative order of elution was $t_{r,BA} < t_{r,OA} < t_{r,UA}$ and selectivities $S_{OA,BA} = 1.05$ and $S_{UA,OA} = 1.06$, for the first set of peaks and $S_{OA,BA} = 1.12$ and $S_{UA,OA} = 1.08$, for the second set of peaks. The two peak phenomenon was attributed to complex effects of the chiral carbon atom of CPMS reagent and the complex structure of the analytes. The presence of two different retention times and two similar peak areas for triterpenic acid doubly ensured the accuracy of HPLC identification by eliminating interferences from fatty acids, since each fatty acid derivative had only one peak. Wu et al. [161] developed a method using 2-(12,13-dihydro-7H-dibenzo[a,g]carbazol-7-yl)ethyl 4-methylbenzenesulfonate (DDCETS) for the analysis of six triterpenic acids (maslinic, corosolic, betulinic, betulonic, oleanolic, and ursolic acids) in traditional Chinese medicinal herbs. A Hypersil ODS C18 (200 × 4.6 mm, 5 µm) column and a gradient of acetonitrile and water were used at 303 K. Selectivities $S_{OA,BA}$ and $S_{UA,OA}$ were, respectively, 1.11 and 1.03, and the analysis run time was above 30 min. The reported limits of detection were between 0.95 ng/mL and 1.36 ng/mL.

You et al. [162] developed a method using 2-(12-benzo[b] acridin-5-(12H)-yl)-acetohydrazide (BAAH) as a labeling marker for the analysis of maslinic, ursolic, oleanolic and betulinic acids. TTAs were separated using a Hypersil BDS C18 column (200 × 4.6 mm, 5 µm) and a gradient of acetonitrile and water at 303 K. Selectivities of $S_{BA,OA}$ and $S_{OA,UA}$ were, respectively, 1.04 and 1.02 and the limit of detection was 0.28 – 0.29 ng/mL. Zeng et al. [163] compared two labeling markers, BAAH and 2-(5H-benzo[a]-carbazol-11(6H)-yl) ethyl hydrazine-carboxylate (BCEHC) for the determination of five triterpenic acids (asiatic, maslinic, corosolic, betulinic, and oleanolic acids). A Hypersil Gold C18 column (250 × 4.6 mm, 5 µm) and a gradient of acetonitrile and water were used at 303 K. While BCEHC showed a lower limit of detection (0.42 – 1.35 ng/mL), BAAH was able to resolve betulinic and oleanolic acids peaks with shorter times simultaneously with higher selectivities ($S_{OA,BA} = 1.17$).

Zheng et al. [164] proposed the use of 2′-carbonyl-piperazine rhodamine B (CPR) as a derivatization agent for the enhanced UHPLC-MS determination of oleanolic and ursolic acids. The separation of betulinic, oleanolic and ursolic acids was performed at 303 K with a Zorbax SB column (50 × 2.1 mm, 1.8 µm) and a gradient of acetonitrile/water, and the selectivities $S_{UA,OA}$ and $S_{OA,BA}$ were 1.05 and 1.11, respectively. The limits of detection of the proposed method were 0.025 and 0.020 ng/mL for oleanolic acid and ursolic acids, respectively. More recently, Wada et al. [165] reported the use of 4-(4,5-diphenyl-1H-imidazole-2-yl)benzoyl chloride (DIB-Cl) for the determination of betulinic, oleanolic and ursolic acids. A Wakopac Handy ODS column (250 × 4.6 mm, 6 µm) provided better results (Daisopak-ODS-120 – 5-BP and ZIC-HILIC columns were also tested) and the samples were eluted isocratically with a mixture of acetonitrile/methanol/water 82/10/8 (%, v/v) with 25 mM of an acetate buffer (pH 4.5). Analysis times of 30 min were obtained and the limit of detection provided by the method was between 0.2 and 0.5 ng/mL.

4.2 Cyclodextrins as mobile-phase modifiers

Cyclodextrins (CDs) are cyclic, toroidally shaped, naturally occurring, and chiral oligosaccharides composed of at least six α-1,4 linked D-glucopyranose units per molecule. The most common are the α-, β-, γ-CDs, which contain six, seven, and eight units of glucose, respectively. The CD interior is hydrophobic and forms a cavity of different sizes (as illustrated in Fig. 8) that may selectively and non-covalently incorporate organic compounds of appropriate size, forming inclusion complexes. Their exterior is hydrophilic and thus CDs may provide a way to carry selectively non-polar solutes in moderately polar environments [167]. Due to their high biocompatibility, versatility, and capacity to complex with a large variety of molecules, over the last two decades non-derivatized and derivatized CDs have received growing research attention, exhibiting numerous applications in various areas, such as pharmaceuticals, cosmetics, food and nutrition, textiles, and separation sciences [168–171].

Regarding their application in liquid chromatography, CDs are greatly used as stationary phases connected to a solid support or as mobile-phase additives as they are able to discriminate between positional isomers, functional groups, and enantiomers [168]. The CD cavity is of primary importance, as only those analytes able to contact intimately with the chiral cavity of the CD will form stable complexes. Accordingly, differences in complexation energies between solutes and CD cavities can be greatly explored with the advantage to design suitable CDs for a specific separation.

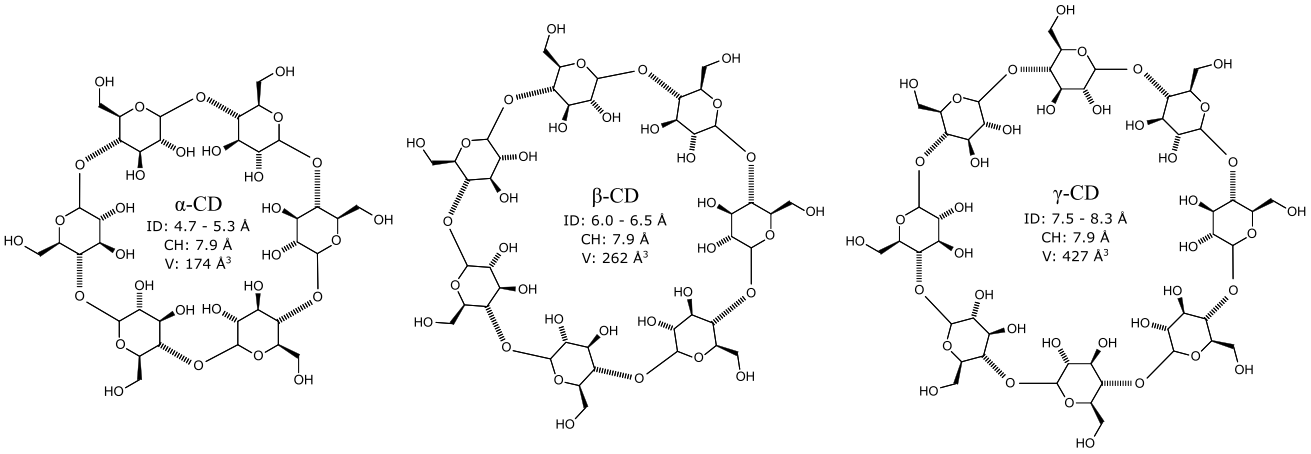

Fig. 8 Structure and approximate geometric dimensions of α-, β-, and γ-cyclodextrin molecules (CD, cyclodextrin; ID, internal diameter; CH, cavity height; V, approximate cavity volume) [166]

Moreover, due to the presence of multiple reactive hydroxyl groups, CD functionality can be finely tuned [172].

The works that have been dealing with the application of CDs to the separation of the three TTAs under analysis in this review, have been considering their use as mobile-phase modifiers and will be discussed in detail in the following paragraphs. These works are also compiled in Table SM7 along with the column packing features, mobile phases, flow rate, temperature, detection conditions, and calculated selectivities and resolutions.

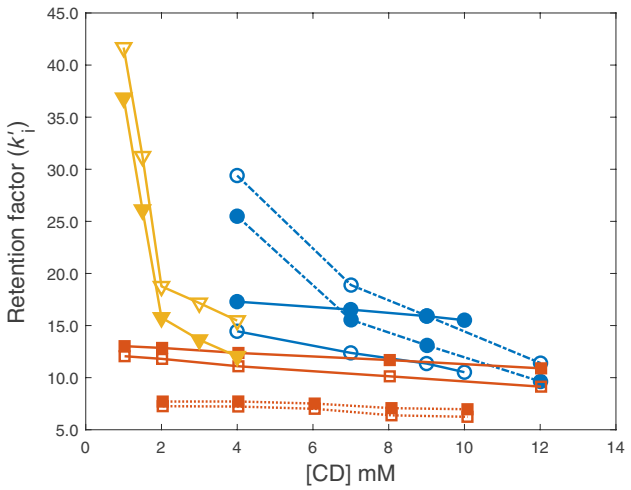

Fig. 9 Retention factors for oleanolic acid (open symbols) and ursolic acid (closed symbols) as a function of CD concentration. Symbols: Circles correspond to data from the work of Claude et al. [173] (continuous lines: acetonitrile/water 70/30 (%, v/v; 0.02 M phosphate buffer) + DM-β-CD; dashed-dotted lines: acetonitrile/water 50/50 (%, v/v; 0.02 M phosphate buffer) + HP-γ-CD); squares correspond to data from the work of Fan et al. [176] (methanol/water 85/15 (%, v/v; 0.2% acetic acid) + HP-β-CD; continuous lines: 293 K; dotted lines: 308 K); triangle symbols are data from the work of Wang et al. [175] (acetonitrile/water 60/40 (%, v/v; 0.2% phosphate buffer) + γ-CD)

The use of cyclodextrins as mobile-phase modifiers for the separation of betulinic, oleanolic, and ursolic acids by liquid chromatography was first reported in 2004 by Claude et al. [173]. In their work, the effect of the type of cyclodextrin such as γ-cyclodextrin (γ-CD), dimethyl-β-cyclodextrin (DM-β-CD), and hydroxypropyl-γ-cyclodextrin (HP-γ-CD), as well as the impact of their concentration on TTA separation, was evaluated with isocratic mobile phases consisting of acetonitrile and water (with a 0.02 mM phosphate buffer, pH 3.5) and a LiChroshper 100 RP-C18 column (125 × 4 mm, 5 µm). Acetonitrile was preferred over methanol as it provided lower retention factors and prevented the collapse of chromatographic peaks. It was found that TTAs retention time decreased with increasing concentration of HP-γ-CD (4 – 12 mM) and DM-β-CD (4 – 10 mM) and that was attributed to the formation of inclusion complexes that, for instance, increased TTAs solubility in the mobile phase and, thus, decreased retention times. Moreover, TTA elution order depended on the type of cyclodextrin used, with an elution order of $t_{r,BA} < t_{r,UA} < t_{r,OA}$ for HP-γ-CD (4 –12 mM) and $t_{r,OA} < t_{r,BA} < t_{r,UA}$ for DM-β-CD (4 – 10 mM). Efficient and extremely well-resolved peaks for BA, OA, and UA were obtained with 7.5 mM of HP-γ-CD in acetonitrile/water 50/50 (%, v/v; 0.02 mM phosphate buffer, pH 3.5) (Table SM7). The use of γ-CD was discarded as it demanded long chromatographic runs. The stoichiometry of complexation was determined as 1:1 between solute and CD, and higher complex formation constants were obtained for HP-γ-CD than DM-β-CD, reflecting different host–guest interactions as a result of different cavity sizes. Lastly, it was found that the complexation constant decreased as the acetonitrile content increased which was ascribed to a possible competition between acetonitrile and CD cavity for

the hydrophobic TTAs. Later, Kontogianni et al. [174] performed the separation of oleanolic and ursolic acids using 7.5 mM of HP-γ-CD in acetonitrile/water 55/45 (%, v/v; 0.02 mM phosphate buffer, pH 3.5) with a Hypersil ODS C18 column (250 × 4.6 mm, 5 µm) obtaining a selectivity $S_{OA,UA}$ of 1.13.

Wang et al. [175] studied the effect of the type of non-derivatized cyclodextrin such as α-, β- and γ-CD as well as the effect of their concentration and the acidity of the mobile phase on the separation of oleanolic and ursolic acids, using a Kromasil C18 column (250 × 4.6 mm, 5 µm) and acetonitrile/water 60/40 (%, v/v) as mobile phase. Baseline separation was only possible with γ-CD while with α-CD the peaks co-eluted together and with β-CD the resolution was low. TTA retention time showed a steep decrease with the increase of γ-CD concentration up to *ca.* 2 mM, and 0.1% of phosphoric acid was found to be the best percentage for a good compromise between selectivity and peak shape. Ursolic acid was eluted first then oleanolic acid with $S_{OA,UA} = 1.19$.

Fan et al. [176] reported a method for the separation of rotungenic, oleanolic, and ursolic acids employing hydroxypropyl-β-cyclodextrin (HP-β-CD). The effect of CD concentration was studied at 293 and 308 K, and a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm) was used with methanol/water 85/15 (%, v/v; 0.2% acetic acid). Similarly to the work of Wang et al. [175], the addition of non-derivatized β-CD to the mobile phase was not sufficient to promote baseline separation between oleanolic and ursolic acids. Moreover, this CD was discarded due to the low solubility in the mobile phase. The separation was improved by the addition of derivatized HP-β-CD both at 293 K (2 – 12 mM) and 308 K (2 – 10 mM), with retention times decreasing with increasing CD concentration. The apparent formation constants of oleanolic and ursolic acids inclusion complexes were higher at 293 K indicating that more stable complexes were established at lower temperatures. A selectivity $S_{UA,OA}$ as high as 1.19 was obtained at 293 K with 12 mM of HP-β-CD. More recently, Wang et al. [177] performed the separation of oleanolic and ursolic acids with an Agilent 5 HC-C18 column (250 × 4.6 mm, 5 µm) with acetonitrile/water 70/30 (%, v/v; 0.5% ammonium acetate) and 40 mM of HP-β-CD at 288 K. The effect of CD concentration, pH, acetonitrile content, and column temperature (278 – 318 K) were studied and it was found that with the increase in CD concentration and pH, the resolution increased, while with the temperature and acetonitrile content increase, resolution decreased. A selectivity $S_{UA,OA} = 1.57$ was obtained with 40 mM of HP-β-CD.

Kai et al. [178] reported the separation of oleanolic and ursolic acids with a Zorbax SB C18 column (150 × 4.6 mm, 5 µm) and a mobile phase of methanol/water 85/15 (%, v/v; pH 4) modified with 4 mM of glucosyl-β-cyclodextrin (Glu-β-CD). Compared with the mobile phase without CD and with non-derivatized β-CD (4 mM), Glu-β-CD (4 mM) was not able to provide baseline separation between oleanolic and ursolic acids ($R_{UA,OA} = 0.83$).

In an attempt to systematize the influence of type and concentration of CD on the separation of oleanolic and ursolic acids (usually the critical pair to isolate/separate), the retention factors of oleanolic and ursolic acids and the corresponding selectivities are represented in Figs. 9 and 10 as a function of CD concentration, respectively. Overall, oleanolic and ursolic acids retention factors decrease with an increase in the concentration of CD for all CDs and a particularly pronounced decrease up to *ca.* 2 mM is observed when γ-CD concentration increases with a mobile phase of acetonitrile/water 60/40 (%, v/v; 0.2% phosphate buffer) and a Kromasil C18 column. Regarding selectivities, all selectivities increase monotonously with CD concentration except HP-γ-CD, which shows a maximum selectivity between 7 and 9 mM for a mobile phase of acetonitrile/water 50/50 (%, v/v; 0.02 M phosphate buffer) and a Lichrospher 100 RP-18 column. Maximum selectivity of 1.47 was obtained with 10 mM of DM-β-CD and acetonitrile/water 70/30 (%, v/v; 0.02 M phosphate buffer) with a Lichrospher 100 RP-18 column. Nonetheless, below 4 mM, γ-CD with acetonitrile/water 60/40 (%, v/v; 0.2% phosphate buffer) and a Kromasil C18 column provides the highest separation selectivities compared with other CDs with their respective mobile

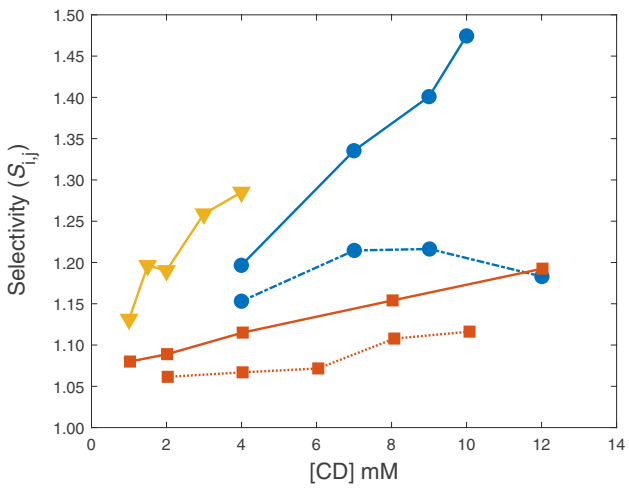

Fig. 10 Calculated selectivity between oleanolic and ursolic acids as a function of CD concentration. Symbols: Circles correspond to data from the work of Claude et al. [173] (continuous lines: acetonitrile/water 70/30 (%, v/v; 0.02 M phosphate buffer) + DM-β-CD; dashed-dotted lines: acetonitrile/water 50/50 (%, v/v; 0.02 M phosphate buffer) + HP-γ-CD); squares correspond to data from the work of Fan et al. [176] (methanol/water 85/15 (%, v/v; 0.2% acetic acid) + HP-β-CD; continuous lines: 293 K; dotted lines: 308 K); triangles correspond to data from the work of Wang et al. [175] (acetonitrile/water 60/40 (%, v/v; 0.2% phosphate buffer) + γ-CD)

phases, though it requires longer analysis times. Increasing the temperature with HP-β-CD and methanol/water 85/15 (%, v/v; 0.2% acetic acid) reduced the separation selectivity. Incrementing temperature from 293 to 308 K, the selectivities provided with HP-β-CD decreased for a mobile phase of methanol/water 85/15 (%, v/v; 0.2% acetic acid) and a Zorbax Eclipse XDB-C18 column.

4.3 Influence of acidic/basic modifiers and *pH* on TTA separation

The elution of ionizable analytes in liquid chromatography depends significantly on the pH and on the nature and content of the organic modifier of the mobile phase, which in turn offers various opportunities to fine-tune their selectivity [179–185]. It is also important to mention that the correct study of the influence of pH on the separation should be carried out looking simultaneously to the temperature [186].

Regarding the betulinic, oleanolic and ursolic acids, most of the works have been performing their separation either by enhancing or by suppressing ionization according to the pKa of analytes ($pKa_{TTAs} = 5.11 - 5.50$ [173]) and column stability restrictions. For instance, Sánchez-Ávila et al. [33] reported that low acidic values favored the separation of triterpenic acids (maslinic, betulinic, oleanolic, and ursolic acids) and high pH values favored the separation of diols (erythrodiol and uvaol). A compromise solution was to set pH 9.1 by defining a mobile phase of acetonitrile/water/methanol 90/8/2 (%, v/v) containing 0.05% ammonia and 0.05% ammonium formate. With an Inertsil ODS-2 C18 column (250×4 mm, 5 µm) at 278 K the separation between all triterpenoids was successful. Liang et al. [187] performed the separation of ursolic and oleanolic acids with a Zorbax Eclipse XDB-C18 column (250×4.6 mm, 5 µm) and various acidic and basic modifiers in different proportions. Buffers containing acetic and phosphoric acid were not able to consistently and completely separate both triterpenic acids. Moreover, mobile phases consisting of methanol, acetic acid, triethylamine and water also did not provide complete separation. With the addition of ammonium acetate to mobile phases consisting of methanol, acetonitrile and water the separation was improved, and a mobile phase consisting of methanol/water 83/17 (%, v/v; 0.2% ammonium acetate) was able to reach baseline separation. Rada et al. [79] compared the performance of 0.1% formic acid, 0.5% phosphoric acid, and 0. % acetic acid (v/v), from which 0.5% (v/v) phosphoric acid was chosen with a mobile phase of acetonitrile/water 85/15 (%, v/v) and a Spherisorb ODS-2 C18 column (250×4.6 mm, 5 µm) to conduct the separation of betulinic, oleanolic and ursolic acids. Xing et al. [78] reported that the modification of the mobile phase with acidic compounds could improve the peak shape of oleanolic and ursolic acids, but the effect of adjusting the pH on the selectivity was negligible. The same finding was previously verified by Xu et al. [74] with an Apollo C18 column (250×4.6 mm, 5 µm). Wang et al. [77] compared the performance of acetic and phosphoric to adjust the mobile phase pH with a Symmetry C18 column (250×4.6 mm, 5 µm) and found that a buffer containing 0.1% (v/v) phosphoric acid was better than acetic acid, as it produced chromatograms exhibiting very sharp peaks without leading or tailing edges. Lee et al. [188] reported that with a Luna C18 column (250×4.6 mm, 5 µm) and mixed mobile phase of acetonitrile/methanol with various aqueous buffer solutions, oleanolic and ursolic acids could not be separated when pH > 3.5 and thus a phosphate buffer was used to decrease pH to 2.8. Sun et al. [83] found out that the best peak shape for pentacyclic triterpenic acids was obtained at pH 3.7 using an Acquity UPLC HSS T3 column (50×2.1 mm, 1.8 µm) and isocratic elution with acetonitrile/methanol/water 49/21/30 (%, v/v) at 313 K. Formic and acetic acids were tested as modifiers and acetic acid was selected as it ensured higher signal to noise ratio.

Hu et al. [189] reported that the separation of eleven triterpenic acids (euscaphic, arjunic, tormentic, arjunolic, asiatic, pomolic, maslinic, corosolic, oleanolic, ursolic, and 2-epitormentic acids) was more easily achieved with an alkaline mobile phase (acetonitrile/water based eluent) and a Shim-pack GIST C18 column (100×2.1 mm, 2 µm). As the pH increased above the pKa of each compound, the retention times were reduced which was attributed to the increased ionization and polarity of each triterpenic acid. The maximum peak resolution was achieved at pH 9, above which arjunolic and asiatic acids as well as oleanolic and ursolic acids started to co-elute.

Olmo-García et al. [68] assessed the influence of different modifiers with methanol/acetonitrile 40/60 (%, v/v) and a Zorbax Eclipse Plus column (150×4.6 mm, 1.8 µm) in the separation of six triterpenoids (maslinic, betulinic, oleanolic, and ursolic acids, and erythrodiol and uvaol). Mobile-phase modifiers were added to enhance triterpenoids' degree of ionization, to reduce run time and increase the resolution of difficult separations such as oleanolic and ursolic acids. Tetrahydrofuran, triethylamine, acetic acid, and ammonium hydroxide were directly used and dissolved in different proportions in water, and the best compromise between retention time, resolution, and signal-to-noise ratio was found with 10% (v/v) basified water. The results obtained for the different modifiers studied by Olmo-García et al. [68] are plotted in Fig. 11 in terms of selectivities ($S_{OA,BA}$ and $S_{UA,OA}$). It is interesting to note how the various modifiers change differently $S_{OA,BA}$ and $S_{UA,OA}$. While 10% (v/v) water results in the highest $S_{OA,BA}$, the opposite is verified for $S_{UA,OA}$. Moreover, the use of basic modifiers resulted in overall higher selectivities when compared to the acidic ones. After that, the pH, a very critical variable for the peak resolution,

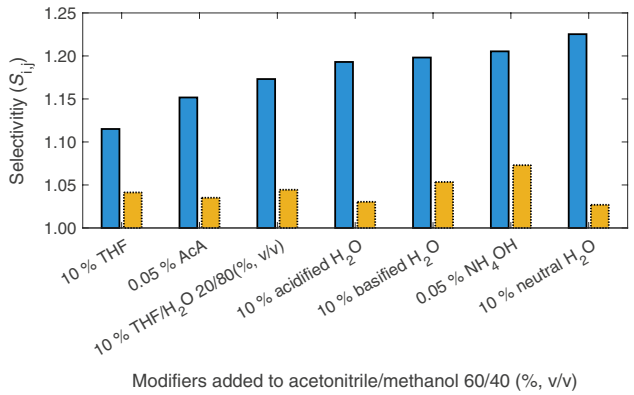

Fig. 11 Selectivities of oleanolic/betulinic acids ($S_{OA,BA}$, blue bars) and ursolic/oleanolic acids ($S_{UA,OA}$, yellow bars) as a function of different neutral, acidic, and basic modifiers of acetonitrile/methanol 60/40 (%, v/v) mobile phase. Selectivities calculated with the retention time of each acid provided by Olmo-García et al. [68] using a Zorbax Eclipse Plus column (150×4.6 mm, 1.8 µm). (THF, tetrahydrofuran; AcA, acetic acid; NH_4OH, ammonium hydroxide; H_2O, water)

was optimized using a Zorbax Extend C18 column (100×4.6 mm, 1.8 µm) as it provided more reproducible results at higher pH. Different mass spectrometry (MS) compatible buffers were tested such as ammonium bicarbonate adjusted to the desired pH with acetic acid, and ammonium formate and ammonium acetate adjusted with ammonium hydroxide at different concentrations levels (1 to 25 mM) in a pH range between 7 and 11. Retention times of all triterpenoids showed a decrease with increasing pH as illustrated in Fig. 12a. It is also possible to see a small increase in both selectivities above pH 9 (more pronounced for $S_{UA,OA}$) in Fig. 12b. It was found that the most appropriate composition was 1.5 mM of ammonium formate in water adjusted at pH 9.6 with ammonium hydroxide. Above that, triterpenic acid peaks started to co-elute. It is also worth to mention that the retention times of uvaol and erythrodiol (not shown here for simplicity) showed negligible variation with pH.

More recently, Falev et al. [84] performed the separation of 10 pentacyclic triterpenoids (betulin, erythrodiol, uvaol, friedelin, lupeol, β-amyrin, α-amyrin, betulinic acid, oleanolic acid and ursolic acid) with an Acclaim Mixed-Mode WAX-1 column with embedded amide and terminal tertiary amino groups (150× 2.1 mm, 3 µm). After selecting the appropriate ratio of acetonitrile/water, the pH conditions were optimized. As shown in Fig. 13a, while monools and diols showed little variation of the retention factors with pH, triterpenic acids showed a steep increase in their retention times, particularly between pH 4 and 5. The increase of the retention factors of these analytes with increasing pH was attributed to the interaction between the anionic form of the acids and the stationary phase, with the decisive contribution of ion exchange interactions to the mechanism of their retention on the stationary phase. The selectivities, for instance, represented in Fig. 13b, did not suffer significant variations, occurring just a subtle decrease.

Furthermore, the existence of additional retention mechanisms besides ion exchange was evaluated by varying the ionic strength of the mobile phase. At fixed pH 4 the logarithm of retention factors of triterpenic acids showed a nonlinear decrease with the increasing logarithm of the concentration of ammonium formate confirming that a significant contribution to the retention mechanism of triterpenic acids belongs to interactions which are not related to ion exchange. The monools and diols showed a subtle increase in the retention times with a decrease in ammonium formate concentration, which was hypothesized as a suppression of ion–dipole and dipole–dipole interactions of the stationary phase with analytes by ammonium formate.

More recently, Wang et al. [177] used HP-β-CD as a mobile-phase modifier and performed the separation of oleanolic and ursolic acids with an Agilent 5 HC-C18 column (250×4.6 mm, 5 µm) with acetonitrile/water 70/30 (%, v/v) with 0.5% ammonium acetate at 288 K. The influence

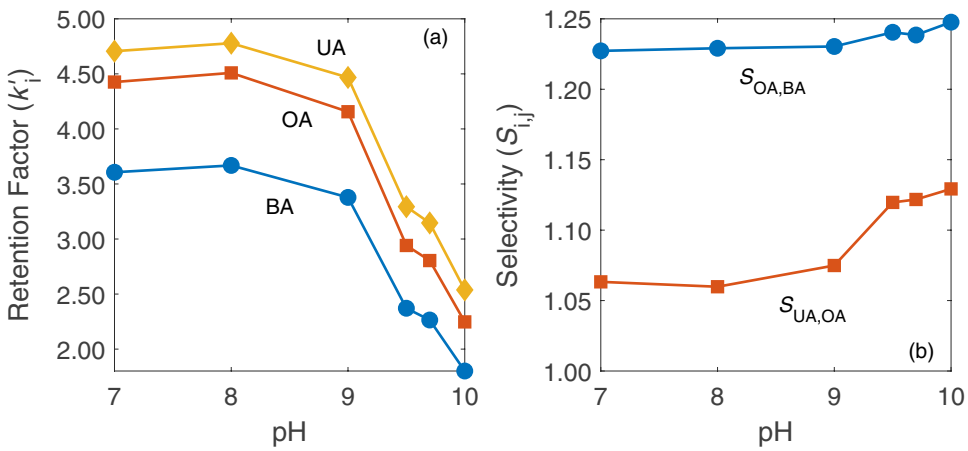

Fig. 12 **a** Retention factors and **b** selectivities as a function of pH calculated from the retention times reported by Olmo-García et al. [68] for a Zorbax Extend C18 column (100×4.6 mm, 1.8 µm) and acetonitrile/methanol 60/40 (%, v/v) as the main mobile phase. (BA, betulinic acid; OA, oleanolic acid; UA, ursolic acid)

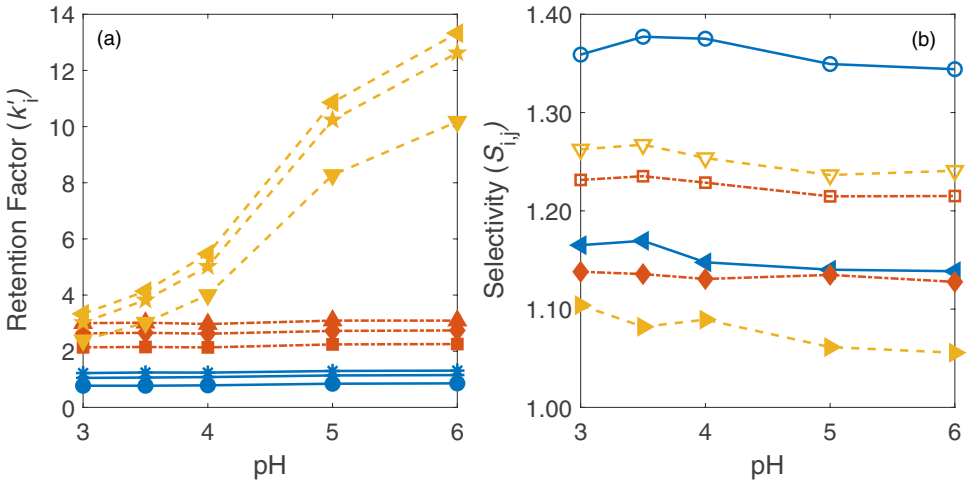

Fig. 13 **a** Retention factors (○—betulin, + —erythrodiol, *—uvaol, □—lupeol, ◇—β-amyrin, △—α-amyrin, ▽—betulinic acid, ⋆—oleanolic acid, ◁—ursolic acid) and **b** calculated selectivities (○—$S_{\text{erythrodiol,betulin}}$; □—$S_{\beta\text{-amyrin,lupeol}}$; ▽—$S_{\text{OA,BA}}$; ◁—$S_{\text{uvaol,erythrodiol}}$; ◇—$S_{\alpha\text{-amyrin,}\beta\text{-amyrin}}$; ▷—$S_{\text{UA,OA}}$) of various triterpenoids as a function of pH. Lines: continuous line—diol class (betulin, erythrodiol, and uvaol); dashed-dotted line—monools class (lupeol, β-amyrin, α-amyrin); dashed line—triterpenic acid class (BA, OA, and UA). Data from Falev et al. [84] with an Acclaim Mixed-Mode WAX-1 with embedded amide and terminal tertiary amino groups (150 × 2.1 mm, 3 µm) and acetonitrile/water 85/15 (%, v/v) with 5 mM ammonium formate. Figure 13 **a** was adapted from Falev et al. [84]; permission from Elsevier

of pH was addressed, and it was found that peak resolution increased with increasing pH between 4 and 6.

4.4 Influence of temperature on TTA separation

Temperature plays a significant role in liquid chromatographic processes both in terms of their transport phenomena and thermodynamics. For instance, as temperature increases column separation efficiency changes as a result of the reduction of eluent viscosity and the increase of diffusion rates, enhancing mass transfer [190–192]. Simultaneously, the pressure drop is reduced and solute solubility is usually incremented. On the other hand, temperature has also a strong effect on the adsorption phenomenon, affecting retention factors, and therefore total analysis time and selectivity, particularly for ionizable compounds [186, 193–199]. In what concerns the separation of betulinic, oleanolic and ursolic acids by liquid chromatography, there is a dearth of literature on the impact of temperature on the process, with most works using room temperatures between 293 and 303 K (as seen in Table SM2–Table SM7). Nonetheless, some authors have reported temperatures at which the separation performs better and this will be briefly discussed here.

Sánchez-Ávila et al. [33] investigated the separation of maslinic, betulinic, oleanolic, and ursolic acids, erythrodiol, and uvaol with an Inertsil ODS-2 C18 column and acetonitrile/water/methanol 90/8/2 (%, v/v; pH adjusted to 9.1) and discussed concisely the effect of temperature on their separation. Temperatures in the range 278 – 308 K were studied and 278 K was reported as the optimum value to conduct the chromatography. Rada et al. [79] published the separation of betulinic, oleanolic, ursolic, and glycyrrhetinic acids with a Spherisorb ODS-2 column (250 × 4.6 mm, 5 µm) and a mobile phase of acetonitrile/water 85/15 (%, v/v, 0.5% phosphoric acid) between 298 and 313 K, and the best operating temperature was 303 K. Wang et al. [77] reported that the separation of betulinic, oleanolic, and ursolic acids was best conducted at 303 K (rather than 293, 298, 308, 313, or 318 K) using C18 columns and acetonitrile/water mixtures as mobile phase. Zhang et al. [70] used a polymeric Ultimate XB-PAH column (250 × 4.6 mm, 5 µm) and a mobile phase of acetonitrile/water 85/15 (%, v/v) and reported a slightly higher resolution $R_{\text{UA,OA}}$ at 293 K than 303 K. Using a polymeric Zorbax Eclipse PAH column (150 × 4.6 mm, 3.5 µm) and methanol/water 83/17 (%, v/v), Giménez et al. [71, 72] published in different works the separation of maslinic, betulinic, oleanolic and ursolic acids, and erythrodiol and uvaol at 293 and 303 K (same mobile-phase conditions, Table SM2). At 293 K, betulinic, oleanolic, and ursolic acids eluted faster with higher selectivities and resolutions. Moreover, at both temperatures, the selectivities $S_{\text{OA,BA}}$ and $S_{\text{UA,OA}}$ remained approximately the same, indicating that with this polymeric column the separation of both TTA pairs is equally difficult to achieve.

Owczarek et al. [200] studied the separation of oleanolic and ursolic acids with a Zorbax Eclipse XDB-C18 column (100 × 3.0 mm, 1.8 µm) and used a central composite design to optimize temperature (288 – 302 K) and flow rate conditions (0.26 – 0.54 mL/min), and the responses were the resolution $R_{\text{UA,OA}}$ and the retention time of the

last eluted compound. Both t_r and $R_{UA,OA}$ showed an increase with decreasing temperature and the best separating conditions were 291 K and 0.44 mL/min. Using a Cosmosil πNAP (naphthalene bonded silica) column (150 × 4.6 mm, 5 µm) with a mobile phase of methanol/water 87/13 (%, v/v), Gleńsk et al. [87] reported complete separation between oleanolic and ursolic acids at 283 K.

Rhourri-Frih et al. [104] studied the separation of betulin, betulinic acid, lupeol, uvaol, α-amyrin, and β-amyrin in a range of 298 to 343 K with a PGC and a gradient of acetonitrile/isopropanol. As illustrated in Fig. 14, temperature had multiple impacts on the separation of each compound. While for betulin and betulinic acid the selectivities increased with increasing temperature up to 332 K, the separation of lupeol from uvaol suffered a decrease in selectivity up to *ca*. 310 K where a change in the order of elution occurred ($S_{lupeol,uvaol} < 1$). At low temperatures, lupeol is the most retained compound and uvaol the least retained one, while the opposite is observed above 312 K. Moreover, above 312 K, temperature did not influence selectivity $S_{lupeol,uvaol}$.

More recently, Wang et al. [177], while using HP-β-CD as a mobile-phase modifier, performed the separation of oleanolic and ursolic acids with an Agilent 5 HC-C18 column (250 × 4.6 mm, 5 µm) with a mobile phase of acetonitrile/water 70/30 (%, v/v; 0.5% ammonium acetate). Peak resolution was studied between 278 and 318 K and it was found to decrease with temperature. A compromise between retention time and backpressure was found at 288 K.

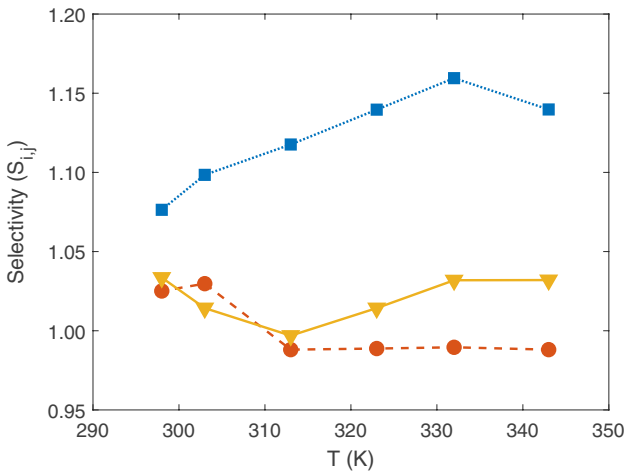

Fig. 14 Temperature dependence of selectivities: □—betulin/betulinic acid ($S_{betulin,BA}$), ○—lupeol/uvaol ($S_{lupeol,uvaol}$), ▽—α-amyrin/β-amyrin ($S_{α-amyrin/β-amyrin}$). Column: Hypercarb PGC; mobile phase: gradient of acetonitrile and isopropanol. Selectivities calculated with the retention factors provided by Rhourri-Frih et al. [104]

5 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) takes advantage of the properties of supercritical fluids, namely, fluids exhibiting liquid-like densities and solvent power, gas-like viscosities, and diffusivities between those of liquids and gases. The advantages of SFC over HPLC are evident and manifold: (i) the higher diffusivities and lower viscosities of supercritical fluids provide faster analysis runs and lower pressure drops; (ii) carbon dioxide is commonly considered an environmentally green solvent (in opposition to methanol and acetonitrile-based mobile phases) [201]; (iii) selectivities that may match those in HPLC, but more easily finely tuned [202, 203].

SFC is nowadays commonly employed for the analysis of non-volatile compounds and it is now established as the primary choice to deal with chiral separations [204, 205]. Carbon dioxide is the main constituent of mobile phases but it is insufficient alone for the elution of moderately polar compounds, and, thus, it demands to be modified to modulate mobile-phase elution strength. Usually, low percentages of methanol, ethanol, isopropanol, and acetonitrile can be used [206]. Even though the addition of modifiers results usually in subcritical operating conditions, an SFC system operating under subcritical conditions holds many of the aforementioned advantages [206].

The use of SFC for the separation of triterpenoids may be particularly advantageous not only for the reasons discussed above, but also due to the possibility of combining supercritical carbon dioxide (SC-CO_2) extraction with SFC. Different natural sources rich in pentacyclic triterpenoids have been studied over the last years, including, for example, *Eucalyptus globulus* bark and leaves [43, 207–212], olive pomace [213], *Alnus glutionsa* (L.) Gaertn [214], among others [215], and an online analysis by SFC coupled to the extraction would allow a fast and expeditious treatment of the resulting extracts.

Literature regarding the separation of betulinic, oleanolic, and ursolic acids using subcritical and/or supercritical fluids is scarce but a few works have dealt with this task and will be discussed. Table SM8 presents a summary of these works along with the respective column packing features, mobile phases flow rate, temperature, detection conditions, and calculated selectivities and resolutions.

The first separation of betulinic, oleanolic and ursolic acids using carbon dioxide as a mobile phase was reported in 2012 by Lesellier et al. [216]. In their work, the separation of oleanane (oleanolic acid, erythrodiol, β-amyrin), ursane (ursolic acid, uvaol), and lupane compounds (betulinic acid, betulin, and lupeol) was performed. Multiple columns were selected for an initial screening of stationary phases: Vision C18 HL (C18), Cosmosil π Napthyl (NAP),

YMC poly(vinyl alcohol) (PVA), Discovery HS F5, Viridis 2-Ethylpyridine (2EP), Synergi Polar-RP (OPHE), and Luna phenylhexyl (all columns 250 × 4.6 mm, 4 – 5 µm). These columns were selected on the basis of a classification based on five main types of interactions: charge transfer, dipole–dipole, hydrogen bond (acceptor and donor), and dispersion. A preliminary analysis with CO_2/methanol 90/10 (%, v/v) at 298 K and 15 MPa indicated that the OPHE and 2EP columns provided the best separations for the target analytes, whereas none of the others achieved the standards separation under the chosen screening conditions. With the selected columns another analysis was performed by reducing methanol content to 5% (%, v/v). Due to the basic character of the 2EP stationary phase, triterpenic acids were the last compounds eluting immediately after diols and monools. With the OPHE column, the order of elution of the different classes of compounds was changed, with diols being the last compounds immediately after triterpenic acids and monools. The 2EP column was, however, discarded since it provided low resolution, while the separation on the OPHE was almost complete, except the acidic compounds. With the OPHE column, the retention order was $t_{r,oleanane} < t_{r,ursane} < t_{r,lupane}$ for all classes of compounds, in opposition to C18 columns with organic solvents where the elution order is typically $t_{r,lupane} < t_{r,oleanane} < t_{r,ursane}$, as discussed in Sect. 3.1.1. Decreasing the temperature from 298 to 293 K improved the separation between betulinic acid and erythrodiol, but a further decrease down to 288 K reduced most selectivities. Additionally, the decrease in backpressure from 18 to 12 MPa improved the separation selectivity between most couples of compounds. Lastly, the methanol content was reduced from 5 to 3% (v/v) and two effects appeared: a near baseline separation was achieved within 16 min, and a strong increase in the detection response was observed.

Later, in 2018, Zhang et al. [217] performed the separation of oleanolic and ursolic acids using SFC. Multiple columns were tested: a Shim-pack UC-X Sil (150 × 2.1 mm, 3 µm), a Shim-pack UC-X Diol (150 × 4.6 mm, 3 µm), a Shim-pack UC-X NH2 (150 × 2.1 mm, 3 µm), an Inertsil CN-3 (250 × 4.6 mm, 2 µm), and a Shim-pack UC-X RP (150 × 4.6 mm, 3 µm). Baseline separation was only achieved by the Shim-pack UC-X Diol and Shim-pack UC-X RP stationary phases with a gradient of CO_2/methanol. Oleanolic acid showed a stronger retention than ursolic acid. The use of methanol, ethanol, and isopropanol as modifiers provided similar selectivities while acetonitrile conducted to long analysis times. Acidic modifiers such as formic acid were also tested but no significant improvements were reported. Retention times and resolution showed a sharp decrease with the increase of pressure (10 – 25 MPa), while resolution increased with increasing temperature.

Ultimately, Falev et al. [218] carried out an analysis of pentacyclic triterpenoids (friedelin, lupeol, β-amyrin, α-amyrin, betulin, erythrodiol, uvaol, betulinic, oleanolic and ursolic acids) separation by supercritical fluid chromatography coupled with mass spectrometry (SFC-MS/MS) using six different silica-based reversed stationary phases. The best compromise between retention and selectivity was found with an Acquity UPC^2 HSS C18 SB column (150 × 3.0 mm, 1.8 µm), which was especially designed for the separation of substances with polar groups in SFC. Further optimization of the separation conditions was performed, and while temperature (293 – 328 K) and backpressure (110 – 190 bar) did not significantly affect the separation, the organic modifiers (co-solvent) content, on the other hand, showed a great impact in the retention of all triterpenoids except friedelin. Using methanol and isopropanol the retention of those triterpenoids decreased sharply with a variation from 6 to 10% of cosolvent (%, v/v). Moreover, by varying methanol concentration, the order of elution of the compounds changes, which may be attributed to the competition of analytes and polar mobile-phase modifiers for the sorption centers (silanol groups) of the stationary phase, changing the overall contributions of hydrophobic and polar interactions to the mixed retention mechanism of PTs, with the possibility of transition from reversed-phase separation to the normal-phase one, and vice versa [218]. This was not verified for friedelin, which does not possess hydroxyl or carboxyl groups. Complete separation was obtained at low methanol contents at the expense of high retention factors for diols and the risk of precipitation of pentacyclic triterpenoids. With isopropanol, a less polar modifier, this issue was avoided. The best mobile phase was established as CO_2/isopropanol 92/8 (%, v/v).

6 Selectivity comparison

In Figs. 15 and 16, the separation selectivities of the pairs oleanolic/betulinic acids and ursolic/oleanolic acids are represented for the different adsorbents and techniques reported by the various works compiled and discussed in the previous sections (Table SM2–Table SM4, Table SM6 –Table SM8). The order of elution is not included in these figures, as all selectivities are above 1.00 and thus it is only intended to illustrate the relative distancing between chromatographic peaks, which is easier to represent and analyze when all selectivities are higher than 1.00. It is possible to see that porous graphitic columns (PGCs) achieve the highest selectivities ($1.92 \leq S_{OA,BA} \leq 4.34$ and $1.20 \leq S_{UA,OA} \leq 1.76$) and that the selected mobile phase to conduct the separation has a big impact. The lowest selectivity provided by PGCs is higher than the highest selectivity provided by C18 columns ($1.00 \leq S_{OA,BA} \leq 1.41$ and $1.00 \leq S_{UA,OA} \leq 1.18$) or other bonded phases different from

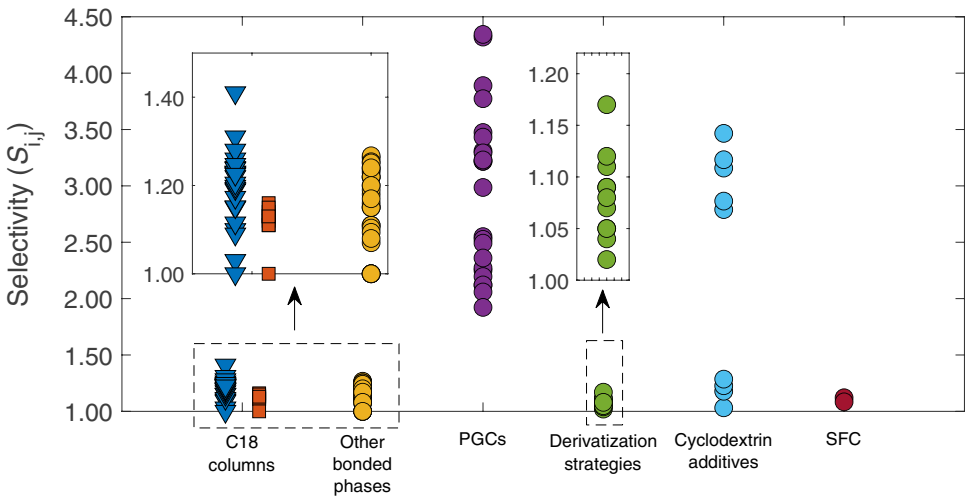

Fig. 15 Compilation of selectivities between oleanolic (OA) and betulinic (BA) acids for the different approaches reported in the literature. Selectivities calculated with data from the works cited in Table SM2–Table SM4, Table SM6–Table SM8. ▽—acetonitrile-based mobile phases, □—methanol-based mobile phases, ○—mobile phases not necessarily based on acetonitrile or methanol, PGCs—porous graphitic columns. SFC, supercritical fluid chromatography. Please see the two inserts with enlarged ordinate scales

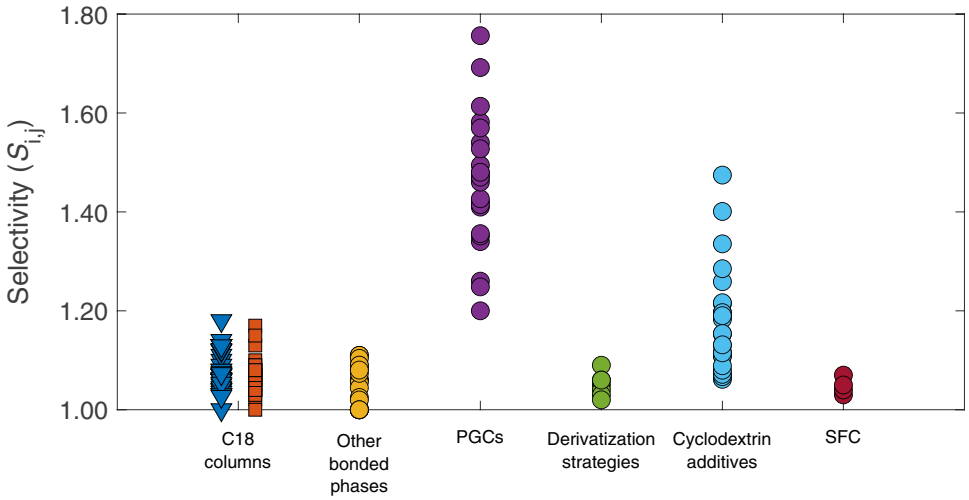

Fig. 16 Compilation of selectivities between ursolic (UA) and oleanolic (OA) acids for the different approaches reported in the literature. Selectivities collected with data from the works cited in Table SM2—Table SM4, Table SM6—Table SM8. ▽—acetonitrile-based mobile phases, □—methanol-based mobile phases, ○—mobile phases not necessarily based on acetonitrile or methanol. PGCs, porous graphitic columns; SFC, supercritical fluid chromatography

C18 ($1.00 \leq S_{OA,BA} \leq 1.27$ and $1.00 \leq S_{UA,OA} \leq 1.11$). Concerning C18 bonded phases, acetonitrile-based eluents provide highest selectivities for the separation of betulinic and oleanolic acids ($1.00 \leq S_{OA,BA} \leq 1.41$) compared to methanol-based mobile phases ($1.00 \leq S_{OA,BA} \leq 1.16$), while the separation of oleanolic and ursolic acids is not as affected by the organic modifier in the mobile phase ($1.00 \leq S_{UA,OA} \leq 1.18$) (methanol/acetonitrile 50/50 (%, v/v) was considered a methanol-based mobile phase). Taking into account the wide variability of mobile phases (composition and modifiers) and temperature conditions, it is not possible to systematize the influence of the different packing materials properties on the separation of TTAs, i.e., the influence of pore size, carbon load, surface area or ligand density. Regarding the other bonded phases, the Acclaim C30 column was able to provide one of the highest selectivities ($S_{OA,BA} = 1.23$ and $S_{UA,OA} = 1.11$) from all columns tested and compiled in Table SM3 with mobile phases suitable for preparative applications. It is worth mentioning that the derivatization strategies and the application of cyclodextrins modifiers (compiled in Table SM6 and Table SM7), respectively, have been performed with C8 and C18 columns. Overall, the pre-column derivatization approaches did not result in any significant selectivity enhancements ($1.02 \leq S_{OA,BA}$ or $S_{BA,OA} \leq 1.17$ and $1.02 \leq S_{UA,OA}$ or $S_{OA,UA} \leq 1.09$) when compared with the regular C18 columns separation of each triterpenic acid. On the other side, cyclodextrins emerged as effective additives to conduct the separations with great selectivity gains ($1.03 \leq S_{OA,BA}$ or $S_{BA,OA} \leq 3.47$ and $1.06 \leq S_{UA,OA}$ or $S_{OA,UA} \leq 1.47$), particularly HP-γ-CD for the separations of betulinic and oleanolic acids, and DM-β-CD for the separation of oleanolic and ursolic acids. Nonetheless, it should be mentioned that the elution order is different from that observed with C18 columns. Lastly, the selectivities provided by subcritical and supercritical fluid chromatography (SFC) are low, in pair with the inferior ones obtained by regular C18 columns ($S_{BA,OA} \leq 1.12$ and $S_{UA,OA}$ or $S_{OA,UA} \leq 1.07$).

7 Final outlook

Betulinic, oleanolic and ursolic acids are ubiquitous compounds with multiple recognized biological and nutraceutical activities. Taking into account their structural similarity and simultaneous occurrence, their separation after extraction is very challenging. Liquid chromatography is a widely used technique for their separation due to well-established procedures, fast analysis times, and relatively straightforward scale-up. The supercritical fluid chromatography (SFC) is not as widespread and the achieved separation is still comparatively inferior. In both cases, the choice of stationary phases, eluents, mobile-phase modifiers, and operating conditions like temperature and pH is of primary importance for the viability of such preparative processes.

The TTAs solubilities are of primary importance to properly design a throughput chromatographic separation method. The low solubilities in most pure and mixed conventional solvents are the current bottleneck for large-scale production of these solutes. In many cases, the addition of water in mobile phases to enhance selectivity conveys a huge negative impact on TTAs dissolution, as small amounts decrease abruptly their solubility. Alternatives to improve solubility such as the use of cyclodextrins, ionic liquids, and natural deep eutectic solvents demonstrated to be effective, but more research is still necessary in this area.

Concerning the separation by liquid chromatography, a vast selection of commercial stationary phases (C18 packings, other bonded phases different from C18, and porous graphitic columns (PGCs)), customized molecularly imprinted polymers (MIPs) and other polymeric adsorbents, and eluents, along with temperature and pH conditions, mobile phases modifiers (cyclodextrins) and different pre-column derivatization strategies have been researched up till now.

Best separations between the analytes are undoubtedly obtained with porous graphitic columns (PGCs), showing a wide range of selectivities with the choice of solvent ($1.92 \leq S_{OA,BA} \leq 4.34$ and $1.20 \leq S_{UA,OA} \leq 1.76$). Regarding C18 bonded phases, acetonitrile-based eluents provide the highest selectivities for the separation of betulinic and oleanolic acids ($1.00 \leq S_{OA,BA} \leq 1.41$) in comparison with methanol-based mobile phases ($1.00 \leq S_{OA,BA} \leq 1.16$), while the separation of oleanolic and ursolic acids is not as affected by the organic modifier in the mobile phase ($1.00 \leq S_{UA,OA} \leq 1.18$). C30 bonded phases show superior separation capacity than C18 phases when operated with the same solvents, particularly in the case of methanol/acetonitrile mixtures. MIPs show encouraging results in terms of oleanolic and ursolic separation but at the expense of excessive peak broadening and tailing. On the whole, after extensive variations of alkyl-bonded stationary phases, in the last 20 years, the research community only witnessed the appearance of the disruptive and successful porous graphitic columns. Recently, mixed-mode packings are under investigation but more efforts are needed to reach the higher efficiencies already prevailing in the market. Hence, there is room for further improvements from the side of materials science and engineering.

Concerning pre-column derivatization strategies, typical approaches have not been improving selectivities ($1.02 \leq S_{OA,BA}$ or $S_{BA,OA} \leq 1.17$ and $1.02 \leq S_{UA,OA}$ or $S_{OA,UA} \leq 1.09$). Nonetheless, the use of cyclodextrins provides great selectivity enhancements depending on the type of cyclodextrin and mobile phase ($1.03 \leq S_{OA,BA}$ or $S_{BA,OA} \leq 3.47$ and $1.06 \leq S_{UA,OA}$ or $S_{OA,UA} \leq 1.47$). Therefore, further advances may be exploited in this area.

Nomenclature

AA: Acrylamide; *Aca*: Acetic acid; *AIBN*: Azobisisobutyronitrile; *BA*: Betulinic acid; *CD*: Cyclodextrin; *C18*: Octadecylsilyl; *C30*: Triacontylsilyl; *DM*: Dimethyl; *DMSO*: Dimethylsulfoxide; *DNF*: N, N-dimethylformamide; *DVB*: Divinylbenzene; d_p : Particle diameter; *EGDMA*: Ethylene glycol dimethacrylate; *ELSD*: Evaporative light scattering detection; *Glu*: Glucosyl; *HP*: Hydroxypropyl; *HPLC*: High-pressure liquid chromatography; *ID*: Internal diameter; k'_i : Retention factor of species i; *L*: Column length; *LC*: Liquid chromatography; *MAA*: Methacrylic acid; *MeOH*: Methanol; *MIM*: Molecularly imprinted polymer microspheres; *MIP*: Molecularly imprinted polymer; *MOF*: Metallic organic framework; *MS*: Mass spectrometry; *MWCNTs*: Multiwalled carbon nanotubes; *NIP*: Non-imprinted polymer; *NIPAAm*: N-Isopropylacrylamide; *NP*: Natural product; *OA*: Oleanolic acid; *PGCs*: Porous graphitic columns; *pKa*: Negative logarithm of the acid ionization constant; *PMMA*: Polymethylmethacrylate; *PT*: Pentacyclic triterpenoids; $R_{i,j}$: Separation resolution between species i and j; *SC-CO*$_2$: Supercritical carbon dioxide; *SEM*: Scanning electron microscopy; *SFC*: Supercritical fluid chromatography; *SPE*: Solid-phase extraction; $S_{i,j}$: Selectivity between species i and j; *T*: Temperature; *TGA*: Thermogravimetric analysis; *THF*: Tetrahydrofuran; *TM*: Template molecule; *TTAs*: Triterpenic acids; $t_{r,i}$: Retention time of species i; t_0 : Column hold-up time; *UA*: Ursolic acid; *UV*: Ultraviolet; $w_{0.5H,i}$: Chromatographic peak width at half height

Greek symbols

ε: Porosity; ϕ : Volumetric fraction

Subscripts

i, j: Species i and j; *T*: Total porosity; *r*: Retention; *0.5H*: Half height

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13399-022-03723-3.

Author contribution Ivo S. Azenha: methodology, investigation, writing—original draft, formal analysis, data curation; Mário M. Q. Simões: writing—review and editing, formal analysis. Adélio Mendes: resources, writing—review and editing, supervision, project administration; Carlos M. Silva: supervision, conceptualization, resources, writing—review and editing, funding acquisition, formal analysis, project administration. All authors have read and agreed to the published version of the manuscript.

Funding Open access funding provided by FCT|FCCN (b-on). This work was developed within the scope of projects: CICECO-Aveiro Institute of Materials, UIDB/50011/2020, UIDP/50011/2020 & LA/P/0006/2020, financed by national funds through the FCT/MCTES (PIDDAC); Multibiorefinery project (POCI-01–0145-FEDER-016403); project LAQV-REQUIMTE (UIDB/QUI/50006/2020) financed through national funds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement; Project POCI-01–0145-FEDER-006939 (Laboratory for Process Engineering, Environment, Biotechnology and Energy—LEPABE funded by FEDER funds through COMPETE2020—Programa Operacional Competitividade e Internacionalização (POCI). I.S. Azenha acknowledges a PhD grant from Fundação para Ciência e a Tecnologia (Portugal) (grant number SFRH/BD/126509/2016).

Data availability All relevant data are included in the article and its supplementary material files.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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