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Evaluation of apparent formation constants of pentacyclic triterpene acids complexes with derivatized β- and γ-cyclodextrins by reversed phase liquid chromatography

B. Claude[a,*], Ph. Morin[a], M. Lafosse[a], P. Andre[b]

[a] *Institut de Chimie Organique et Analytique, UMR CNRS 6005, Université d'Orléans, B.P. 6759, 45067 Orléans Cedex 2, France*
[b] *Laboratoire Actifs, Biologie et Cosmétique, Parfums Christian DIOR, St-Jean-de-Braye 45800, France*

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Abstract

A reversed phase HPLC method has been investigated in order to resolve three main pentacyclic triterpene acids (oleanolic-, betulinic- and ursolic acid) found in a lot of plants. Some of them (oleanolic and ursolic acids) are position isomers and their resolution is highly improved by the addition of derivatized cyclodextrins in mobile phase. The formation of 1:1 inclusion complexes was assumed. Apparent formation constants of triterpene acids with DM-β-CD and HP-γ-CD were determined by HPLC method. Experimental results confirmed the complexation model and explained the modification of elution order according to the type of cyclodextrin added to the mobile phase. The influence of mobile phase organic modifier on apparent formation constants was also investigated. Results proved the competition between cyclodextrins hydrophobic cavity and organic solvent towards triterpene acids affinity.

Keywords: Derivatization, LC; Inclusion complexation; Betulinic acid; Oleanolic acid; Ursolic acid; Triterpenes; Cyclodextrins

1. Introduction

Triterpenoids, such as oleanolic, ursolic or betulinic acids are pentacyclic molecules (Fig. 1). As secondary metabolites of some plants, they possess pharmacological properties. The triterpene acids have been shown to exhibit significant anti-HIV activity [1].

Betulinic acid can act as a selective inhibitor of human melanoma in cell culture and animal models that function by induction apoptosis, whereas other compounds currently used in cancer therapy (taxol, vinblastine) inhibit the replication of both cancer and normal cells [2]. Ursolic acid has also shown significant cytotoxicity in lymphocytic leukemia cells [3]. As this molecule is relatively non-toxic and possesses anti-inflammatory and antihyperlipidemic properties, it has been used in cosmetics. Oleanolic acid has also been proposed as an anti-inflammatory and antiarthritic agent.

Oleanolic and ursolic acids occur especially in the waxy coatings of leaves and on fruits such as apple and pear. Betulinic acid can be found in birch, plane and cork barks [4,5]. Betulinic, oleanolic and ursolic acids have been identified in almond hulls [6].

The most common analytical methods of triterpenoids in plants are liquid chromatography [7–9], gas chromatography after silylation [10,11] or methylation [6]. The identification of betulinic, oleanolic and ursolic acids in natural extracts has been performed by GC–MS after derivatisation [4,12,13]. Capillary supercritical fluid chromatography [14] and cyclodextrin-modified micellar electrokinetic chromatography [15] have also been used for plant analysis.

Liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) has already been used to identify betulinic acid [7] and liquid chromatography–atmospheric pressure chem-

* Corresponding author. Fax: +33 2 3841 7281.
E-mail address: berengere.claude@univ-orleans.fr (B. Claude).

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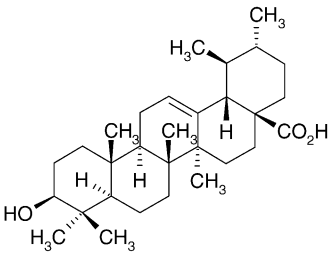
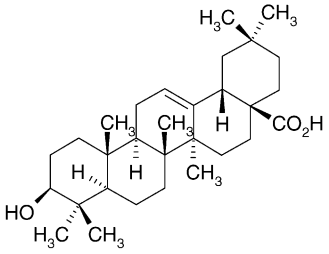


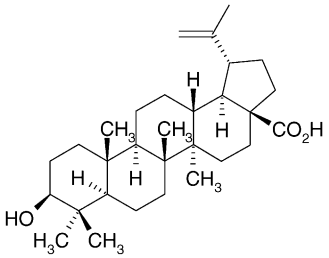
ursolic acid

(pK_A = 5.29, log P = 6.46)



oleanolic acid

(pK_A = 5.11, log P = 6.32)



betulinic acid

(pK_A = 5.50, log P = 6.85)

Fig. 1. Chemical stuctures of studied triterpene acids with their pK_a and log P values.

ical ionisation mass spectrometry (LC–APCI-MS) to quantify ursolic acid [16], as triterpene acids have weak chromophores.

The resolution of oleanolic and ursolic acids by LC seems difficult on reversed phase as these molecules are position isomers. The addition of cyclodextrins (CD) to the mobile phase was therefore investigated to improve the separation. In fact, cyclodextrins have already been used as mobile phase modifiers in HPLC applications for the analysis of steroids [17].

Cyclodextrins (CDs, α, β, γ) are torus-shaped, naturally occurring, enzymatically synthesized, cyclic oligosaccharides composed of six to nine α-1,4 linked D-glucopyranose units per molecule (α-, β-, γ- and δ-cyclodextrin, respectively). While the exterior of the molecule is hydrophilic, its hydrophobic cavity may selectively include molecules with appropriately sized organic compounds by forming non-covalent inclusion complexes. Besides, the internal cavity being less polar than the surrounding water molecules, chemical properties of the guest, once included, may be dramatically affected. For the following of this study, it is interesting to note the slight solubility of cyclodextrins in water and more particularly in organic solvent–water mixtures. Our study was therefore performed with cyclodextrins derivatized by dimethyl- or hydroxypropyl groups.

In the present paper, a simple and fast high-performance liquid chromatographic method for the separation of the most common triterpene acids is reported. The use of cyclodextrins as HPLC modifier has never been reported in the literature for the separation of triterpene acids. The resolution of these closely related hydrophobic triterpene acids requires an HPLC mobile phase containing a high percentage of acetonitrile and one derivatized cyclodextrin to obtain sufficient selectivity. Thus the selectivity was assessed by the addition of dimethyl-β or hydroxypropyl-γ-cyclodextrin to the mobile phase to promote hydrophobic interactions in order to differentiate structurally similar molecules.

The aim of our research was also to assess the triterpene acid–cyclodextrin inclusion complex by HPLC. Indeed, cyclodextrins are known to improve some properties of drugs (solubility, bioavailability) and enhance drug activity by encapsulation of the active molecule. The stoichiometry of the complex and the corresponding apparent formation constant (K_f) have been determined from the change in retention factors as the concentration of cyclodextrin in the mobile phase varied. This paper deals with the choice of a cyclodextrin for a C_{18} stationary phase. Complex apparent formation constants (CD-triterpene acid) have been determined experimentally at several compositions of the mobile phase.

2. Experimental

2.1. Apparatus

The HPLC system used was a Varian model 9012 ternary pump (Les Ulis, France), equipped with a Rheodyne (Cotati, CA, USA) model 7125 injection valve fitted with a 20 μL injection loop. The triterpene acids possess a UV absorption maximum at 225 nm, so this wavelength was used for detection. A Shimadzu SPD-6A spectrophotometric detector was connected to a computer equipped with EZChrom-Elit software version 2.5 (Scientific Software Inc., Pleasanton, CA, USA). A C_{18} silica Lichrospher® 100 RP-18 column (125 × 4 mm i.d., 5 μm particle size, 100 Å pore size, 350 m^2/g surface area, 21% carbon load and no endcapping) provided by Merck (Darmstadt, Germany) was used for HPLC separations of these triterpene acids. An isocratic mobile phase elution was performed at 1 mL/min flow-rate. The column was thermostated at 25 °C with a Croco-Cil column oven (Cluzeau, Ste Foy-La-Grande, France) to control temperature column. The pK_a and log P values of the triterpene acids were calculated by using Pallas Prolog D software version 2.0 (Compudrug Chemistry Ltd., Budapest, Hungary).

2.2. Chemicals

Betulinic, oleanolic and ursolic acids were purchased from ExtraSynthese (Genay, France). Dimethyl-β-cyclodextrin (DM-β-CD, average degree of substitution 1.8) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD, average degree of

substitution 0.6) were provided by Wacker Chemie (Munich, Germany). HPLC grade acetonitrile (Carlo Erba, Milan, Italy) was used without further purification. Other chemicals (*ortho*-phosphoric acid, sodium hydroxide) were of analytical reagent-grade, from Carlo Erba (Milano, Italy) and used without further purification. 18 MΩ deionized water was obtained from Elgastat UHQ II system (Elga, Antony, France).

Mobile phases were prepared by mixing the appropriate amount of acetonitrile and 0.02 M phosphate buffer (pH 3.5) and then by dissolving cyclodextrin in the resulting solution with the help of a Branson 1200 ultrasonic bath (Branson, Danbury, USA). The phosphate buffer was prepared at fixed pH and concentration with the help of Phoebus software (Analis, Namur, Belgium); its composition included 20 mM phosphoric acid with 18.9 mM sodium hydroxide. Cyclodextrin was then dissolved in the buffer at the required final concentration; their pH and ionic strength values were equal to 3.5 and 19.3 mM, respectively. The buffer capacity, calculated with this software, was equal to 3 mM/pH unity. Subsequently, electrolyte solutions were filtered through a nylon filter membrane with a 0.2 μm porosity (Whatman, VWR International, Fontenay-sous-Bois, France) and the pH of the buffer was checked using a Beckman pH meter (Model Φ200, Fullerton, CA, USA).

The stock solution of each analyte was prepared by dissolving 1 mg-amount of triterpene acid into 1 ml methanol; these solutions were stored at −20 °C and found to be stable for at least 1 month. Otherwise, standard solutions (200 μg/ml concentration) were prepared daily by dilution of related stock solutions with free cyclodextrin mobile phase and stored at −16 °C during the day. The void volume of the C_{18} column was obtained by monitoring the retention time of a 5 μg/ml KNO_3 solution.

3. Results and discussion

Acetonitrile–phosphate buffer (pH 3.5) was selected as the mobile phase because of the good elution of the triterpene acids without cyclodextrins. With methanol as organic modifier in mobile phase, the cyclodextrins solubility is improved but the retention of triterpene acids was dramatically increased and a collapse of chromatographic peaks was observed.

3.1. Influence of cyclodextrin nature and concentration

The HPLC separation of a standard mixture of betulinic, oleanolic and ursolic acids was performed by using the acetonitrile–0.02 M phosphate buffer, pH 3.5 (70/30, v/v) mixture as mobile phase. The separation was studied on a silica-based C_{18} Lichrospher column. Fig. 2a displays the separation of the triterpene acids mixture; betulinic acid was eluted first with a good peak shape, but oleanolic and ursolic acids were coeluted. Due to the similar structure of some of these triterpene acids (oleanolic and ursolic acids only differ

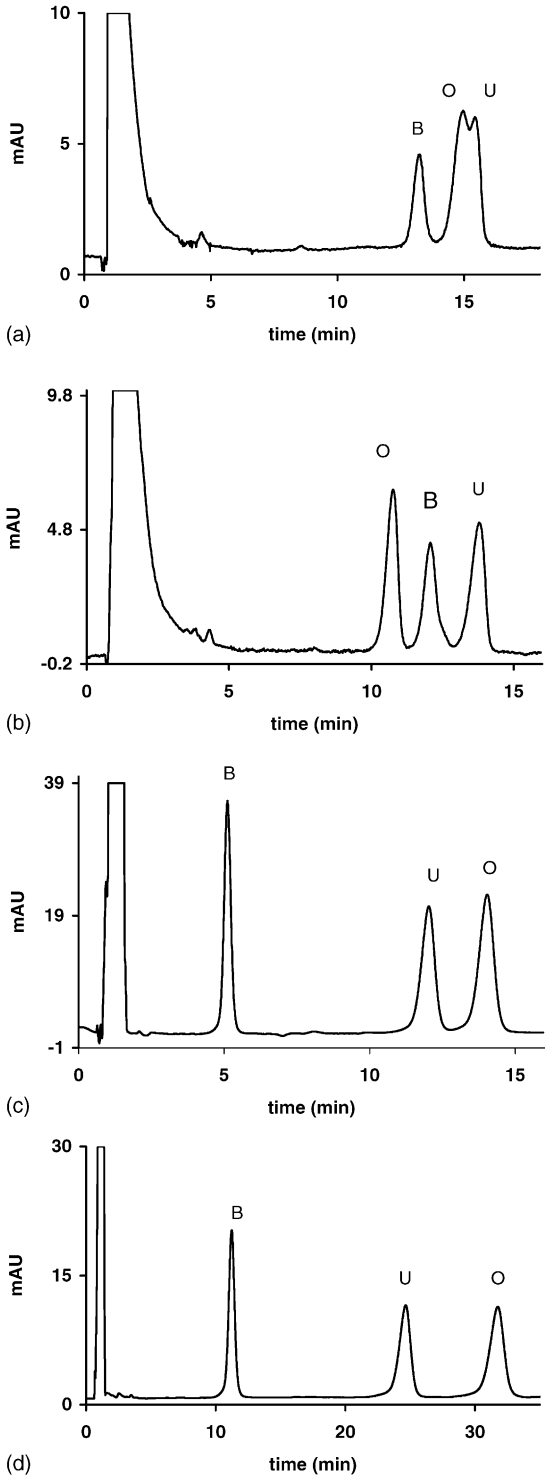


Fig. 2. Separation of a standard mixture of betulinic, oleanolic and ursolic acids by HPLC. Column Lichrospher 100 RP-18: 125 mm × 4 mm i.d. (5 μm); flow-rate: 1 ml/min; solute concentration: 200 μg/ml dissolved in free cyclodextrin mobile phase; sample loop volume: 20 μl; temperature: 25 °C. Mobile phase: (a) acetonitrile–0.02 M phosphate buffer pH 3.5 (70/30, v/v); (b) acetonitrile–0.02 M phosphate buffer pH 3.5 (70/30, v/v) + 6 mM DM-β-CD; (c) acetonitrile–0.02 M phosphate buffer pH 3.5 (50/50, v/v) + 7.5 mM HP-γ-CD; (d) acetonitrile–0.02 M phosphate buffer pH 3.5 (50/50, v/v) + 2.5 mM γ-CD. Solutes (200 μg/mL): B: betulinic acid; O: oleanolic acid, U: ursolic acid.

by the position of two methyl groups), the overall resolution of the chromatographic peaks presents certain difficulties. A slight improvement in the resolution of oleanolic and ursolic acids was obtained with an increasing phosphate buffer concentration but at the expense of a longer analysis time and lower peak efficiency. The weak solubility of triterpene acids in aqueous medium can be ascribed to their extreme hydrophobicity ($\log P \approx 6.5$).

The resolution of closely related hydrophobic triterpene acids may be improved by a second mechanism based on the differences in hydrophobicity between solutes. Thus, a cyclodextrin was added to the HPLC mobile phase in order to promote hydrophobic interactions. We also investigated by HPLC the inclusion complexes of triterpene acids with different cyclodextrins.

The acetonitrile–phosphate buffer mobile phase was modified by the addition of a neutral cyclodextrin (γ-CD, dimethyl-β-CD or hydroxypropyl-γ-CD) at a low concentration. Each cyclodextrin was initially injected into the chromatographic system to check that cyclodextrin-stationary phase interactions were negligible. The chromatographic system was quickly equilibrated (about 20 min). Fig. 3 reports the variation of retention factors versus DM-β-CD and HP-γ-CD concentrations. As can be seen, the retention factors of all the triterpenes under study decreased when DM-β-CD (Fig. 3a) or HP-γ-CD (Fig. 3b) concentrations increased. This phenomenon can be interpreted by the formation of hydrophilic inclusion complexes between cyclodextrin and triterpene acid. Indeed, the complexation phenomenon increases the solubility of the analyte in the mobile phase and then decreases its retention time in the column.

The most suitable CD concentrations were found to be 6 mM for DM-β-CD and 7.5 mM for HP-γ-CD. Fig. 2a–d show chromatograms obtained using various mobile phases prepared in the absence or presence of several cyclodextrins.

The elution order changes as cyclodextrin is added to the mobile phase and depends on the nature of this retention modifier. Thus, betulinic acid elutes before oleanolic acid with a free cyclodextrin mobile phase, but the elution order is reversed when 6 mM DM-β-CD is added to the mobile phase. So, more stable DM-β-CD complexes are formed with oleanolic than with betulinic acids. Otherwise, the elution order between ursolic and oleanolic acids is dependent on the nature of the cyclodextrin. Thus, weaker inclusion complexes were obtained for ursolic acid compared to oleanolic acid with DM-β-CD than with HP-γ-CD. Efficient, selective and well-resolved separations of these three triterpene acids could be achieved by adding 7.5 mM HP-γ-CD to acetonitrile–0.02 M phosphate buffer pH 3.5 (50/50, v/v) (Fig. 2c). Oleanolic and ursolic acids are well resolved, betulinic acid enhances a retention much lower than before. Thus, the betulinic acid/HP-γ-CD complex is assumed to have a high formation constant. Lastly, γ-cyclodextrin was rejected because of an excessive run time.

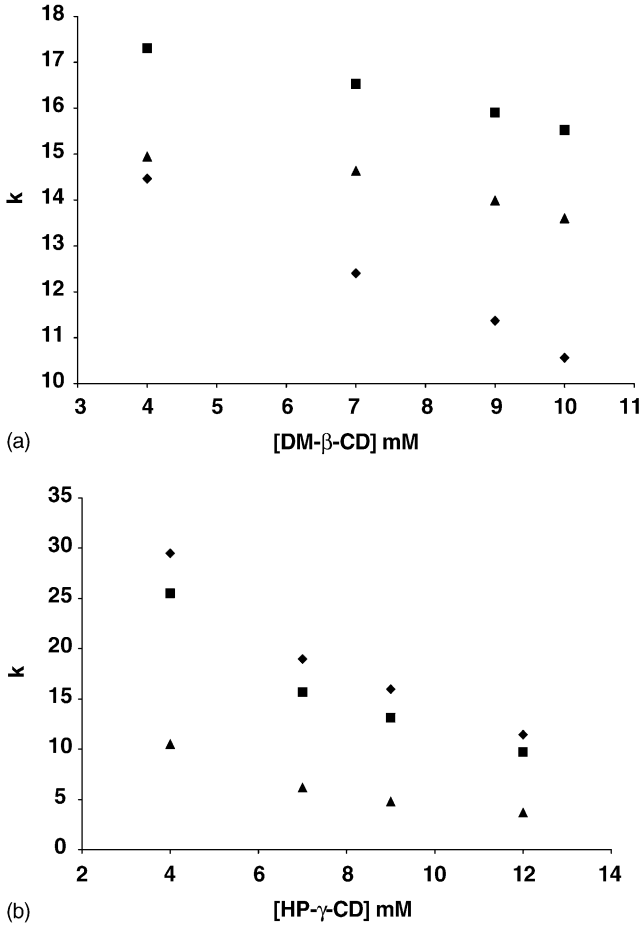


Fig. 3. Effect of cyclodextrin concentration on retention factor of triterpene acids in HPLC. Same experimental conditions as in Fig. 2 except the mobile phase composition. Mobile phase: (a) acetonitrile–0.02 M phosphate buffer pH 3.5 (70/30, v/v) + DM-β-CD; (b) acetonitrile–0.02 M phosphate buffer pH 3.5 (50/50, v/v) + HP-γ-CD. Solutes: (♦) oleanolic acid, (■) ursolic acid, (▲) betulinic acid.

3.2. Stoichiometry and apparent formation constants of the complexes

Assuming that each triterpene acid (TA) forms a 1:1 inclusion complex with the cyclodextrin (CD), the following equilibrium can be written:

$$\mathrm{TA} + \mathrm{CD} \rightleftharpoons \mathrm{TA{-}CD} \quad (1)$$

and the formation constant (K_f) of the complex is given by:

$$K_f = \frac{[\mathrm{TA{-}CD}]}{[\mathrm{TA}][\mathrm{CD}]} \quad (2)$$

where [TA–CD], [TA] and [CD] are equilibrium concentrations.

Then, according to [18], the relationship between the retention factor and the cyclodextrin concentration $[(\mathrm{CD})_m]$ in mobile phase is given by:

$$\frac{1}{k} = \frac{1}{k_0} + K_f \frac{[(\mathrm{CD})_m]}{k_0} \quad (3)$$

where k is the retention factor of the solute at the concentration of the cyclodextrin in mobile phase, k_0 the retention factor in the absence of cyclodextrin and K_f the apparent formation constant.

When $1/k$ is plotted versus $[(CD)_m]$, a linear relationship was observed, reflecting a solute with a 1:1 stoichiometry with cyclodextrin and the (slope/intercept) ratio gives the value of K_f. The cyclodextrin concentration $[(CD)_m]$ used in Eq. (3) is not the analytical cyclodextrin one $[(CD)_{tot}]$ because acetonitrile can form a weak complex with cyclodextrins in competition with triterpene acids [19]. We assume that $[(CD)_m]$ and $[(CD)_{tot}]$ have the same value in this study. These experiments have been performed for DM-β-CD dissolved in acetonitrile/phosphate buffer pH 3.5 (70/30, v/v) (Fig. 4a) and for HP-γ-CD dissolved in acetonitrile/phosphate buffer pH 3.5 (50/50, v/v) (Fig. 4b). In these two cases, linear relationships were observed for each triterpene acid (Table 1), reflecting a 1:1 inclusion complex.

Once the stoichiometric ratio had been determined, the reciprocal plot method ($1/k$) veusus [CD] was used to calculate the apparent formation constant (K_f) of the inclusion complex. Indeed, least squares regression resulted in a linear relationship, where the apparent formation constant (K_f) is given by the ratio of the intercept to the slope. Table 1 reports the K_f values of complexes formed between triterpene acid and cyclodextrin (DM-β-CD, HP-γ-CD) at several acetonitrile contents in the mobile phase.

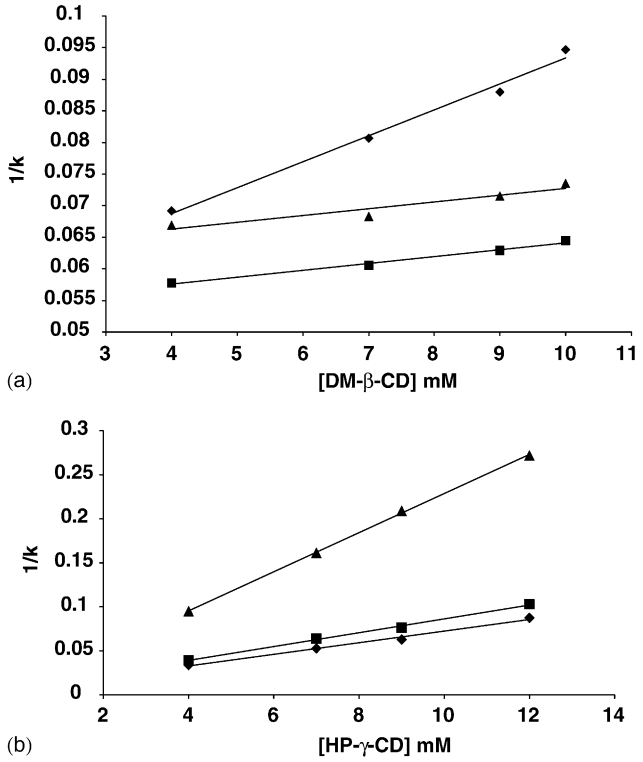


Fig. 4. Reciprocal plots $1/k$ vs. [CD] for the study of triterpene acid−cyclodextrin inclusion complex (assumption of 1:1 complex). Same experimental conditions as in Fig. 2 except the mobile phase composition. (a) Cyclodextrin: DM-β-CD; mobile phase: acetonitrile–0.02 M phosphate buffer pH 3.5 (70/30, v/v). (b) Cyclodextrin: HP-γ-CD; mobile phase: acetonitrile–0.02 M phosphate buffer pH 3.5 (50/50, v/v). Solutes: (♦) oleanolic acid, (■) ursolic acid, (▲) betulinic acid.

Table 1
Linear regression analysis of $1/k$ vs. [CD] curves and K_f values (M^{-1}) of triterpene acid cyclodextrin inclusion complex at several acetonitrile volume fractions

Acetonitrile (%, v/v)	Solute	Slope $\times 10^3$	Intercept $\times 10^2$	r^2	K_f (M^{-1})
DM-β-CD					
55	B	0.6	1.48	0.9812	40
	O	2.2	1.35	0.9928	162
	U	0.6	1.38	0.9648	43
60	B	1.0	2.34	0.993	43
	O	3.1	2.00	0.9975	155
	U	1.0	2.00	0.9987	50
65	B	1.2	3.81	0.8566	31
	O	3.4	3.43	0.9672	99
	U	1.0	3.40	0.8054	29
70	B	1.1	6.20	0.9096	18
	O	4.1	5.23	0.9895	78
	U	1.1	5.32	0.9888	21
HP-γ-CD					
50	B	22.2	0.64	0.9995	3469
	O	6.6	0.64	0.9905	1031
	U	7.9	0.76	0.9964	1039
60	B	27.8	3.15	1	883
	O	8.8	2.71	0.9987	325
	U	10.5	2.70	0.9990	389
65	B	30.1	5.08	0.9995	593
	O	10.1	3.99	0.9982	253
	U	11.8	4.03	0.9985	293
70	B	35.2	5.27	0.9708	668
	O	10.9	6.3	0.9951	173
	U	12.4	6.67	0.9939	186

Same experimental conditions as in Fig. 2 except the mobile phase composition.

As expected, larger K_f values for all triterpene acids are obtained with HP-γ-CD than with DM-β-CD (for example, 668 and 18 M^{-1}, respectively in acetonitrile–phosphate buffer (70/30, v/v) for betulinic acid). The low values obtained for DM-β-CD are related to the small cavity size of this CD, which prevents strong host–guest interactions.

As observed previously in Fig. 2b and c, triterpene acid/DM-β-CD complexes are more stable with oleanolic than with betulinic or ursolic acids. In contrast, betulinic acid formed the more stable complex with HP-γ-CD.

3.3. Influence of mobile phase composition on apparent formation constants values

Apparent formation constants between triterpene acids and cyclodextrins in various binary aqueous-organic solvent mixtures have been evaluated in the 50–70% solvent com-

position range. Linear graphs of $1/k$ versus $[(CD)_m]$ were plotted with the acetonitrile–phosphate buffer mobile phases containing between 50 and 70% (v/v) of solvent. Experimental values of apparent formation constants reported in Table 1 show the increase in K_f as a function of the mobile phase water content. This may be due to an enhanced competition of acetonitrile and hydrophobic cyclodextrin cavity for triterpene acids [20].

4. Conclusion

The separation of triterpene acids by HPLC was improved by addition of cyclodextrins (DM-β-CD, HP-γ-CD) to the acetonitrile–phosphate buffer mobile phase. The formation of inclusion complexes between triterpene acids and cyclodextrins explains the selectivity modification and the elution order of analytes. The stability of these complexes depends on the size and conformation of triterpene acids as well as on the hydrophobic cavity size of cyclodextrins.

Values of the complex apparent formation constants were determined by HPLC and experimental data confirmed the 1:1 stoichiometry of solute/CD complexes.

The apparent formation constant values decrease when the acetonitrile percent increases in mobile phase, whatever the nature of the cyclodextrin. This proves the competition of organic solvent and cyclodextrin cavity for hydrophobic solutes.

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