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Separation of 9,10-anthraquinone derivatives: Evaluation of functionalised stationary phases in reversed phase mode

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ABSTRACT

A series of reversed phases bonded with several functional groups was investigated for separation of anthraquinone derivatives, following the previous work, dedicated to the selectivity of octadecyl silica bonded phases. Considering wide diversity of substitutions in hydrophobic anthraquinone skeleton, interactions like dipole–dipole, π–π or H-bond acceptor/donor, as well as inclusion complexes formation can be employed to improve separation. In this study, several phases with grafts like cyano, nitro, aromatic, PEG, diol, calixarene and cyclodextrin were used with water–acetonitrile gradient for separation of thirty anthraquinoids' standards. The evaluation of performances was measured using the symmetry parameter and the number of critical pairs of peaks formed. The results point out the aromatic and calixarene bonded silica as the most interesting in terms of symmetry and critical pairs number. Finally we tested the performance of *Caltrex Resorcinaren*, *Pursuit XRs DP* and *Luna Phenyl-Hexyl* on real samples of anthraquinone natural dye extracted from a red thread taken from a 15th C. tapestry. We observed and compared the retention behaviour of some new anthraquinoids additional to our standards set and showing behaviour particular to substituted anthraquinone carboxylic acids.

1. Introduction

Octadecyl bonded stationary phases are the most popular for HPLC separation of naturally occurring anthraquinones [1–11], but the systematic evaluation of C18 stationary phases for these applications was published only recently [12]. This work shows that a lot of C18 bonded phases can be used, but their properties are more or less adapted to the analysis of anthraquinones. Some anthraquinone derivatives give asymmetric peaks on several stationary phases, sometimes so extremely tailed, that their detection is impossible, especially when these compounds are present as traces.

The separation of 30 representative anthraquinone derivatives on various C18 columns brands displays a number of critical pairs [12]. It is notable that our set of studied standards represents randomly 5% of structures from about 500 existing natural anthraquinoids [13,14]. That series was extended by some synthetic ones. The separation of these compounds gives a fairly well image of potential separation problems which can be observed on any other statistically important series of anthraquinone derivatives: peak tailing and the existence of critical pairs.

The full separation of complex mixtures is impossible from a theoretical and practical point of view in reasonable gradient analysis conditions without particular optimisation [15–18]. Our target was, then, to evaluate the most efficient stationary phase adapted to the analysis of anthraquinoids. The previous study showed that C18 phases give a weak change of selectivity towards all standards of anthraquinone derivatives [12].

Analyses in reversed phase mode are still interesting because of the existence of a huge number of available stationary phases compatible with hydro-organic eluents, of an efficient separation of compounds included in the wide range of hydrophobicity, of the use of less polluting solvents than those used in normal phase separations and of the compatibility with water-containing injection solutions.

The molecules of anthraquinone derivatives have a large skeletal hydrocarbon volume with three condensed aromatic rings ensuring their hydrophobicity. The various attached functional groups have different properties: H-bonds donor or acceptor, polarity, polarisability, etc. It seemed interesting to introduce in the separation process specific solute–stationary phase interactions other than hydrophobic ones.

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Table 1
Anthraquinone derivatives. Functional groups R1–R8 position – see Fig. 1.

Compound	Abbreviation	Origin[a]	*M* [g/mol]	R1	R2	R3	R4	R5	R6	R7	R8
Anthraquinone	Anq	N	208	–H	–H	–H	–H	–H	–H	–H	–H
Tectoquinone	Tec	N	222	–H	$-CH_3$	–H	–H	–H	–H	–H	–H
1,4-Dimethylanthraquinone	Dma	S	236	$-CH_3$	–H	–H	$-CH_3$	–H	–H	–H	–H
2-Ethylanthraquinone	Eta	S	236	–H	$-C_2H_5$	–H	–H	–H	–H	–H	–H
2,3-Dimethylanthraquinone	Oma	N	236	–H	$-CH_3$	$-CH_3$	–H	–H	–H	–H	–H
2-Hydroxymethylanthraquinone	Hma	S	238	–H	$-CH_2OH$	–H	–H	–H	–H	–H	–H
Anthraflavic acid	Afv	N	240	–H	–OH	–H	–H	–H	–OH	–H	–H
Alizarin	Ali	N	240	–OH	–OH	–H	–H	–H	–H	–H	–H
Anthrarufin	Arf	S	240	–OH	–H	–H	–H	–OH	–H	–H	–H
Danthron (= Chrysazin)	Dan	N	240	–OH	–H	–H	–H	–H	–H	–H	–OH
Hystazarin	Hys	S	240	–H	–OH	–OH	–H	–H	–H	–H	–H
Quinizarin	Qza	N	240	–OH	–H	–H	–OH	–H	–H	–H	–H
Xanthopurpurin (= Purpuroxanthin)	Xpu	N	240	–OH	–H	–OH	–H	–H	–H	–H	–H
Anthraquinone-2-carboxylic acid	Can	S	252	–H	–COOH	–H	–H	–H	–H	–H	–H
Chrysophanol (= Chrysophanic acid)	Chr	N	254	–OH	–H	$-CH_3$	–H	–H	–H	–H	–OH
3-Methoxyhystazarin	Moh	N	254	–H	–OH	$-OCH_3$	–H	–H	–H	–H	–H
Anthragallol	Agl	N	256	–OH	–OH	–OH	–H	–H	–H	–H	–H
Purpurin	Pur	N	256	–OH	–OH	–H	–OH	–H	–H	–H	–H
2-*tert*-Butylanthraquinone	Bua	S	264	–H	$-C(CH_3)3$	–H	–H	–H	–H	–H	–H
2,3-Dimethylquinizarine	Dmq	S	268	–OH	$-CH_3$	$-CH_3$	–OH	–H	–H	–H	–H
Nordamnacanthal[b]	Nor	N	268	–OH	–CHO	–OH	–H	–H	–H	–H	–H
Aloe-emodin	Ale	N	270	–OH	–H	$-CH_2OH$	–H	–H	–H	–H	–OH
Emodin	Emo	N	270	–OH	–H	–OH	–H	–H	$-CH_3$	–H	–OH
Quinalizarin	Qlz	S	272	–OH	–OH	–H	–H	–OH	–H	–H	–OH
Physcion (= Parietin)	Phy	N	284	–OH	–H	$-OCH_3$	–H	–H	$-CH_3$	–H	–OH
Rhein	Rhe	N	284	–OH	–H	–COOH	–H	–H	–H	–H	–OH
Munjistin[b]	Mun	N	284	–OH	–COOH	–OH	–H	–H	–H	–H	–H
Pseudopurpurin[b]	Psp	N	300	–OH	–COOH	–OH	–OH	–H	–H	–H	–H
Flavokermesic acid (= Laccaic acid D)	Flk	N	314	–OH	–H	–OH	–H	–H	–OH	–COOH	$-CH_3$
Kermesic acid	Ker	N	330	–OH	–H	–OH	–OH	–H	–OH	–COOH	$-CH_3$
Frangulin	Fra	N	416	–OH	–H	–O-rhamnose	–H	–H	–OH	–H	–OH
Flavokermesic acid glycoside[b]	Flk-gly	N	476	–OH	–Glucose	–OH	–H	–H	–OH	–COOH	$-CH_3$
Carminic acid	Car	N	492	–OH	–Glucose	–OH	–OH	–H	–OH	–COOH	$-CH_3$
Ruberythric acid	Rba	N	534	–OH	–O-primeverose	–H	–H	–H	–H	–H	–H
Lucidin primeveroside[b]	Luc-gly	N	564	–OH	$-CH_2OH$	–O-primeverose	–H	–H	–H	–H	–H

[a] N – present in nature, S – absent in nature.
[b] Supplementary compounds identified in historical sample of dyed wool.

Following this approach, we turned to the investigation of functionalised reversed phases.

A number of functionalised phases of many brands are proposed by manufacturers. They are frequently bonded with phenyl, halogenated phenyl, nitryl, amine, glyceryl, etc. through an alkyl spacer. Some grafts are unique, according to proprietary formulations [19].

The functionalised, silica bonded stationary phases are mostly used for special separation issues, especially when octadecyl bonded ones do not give satisfactory results in fixed analysis conditions. They work basically through a hydrophobic effect with water containing mobile phase, but offer supplementary interactions with analysed compounds. These additional interactions could change selectivity and potentially improve the resolution of critical pairs observed on C18 stationary phases.

The pre-selection of these phases for a given application is more dependent on graft type than on fine characteristics of support or complementary treatment [20–23]. A comparative study of the selectivity was done with cyano, phenyl and alkyl groups bonded on the same silica support [24]. An interesting comparison of C18 and phenyl-type phases for separation of PAHs was published [25].

Several publications were devoted to the behaviour of cyano phases in reversed phase mode. The general evaluation of their properties according to the hydrophobic-subtraction model was published by [26]. The phases of different brands were compared for alkylarylketones and column test compounds [27]. More systematic study of the influence of graft functionality (mono-, tri-), bonding density and end-capping on selectivity for polyaromatics, substituted monoaromatics and basic compounds was also done [28].

The hydrophobic-subtraction retention model, extended by π–π phase–solute interactions, was used to describe the properties of arylsiloxane bonded phases [29]. Reubsaet and Vieskar published a work concerning the implication of π–π interactions in the retention of aromatic compounds on various raw and functionalised poly(styrene-divinylbenzene) copolymer based phases [30]. A series of recent papers was dedicated to in depth study of phenyl phases [31–34]. They focused comprehending the influence of phenyl spacer length and bonding density on selectivity.

The hydrogen donor or acceptor phases seem to have been less studied in a systematic way. However, the diol phase selectivity was quite early examined by Jansen et al. [35]. The comparison of retention mechanism on PEG and diol stationary phases using aqueous/MeCN mobile phase was done with phenolic acids and flavones [36].

The silica bonded with electron-acceptor groups were extensively studied by Welch and Hoffman [37]. Their work covered several nitro substituted aromatic grafts as well as phenyl, pentafluorophenyl and aminoalkyl ones and concerned the retention behaviour of a series of planar and non-planar aromatic solutes.

Inclusion complex phases exhibit multiple interactions with solutes and their behaviour was largely studied for calixarenes [38,39]. The cyclodextrin bonded silicas were proposed for a multitude of practical applications especially for all kinds of isomers [40–42]. Currently a large variety of native and modified grafts is used and continuously introduced in HPLC. However, few mechanistic studies exist on commercial phases. They are mainly focused on solvent influence on chiral separations [43–45].

So, the variety of stationary phases which are interesting because of their possible interactions is broad and the system-

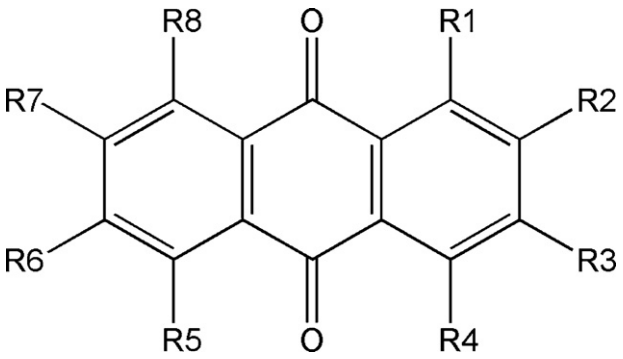

Fig. 1. Anthraquinoid skeleton (see Table 1 for R1–R8 characteristics).

atic study of them for separation improvement of anthraquinone derivatives seems to be imperative. This study is a pioneer application for that class of compounds.

2. Materials and methods

2.1. Standards and samples

Anthraquinoids standards (Table 1, Fig. 1) and their preparation for analysis were the same as in the previous publication [12].

Commercial standards were obtained from several suppliers. Alizarin, emodin, anthraflavic acid and 2-hydroxymethylanthraquinone were purchased from Acros Organics (Geel, Belgium). Purpurin, anthraquinone, tectoquinone, 2-*tert*-butyl anthraquinone, 2-ethylanthraquinone, anthraquinone-2-carboxylic acid, danthron, anthrarufin, quinizarin and 2,3-dimethylquinizarin were from Sigma–Aldrich (Saint Louis, MO, USA). Physcion, 2,3-dimethylanthraquinone, 1,4-dimethylanthraquinone, chrysophanol, rhein and aloe-emodin were from Extrasynthese (Genay, France). Some of the rare standards were provided by Helmut Schweppe (Frankenthal, Germany): hystazarin, 3-methoxyhystazarin, xanthopurpurin, anthragallol, frangulin, ruberythric acid, carminic acid (actually dried extract from the cochineal *Dactylopius coccus*) and a mix of kermesic and flavokermesic acids (dried extract from *Kermes verilio* insect).

The stock solutions of pure standards and dried extracts were prepared in an acetonitrile/water (50/50) solution at an average concentration of 12 ppm. These solutions were stored at 4 °C. For some of these stock solutions the solubility was not complete but was sufficient for detection of those compounds. All solutions were heated up to room temperature and vortexed prior to filtration and injection. For analysis purposes they were sampled and mixed by 3, choosing the compounds differing by UV–Vis spectra [12] in order to reduce number of injections. The obtained solutions were also stored in a refrigerator for further use.

A sample of red colored wool was taken from a tapestry « The life of the Holy Virgin » dated at the end of 15th Century from L'Eglise Notre-Dame in Beaune (Burgundy, France). The extraction conditions were adapted from protocol elaborated by Sanyova and Reisse [46] and described in detail in our former work [12].

The supplementary anthraquinoids found in the real sample were identified according to previously collected chromatographic and spectral data [10,12] and marked "b" in Table 1.

2.2. Chemicals

Acetonitrile (MeCN, Chromasolv) and acetone (Me_2CO, Chromasolv Plus), both HPLC gradient grade were supplied by Sigma–Aldrich (Saint-Quentin Fallavier, France). Formic acid (HCOOH, 99%, RPE-ACS) was obtained from Carlo Erba (Milan, Italy). Hydrofluoric acid (HF, 40%, Suprapur) was provided by Merck (Darmstadt, Germany). All purpose ultrapure water was obtained from Milli-Q Plus system (Millipore, Molsheim, France).

2.3. Instruments

The Agilent HP 1100 series modular system (Agilent, Walbronn, Germany) consisted of a vacuum solvent degasser, quaternary pump, autosampler, thermostated column compartment and diode array detector. The system worked under ChemStation environment.

The mixtures of anthraquinone compounds were injected by autosampler at 5 μl (2.0 and 2.1 mm i.d. columns) or 20 μl (4.0 and 4.6 mm i.d. columns).

2.4. Stationary phases

Considering anthraquinone derivatives structures (Fig. 1), especially the presence of their polyaromatic core and the properties of attached groups, and also general molecular shape, we selected a series of functionalised stationary phases (Table 2). These phases, in addition to the general, hydrophobic retention mechanism, are able to retain the analysed compounds by dipole–dipole interactions (cyano and nitro groups), hydrogen bond creation (some embedded or terminal polar groups), π–π interactions (aromatic and nitro groups) or to form the inclusion complexes with analytes (calixarenes and cyclodextrins).

As we looked for the greatest selectivity differences, our choice of phases thus followed first the graft variability (Fig. 2). Only cyano phases are represented by a larger group with various grafting and end-capping characteristics on silica support (#F-14, F-15 and F-17 to F-21).

The aromatic phases tested differ by graft group type: phenyl (#F-02, F-06, F-07 and F-08), fluorinated phenyl (#F-05), brominated benzoyl (#F-03), bi-phenyl (#F-01), di-phenyl (#F-11), pyrenyl (#F-04), dinitroanilide (#F-12), nitrophenyl (#F-09) or by phase structure: bonded silica, polymer embedded Al_2O_3 (#F-06) and PS-DVB (#F-10).

Two alkyl phases containing polar groups were included in this study: embedded ether oxygen (polyethyleneglycol, #F-16), and terminal hydroxyl (dodecyldiol, #F-13). These phases and the mixed hydroxyalkyl/C18 phases studied in previous work, namely Nucleodur Isis and Aquasil [12], were similar in nature.

Among the inclusion complex phases selected, three of them contain structures with additional hydroxyl groups (resorcincalixarene and cyclodextrins, #F-22, F-24 and F-25) and one of them does not (calixarene, #F-23). The internal diameter of cavities formed by their cycles is also different.

2.5. Analysis conditions

Analyses were performed with mobile phase suitable for the MS detection anthraquinone derivatives [10] using linear gradient I shown in Table 3 [12]. The flow-rates corresponded to linear velocity about 0.16 cm/s which is equivalent, for example, to 1.0 ml/min for 4.6 mm internal diameter columns. An exception was made for *PLRP-S* column. Because of its high backpressure generation with standard flow-rate, mobile phase pumping was set at 0.8 ml/min.

In the case of highly retentive stationary phases (*Cosmosil PBB-R* and *PYE*, respectively #F-03 and #F-04), the gradient of acetonitrile (MeCN) was not sufficient to elute the strongly retained compounds. We thus tested an elution gradient replacing the acetonitrile (MeCN, gradient component B) by acetone (Me_2CO) and keeping other parameters unchanged. This new gradient did not

Table 2
Characteristics of columns.

No.	Stationary phase	Manufacturer	Column L × i.d. [mm]	Support material	Carbon load [%]	Specific area [m^2/g]	Particle size [μm]	Pore diameter [Å]	Graft	Endcapping
F-01	Allure Biphenyl	Restek	250 × 4.6	SiO_2	23	*No data*	5	60	Biphenyl	Yes
F-02	Ascentis Phenyl	Supelco	250 × 4.6	SiO_2	19	450	5	100	Phenyl-butyl	*No data*
F-03	Cosmosil 5PBB-R	Nacalai-Tesque	250 × 4.6	SiO_2	8	300	5	120	Pentabromobenzyl	Yes
F-04	Cosmosil 5PYE	Nacalai-Tesque	250 × 4.6	SiO_2	18	300	5	120	2-(1-Pyrenyl)ethyl	Yes
F-05	Fluorosep-RP Phenyl	ES Industries	250 × 4.6	SiO_2	*No data*	350	5	60	Pentafluorophenyl	*No data*
F-06	Gammabond A Phenyl	ES Industries	250 × 4.6	Al_2O_3, polymer embedded	–	*No data*	5	130	Phenyl	-
F-07	Luna Phenyl-Hexyl	Phenomenex	250 × 2.0	SiO_2	17.5	400	5	100	Phenyl-hexyl	*No data*
F-08	Nucleodur Sphinx	Macherey-Nagel	250 × 4.6	SiO_2	15	*No data*	5	110	Phenyl-propyl/C18 mixed phase	Yes
F-09	Nucleosil NO2	Macherey-Nagel	250 × 4.6	SiO_2	*No data*	350	5	100	Nitrophenylpropyl	No
F-10	PLRP-S	Polymer Laboratories	150 × 4.6	PS-DVB copolymer	–	500	3	100	–	–
F-11	Pursuit XRs DP	Varian	250 × 4.6	SiO_2	15	440	5	100	Diphenyl	Yes
F-12	Uptisphere DNAP	Interchim	250 × 4.6	SiO_2	*No data*	320	5	120	Dinitroanilinopropyl phenyl	No
F-13	Acclaim Mixed-Mode HILIC-1	Dionex	150 × 4.6	SiO_2	18	300	5	120	Dodecyl-11,12-diol	Yes
F-14	Alltima Cyano	Alltech	250 × 2.1	SiO_2	*No data*	340	5	100	Cyano, polyfunctional	Yes
F-15	Alltima HP Cyano	Alltech	250 × 2.1	SiO_2	4	200	5	190	Cyano, monofunctional	Yes
F-16	Discovery HS-PEG	Supelco	250 × 4.6	SiO_2	12	300	5	120	Polyethyleneglycol	No
F-17	Grom-Sil Cyano-1ST	Alltech	250 × 2.0	SiO_2	4.8	300	5	120	Cyano, monofunctional	Yes
F-18	Grom-Sil Cyano-3CP	Alltech	250 × 2.0	SiO_2, encapsulated	4	320	5	120	Cyano, polyfunctional	No
F-19	LiChrospher CN	Merck	250 × 4.0	SiO_2	6.6	360	5	100	Cyano, monofunctional	No
F-20	Nucleosil CN	Macherey-Nagel	250 × 4.6	SiO_2	4	350	5	100	Cyanopropyl	No
F-21	Uptisphere CN	Interchim	250 × 2.0	SiO_2	8	320	5	120	Cyano, monofunctional	One step
F-22	Caltrex Resorcinaren	Synaptec	250 × 4.0	SiO_2	*No data*	340	5	100	Calixarene Resorcinol	*No data*
F-23	Caltrex Science	Synaptec	250 × 4.0	SiO_2	*No data*	340	5	100	Calixarene BI/AIII	*No data*
F-24	ChiraDex Beta	Merck	250 × 2.0	SiO_2	*No data*	300–360	5	100	Beta Cyclodextrin	*No data*
F-25	ChiraDex Gamma	Merck	250 × 2.0	SiO_2	*No data*	300–360	5	100	Gamma Cyclodextrin	*No data*

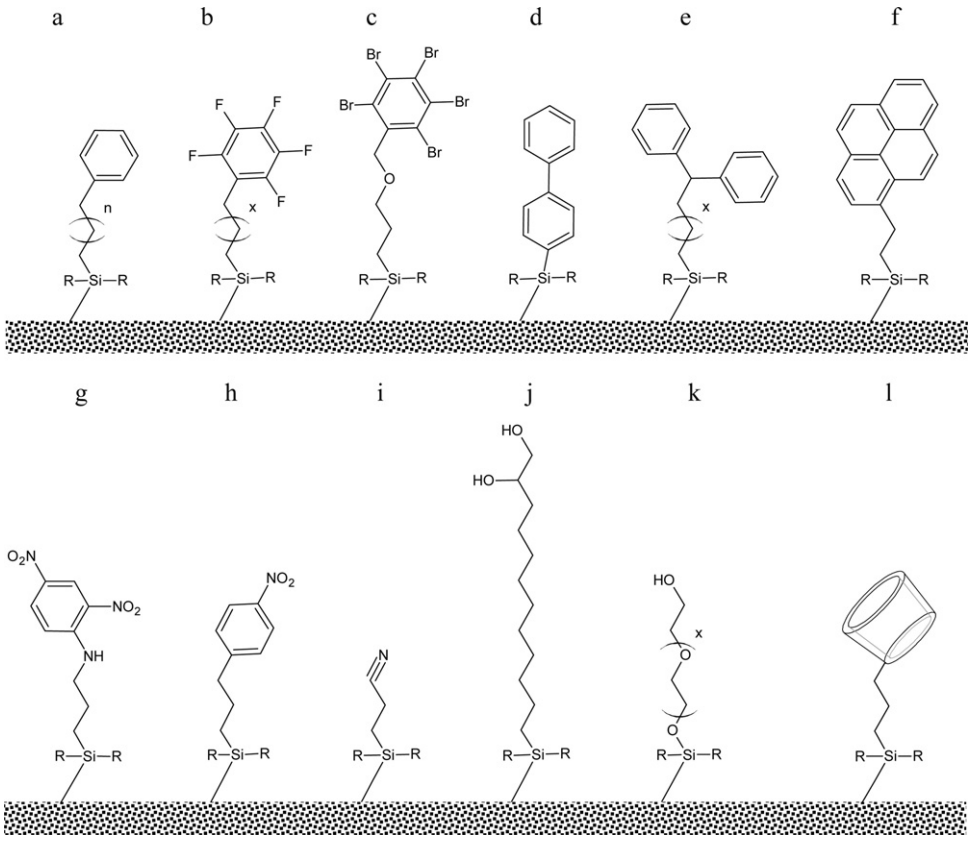

Fig. 2. Grafts bonded to silica support in studied phases. (a) Phenyl, $n = 1, 2$ or 4 (Ascentis Phenyl, Gammabond A Phenyl, Luna Phenyl, Nucleodur Sphinx); (b) pentafluorophenyl, x = unknown (Fluorosep-RP Phenyl); (c) pentabromobenzoyl (Cosmosil PBB-R); (d) biphenyl (Allure Biphenyl); (e) diphenyl, x = unknown (Pursuit XRs DP); (f) pyrenylethyl (Cosmosil PYE); (g) dinitroanilinopropyl (Uptisphere DNAP); (h) nitrophenyl (Nucleosil NO2); (i) cyano (Alltima CN, Alltima HP CN, Grom-Sil CN-1ST, Grom-Sil CN-3CP, LiChrospher CN, Nucleosil CN and Uptisphere CN); (j) diol (Acclaim Mixed-Mode); (k) poly(ethyleneglycol), x = unknown (Discovery HS-PEG); (l) inclusion complex; the ring is a calixarene (Caltrex Resorcinaren and Caltrex Science) or a cyclodextrin (ChiraDex Beta and ChiraDex Gamma).

change the selectivity of tested *Cosmosil* phases (*PBB-R* and *PYE*) towards our anthraquinone derivatives standards.

All columns were thermostated at 30 °C.

Data statistical processing has been done with Microsoft Office Excel software with the extension XLStat (Addinsoft, Paris, France).

3. Results and discussion

3.1. Pre-selection of stationary phases

The number of existing functionalised phases is important, but each grafting type is not as well represented as alkyl bonded phases from the point of view of bonded structures (mono- and poly-layer, sandwich, mixed mode), grafting mode (mono-, bi- and tri-anchored) and complementary treatment (end-capping, base deactivation, etc.) [47]. The availability of phases in our laboratory allowed the study of just some of them, far from covering the entire range of functionalised phase possible characteristics. Starting from that point, no systematic study could be performed, but some axes for further in depth evaluations may be drawn.

After the injection of all the mixtures of standards of anthraquinone derivatives on respective columns and elution with gradient I (Table 3), we observed that several compounds were not detected on some of them.

More than 10 compounds "disappeared" from chromatograms obtained with *Gammabond A Phenyl* (#F-06), *Nucleosil NO2* (#F-09), *Uptisphere DNAP* (#F-12) and *Lichrospher CN* (#F-19). The majority of these compounds are anthraquinoids with strong chelating potential, hydroxyl substituted at least at R1, R2 or R1, R4 or R2, R3 positions (Ali, Qza, Hys, Agl, Pur, Qlz) [48]. It has been shown that the chelating anthraquinones are sensitive to the presence of metallic impurities in the silica support, giving tailing peaks [49]. This is probably a reason for irreversible adsorption or excessive tailing on type A silica of column *LiChrospher CN* (#F-19), leading to peak disappearance for these compounds. The presence of alumina as phase support could explain in the same way the problems

Table 3
Elution gradients.

	Mobile phase composition			Gradient I	Gradient II	Gradient III
	A (H_2O) [%]	B (MeCN) [%]	C (1% HCOOH in H_2O) [%]	min	min	min
Program	85	5	10	0.0	0.0	0.0
	85	5	10	1.0	2.7	2.3
	0	90	10	66.0	88.5	83.7
	0	90	10	68.0	90.7	85.7
Equilibration period [min]				12	17	16
Flow-rate [ml/min]				1.00	1.00	0.20

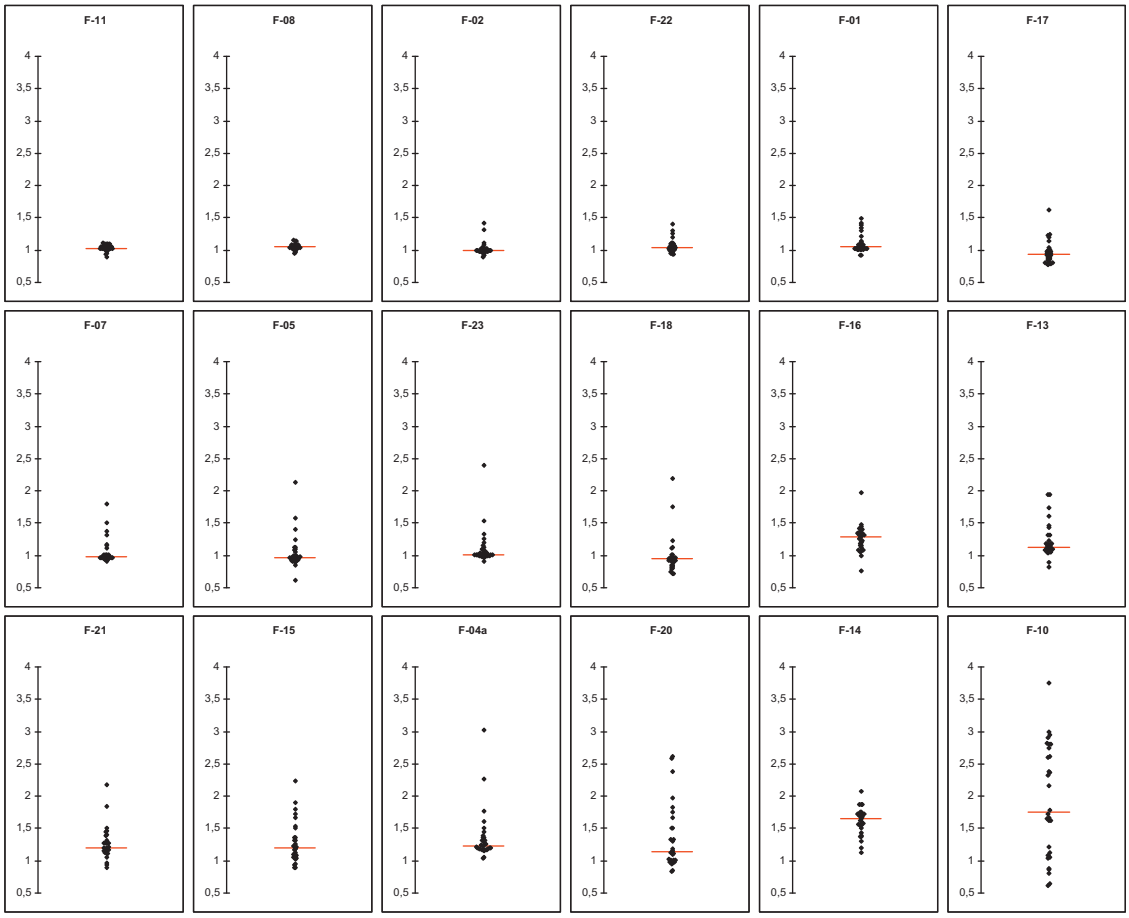

Fig. 3. Scattergrams of tailing factor (TF) of individual compounds on respective stationary phases. Horizontal bars represent the average values ($\overline{TF}$) for each stationary phase.

observed on *Gammabond A Phenyl* column (#F-06). The alumina encapsulation for that phase seems to be insufficiently isolating, maybe because of polymer ageing.

Other disappearing compounds are anthraquinone carboxylic acids (Car, Flk, Ker, Rhe, Can). They develop very strong interactions with bonded functional groups of two stationary phases: *Uptisphere DNAP* (#F-12) and *Nucleosil NO2* (#F-09).

Carboxylic acids also present problems of elution on cyclodextrin bonded silica. Carminic acid on both #F-24 and #F-25 *ChiraDex* phases, and kermesic acid on the first of them were not eluted. However, that problem arises from particularly high retention of all anthraquinone carboxylic acids and not from their irreversible absorption. In substance, other carboxyanthraquinones: Flk, Rhe and Can are also much more retained on these stationary phases than on aromatic or polar ones.

The high retention of all anthraquinoids is observed on *Cosmosil PBB-R* and *PYE* (respectively #F-03 and #F-04) which is consistent with previously published observations made on aromatic solutes [20]. This effect is so strong that the most retained compounds Dmq and Phy are eluted only when acetonitrile is replaced by acetone as B solvent in gradient I (Table 3). Thus, the obtained separation systems were named respectively #F-03a and #F-04a. Unfortunately, Dmq remains not eluted even in #F-03a system.

According to these observations, columns *Cosmosil PBB-R* (#F-03), *Cosmosil PYE* (#F-04), *Gammabond A Phenyl* (#F-06), *Nucleosil NO2* (#F-09), *Uptisphere DNAP* (#F-12), *LiChrospher CN* (#F-19), *ChiraDex Beta* (#F-24) and *ChiraDex Gamma* (#F-25) were excluded from further evaluation. The remaining 18 other phases were evaluated for peaks symmetry and critical pairs number, following the approach used for C18 bonded silica [12].

3.2. Symmetry parameter

Obtaining symmetric peaks is important for accurate integration by precise location of the start and end of the peak. It is also essential for detection and quantification limits, because the height for large tailing peaks for the same quantity injected is smaller than for symmetric and thin ones. Symmetry also has an indirect impact on efficient resolution, especially when baseline resolution is needed, and on peak purity estimation, when the spectra of the compound and its impurity are close [50,51].

Using the asymmetry expressed as a tailing factor of all analysed standards, it is possible to survey the efficiency of every solute exchange or problems between mobile phase and tested columns. The scattergrams illustrating the tailing factor of respective peaks (*TF*) (Eq. (1)) obtained for each stationary phase are presented in Fig. 3.

$$TF = \frac{WL_5 + WR_5}{2 \times WL_5} \quad (1)$$

where WL_5 and WR_5 are the left (front) half-width and the right (rear) half-width of the peak at 5% of the peak height, respectively.

The average tailing factor ($\overline{TF}$) value was calculated and represented as bars in Fig. 3.

The tailing of a single peak (*TF*) above 1.5 may be considered as undesirable, because of an important decrease of sensitivity of detection and the loss of resolution [52–54].

If we apply that criterion to our series of anthraquinoids, only 5 columns have all peaks meeting this condition, in order of minimum tailing: *Pursuit XRs DP* (#F-11), *Nucleodur Sphinx* (#F-08), *Ascentis Phenyl* (#F-02), *Caltrex Resorcinaren* (#F-22) and *Allure Biphenyl* (#F-01).

Almost all other stationary phases present good peak shape for quinizarin (Qza) – the marker of silica metal residue [49]. This compound gives peaks of acceptable symmetry on all bonded silica phases, except *Nucleosil CN* (#F-20), showing the excellent quality of silica supports used by all manufacturers.

According to raw data, not detailed in Fig. 3, usually only few compounds per phase tail: acids (Car, Ker, Flk), strong complexing ligands (Pur, Qlz, Qza, Hys, Agl) and also Phy or Oma.

The set of tailing compounds seem to depend partially on graft nature. For example, the acids give strongly asymmetric peaks on all cyano phases. On the other hand they also tail on *Fluorosep-RP Phenyl* (#F-05) and *Cosmosil PYE* (#F-04a).

The peak of Phy is asymmetric on *Caltrex Science* (#F-23) and *Alltima CN* (#F-14); Oma peak on *Luna Phenyl-Hexyl* (#F-07) and *Discovery HS-PEG* (#F-16). The tailing of these two compounds thus does not depend on the graft type.

The stationary phases giving a lot of excessively tailing peaks are cyano bonded: *Alltima CN* (#F-14), *Nucleosil CN* (#F-20) as well as *PLRP-S* (#F-10)

It should be pointed out that *Alltima CN* (#F-14) and *PLRP-S* (#F-10) display an average tailing ($\overline{TF}$) above 1.5, which is an unacceptable value even for a single compound, with respectively narrow and wide dispersion of asymmetry.

PLRP-S (#F-10) gives unacceptable tailing factor values for the majority of compounds (20/30). That behaviour was unexpected. The tailing mechanism should not be related there to metal impurities, as observed for bonded silica, because the particles are entirely polymeric (cross-linked PS-DVB). Actually, on this phase the tailing peaks were observed for hydrophobic anthraquinoids, and the polar compounds have peaks of relatively better symmetry. It is thus independent of complexing ability of anthraquinone derivatives.

The peaks on *Discovery HS-PEG* (#F-16) have particular behaviour: nearly all compounds tail a little, so the average tailing ($\overline{TF} = 1.27$) is higher than for many other columns, but individual peak asymmetries are not scattered much.

For easier comparison between 18 stationary phases, they were classified according to symmetry parameter $d(TF;1)$ calculated from equation (Eq. (2)) [12].

$$d(TF;1) = \sqrt{{\sigma_{TF}}^2 + (\overline{TF} - 1)^2} \quad (2)$$

where σ_{TF} is standard deviation of the tailing factor calculated for each standard and $\overline{TF}$ is the average symmetry of the whole set of standards.

This parameter takes into account with the same weight the dispersion of results (σ_{TF}) and their global distance from the perfect average symmetry ($\overline{TF} - 1$).

The obtained numerical values are reported in Table 4.

When all peaks of a series of compounds are fully symmetric, on an ideal column, the $d(TF;1) = 0$. Of course that situation is difficult to reach and as it can be seen in the results, the peaks tailing is more or less significant and concerns different numbers of compounds per column. In common applications, the parameter is usually $d(TF;1) > 0$. It allows classification of the columns: the higher is the symmetry parameter, the less suitable is the column. However, it is important to define the absolute upper limit of value for this parameter to see how many tested columns give an acceptable tailing.

Table 4
Symmetry parameter $d(TF;1)$.

Stationary phase	Column number	Symmetry parameter $d(TF;1)$	Rank
Pursuit XRs DP	F-11	0.06	A+
Nucleodur Sphinx	F-08	0.07	A+
Ascentis Phenyl	F-02	0.11	A+
Caltrex Resorcinaren	F-22	0.13	A+
Allure Biphenyl	F-01	0.18	A+
Grom-Sil Cyano-1ST	F-17	0.18	A+
Luna Phenyl-hexyl	F-07	0.21	A+
Fluorosep-RP Phenyl	F-05	0.28	A+
Caltrex Science	F-23	0.30	A+
Grom-Sil Cyano-3CP	F-18	0.31	A
Discovery HS-PEG	F-16	0.33	A
Acclaim Mixed-Mode HILIC-1	F-13	0.35	A
Uptisphere CN	F-21	0.37	A
Alltima HP CN	F-15	0.42	A
Cosmosil PYE	F-04a[a]	0.54	
Nucleosil CN	F-20	0.63	
Alltima CN	F-14	0.64	
PLRP-S	F-10	1.26	

[a] Elution gradient with acetone as organic modifier.

The individual peak tailing acceptability criterion ($TF_{max} = 1.5$) can be used to define that limit by extension to the population of many peaks. The prerequisite is that the worse, but still satisfactory situation occurs when all peaks display the maximum acceptable tailing. The average tailing factor is thus $\overline{TF} = 1.5$. In this case, dispersion must be inexistent and $\sigma_{TF} = 0$, otherwise tailing limits will be overstepped.

When these values are used in Eq. (2), the symmetry parameter gives: $d(TF;1) = 0.5$.

Thus, the symmetry parameter can be considered as suitable for any pair of average tailing and tailing standard deviation values fulfilling the condition: $d(TF;1) \leq 0.5$. In reality, this condition is assumed even by the series of compounds which contains few peaks with large tailing ($TF > 1.5$), if the average tailing for whole population is very low ($\overline{TF} \sim 1.0$). That is an important property which should be kept in mind when the comparison of columns is done using the symmetry parameter.

The comparison of the symmetry parameters from Table 4 points out the series of columns giving excellent results. Fourteen heading phases in this table, from *Pursuit XRs DP* (#F-11) to *Alltima HP CN* (#F-15), display the symmetry parameter below 0.50 and are classified "A+" and "A".

The three following columns – *Cosmosil PYE* with acetone containing mobile phase (#F-04a), *Nucleosil CN* (#F-20) and *Alltima CN* (#F-14) – gave higher values, up to 0.64, but not far from the defined limit. Only one column – *PLRP-S* (#F-10) – falls out of rank with excessive symmetry parameter value of 1.26. Because of the low general symmetry of peaks obtained on *PLRP-S*, this phase was discharged from further considerations.

It could be noticed that stationary phases from "A+" class have aromatic grafts and those from class "A" are polar bonded.

3.3. Separation capacity parameter

In general, the efficiency of separation of the large number of compounds can be evaluated by the number of critical pairs they form [55,56] in given conditions. These conditions in gradient elution concern: gradient parameters, mobile phase composition and linear velocity, temperature of separation, stationary phase characteristics and column dimensions. In the present work, the evaluation of separation capacity of stationary phases was performed in comparable conditions of analysis.

Table 5
Separation capacity – number of critical pairs on each stationary phase.

Column	Column number	Total pairs	Rank
Caltrex Resorcinaren	F-22	9	A
Cosmosil PYE	F-04a[a]	12	B
Caltrex Science	F-23	12	B
Luna Phenyl-Hexyl	F-07	13	B
Pursuit XRs DP	F-11	14	B
Nucleodur Sphinx	F-08	15	B
Allure Biphenyl	F-01	15	B
Ascentis Phenyl	F-02	16	C
Alltima CN	F-14	17	C
Fluorosep-RP Phenyl	F-05	18	C
Grom-Sil Cyano-3CP	F-18	20	C
Alltima HP CN	F-15	20	C
Uptisphere CN	F-21	22	D
Grom-Sil Cyano-1ST	F-17	22	D
Nucleosil CN	F-20	24	D
Acclaim Mixed-Mode	F-13	27	E
Discovery HS-PEG	F-16	31	E

[a] Elution gradient with acetone modifier.

For each column, critical pairs were determined for every two compounds with resolution value $R_s < 1.5$ [50,57]. The number of critical pairs obtained for each column is reported in Table 5, along with column ranking. The results are classified according to previously used criteria [12].

The column ranked "A" (<10 critical pairs) and "B" (from 11 to 15 pairs), plus the first from "C" (16 pairs), have aromatic grafting. These columns retains compounds by hydrophobic and π–π interactions.

The best performance is achieved by poly-aromatic phases *Caltrex* (#F-22 and F-23) as well as *Cosmosil PYE* (#F-04a) tested with acetone as mobile phase modifier.

The separation on aromatic bonded phases: phenyl (*Ascentis Phenyl*, #F-02 and Luna *Phenyl-Hexyl*, #F-07), diphenyl (*Pursuit XRs DP*, #F-11), biphenyl (*Allure Biphenyl*, #F-01) and mixed C18/phenyl (*Nucleodur Sphinx*, #F-08) is similar.

The fluorinated phenyl phase (*Fluorosep-RP Phenyl*, #F-05) is the least efficient with 18 critical pairs and was classified in the "C" group. The retention mechanism on this electron-acceptor phase [37] includes other, not clearly identified interactions [31], which should explain its difference compared to aromatic hydrocarbon bonded ones.

Most phases from classes "C" (from 16 to 20 pairs) and "D" (from 21 to 25 pairs) are polar hydrophobic – owning grafts with nitrile group. The lowest pair number (17) is observed on polyfunctional end-capped *Alltima CN* (#F-14) whereas the highest (24) is on non end-capped *Nucleosil CN* (#F-20).

The less efficient are H-bond acceptor/donor phases: (poly)ethyleneglycol (*Discovery HS-PEG*, #F-16) and diol (*Acclaim Mixed-Mode*, #F-13). They were classified "E" and produce over 26 critical pairs with 30 standards, which means: the compounds are co-eluted by "bunches" and not like the isolated pairs. These phases also give the weakest retention.

The most permanent critical pairs (stated for 9 or more phases) are Flk–Ker, Pur–Xpu, Arf–Qza, Pur–Qlz, Ali–Ale, Can–Moh, Ali–Moh and Ali–Can.

Flk and Ker differ only by the presence of hydroxyl group in position R4 for the latter (see Table 1). The investigated stationary phases are globally not very selective towards these structural differences, except *Cosmosil PYE* (#F-04a), *Alltima HP-CN* (#F-15), *Discovery HS-PEG* (#F-16) and *Caltrex Resorcinaren* (#F-22).

The same structural difference, concerning R4 position, exists for Xpu and Pur (see Table 1), however these compounds are substituted by fewer polar groups than precedents. Nevertheless, the same phases are efficient for their separation, except *Alltima CN* replaced by *Uptisphere CN* (#F-21) and *Nucleosil CN* (#F-20), and two other additional aromatic bonded phases *Allure Biphenyl* (#F-01) and *Fluorosep-RP Phenyl* (#F-05).

Arf and Qza are isomers substituted by two hydroxyl groups respectively in positions 1,5- and 1,4- (see Table 1). They are differentiated only by *Fluorosep-RP Phenyl* (#F-05), both *Alltima CN* (#F-14 and F15) and *Uptisphere CN* (#F-21).

The phases selective for the pair of 1,2,4-trihydroxyanthraquinone (Pur) and the 1,2,5,8-tetrahydroxyanthraquinone (Qlz) (see Table 1) are *Cosmosil PYE* (#F-04a), *Fluorosep-RP Phenyl* (#F-05), *Discovery HS-PEG* (#F-16), *Nucleosil CN* (#F-20), and *Caltrex Resorcinaren* (#F-22) as well as *Acclaim Mixed-Mode* (#F-13).

Ali, Ale, Moh and Can have very different structures (Table 1) and their recurrent co-elution is probably due more to compensation of their interactions with stationary and mobile phases under study. Then 1,2-dihydroxy substitution (Ali) seems to produce the same effect as substitutions like 1,8-dihyroxy-3-hydroxymethyl (Ale), 2-hydroxy-3-methoxy (Moh) or 2-carboxy (Can).

The Ali–Moh pair is separated by *Cosmosil PYE* (#F-04a), *Alltima CN* (#F-14), *Discovery HS-PEG* (#F-16) and *Nucleosil CN* (#F-20).

The Ali–Can pair is separated by *Cosmosil PYE* (#F-04a), *Fluorosep-RP Phenyl* (#F-05), *Acclaim Mixed-Mode* (#F-13), *Nucleosil CN* (#F-20) and *Caltrex Resorcinaren* (#F-22).

For the resolution of the Ale–Ali pair, aromatic and calixarene phases (except *Fluorosep-RP Phenyl*, *Pursuit XRs DP* and *Nucleodur Sphinx*) could be used. *Alltima CN* (#F-14) and *Discovery HS-PEG* (#F-16) may be also applied.

Can–Moh are resolved by aromatic grafted Cosmosil PYE, Fluorosep-RP Phenyl, Pursuit XRs DP, H-bond donor/acceptor Acclaim Mixed-Mode, Discovery HS-PEG, polar Nucleosil CN and calixarene Caltrex Resorcinaren.

As it can be globally observed, the cyano phases have difficulty separating compounds with hydroxyl substitutions in positions R1, R4, R5 and R8 from their "homologues" not substituted in that positions, as pairs: Pur–Qlz, Pur–Xpu, Flk–Ker, Agl–Hys.

The aromatic phases are not as selective for isomers substituted by two hydroxyl groups in positions R1 and R4, R5 or R8, which means Arf, Qza and Dan or for compounds with additional intramolecular hydrogen bond, as the series Pur–Qlz–Xpu (see Table 1).

Hydrogen bonds acceptor and donor phases are not very selective like *Discovery HS-PEG* or behave similarly to cyano phases like *Acclaim Mixed-Mode*. The *Acclaim* phase also gives a selectivity close to that formerly tested with the same set of compounds hydroxyloctyl/C18 mixed phase *Nucleodur Isis* [12].

Taking in account the above observations, the selectivity power in the separation of anthraquinones using MeCN containing mobile phase seems to depend basically on hydrophobicity, H-bond creation and π–π interactions, and much less on dipole–dipole interactions.

The retention on alkylnitrile phases is globally weaker and especially for hydrophobic anthraquinoids. In acetonitrile modified mobile phase, the cyano phases tend to work like alkyl bonded phases [26,58].

Although MeCN tends to limit π–π interactions between the solutes and phenyl phases [58,59], the condensed polyaromatic ligands may efficiently change selectivity in anthraquinone derivatives separation.

Hydrogen bond formation with donor or acceptor phases reduces the selectivity because this retention mechanism probably minimizes the hydrophobic interactions imposed by mobile phase in gradient elution. On the PEG and diol phases, the compounds with groups able to generate hydrogen bonding are retained a little more than on alkyl-siloxane bonded phases, due to the phase basicity [60], and the structures with hydrophobic groups are a little less retained. This effect "squeezes" the retention time range

and the peak accumulation in this narrower time window leads to the crowded chromatograms.

3.4. Separation complementarity

As none of the columns allow for the full standard separation, one selected column may also be inefficient for any series of anthraquinone derivatives. The larger is the series, the higher is the probability of unsatisfactory separation. The advantage of testing the wide range of functionalised stationary phases was that we observed that many of them are able to resolve the compounds from critical pairs obtained on others. The use for the analyses of two or three columns with alternative resolution should help to overcome most problems in current analysis of anthraquinoids.

In Table 6, we presented nine unresolved pairs of peaks obtained on the most efficient stationary phase *Caltrex Resorcinaren* (#F-22).

Among the 16 other tested stationary phases only one presents the capacity of resolution of 8 pairs unresolved on #F-22: *Cosmosil PYE* with acetone organic modifier of mobile phase (#F-04a). The important naturally occurring anthraquinoids are separated from their pairs (Fra, Rhe, Xpu). Five stationary phases are able to resolve 7 pairs from Caltrex Resorcinaren, and seven of them 6 pairs.

The combination of *Caltrex Resorcinaren* (#F-22) with two other stationary phases may ensure the alternative separation of all critical pairs. For instance, the alternative solutions are: 2nd phase: *Cosmosil PYE* (#F-04a) or *Discovery HS-PEG* (#F-16), 3rd phase: *Fluorosep-RP Phenyl* (#F-05), one of *Alltima CN* (#F-14 or #F-15), or *Uptisphere CN* (#F-21).

From the point of view of symmetry, the set that is most suitable to *Caltrex Resorcinaren* complementary phases is formed by *Fluorosep-RP Phenyl* and *Discovery HS-PEG*; however the last column displays the highest number of critical pairs (31) of all those studied. The *Alltima CN* and *HP CN* get similar performances to *Grom-Sil CN-3CP*, but the symmetry of peaks on these phases are worse. The excellent symmetry with similar to *Grom-Sil CN-3CP* selectivity is also displayed by *Pursuit XRs DP*. This column also gives less critical pairs (14) than *Grom-Sil* (20). The *Cosmosil PYE* might seem to be the first choice phase because of its separation power. Unfortunately, it gives a lot of tailing peaks and bleeds in gradient elution.

3.5. Application to analysis of dye from a historical sample

The separation of anthraquinones from the real sample of dyed wool from a 15th Century tapestry [12] was performed using the most effective functionalised phases: *Caltrex Resorcinaren* (#F-22), *Pursuit XRs DP* (#F-11) and *Luna Phenyl-Hexyl* (#F-07).

The separation was performed using on columns 4.6 mm i.d. (*Pursuit XRs DP*) and 4.0 mm i.d. (*Caltrex Resorcinaren*) with gradient II detailed in Table 3 at mobile phase flow-rate of 1.0 ml/min, and on column 2.0 mm i.d. (*Luna Phenyl-Hexyl*) with gradient III at 0.2 ml/min. The injected volumes were 20 μl and 5 μl respectively.

The obtained chromatograms are displayed Fig. 4.

In the chromatograms recorded at 254 nm we looked for nine compounds previously detected in this extract [12]. Their characteristics are presented in Table 1. Compound "A" visible in chromatograms is an artefact from formic acid used in mobile phase, accumulated during column equilibration period and eluted gradient run.

The comparison of results shows the differences in selectivity of aromatic and inclusion complex phases.

The obtained peak symmetry is globally excellent on three phases used for historical sample analysis, also for pseudopurpurin (Psp) and munjistin (Mun). Actually, the peak symmetry of these both compounds, as well as their separation, is often problematic on some stationary phases [12].

Table 6
Complementary separation capacity. R – resolved critical pairs from *Caltrex Resorcinaren* phase (*#F-22*).

Critical pair on Caltrex Resorcinaren F-22	Cosmosil PYE F-04a[a]	Fluorosep-RP Phenyl F-05	Alltima CN F-14	Alltima HP CN F-15	Discovery HS-PEG F-16	Grom-Sil Cyano-3CP F-18	Allure Biphenyl F-01	Ascentis Phenyl F-02	Nucleodur Sphinx F-08	Pursuit XRs DP F-11	Grom-Sil Cyano-1ST F-17	Uptisphere CN F-21	Nucleosil CN F-20	Luna Phenyl-Hexyl F-07	Acclaim Mixed-Mode F-13	Caltrex Science F-23
Afv–Ker	R	R		R	R	R					R		R	R	R	
Ali–Moh	R		R		R								R			
Arf–Qza		R	R	R								R				
Chr–Dma	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Dan–Tec	R		R	R	R	R	R	R	R	R	R	R	R		R	
Eta–Oma	R	R	R	R		R	R	R	R	R	R	R		R		R
Fra–Rhe	R	R			R	R	R	R	R	R		R	R	R	R	R
Fra–Xpu	R	R	R	R	R	R	R	R	R	R	R	R		R		R
Rhe–Xpu	R	R	R	R	R	R	R	R	R	R	R		R		R	R
Number of resolved pairs (R)	8	7	7	7	7	7	6	6	6	6	6	6	6	5	5	5

[a] Elution gradient with acetone organic modifier.

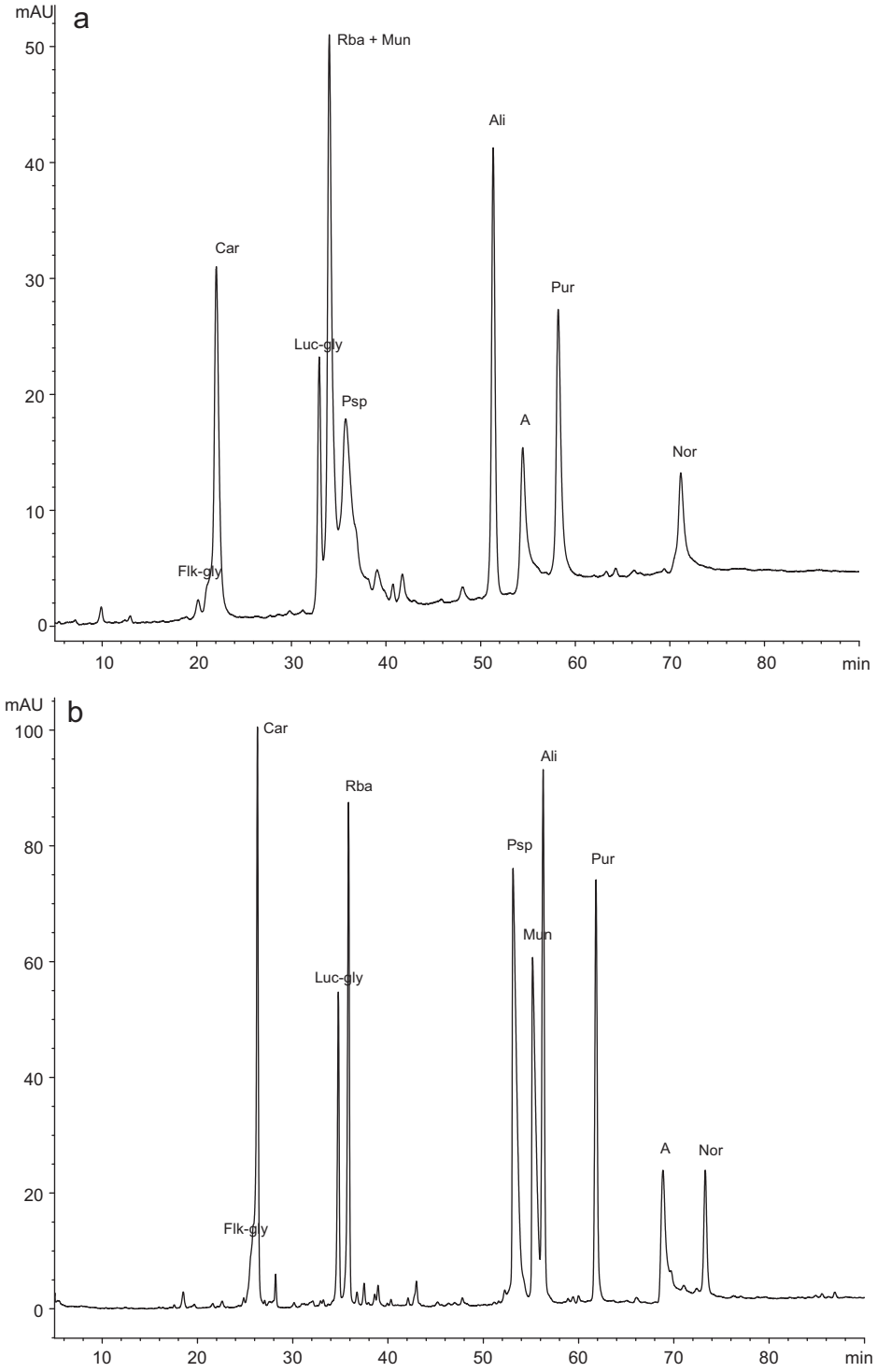

Fig. 4. Chromatograms of a red wool sample from 15th C. tapestry. Detection wavelength: 254 nm. For experimental conditions – see Section 2.1 and Table 3, and for peak identification – see Table 1. Peak "A" is an impurity of formic acid used in mobile phase. (a) Caltrex Resorcinaren; (b) Pursuit XRs DP; (c) Luna Phenyl-Hexyl.

The aromatic grafts of *Pursuit XRs DP* (#F-11) and *Luna Phenyl-Hexyl* (#F-07) are relatively more retentive for pseudopurpurin and munjistin. Both, Psp and Mun have a carboxyl group in position R2 and the difference between them is the hydroxyl group in R4 for pseudopurpurin forming an additional intramolecular H-bond, inexistent in munjistin. The carboxyl group should specifically interact with phenyl moieties of stationary phases while calixarene bonded silica reveals to a much greater extent the low hydrophobicity of these compounds. *Caltrex Resorcinaren* (#F-22) does not retain Psp and Mun as much and enables elution together with O-glycosides: ruberythric acid (Rba, actually: alizarin-glycoside) and lucidin (Luc-gly). So, in applied conditions the co-elution of Rba and Mun occurs. Also the elution order of Psp and Mun is inversed.

That retention behaviour change is not observed for carminic acid (Car) which, aside of the carboxyl function it possesses in R7, owns the C-glycoside in R2. This glycoside clearly determines the chromatographic properties of Car on tested stationary phases. However, the separation of carminic acid and flavokermesic acid

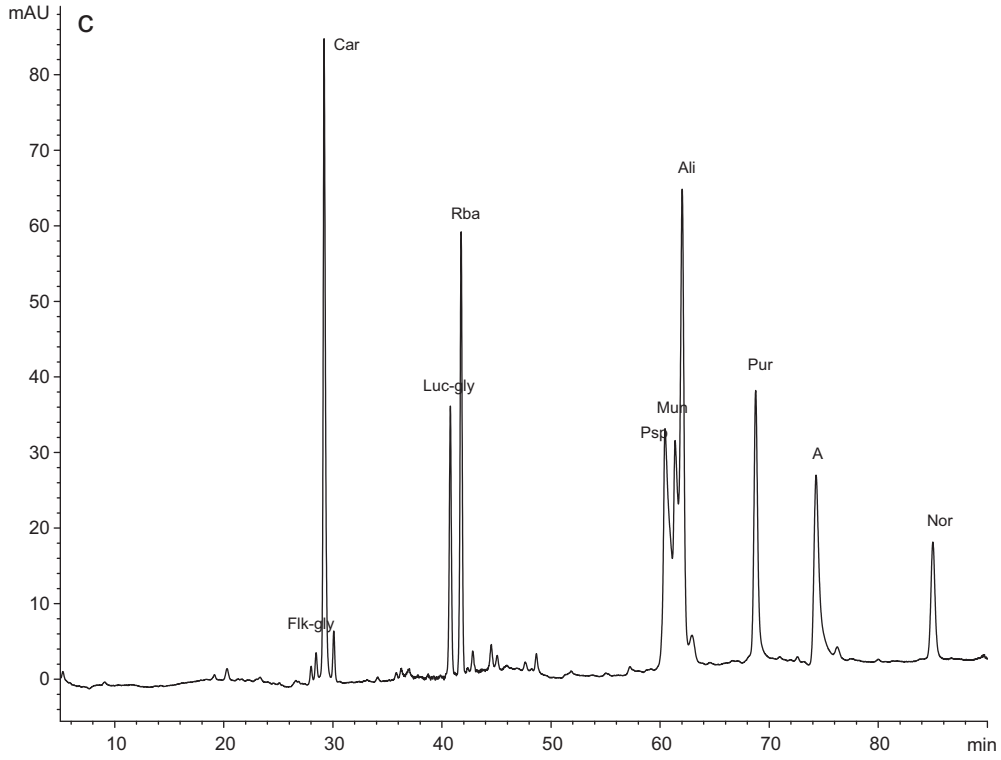

Fig. 4. (*Continued*).

glycoside (Flk-gly) – also the R2 substituted C-glycoside – is good, except on *Pursuit XRs DP*. The difference between these compounds is the same as for the Psp and Mun couple: presence/absence of hydroxyl group in R4. Thus, the selectivity of these stationary phases is quite sensitive to structural differences of compounds.

The calixarene and aromatic bonded phases give a somewhat different resolution and elution order of anthraquinone derivatives. The use of one of them, *Caltrex Resorcinaren* (#F-22), could be completed by an alternative *Pursuit XRs DP* (#F-11) or *Luna Phenyl-Hexyl* (#F-07) in the case of difficult separations. For the presented example, the *Pursuit XRs DP* phase seems to be the most effective; however, if the sample composition was different it could be necessary to check the separation efficiency on *Caltrex Resorcinaren*. Both phases could be thus recommended as complementary.

4. Conclusion

The evaluation of the efficiency of functionalised stationary phases for separation of anthraquinoids highlights the good performances of some calixarene and aromatic bonded phases. They are usually superior to other types of grafts studied here: nitro, cyano, PEG, diol or cyclodextrin.

The anthraquinone derivatives represent a huge family of compounds and the proposed reference columns set may not resolve every practical problem. However, the complementary selectivity of these phases allows two or three phases to be found, resolving all of the critical pairs of representative compounds we studied. Of course, the studied phases represent only some commercially available products and further phases can be evaluated and compared with reference phases we propose.

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