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ROZPRAWA DOKTORSKA

Charakterystyka oraz badania adsorpcji i mechanizmu retencji faz stacjonarnych z wbudowaną grupą fosfodiestrową

Praca wykonana
w Katedrze Chemii Środowiska i Bioanalityki
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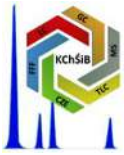

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Characterization and study of adsorption and retention mechanism of stationary phases with embedded phosphodiester group

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Spis treści

Wykaz skrótów i oznaczeń

ACN Acetonitryl (ang. *acetonitrile*)

BET Model izotermy Brunauera-Emmetta-Tellera

CHN Analiza elementarna (ang. *elemental analysis*)

DVLO Skrót teorii pochodzący od jej twórców: Derjauguina, Landau'a, Verwey'a i Overbeeka

FA Analiza czołowa (ang. *frontal analysis*)

HETP Wysokość równoważna półce teoretycznej (ang. *height equivalent to a theoretical plate*)

HILIC Chromatografia cieczowa oddziaływań hydrofilowych (ang. *hydrophilic interaction liquid chromatography*)

HPLC Wysokosprawna chromatografia cieczowa (ang. *high-performance liquid chromatography*)

HTLC Wysokotemperaturowa chromatografia cieczowa* (ang. high temperature liquid chromatography)

IM Metoda inwersyjna (ang. *inverse method*)

i-PDeA Inteligentna analiza dekonwolucji pików (ang. *intelligent peak deconvolution analysis*)

IPA Alkohol izopropylowy (ang. isopropyl alcohol)

ISEC Odwrócona chromatografia wykluczenia (ang. *inverse size exclusion chromatography*)

MDM Metoda zaburzeniowa (ang. *minor disturbance method*)

µHPLC Kapilarna wysokosprawna chromatografia cieczowa (ang. *micro-high performance liquid chromatography*)

ODS Oktadecylosilan (ang. *octadecylsilane*)

PALC Wodna chromatografia cieczowa* (ang. *per aqueous liquid chromatography*)

PHW-LC Chromatografia cieczowa z gorącą wodą pod ciśnieniem (ang. *pressurized hot water liquid chromatography*)

POPLC Zoptymalizowana fazowo chromatografia cieczowa (ang. *phase optimized liquid chromatography*)

RP LC Chromatografia cieczowa w odwróconym układzie faz (ang. *reversed phase liquid chromatography*)

SBWC Chromatografia z wodą w stanie podkrytycznym* (ang. *subcritical water chromatography*)

SHWC Chromatografia w przegrzanej wodzie (ang. superheated water chromatography)

TGA Analiza termograwimetryczna (ang. *thermogravimetric analysis*)

THF Tetrahydrofuran (ang. *tetrahydrofuran*)

UHPLC Ultra-wysokosprawna chromatografia cieczowa (ang. *ultra-high performance liquid chromatography*)

Wprowadzenie

We współczesnym świecie rośnie świadomość oraz praktyka związana z ekologicznym podejściem do życia. Coraz większy nacisk wywierany jest w kierunku eliminacji lub znacznej redukcji produkowania odpadów. Świat nauki również dąży do tego, aby podejście do planowania i realizacji badań było jak najbardziej „zielone". W przypadku chemii za swoisty początek większej świadomości ekologicznej oraz przełożenia tego na praktykę można uznać wprowadzanie przez Anastas'a oraz Werner'a w 1999 roku pojęcia „zielonej chemii" i zdefiniowanie jej 12 zasad. Również w chromatografii cieczowej coraz istotniejsze jest poszukiwanie i wdrażanie rozwiązań pozwalających na zmniejszenie lub całkowitą redukcję wytwarzania szkodliwych odpadów w czasie wykonywania analiz. W przypadku chromatografii, jednostkowo chromatograf cieczowy nie produkuje znacznych ilości odpadów, jednakże biorąc pod uwagę szerokie zastosowanie tego typu aparatury w laboratoriach farmaceutycznych, analitycznych, medycznych czy związanych z żywnością oraz mnożąc przez czas, w jakim wykonują swoją pracę, ilość generowanych odpadów znacznie rośnie.

Do możliwych rozwiązań „zielonej" chromatografii cieczowej pozwalających na redukcję wytwarzanych szkodliwych dla środowiska odpadów można zaliczyć miniaturyzację aparatury chromatograficznej lub skrócenie czasu wykonania analiz. Taki efekt może zapewnić także zastosowanie faz stacjonarnych umożliwiających wykonywanie rozdzieleń w więcej niż jednym trybie chromatograficznym. Nie pojawia się konieczność posiadania wielu kolumn chromatograficznych wypełnionych różnymi fazami stacjonarnymi, jeżeli jeden materiał może zapewnić ich zastosowania i właściwości. Ogranicza się także ilość rozpuszczalników potrzebnych na przepłukiwanie i kondycjonowanie kolumn.

Całkowitą eliminację rozpuszczalników organicznych zapewni jedynie zamiana obecnie wykorzystywanych eluentów jako faz ruchomych na nietoksyczne, biodegradowalne i „zielone" np. czystą wodę, etanol lub dwutlenek węgla w stanie nadkrytycznym. Taką możliwość zapewnia odpowiedni dobór fazy stacjonarnej. Konieczne jest zatem poszukiwanie nowych materiałów pozwalających na wydajne i selektywne rozdzielenie analitów w „zielonej" fazie ruchomej. Powstające wtedy zanieczyszczenia zminimalizowane są jedynie do niewielkich ilości analitów eluujących z kolumny.

Możliwość pracy w warunkach nietoksycznych rozpuszczalników jako fazy ruchomej stwarzają fazy stacjonarne, które jednocześnie posiadają grupę polarną oraz niepolarną

przyłączoną do powierzchni krzemionki lub innego nośnika. Pierwsze wzmianki literaturowe opisujące próby syntezy takich materiałów do chromatografii cieczowej pojawiają się w latach 90-tych ubiegłego wieku, co świadczy o tym, iż są to materiały stosunkowo nowe oraz wymagające szerszej charakteryzacji. Dzięki obecności zarówno grup hydrofobowych jak i hydrofilowych takie fazy stacjonarne charakteryzują się mieszanym mechanizmem retencji, co umożliwia rozdzielanie mieszanin związków o szerokim spektrum polarności oraz pracę w nietypowych warunkach składu fazy ruchomej, w tym całkowicie wodnych.

Publikowane prace związane z fazami stacjonarnymi z wbudowanymi grupami polarnymi lub grupami polarnymi dołączonymi na etapie wtórnej silanizacji, dotyczą głównie opisu syntezy i przykładów zastosowań oraz mechanizmu retencji. Szeroka możliwość doboru grupy polarnej oraz dołączanej części niepolarnej sprawia, iż badania dotyczące przygotowania nowych faz stacjonarnych intensywnie rozwijają się w ostatnich latach. Pierwsze fazy stacjonarne z wbudowanymi grupami fosfodiestrowymi pojawiły się w 2015 roku. Od tego czasu brakowało pełnej charakterystyki tych faz, opisu mechanizmu retencji oraz ich możliwości chromatograficznych.

Przedstawione w niniejszej pracy dogłębne badania analityczne nad fazami stacjonarnymi do wysokosprawnej chromatografii cieczowej pozwoliły na poszerzenie grona otrzymywanych materiałów o kolumny posiadające zastosowanie w różnych trybach chromatograficznych. Wśród zsyntezowanych faz stacjonarnych, dwie są całkowicie nowe, natomiast dwie były wcześniej prezentowane w publikacjach naukowych, jednakże brakowało ich pełnej charakterystyki. Z sukcesem wykonano analizy chromatograficzne potwierdzające zastosowanie przygotowanych materiałów zarówno w układzie RP LC, jak i HILIC, a także w warunkach czystej wody. Po raz pierwszy została wykonana separacja związków całkowicie hydrofobowych, jakimi są wielopierścieniowe węglowodory aromatyczne, stosując wodę jako jedyny składnik fazy ruchomej. Dzięki przeprowadzonym badaniom i kompleksowej charakterystyce pojawia się możliwość wykorzystania spreparowanych faz stacjonarnych w analizach związków farmakologicznie oraz biologicznie istotnych. Zachowując zasady „zielonej chemii”, przygotowane materiały pozwalają na zwiększenie ekologii analiz chromatograficznych.

Niniejszą pracę doktorską stanowi zbiór sześciu artykułów naukowych, w tym dwóch przeglądowych oraz czterech eksperymentalnych, opublikowanych w specjalistycznych czasopismach naukowych:

[D1] M. Dembek, S. Bocian, *Pure water as a mobile phase in liquid chromatography techniques*, TrAC - Trends Anal. Chem. 123 (2020) 115793. **IF = 12,196; MEiN = 140.**

[D2] M. Dembek, S. Bocian, *Stationary Phases for Green Liquid Chromatography*, Materials (Basel). 15 (2022) 419. **IF = 3,748; MEiN = 140.**

[D3] M. Dembek, S. Bocian, B. Buszewski, *Solvent Influence on Zeta Potential of Stationary Phase—Mobile Phase Interface*, Molecules. 27 (2022) 968. **IF = 4,927; MEiN = 140.**

[D4] M. Dembek, M. Szumski, S. Bocian, B. Buszewski, *Optimization of the packing process of microcolumns with the embedded phosphodiester stationary phases*, J. Sep. Sci. 45 (2022) 3310–3318. **IF = 3,1; MEiN = 70.**

[D5] M. Dembek, S. Bocian, *Phosphodiester stationary phases as universal chromatographic materials for separation in RP LC, HILIC, and pure aqueous mobile phase*, Materials (Basel). 16 (2023) 3539. **IF = 3,748; MEiN = 140.**

[D6] O. Kalisz, **M. Dembek**, S. Studzińska, S. Bocian, *Beta-blockers separation on phosphodiester stationary phases - the application of Intelligent Peak Deconvolution Analysis*, Molecules. 28 (2023) 3249. **IF = 4,927; MEiN = 140.**

1. Cele i założenia rozprawy

Celem nadrzędnym dysertacji było otrzymanie, charakterystyka oraz wykorzystanie chemicznie związanych faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi jako materiałów wykorzystywanych zarówno w odwróconym układzie faz (RP LC), jak i w chromatografii cieczowej oddziaływań hydrofilowych (HILIC) do rozdzielania związków o szerokim zakresie polarności, a także przy zastosowaniu czystej wody jako jedynego składnika fazy ruchomej, co wpisuje się w założenia „zielonej chromatografii". W ramach realizacji postawionych zadań wykorzystano techniki syntezy i pakowania faz stacjonarnych, analizy instrumentalne, fizykochemiczne i chromatograficzne oraz obliczenia komputerowe.

Powyższe cele zostały zrealizowane poprzez:

- przegląd literatury oraz systematyczna analizę doniesień naukowych dotyczących zastosowania czystej wody w wysokosprawnej chromatografii cieczowej oraz faz stacjonarnych z wbudowanymi grupami polarnymi **[D1, D2]**,
- syntezę serii faz stacjonarnych z dołączonymi grupami niepolarnymi oraz grupą fosfodiestrową i grupami hydroksylowymi, jako częściami polarnymi **[D3]**,
- optymalizację procesu zawiesinowego pakowania kolumn chromatograficznych **[D4]**,
- scharakteryzowanie mechanizmu retencji zachodzącego na przygotowanych fazach stacjonarnych zarówno w czystej wodzie jak i w układzie chromatografii cieczowej oddziaływań hydrofilowych (obecnie dwa manuskrypty są po pierwszej recenzji w Journal of Chrmatography A),
- badanie właściwości chromatograficznych faz stacjonarnych do rozdzielania mieszanin związków o różnej polarności z wykorzystaniem chromatografii cieczowej w dwóch układach (RP LC i HILIC) **[D5],**
- wykorzystanie spreparowanych kolumn do analizy mieszanin leków beta-adrenolitycznych z zastosowaniem programowania metod HPLC oraz inteligentnej analizy dekonwolucji pików (i-PDeA II) **[D6]**.

Dwie prace przeglądowe **[D1, D2]** oraz cztery prace badawcze **[D3-D6]** stanowią spójną całość niniejszej dysertacji i szczegółowo przedstawiono w nich realizację zamierzonych celów, otrzymane wyniki oraz wykorzystane metody badań.

2. Problem badawczy

2.1. Założenia zielonej chemii

Współczesne badania chemii coraz częściej dotyczą ekologicznego aspektu działań. Przełom w rozwoju nastąpił po wprowadzeniu przez Anastas'a oraz Werner'a 12 zasad zielonej chemii [1]. Opisują one, z punktu widzenia chemii, odpowiednie działania mające na celu zmniejszenie lub całkowite pozbycie się niekorzystnego wpływu działalności człowieka na środowisko. Oczywisty jest związek zielonej chemii z chemią analityczną, jednakże jest on dwojaki. Z jednej strony chemia analityczna umożliwia badanie negatywnych wpływów technologii, produkcji i produktów na środowisko oraz pomiar emitowanych zanieczyszczeń. Jednocześnie jest celem działań zielonej chemii, gdyż pojawiają się możliwości wdrażania kilku jej zasad w samej chemii analitycznej. Główne z nich to ograniczenie zużycia substancji niebezpiecznych (głównie rozpuszczalników organicznych), możliwość degradacji zużytych produktów oraz efektywne wykorzystanie energii [2,3].

Przełożenie tych zasad na praktykę znajduje również zastosowanie w chromatografii cieczowej, a pojęcie zielonej chromatografii jest już powszechnie stosowanym terminem. W tej technice działania skoncentrowane są na chromatograficznym rozdzielaniu składników mieszaniny, dlatego w dalszej części skupiono się na samych analizach chromatograficznych. Zestawiając syntezę chemiczną z analizami, ilość produkowanych szkodliwych odpadów może wydawać się nieznaczna. Przy stosowaniu standardowego przepływu 1 ml/min szacuje się produkcję jednego litra odpadów organicznych przypadających na dzienną pracę wysokosprawnego chromatografu cieczowego. Jednakże biorąc pod uwagę ilość aparatów wykorzystywanych w przemyśle oraz ich ciągłą pracę, wartości te kumulatywnie rosną. Dla przykładu w przemyśle farmaceutycznym firmy mogą posiadać ponad 1000 chromatografów. Coraz częściej wykorzystywana jest również ultra-wysokosprawna chromatografia cieczowa (UHPLC), która dzięki wykorzystaniu kolumn chromatograficznych o mniejszych średnicach, umożliwia przeprowadzanie analiz w krótszym czasie i przy mniejszych przepływach fazy ruchomej w porównaniu do wysokosprawnej chromatografii cieczowej (HPLC). Zmniejsza to ilość generowanych odpadów. Jednakże w farmacji nadal HPLC jest dominującą techniką [2,4].

Obecnie wdrażane są już rozwiązania, które skupiają się wokół trzech grup działań:

- ograniczenie wytwarzania odpadów w postaci rozpuszczalników organicznych – skrócenie czasu analiz,
- zmniejszenie wymiarów układu chromatograficznego – zmniejszenie średnic kapilar i kolumn, a w konsekwencji przepływu fazy ruchomej,
- zamiana rozpuszczalników organicznych na alternatywne – dodatki do fazy ruchomej, zmiany w aparaturze lub warunków prowadzenia analiz.

Spośród wyżej przedstawionych rozwiązań pierwsze zakłada zastosowanie wyższych ciśnień, temperatury, zastosowanie odpowiednich kolumn tak, aby maksymalnie skrócić czas analizy, a tym samym zredukować ilość tworzonych odpadów, nie tracąc na jakości wyników. Drugie - opiera się na takiej modyfikacji aparatury, aby nie było konieczności stosowania dużych ilości rozpuszczalników organicznych bez zmiany warunków analiz. Jest to możliwe dzięki np. zmniejszeniu wymiarów kolumny, redukcji wielkości ziarna stanowiącego wypełnienie czy zastosowanie miniaturyzacji HPLC do mikro chromatografii (μHPLC), gdzie wymiary kapilar są zredukowane, a prędkości przepływu fazy ruchomej zmniejszają się z mililitrów na mikrolitry na minutę. Rozwiązania te nadal nie eliminują tworzenia odpadów, a jedynie redukują ich ilość. Jedynym rozwiązaniem pozwalającym na całkowite pozbycie się szkodliwych odpadów jest zamiana obecnie używanych rozpuszczalników, takich jak acetonitryl czy metanol, na bardziej przyjazne środowisku. Do ich grona należą głównie woda, etanol i dwutlenek węgla w stanie nadkrytycznym [5,6].

Zastosowanie alternatywnych i jednocześnie „zielonych” rozpuszczalników wymaga odpowiednich zmian w analizie chemicznej. Jedną z możliwości jest zastosowanie podwyższonej temperatury, która powoduje, iż czysta woda może posiadać siłę elucyjną odpowiadającą jej mieszaninom z acetonitrylem lub metanolem. Literatura podaje, iż podwyższenie temperatury o 3,5°C odpowiada wzrostowi stężenia metanolu w fazie ruchomej o 1%, natomiast zwiększenie temperatury o 5-8°C odpowiada zwiększeniu stężenia acetonitrylu o 1% [7,8]. Jednakże, aby móc przeprowadzać analizy w temperaturze otoczenia, przy jednoczesnym zastosowaniu „zielonych” zamienników rozpuszczalników organicznych, konieczne jest wykorzystanie odpowiednio modyfikowanych faz stacjonarnych. Główną grupę stanowią fazy stacjonarne, których powierzchnia jest modyfikowana zarówno grupami polarnymi, jak i niepolarnymi. Taka modyfikacja zapewnia możliwość prowadzenia analiz chromatograficznych z wykorzystaniem fazy ruchomej, w skład której wchodzi tylko woda

nie powodując degradacji fazy stacjonarnej. Dodatkowo, przyłączone grupy funkcyjne odpowiadają za retencję i selektywność podczas analiz chromatograficznych **[D1]**.

Powyższe rozważania stały się powodem podjęcia działań i badań wykonanych w niniejszej pracy doktorskiej. Wykonano zatem szczegółowy przegląd literatury pod kątem wykorzystania czystej wody jako jedynego składnika fazy ruchomej w HPLC. Zbiór rozwiązań został zaprezentowany w pracy przeglądowej pt. *Pure water as a mobile phase in liquid chromatography techniques* (**D1**). Opisano w niej zastosowanie wody w stanie podkrytycznym, gdzie woda o wysokiej temperaturze utrzymywana jest pod wysokim ciśnieniem, aby nie doprowadzić do jej wrzenia. Takie techniki określane są w literaturze wieloma określeniami takimi jak: chromatografia z wodą w stanie podkrytycznym (SBWC - Subcritical Water Chromatography), chromatografia w bardzo gorącej wodzie (Chromatography in very hot water), chromatografia w przegrzanej wodzie (SHWC - Superheated Water Chromatography), wysokotemperaturowa chromatografia cieczowa (HTLC - High Temperature Liquid Chromatography) czy chromatografia cieczowa z gorącą wodą pod ciśnieniem (PHW-LC - Pressurized Hot Water Liquid Chromatography)*. Mnogość wykorzystywanych określeń i towarzyszących im skrótów wprowadza zamęt w systematyce technik chromatograficznych oraz negatywnie wpływa na możliwości poszukiwania nowych badań związanych z ich wykorzystaniem. Ze względu na ilość badań i publikacji związanych z szeroko pojętą chromatografią cieczową z wykorzystaniem podgrzanej wody w pracy **D1** szczegółowo opisano tę technikę. Skupiono się na podstawach teoretycznych, konstrukcji aparatury, możliwościach zastosowania SHWC, wykorzystywanych fazach stacjonarnych oraz rodzajach detekcji.

Spośród technik wykorzystujących czystą wodę stosunkowo młodą, ale powszechną jest wodna chromatografia cieczowa* (PALC – *per aqueous* liquid chromatography). Wykorzystuje ona żel krzemionkowy, na powierzchni którego występują wolne silanole, dzięki czemu możliwe jest rozdzielanie związków polarnych. Zastosowanie czystej wody jako fazy ruchomej powoduje jednak zmianę charakteru tej fazy stacjonarnej. [9–11]. Z tego względu w praktyce częściej stosowana jest faza ruchoma o dużej zawartości wody, a nie czysta woda [12–18]. Zastosowanie czystej wody jako jedynego eluentu w fazie ruchomej w temperaturze otoczenia wymagało wyłonienia nowej podkategorii nazwanej „tylko wodną" chromatografią* cieczową w odwróconym układzie faz (WRP-LC – water-only reversed phase liquid chromatography) [19]. Zaliczają się do niej również analizy z wykorzystaniem techniki PALC, ale tylko gdy wykorzystywana jest czysta woda. Już ponad 30 lat temu

*tłumaczenie autora pracy doktorskiej

Colwell i współpr. [20] wykorzystali czystą wodę jako fazę ruchomą w odwróconym układzie faz. Wstępnie otrzymano zadowalające wyniki dla faz stacjonarnych modyfikowanych krótkimi łańcuchami węglowymi (C8). Wykorzystywanie dłuższych łańcuchów powodowało zmiany konformacyjne hydrofobowych ligandów fazy stacjonarnej [21,22]. Jednakże w późniejszych badaniach okazało się, iż obecność wolnych silanoli ma znaczący wpływ na mechanizm rozdzielania. Podejmowano próby funkcjonalizacji powierzchni krzemionki krótszymi łańcuchami alkilowymi (C4), co zapewniało większą gęstość pokrycia i stabilność fazy stacjonarnej. Najnowsze doniesienia literaturowe potwierdzają, iż w warunkach czystej wody nie dochodzi do zmian konformacji ligandów hydrofobowych przyłączonych do powierzchni, a do blokowania całych porów, przez co ligandy te stają się niedostępne. Retencja jest wtedy spowodowana oddziaływaniem analitów z powierzchnią fazy stacjonarnej z wyłączeniem porów, co powoduje jej drastyczny spadek [23,24].

W konsekwencji, aby znacznie ograniczyć wpływ wolnych silanoli na retencję, powstała koncepcja faz stacjonarnych z wbudowanymi grupami polarnymi (ang. polar-embedded stationary phases) lub grupami polarnymi dołączonymi na etapie wtórnej silanizacji (ang. polar-endcapped stationary phases). Materiały takie są obecnie stosowane jako fazy stacjonarne dedykowane rozdzieleniom prowadzonym z wykorzystaniem faz ruchomych o dużej zawartości wody. Zwiększają one znacząco stabilność fazy stacjonarnej podczas procesu rozdzielenia. Przegląd literatury związanej z wdrażaniem zasad „zielonej chemii", a w tym zastosowanie czystej wody jako jedynego eluentu fazy ruchomej w analizach chromatograficznych, potwierdził konieczność charakterystyki, opisu mechanizmu retencji oraz podjęcia prób analiz chromatograficznych na fazach stacjonarnych z wbudowanymi grupami polarnymi. Zdecydowano się na syntezę faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi, ze względu na ich obiecujące właściwości opublikowane przez zespół z naszej Katedry [25–27], a także na brak ich pełnego opisu właściwości, charakterystyki powierzchni i możliwości chromatograficznych. Dodatkowo zdecydowano się na przygotowanie dwóch całkowicie nowych faz stacjonarnych **[D1]**.

2.2. Fazy stacjonarne z wbudowanymi grupami polarnymi

Fazy stacjonarne umożliwiające rozdzielanie substancji w czystej wodzie jako fazie ruchomej muszą charakteryzować się pewnymi cechami. Konieczna jest ich stabilność w silnie polarnych warunkach. W przypadku stosowania wody w podwyższonej temperaturze istotna jest również stabilność termiczna fazy stacjonarnej. Jednocześnie muszą zapewniać

selektywność, zadowalającą sprawność oraz retencję, przy czym ta ostatnia nie może być przesadnie duża. Obecnie na rynku dostępnych jest wiele kolumn bazujących na materiałach różnego typu [8,28]. Do najpopularniejszych należą: chemicznie związane fazy stacjonarne na nośniku krzemionkowym [10,15,18,29–33], fazy stacjonarne wykorzystujące tlenek cyrkonu lub tlenków innych metali jako nośnik [34–37], fazy polimerowe [7,38], węglowe [39,40] oraz hybrydowe - organiczno-nieorganiczne [41,42].

Komercyjnie używane wypełnienia kolumn chromatograficznych zawierają materiał o niemodyfikowanej lub modyfikowanej powierzchni zapewniający retencję oraz selektywność. Właściwości fazy stacjonarnej determinują możliwą do zastosowania fazę ruchomą, z tego względu różne wypełnienia kolumn chromatograficznych będą wykorzystywane w różnych trybach chromatografii cieczowej, np. w HILIC i RP LC. W pierwszym przypadku stosowane są polarne fazy stacjonarne do rozdzielania związków o średniej i dużej polarności w warunkach dużej zawartości rozpuszczalników organicznych sięgających nawet 98% zawartości acetonitrylu. W przypadku RP LC wypełnienia kolumn chromatograficznych są niepolarne, dlatego też stosowane są do rozdzieleń mieszanin związków hydrofobowych. Natomiast w składzie fazy ruchomej zawartość rozpuszczalników organicznych może być niska (np. 5%), niemniej w niektórym metodach może sięgać nawet 95%, przy jednoczesnym zapewnieniu rozdzielenia mieszanin związków chemicznych [43].

Fazy stacjonarne wykorzystywane w warunkach czystej wody zostały opisane w pracy przeglądowej pt. *Stationary phases for green liquid chromatography* (**D2**). Wiele z nich stosowanych jest w warunkach podwyższonej temperatury kolumny chromatograficznej. Wymaga to, oprócz wcześniej wspomnianej stabilności termicznej, również odpowiednio dostosowanej aparatury, która zapewni równomierne ogrzanie zarówno fazy ruchomej, jak i samej kolumny. Konieczne jest wykorzystanie specjalnych termostatów, systemów wstępnego podgrzania fazy ruchomej oraz jej chłodzenia przed detektorem. Ze względu na konieczność stosowania wysokich ciśnień zachodzi również potrzeba zastosowania odpowiednich kapilar, aby zapewnić utrzymanie wody w stanie ciekłym **[D2]**.

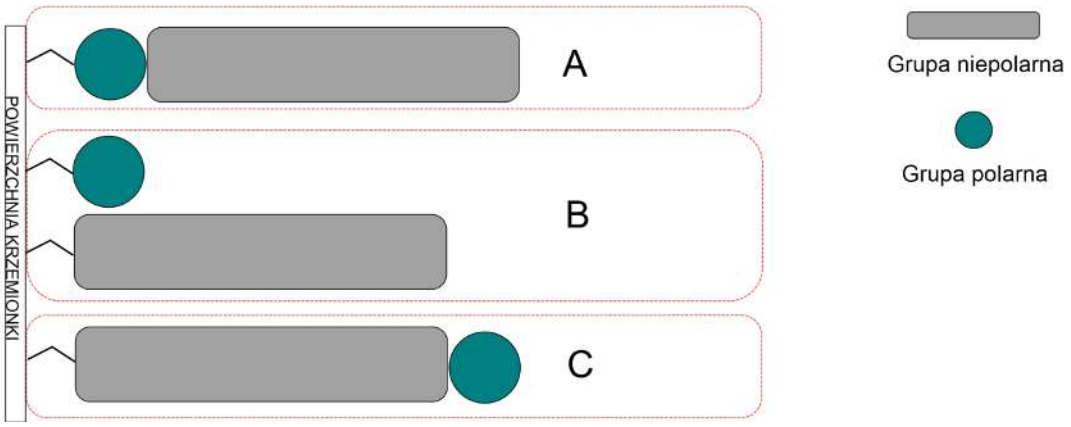
Istotne stało się poszukiwanie takiego rozwiązania, które zapewni korzystanie ze standardowej aparatury do chromatografii cieczowej, a jednocześnie umożliwi rozdzielanie analitów z zastosowaniem wody jako jedynego składnika fazy ruchomej. Pod koniec lat 80-tych poprzedniego wieku po raz pierwszy zaczęto syntezować fazy stacjonarne zawierające w swojej strukturze jednocześnie grupy polarne, jak i niepolarne. Celem była ich aplikacyjność w bardzo szerokim zakresie stężeń modyfikatora organicznego w fazie

ruchomej przy jednoczesnym zapewnieniu odpowiedniej sprawności i selektywności. Z założenia miały też być wykorzystywane do rozdzielania zarówno związków polarnych, jak i niepolarnych ze względu na dwojaką, pod względem polarności, budowę dołączonych do powierzchni ligandów [44–46].

Spośród faz stacjonarnych zawierających dołączone grupy polarne wyróżniamy trzy typy:

- fazy stacjonarne z wbudowanymi grupami polarnymi,
- fazy stacjonarne z grupami polarnymi dołączonymi na etapie wtórnej silanizacji,
- niepolarne fazy stacjonarne z terminalną grupą polarną (ang. polar-headed stationary phases) [47].

Schematy tych faz stacjonarnych zostały przedstawione na **Rysunku 1**. Każda z nich zawiera zarówno grupę polarną, jak i grupę niepolarną w postaci łańcucha alkilowego lub innej cząsteczki hydrofobowej. Do najbardziej popularnych grup polarnych stosowanych w tego typu wypełnieniach kolumn chromatograficznych należą grupy amidowe, karbaminianowe, eterowe, estrowe, fosfoestrowe oraz cząsteczki mocznika. Różnica w ich budowie między wymienionymi rodzajami faz stacjonarnych, wynika z metody syntezy. Fazy stacjonarne z wbudowanymi grupami polarnymi syntezuje się, wykorzystując żel krzemionkowy, do którego w pierwszym etapie przyłącza się krótki łącznik alkilowy z reaktywną grupą funkcyjną, w drugim etapie dołącza się grupę polarną, a następnie reaguje ona z niepolarnym ligandem. Fazy stacjonarne z terminalną grupą polarną przyłączoną do ligandu hydrofobowego syntezuje się w podobny sposób, jednakże odwrócona jest kolejność przyłączania kolejnych cząsteczek [47]. W przypadku faz z grupami polarnymi dołączonymi na etapie wtórnej silanizacji pierwszy etap przebiega jak w przypadku syntezy oktadecylowej fazy stacjonarnej ODS (ang. octadecylsilane). Drugim etapem jest wykorzystanie resztkowych, wolnych silanoli do przyłączenia grup polarnych [27,48,49].



Rysunek 1. Schematyczne przedstawienie struktur faz stacjonarnych posiadających zarówno grupy polarne, jak i niepolarne w swojej strukturze A – faza stacjonarna z wbudowaną grupą polarną, B – faza stacjonarna z grupą polarną dołączoną na etapie wtórnej silanizacji, C – niepolarna faza stacjonarna z grupą polarną przyłączoną na końcu części hydrofobowej.

Zaletą faz stacjonarnych posiadających oba rodzaje grup jest możliwość ich zastosowania do rozdzieleń mieszanin analitów w najbardziej „zielonych" warunkach w chromatografii cieczowej. Należą do nich między innymi przygotowane w niniejszej pracy fazy stacjonarne z wbudowanymi grupami fosfodiestrowymi. Czysta woda w temperaturze pokojowej zapewnia możliwość korzystania z klasycznych chromatografów cieczowych bez konieczności ich dostosowania do podwyższonych temperatur. Możliwość doboru struktury grupy polarnej oraz niepolarnej budujących fazę stacjonarną oraz sposobu modyfikacji powierzchni nośnika sprawia, iż ilość kombinacji jest praktycznie nieograniczona. Dzięki temu można uzyskiwać różną selektywność tego typu faz stacjonarnych. Wadą przy analizach wykorzystujących jednoskładnikową, wodną fazę ruchomą jest to, że praktycznie tylko struktura fazy stacjonarnej odpowiada za selektywność rozdzielenia, a wpływ składu fazy ruchomej jest wyeliminowany. Nie ma możliwości zastosowania analizy w warunkach elucji gradientowej. Jedyną możliwość optymalizacji daje zmiana temperatury, a w przypadku stabilności termicznej fazy stacjonarnej można posiłkować się gradientem temperatury [**D2**].

2.3. Synteza faz stacjonarnych

Oprócz większej stabilności w porównaniu do klasycznych alkilowych faz stacjonarnych wykorzystywanych w RP LC fazy stacjonarne z wbudowanymi grupami polarnymi w warunkach dużej zawartości wody dodatkowo pozwalają na otrzymanie bardziej symetrycznych pików na chromatogramie, szczególnie dla substancji o charakterze

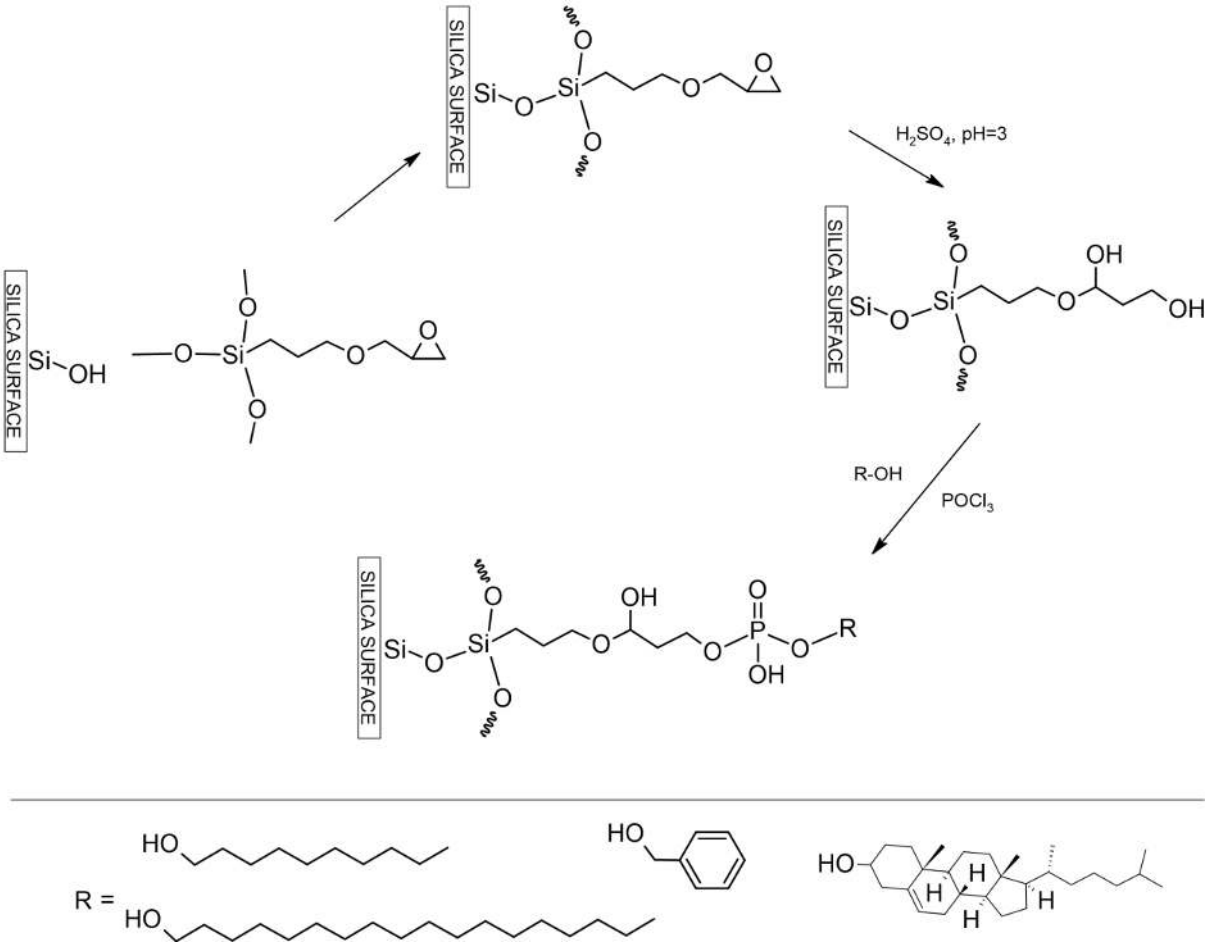
zasadowym. Wolne silanole nie wpływają na retencję, ze względu na ekranowanie ich przez warstwę wodną zaadsorbowaną wokół grup polarnych. Warstwa ta sprzyja również zmniejszeniu wytworzonej energii swobodnej dla transferu analitu. Ich specyficzna selektywność sprawia, iż stają się pożądane w badaniach metodą POPLC (ang. Phase Optimized Liquid Chromatography), w której przeprowadza się wstępnie dobór odpowiedniej fazy stacjonarnej do analitu, aby później optymalizować skład fazy ruchomej. Stanowią one zatem uzupełnienie pod względem selektywności dla alkilowych i polarnych faz stacjonarnych [21,50–55].

Istnieją dwie procedury syntezy faz stacjonarnych pozwalających na wbudowanie grupy polarnej. Historycznie pierwsza, w której otrzymano fazę amidową, polega na przyłączeniu do powierzchni nośnika aminopropylowego silanu, który następnie w reakcji z chlorkiem kwasowym lub izocyjanianem formuje grupę amidową lub mocznikową [56]. Ze względów sterycznych niemożliwa jest jednak modyfikacja wszystkich grup aminowych w drugim etapie syntezy fazy stacjonarnej, co prowadzi do utworzenia powierzchni zawierającej jednocześnie grupy aminowe oraz amidowe [57]. Jako rozwiązanie została zaproponowana druga metoda przeprowadzana jednoetapowo [53,58]. Do powierzchni krzemionki przyłączany jest odpowiedni silan z wewnętrznie wbudowaną grupą polarną. Wadą tej metody jest konieczność posiadania pożądanego silanu, zatem możliwości swobodnego doboru grupy polarnej i niepolarnej są znacząco ograniczone [50].

Stosunkowa nowość faz stacjonarnych z wbudowanymi grupami polarnymi oraz duża ilość możliwości doboru grup polarnych oraz niepolarnych spowodowała zainteresowanie takimi materiałami w analizach chromatograficznych. Początkowo synteza nowych materiałów w ramach niniejszej pracy doktorskiej miała na celu otrzymanie kolumn, które umożliwią efektywną pracę w czystej wodzie, co zostało z sukcesem osiągnięte. W trakcie prowadzenia badań okazało się, iż możliwości są o wiele szersze, zatem cel został zmodyfikowany na otrzymanie faz stacjonarnych umożliwiających rozdzielenie w różnych trybach chromatograficznych (RP LC i HILIC) oraz ich charakteryzacji z wykazaniem aplikacyjności w rozdzielaniu grup małocząsteczkowych związków polarnych i niepolarnych, a w tym alkaloidów purynowych, zasad azotowych, benzenu i wielopierścieniowych węglowodorów aromatycznych a także leków beta-adrenolitycznych **[D5,D6]**.

Procedurę syntezy czterech faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi przedstawiono w pracy pt. *Solvent influence on zeta potential of stationary phase—mobile phase interface* (**D3**). Początkowo powierzchnię krzemionki funkcjonalizuje się silanem

zawierającym krótki łańcuch alkilowy zakończony grupą epoksydową. W następnym etapie pierścień epoksydowy zostaje otwarty w reakcji hydrolizy katalizowanej kwasem. Otrzymana zostaje faza stacjonarna zawierająca dwie grupy hydroksylowe - diol. W kolejnych etapach do grup hydroksylowych przyłączana jest grupa polarna poprzez reakcję z trichlorkiem fosforylu, a następnie część hydrofobowa poprzez reakcję z odpowiednim alkoholem. **Rysunek 2** przedstawia schemat syntezy fazy stacjonarnej z wbudowaną grupą fosfodiestrową w oparciu o zastosowaną w badaniach procedurę. Ostatecznie otrzymano dwie fazy stacjonarne, których struktury zostały opublikowane wcześniej w literaturze naukowej: Diol-P-C10 oraz Diol-P-C18 (obie fazy zostały opracowane przez nasz zespół i przygotowane zgodnie z wcześniejszym opisem syntezy) [26,27,59] oraz dwie całkowicie nowe fazy: Diol-P-benzyl oraz Diol-P-chol.



Rysunek 2. Schemat syntezy faz stacjonarnych z wbudowaną grupą fosfodiestrową przygotowanych w ramach badań.

2.4. Charakterystyka faz stacjonarnych

2.4.1. Analiza elementarna i gęstość pokrycia

Synteza nowych faz stacjonarnych zawsze powiązana jest z charakterystyką powierzchni materiału. W tym celu przeprowadza się szereg analiz instrumentalnych oraz obliczeń pozwalających w jak najlepszy sposób jakościowo oraz ilościowo opisać powierzchnię sorbentu. Włączają się w to również analizy chromatograficzne pozwalające na określenie i wyznaczenie parametrów chromatograficznych kolumny, takich jak sprawność, rozdzielczość, selektywność czy mechanizm retencji [60].

Metody termoanalityczne, w tym analiza elementarna (CHN) oraz analiza termograwimetryczna (TGA) wykonywane są w celu charakterystyki powierzchni oraz określenia składu pierwiastkowego fazy stacjonarnej. Termograwimetria pozwala na określenie stabilności termicznej otrzymanych materiałów, jednakże nie daje odpowiedzi dotyczących struktury związanych ligandów na powierzchni krzemionki. Analiza elementarna pozwala na określenie zawartości procentowej węgla, wodoru oraz azotu w badanej próbce. Takie badania wykonane na poszczególnych etapach syntezy mogą potwierdzać przyłączanie się kolejnych cząsteczek do powierzchni fazy stacjonarnej. Służą one również w obliczeniu gęstości pokrycia przyłączonymi ligandami zgodnie z równaniem wyznaczonym przez Berendsen'a oraz de Galan'a [54,55,**D3**]:

$$\alpha = \frac{10^6 P_c}{1200 n_c - P_c(M - n_x)} \cdot \frac{1}{S_{BET}} \quad (1)$$

gdzie: α - gęstość pokrycia [µmol/cm^2]; n_c – liczba atomów węgla w dołączonej do powierzchni cząsteczce; n_x – liczba reaktywnych grup w silanie; M – masa molowa dołączonego ligandu [g/mol]; S_{BET} – powierzchnia właściwa wyznaczona z modelu BET [m^2/g]; P_c – zawartość procentowa węgla wyznaczona podczas analizy elementarnej [%].

Wyniki analizy elementarnej dla poszczególnych faz stacjonarnych przedstawiono w **Tabeli 1**. Analizy wykonano po pierwszym etapie syntezy, gdzie powierzchnia krzemionki została zmodyfikowana z otrzymaniem dioli oraz po drugim etapie, w którym dołączona została grupa fosfodiestrowa z 4 różnymi cząsteczkami hydrofobowymi: łańcuchem decylowym (C10), łańcuchem oktadecylowym (C18), grupą benzylową (benzyl) oraz cząsteczką cholesterolu (chol). Widoczny jest wzrost zawartości węgla po pierwszym etapie, co sugeruje przyłączenie się krótkiego łącznika węglowego do powierzchni. Po drugim

etapie również następuje wzrost, który jest różny w zależności od syntezowanej fazy stacjonarnej, co sugeruje pomyślne zajście dalszych etapów syntezy **[D3]**.

Tabela 1. Wyniki analizy elementarnej dla poszczególnych faz stacjonarnych oraz dla fazy diolowej.

PROCENTOWA ZAWARTOŚĆ PIERWIASTKA NA POWIERZCHNI ZMODYFIKOWANEJ FAZY STACJONANREJ	DIOL	DIOL-P-C10	DIOL-P-C18	DIOL-P-BENZYL	DIOL-P-CHOL
C [%]	1,438	3,438	4,177	2,865	9,308
N [%]	0,062	0,108	0,187	0,456	0,355
H [%]	1,132	1,234	1,029	1,319	2,105

Gęstość pokrycia sorbentu dołączonymi ligandami wpływa na dostępność do powierzchni krzemionki dla cząsteczek rozpuszczalnika oraz analitu. Im jest ona mniejsza, tym dostępność ta jest większa, a co za tym idzie, oddziaływania analitu z nieprzereagowanymi silanolami są bardziej prawdopodobne. Zatem wartości gęstości pokrycia mogą stanowić podstawę do wyjaśniania tendencji w zmianach retencji występujących dla różnych analitów przy opisie mechanizmu retencji w układzie HILIC. W RP LC rozdzielane są związki o niskiej polarności, zatem wpływ interakcji wolnych silanoli z analitami na retencję jest zaniedbywalny, chyba że anality zawierają grupy o charakterze zasad Lewisa, np. grupy aminowe. W przypadku chromatografii oddziaływań hydrofilowych zawartość wody w składzie fazy ruchomej jest niższa niż zawartość modyfikatora organicznego, a anality są polarne i dobrze rozpuszczalne w wodzie. Zatem oddziaływania analit-silanole mają istotny wpływ na retencję. Niewielka gęstość pokrycia w takim przypadku może niekorzystnie wpływać na występowanie tych oddziaływań. W konsekwencji skutkuje to wydłużeniem czasu retencji oraz ogonowaniem pików [56,57,**D6**].

2.4.2. Hydrofobowość

Ze względu na złożony charakter faz stacjonarnych z wbudowanymi grupami polarnymi pod względem ich polarności istotne jest wyznaczenie ich hydrofobowości. Standardowo stosowany jest test Galushko, który pozwala na wyznaczenie zarówno hydrofobowości, jak i aktywności wolnych silanoli. Test na hydrofobowość polega na wyznaczeniu średniej

arytmetycznej z wartości współczynników retencji dla toluenu i benzenu w określonych warunkach [65]:

$$H_G = \frac{k_{toluene} + k_{beznene}}{2} \quad (2)$$

gdzie H_G – hydrofobowość; $k_{toluene}$, $k_{benzene}$ – współczynniki retencji odpowiednio toluenu i benzenu.

Aktywność silanolowa jest testem pozwalającym na określenie udziału niezwiązanych grup silanolowych w mechanizmie retencji analitu. W tym celu wyznacza się współczynniki retencji aniliny i fenolu oraz oblicza ją zgodnie ze wzorem [65]:

$$SA_G = 1 + 3 \cdot \left[\frac{k_{aniline}}{k_{phenol}} - 1\right] \quad (3)$$

gdzie SA_G – aktywność silanoli, $k_{aniline}$, k_{phenol} – współczynniki retencji odpowiednio aniliny i fenolu.

Jednakże w przypadku związanych faz stacjonarnych, które posiadają w swojej strukturze grupy hydroksylowe, aktywność ta jest sumą udziałów resztkowych silanoli oraz np. dioli. Dodatkowo fazy stacjonarne wykazujące możliwości mechanizmu kationowymiennego, uniemożliwiają przeprowadzenie tego testu ze względu na oddziaływania elektrostatyczne z aniliną **[D5]**.

Wyznaczenie hydrofobowości oraz aktywności silanoli dla związanych faz stacjonarnych jest istotnym etapem charakterystyki otrzymanych materiałów. Pierwszy parametr pozwala na określenie w jakim stopniu modyfikacja powierzchni wpłynęła na niepolarny charakter materiału. Drugi - opisuje stopień wpływu aktywnych i swobodnych silanoli na retencję. Dzięki niemu można określić, jaka część grup hydroksylowych przy silanolach nie uległa modyfikacji cząsteczkami niepolarnymi, jak ma to miejsce w przypadku syntezy faz ODS. Ma to szczególne znaczenie przy charakterystyce wszystkich kolumn używanych w RP LC [65–67].

W przypadku kolumn wykorzystywanych w różnych trybach chromatografii cieczowej wyznaczenie tych parametrów pozwala na szerszy opis tendencji w retencji związków o różnej polarności. W pracy pt. *Phosphodiester stationary phases as universal chromatographic materials for separation in RP LC, HILIC, and pure aqueous mobile phase* (**D5**) wykonano test Galushko, w którym określono hydrofobowość przygotowanych materiałów. Aktywność silanoli nie mogła zostać wyznaczona z dwóch względów. Jednym

jest obecność dodatkowych grup hydroksylowych pochodzących od dioli i grupy fosfodiestrowej, których charakter jest inny niż resztkowych silanoli. Drugim powodem jest fakt, iż otrzymane fazy stacjonarne mają charakter słabych wymieniaczy jonowych, co uniemożliwia otrzymanie powtarzalnego i wiarygodnego wyniku badania retencji aniliny, która w warunkach pomiaru jest częściowo protonowana. W pracy pt. *Beta-blockers separation on phosphodiester stationary phases - the application of Intelligent Peak Deconvolution Analysis* (**D6**) wykazano fakt, iż właściwość słabego wymieniacza kationowego faz stacjonarnych ma rzeczywisty wpływ na retencję związków posiadających wiele grup funkcyjnych o zróżnicowanej polarności. Dla grupy fosfodiestrowej wartość pKa równa 1,45 ± 0,5 sprawia, iż w fazie ruchomej o pH równym 7,5 grupa ta jest zdysocjowana i jest miejscem wymiany kationów. Powoduje to występowanie mieszanego mechanizmu retencji dla większości stosowanych składów fazy ruchomej oraz uniemożliwia przeprowadzenie analizy beta-blokerów bez wykorzystania soli w składzie fazy ruchomej. Obecność oddziaływań jonowych wpływa również na poszerzenie pasma chromatograficznego i zmniejszenie rozdzielczości, co utrudnia analizę w warunkach izokratycznych, dlatego też najlepsze rezultaty otrzymano przy wykorzystaniu elucji gradientowej [**D6**].

2.4.3. Potencjał elektrokinetyczny – stabilność zawiesin faz stacjonarnych

Powierzchnia związanych faz stacjonarnych charakteryzuje się heterogenicznością. Początkowo czysta krzemionka posiada grupy silanolowe (wolne i bliźniacze) i siloksanowe [68]. W wyniku kilkuetapowej modyfikacji dołączane są kolejne cząsteczki. Zakładając, iż żaden z etapów nie zachodzi z wydajnością 100%, na powierzchni przygotowanych faz istnieją przyłączone cząsteczki zawierające grupy diolowe, ligandy z dołączoną tylko grupą fosforanową, a także kompletne ligandy zawierające grupę polarną i niepolarną **[D5]**.

Na powierzchni faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi znajdują się resztkowe silanole oraz dołączone grupy polarne, które mogą ulegać dysocjacji, solwatacji, a także asocjacji polarnych cząsteczek. Wolne silanole ulegają dysocjacji, tworząc ujemne ładunki na powierzchni. Również grupy hydroksylowe znajdujące się w polarnej grupie fosfodiestrowej mogą ulegać jonizacji z utworzeniem grup o ładunku ujemnym (w zależności od pH fazy ruchomej). W konsekwencji następuje przyciąganie ładunków dodatnich znajdujących się w rozpuszczalniku solwatującym fazę stacjonarną. Skutkiem jest utworzenie warstwy elektrycznej otaczającej każdą cząstkę fazy stacjonarnej

znajdującą się roztworze. Utworzona przestrzeń przy powierzchni fazy nosi nazwę podwójnej warstwy elektrycznej [69,70].

Naładowana powierzchnia krzemionki przyciąga i unieruchamia blisko swojej powierzchni jony o przeciwnym znaku. Warstwa ta nosi nazwę warstwy adsorpcyjnej (inaczej nazywaną warstwą Sterna). Powstaje zatem potencjał powierzchniowy (ϕ_O), który maleje wraz ze wzrostem odległości od powierzchni. Na granicy warstwy Sterna można wyróżnić potencjał Sterna (ϕ_S), który dalej maleje w miarę oddalania się od warstwy adsorpcyjnej. Drugą warstwą jest warstwa dyfuzyjna, gdzie cząstki w postaci rozmytej nadal oddziałują z powierzchnią. Zatem w tej przestrzeni znajduje się większe nagromadzenie molekuł o znaku przeciwnym do powierzchniowego. W warstwie dyfuzyjnej znajduje się płaszczyzna poślizgu, która graniczy z głębią fazy ruchomej, a granica ta określana jest mianem potencjału elektrokinetycznego zwanego potencjałem zeta (ζ). [69–73].

Potencjał zeta ma praktyczne zastosowanie przy opisie dwuwarstwy elektrycznej tworzącej się przy powierzchni fazy stacjonarnej. Ze względu na stały kontakt fazy ruchomej z powierzchnią fazy stacjonarnej określenie potencjału zeta będzie miało istotny wpływ przy zrozumieniu mechanizmów zachodzących na powierzchni w trakcie separacji chromatograficznej oraz wpływających na selektywność rozdzielenia. Nie tylko charakter powierzchni ma wpływ na utworzony potencjał, ale również rodzaj rozpuszczalnika, który solwatuje modyfikowaną krzemionkę. Rozpuszczalniki o różnej polarności, tendencji do polaryzacji oraz właściwościach fizykochemicznych będą w różny sposób wpływać na zjawiska powierzchniowe [74–77].

Najbardziej powszechnym sposobem wyznaczenia potencjału zeta jest badanie ruchliwości elektroforetycznej (μ), która następnie w oparciu o odpowiednie założenia teoretyczne jest przeliczana na potencjał zeta. Wśród równań pozwalających na wyznaczenie tego parametru znajduje się równanie Smoluchowskiego, Hückel'a oraz Henry'ego. Równanie Henry'ego prezentuje się następująco:

$$\mu = \frac{2}{3}\frac{\varepsilon_r \varepsilon_0 \zeta}{\eta} \cdot F(\kappa a) \tag{4}$$

gdzie μ – ruchliwość elektroforetyczna [μm·cm/Vs], η – lepkość rozpuszczalnika [Pa·s], ε_r – stała dielektryczna rozpuszczalnika, ε_0 – przenikalność elektryczna próżni [C^2/J·m], ζ – potencjał zeta [mV] oraz F(κa) – funkcja Henry'ego.

Równanie to uwzględnia każdą zależność między grubością podwójnej warstwy elektrycznej a promieniem cząstki. Jednakże w granicznych przypadkach może ono zostać uproszczone. Gdy grubość dwuwarstwy elektrycznej jest znacznie większa od promienia cząstki, wartość κa jest znacznie mniejsza od jedności (κ^{-1} - długość ekranowania Debye'a-Hückel'a, a – promień cząstki), a funkcja F(κa) przyjmuje wartość 1. Otrzymuje się wtedy równanie Hückel'a:

$$\mu = \frac{2}{3}\frac{\varepsilon_r \varepsilon_0 \zeta}{\eta} \tag{5}$$

Jednakże gdy sytuacja jest całkowicie odwrotna i wielkość cząstki znacznie przekracza grubość utworzonej podwójnej warstwy elektrycznej, wartość κa jest znacznie większa od 1, a funkcja Henry'ego równa jest 1,5. Pozwala to na zastosowanie matematycznego przybliżenia w postaci równania Smoluchowskiego:

$$\mu = \frac{\varepsilon_r \varepsilon_0 \zeta}{\eta} \tag{6}$$

Wpływ na grubość dwuwarstwy elektrycznej tworzącej się przy powierzchni cząstki krzemionki ma rodzaj rozpuszczalnika, a w szczególności obecne w nim jony. Większe stężenie jonów będzie sprzyjać tworzeniu się mniejszej warstwy, a niższe będzie powodować utworzenie grubej warstwy. Również siła jonowa będzie wpływać na grubość podwójnej warstwy elektrycznej. W trakcie badań przy pracy **D3** wykorzystywane były głównie rozpuszczalniki organiczne oraz krzemionka o wielkości ziarna 5 μm. Tak duże ziarna oraz słaba jonizacja rozpuszczalników uzasadnia wykorzystanie uproszczonego równania Smoluchowskiego [70,78–81].

Podczas badań potencjału zeta należy wziąć pod uwagę istotny fakt, iż jest to parametr globalny i odnosi się do całej cząstki oraz przestrzeni występujących pomiędzy cząstkami w ewentualnie utworzonym agregacie. Uznając, iż odległość od powierzchni krzemionki, w której potencjał spada do zera, może osiągać wartość 30 nm oraz fakt, iż pory w standardowo stosowanej krzemionce w HPLC mają średnicę około 10 nm, utworzona podwójna warstwa elektryczna nie tylko może zachodzić na siebie z przeciwległych ścian poru, ale również wypełniać całą jego objętość. Powierzchnia właściwa krzemionki głównie zlokalizowana jest w porach i to tam dochodzi do wielu modyfikacji powierzchniowych silanoli. Na podstawie opisanych zjawisk można wnioskować, że lokalnie potencjał zeta może być zróżnicowany, a jego wartość może być zaburzona i nie odzwierciedlać faktycznych oddziaływań przyłączonych ligandów z cząsteczkami rozpuszczalnika [75, **D3**].

Teoria DVLO (skrót pochodzący od jej twórców: Derjauguina, Landau'a, Verwey'a i Overbeeka) określa, iż za stabilność cząstek w roztworze odpowiadają siły odpychania i przyciągania. Pierwsze z nich polegają na sterycznym lub elektrostatycznym odpychaniu się podwójnej warstwy elektrycznej. W przypadku krzemionki o średnicy 5 mikronów modyfikowanej grupami polarnymi i niepolarnymi nie występuje odpychanie steryczne, zatem odpychanie elektrostatyczne będzie kluczowe. Przyciąganie następuje na skutek oddziaływań van der Waals'a. Jeżeli siły te pozostają w równowadze, to nie zachodzi agregacja, a cząstki w roztworze pozostają zawieszone. Jednakże jeżeli siły przyciągania przeważają, nastąpi agregacja cząstek. Zatem pomiar potencjału zeta pozwala oszacować stabilność zawiesiny. Krytyczna wartość potencjału zeta, po której przekroczeniu można mówić o trwałości, wynosi ±30 mV. Nie oznacza to braku agregacji, gdyż w zawiesinie mogą utworzyć się niewielkie agregaty przy jednoczesnym zachowaniu stabilności [72,83–85].

W pracy pt. *Solvent influence on zeta potential of stationary phase—mobile phase interface* (**D3)** wykonano pomiary zeta potencjału dla czterech przygotowanych faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi dla 10 rozpuszczalników i 6 mieszanin dwuskładnikowych. Potwierdzono, iż dla większości faz rozpuszczalnikiem zapewniającym najlepszą stabilność zawiesiny jest chloroform, a także dichlorometan. Oba dawały wysoce ujemne wartości zeta potencjału. Taka wartość może wynikać z adsorpcji cząsteczek na powierzchni krzemionki. Chloroform i dichlorometan są cząsteczkami o niezerowym momencie dipolowym wynoszącym odpowiednio 1,04 ± 0,02 C·m i 1,60 ± 0,03 C·m [86], oraz posiadającymi atomy chloru z trzema niesparowanymi parami elektronowymi, co w konsekwencji zapewnia wysoce ujemny ładunek powierzchni. Większość otrzymanych wyników potencjału zeta była ujemna, co potwierdza obecność zjonizowanych grup silanolowych, a także pozwala domniemać, iż zachodzi dysocjacja grupy hydroksylowej znajdującej się przy atomie fosforu w grupie fosfodiestrowej. Wpływ na otrzymane wyniki ma również obecność wysoce elektroujemnych atomów tlenów w strukturze każdej fazy [**D3**].

W celu porównania wpływu rodzaju sorbentu na otrzymywane wyniki przygotowano piątą fazę stacjonarną, która została zsyntezowana w ten sam sposób co Diol-P-C10, jednakże użyto krzemionki Luna zamiast Kromasil. Prezentowane w pracy **D3** wykresy radarowe oraz poszczególne wyniki zeta potencjału potwierdzają, iż nie zachodzi znaczący wpływ rodzaju krzemionki na wartości potencjału zeta fazy stacjonarnej. Większą rolę spełnia modyfikacja powierzchni podłoża krzemionkowego [**D3**].

Całość pracy **D3,** oprócz bezpośrednich wyników zeta potencjału oraz wniosków z nich płynących, została wykonana w celu określenia stabilności zawiesin. Było to konieczne w odniesieniu do kolejnych etapów badań, w których starano się optymalizować proces pakowania kolumn chromatograficznych otrzymanymi fazami stacjonarnymi. Jednym z kryteriów zapewniających efektywne pakowanie metodą zawiesinową jest dobór odpowiedniego rozpuszczalnika pchającego oraz zawiesinowego. Selekcję taką można wykonać w oparciu o badania stabilności i agregacji faz stacjonarnych w różnych rozpuszczalnikach, pomiary zeta potencjału, obserwacje mikroskopowe oraz zestawienie tych wyników z lepkością i gęstością zawiesiny. Szczegółowe badania, wyniki i wnioski opisano w pracy **[D4]**.

2.5. Optymalizacja pakowania faz stacjonarnych metodą zawiesinową

W przypadku przygotowania nowych faz stacjonarnych stanowiących wypełnienie kolumn chromatograficznych istotne jest zadbanie o zoptymalizowanie procesu pakowania. To właśnie wypełnienie pełni kluczową rolę podczas rozdzielenia chromatograficznego, gdyż to faza stacjonarna odpowiada za selektywną retencję związków wchodzących w skład mieszaniny, pozwalając na ich efektywne rozdzielenie. Jakość kolumny określana jest poprzez jej rozdzielczość, co zgodnie z równaniem Purnell'a zależy od selektywności oraz sprawności [87]. Podczas gdy selektywność jest parametrem zależącym od oddziaływań, faza stacjonarna-anality, sprawność jest parametrem fizycznym opisującym liczbę jednostkowych stanów równowagi pozwalających na retencję analitu. Składa się na to zarówno charakter chemiczny powierzchni fazy stacjonarnej, jak i fizyczne parametry opisujące złoże wewnątrz kolumny chromatograficznej. Własność ta uwzględnia zarówno rozmiar, rozrzut wielkości, sferyczność, porowatość ziaren fazy stacjonarnej, jak i ich ułożenie wewnątrz kolumny, a zatem gęstość i homogeniczność upakowania zarówno w przekroju podłużnym, jak i poprzecznym [88,89].

Wielkość fizyczna pozwalająca na liczbowe określenie sprawności w chromatografii cieczowej to wysokość równoważna półce teoretycznej HETP (ang. height equivalent to a theoretical plate). Wysokość ta powinna być jak najmniejsza, aby zapewnić jak największą ilość jednostkowych stanów równowagi między analitem a fazą stacjonarną wzdłuż całej kolumny. Powszechnie stosowane równanie van Deemter'a pozwalające na wyznaczenie HETP posiada następującą postać:

$$HETP = A + \frac{B}{u} + Cu \quad (7)$$

gdzie A – dyfuzja wirowa, B – dyfuzja wzdłużna, C – opór przenoszenia masy między fazą stacjonarną a ruchomą, u – prędkość liniowa fazy ruchomej [$cm \cdot s^{-1}$].

Tylko parametr A jest stały i niemożliwy do optymalizacji podczas analizy chromatograficznej. Zatem jakość zapakowania złoża w kolumnie chromatograficznej będzie determinować wartość parametru A, a w konsekwencji jego wpływ na HEPT. Dyfuzja wirowa to szereg zjawisk wpływających na rozmycie pasma spowodowanego różnymi prędkościami i drogami przepływu analitu przez złoże kolumny. Różnice te wynikają z nierównomiernego rozłożenia wielkości, różnic w rozmiarze i kształtach porów, a także samego kształtu kolumny. Jeżeli ziarna krzemionki zapewniają analitowi łatwą i swobodną drogę oraz niewiele interakcji z powierzchnią fazy stacjonarnej, wartość współczynnika A będzie wysoka, a co za tym idzie, uzyskana zostanie wysoka wartość HETP nawet dla optymalnej prędkości przepływu. Oprócz odpowiedniej gęstości upakowania złoża, która zapewnia jego stabilność i brak osadzania się w trakcie pracy, istotne jest też zapewnienie jego homogeniczności przekrojowej [90].

Czynniki wpływające na jakość wypełnienia kolumny fazą stacjonarną zależą od kilku kluczowych parametrów. Konieczne jest zapewnienie jak największej gęstości, a przestrzenie między cząstkami powinny być zbliżonej wielkości, aby zapewnić homogeniczność przepływu. Należy unikać większych zagęszczeń w kolumnie, a zatem istotna jest jednorodność w przekroju poprzecznym i podłużnym. Podczas pakowania metodą zawiesinową istotne staje się zatem zadbanie o odpowiednie oddziaływania między cząstkami – odpowiednia agregacja – oraz brak sedymentacji zawiesiny. Rozpuszczalnik zawiesinowy powinien zatem dobrze zwilżać powierzchnię materiału, aby zapewnić utworzenie agregatów oraz zapobiec pozostawaniu pęcherzyków gazu w porach krzemionki. Zatem fazy stacjonarne o charakterze hydrofobowym stosowane w RP LC pakowane są zazwyczaj przy użyciu niepolarnych rozpuszczalników, natomiast fazy przeznaczone do HILIC - na odwrót. W przypadku faz stacjonarnych z wbudowanymi grupami polarnymi dobór rozpuszczalnika jest trudniejszy i warto go przeprowadzić eksperymentalnie [83,**D4**].

Historyczne podejście do agregacji lub jej braku podczas stosowania metody zawiesinowej uległo zmianie. Początkowo twierdzono, iż jest to proces niepożądany, gdyż zwiększa oddziaływania międzycząsteczkowe, co prowadzi do szybszej sedymentacji. W latach 90-tych ubiegłego wieku Vissers i współpr. [92–94] przeprowadzili serię szerokich

badań pod kątem wpływu wielu parametrów na pakowanie kolumn. Dowiedli oni, iż występowanie agregacji jest kluczowe, gdyż ogranicza selektywne układanie się cząstek fazy stacjonarnej pod względem wielkości w przekroju złoża. Zauważyli, iż brak agregacji powoduje gromadzenie się większych cząstek w wewnętrznej części kolumny, natomiast mniejszych - przy ściankach. Występuje również zjawisko wypierania mniejszych cząstek przez większe. Oba te procesy negatywnie wpływają na homogeniczność przekrojową złoża, w konsekwencji powodują większą dyfuzję wirową, a ostatecznie mniejszą sprawność kolumny chromatograficznej. Potwierdzona została również praktyka kondycjonowania złoża bezpośrednio po pakowaniu z zastosowaniem faz ruchomych o składzie odpowiadającym prowadzonym w przyszłości analizom. Zapobiega to zachodzeniu zjawiska osiadania złoża po zamknięciu kolumny, co mogłoby skutkować wytworzeniem się pustej przestrzeni w kolumnie. Zatem stosując różnego rodzaju pomiary, należy zadbać o dobór rozpuszczalnika, który zapewni pojawienie się agregacji przy jednoczesnym zachowaniu stabilności zawiesiny. Druga właściwość najczęściej osiągana jest poprzez zastosowanie rozpuszczalnika o zrównoważonej gęstości, co zapobiegnie opadaniu fazy lub poprzez wykorzystanie wysoce lepkiego rozpuszczalnika. Do określania stabilności i agregacji lub ich braku najczęściej stosowane są pomiary zeta potencjału, obserwacje mikroskopowe oraz wyznaczenie prędkości sedymentacji [92–98].

W trakcie realizacji pracy pt. *Optimization of the packing process of microcolumns with the embedded phosphodiester stationary phases* (**D4**) wykonano badania mające na celu wybór odpowiedniego rozpuszczalnika zawiesinowego do pakowania 4 kolumn fazami stacjonarnymi z wbudowanymi grupami fosfodiestrowymi. Wykorzystano do tego otrzymane w pracy **D3** wyniki pomiaru zeta potencjału oraz dokonano obserwacji mikroskopowych. Zestawiając wyniki z wartościami lepkości rozpuszczalników oraz wykonując standardowe analizy chromatograficzne z wykorzystaniem kolumn kapilarnych o średnicy wewnętrznej 400 μm pozwalające na określenie sprawności, dokonano wyboru odpowiedniego rozpuszczalnika zawiesinowego. Proces ten był przeprowadzony dedukcyjnie i w wyniku stosowania rozpuszczalników o granicznych właściwościach lub charakteryzujących się przeciwstawnymi wynikami pomiarów. Zastosowano także eliminację tych, które nie zapewniały uzyskania wysokiej sprawności. Badania wykazały, iż pomiary potencjału zeta nie pozwalają na odpowiedni dobór rozpuszczalnika zawiesinowego. Obecność agregacji obserwowanej pod mikroskopem pozwalała na najlepszy wybór. W zestawieniu z wartościami lepkości izopropanol (IPA) okazał się najlepszym rozpuszczalnikiem

zawiesinowym dla wszystkich czterech faz stacjonarnych. Dla fazy Diol-P-C10 wykazano, iż przy zastosowaniu mieszaniny IPA/H_2O jako rozpuszczalnika nieagregującego, ale o zbliżonej lepkości do izopropanolu, obecność agregacji jest kluczowa dla osiągnięcia wysokiej sprawności zapakowanego złoża. Dla fazy Diol-P-chol zbadano wodę i IPA jako rozpuszczalniki agregujące, jednakże różniące się lepkością. Wyniki wykazały, iż wysoka lepkość rozpuszczalnika zawiesinowego bardzo korzystnie wpływa na zapakowanie kolumn, które w analizach chromatograficznych charakteryzują się wysoką sprawnością [**D4**].

Analizy chromatograficzne przeprowadzono, badając retencję naftalenu w układzie RP LC oraz tymidyny w układzie HILIC, a także wyznaczając sprawność kolumn. Są to pierwsze badania w trakcie realizacji pracy doktorskiej potwierdzające wstępne założenie o możliwości zastosowania otrzymanych faz w różnych trybach wysokosprawnej chromatografii cieczowej. Obie te substancje wykazywały retencję w badanych układach chromatograficznych. W obu przypadkach sprawność układu była wysoka, co potwierdza zredukowana wysokość równoważna półce teoretycznej *h* wynosząca dla fazy Diol-P-benzyl 2,85 w przypadku naftalenu oraz 2,33 dla tymidyny. Wartości *h* między 2, a 3 dla 15 cm kapilar i wielkości ziarna 5 mikronów pozwalają na osiągnięcie 10000 – 15000 półek teoretycznych, co w wysokosprawnej chromatografii cieczowej osiągane jest dla komercyjnych kolumn. Wszystkie fazy stacjonarne zostały zapakowane przy użyciu izopropanolu jako rozpuszczalnika zawiesinowego oraz metanolu jako rozpuszczalnika pchającego. Całość badań wykonanych w ramach pracy [**D4**] potwierdza, iż zaplanowany i zoptymalizowany proces pakowania kolumn chromatograficznych przynosi pożądane efekty w postaci wysokiej sprawności, co często jest trudne do osiągnięcia, w szczególności dla kolumn przygotowanych niekomercyjnie, a także dla nowych faz stacjonarnych. Optymalizacja doboru rozpuszczalnika zawiesinowego pozwoliła na otrzymanie kolumn charakteryzujących się dwukrotnie lepszą sprawnością, niż przy standardowych rozpuszczalnikach zawiesinowych używanych podczas pakowania hydrofobowych kolumn używanych w RP LC [87,**D4**].

2.6. Problem pomiaru objętości martwej

Dokładne wyznaczenie czasu martwego, a co za tym idzie objętości martwej kolumny, jest istotne dla prawidłowego wyznaczenia współczynnika retencji, a także opisu mechanizmu retencji w szczególności dla nowo otrzymanych faz stacjonarnych. Opis właściwości fizykochemicznych powierzchni adsorbentu poprzez wyznaczenie izoterm adsorpcji

wykonywany jest powszechnie z zastosowaniem dwóch metod: analizy czołowej (FA – ang. frontal analysis) oraz metody inwersyjnej (IM – ang. inverse method). Każda z nich bazuje na innym rodzaju eksperymentu. Pierwsza polega na pomiarze serii krzywych przebicia, dzięki czemu zestawienie z objętością martwą daje informacje o adsorpcji analitu, druga natomiast opiera się na obliczeniach wykonywanych na podstawie profilu pików otrzymanych w wyniku przeładowania kolumny. Obie wymagają precyzyjnego wyznaczenia objętości martwej, gdyż błąd na tym etapie skutkuje błędami w wyznaczaniu izoterm adsorpcji, a w konsekwencji interpretacji mechanizmu adsorpcji i retencji. W takich przypadkach izotermy zazwyczaj mają nieliniowy charakter, z tego względu dokładny pomiar objętości martwej jest bardzo istotny w badaniach chromatograficznych. Podczas gdy niedoszacowana wartość ma niewielki wpływ na błąd modelowania, przeszacowanie wartości objętości martwej powoduje duże błędy w wyznaczonych izotermach [99–101].

Objętość całego systemu chromatograficznego (V_{system}) jest opisana trzema czynnikami:

$$V_{system} = V_{extra} + V_S + V_M \tag{8}$$

gdzie V_{extra} – to objętość dodatkowa systemu znajdująca się poza kolumną, a mianowicie, objętość wszystkich kapilar, przestrzeni martwych na złączkach, w detektorze i innych, V_S – to objętość, jaką zajmuje faza stacjonarna w kolumnie, V_M – to objętość fazy ruchomej (objętość martwa) znajdującej się w kolumnie.

Istotne jest rozdzielenie V_M na dwie składowe, gdyż każdą jej część można określić, wychodząc z innych założeń teoretycznych. Zatem V_M opisana jest sumą:

$$V_M = V_i + V_p \tag{9}$$

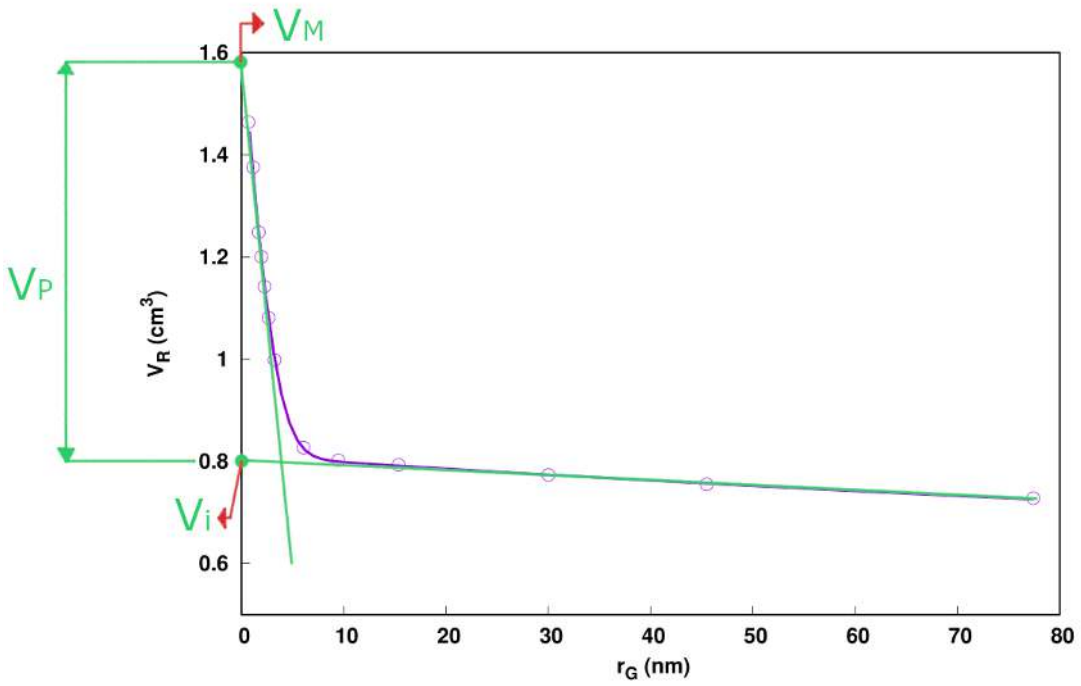
gdzie V_i – to objętość znajdująca się między ziarnami krzemionki zapakowanej w kolumnie, V_p – objętość znajdująca się w porach krzemionki.

W przypadku chromatografii cieczowej, gdzie stosuje się gotowy materiał w postaci zapakowanej kolumny, ważne jest, aby w sposób niedestrukcyjny wyznaczyć objętość martwą kolumny. Następnie z różnicy pomiędzy wewnętrzną objętością pustej kolumny a objętością martwą wyznaczana jest objętość fazy stacjonarnej. V_{extra} możliwa jest do wyznaczenia, stosując te same metody pomiaru objętości martwej, jednakże przy zastosowaniu złączki o zerowej objętości w zamian za kolumnę chromatograficzną [102].

Do metod nieinwazyjnych dla kolumny zalicza się piknometrię jako metodę niechromatograficzną oraz odwróconą chromatografię wykluczenia (ISEC – ang. inverse size exclusion chromatography), metodę zaburzeniową (MDM – ang. minor disturbance method), a także dozowanie substancji nieoddziałującej z powierzchnią fazy stacjonarnej (tzw. marker) lub dozowanie serii homologów [103,104]. Podczas realizacji niniejszej pracy wykorzystano metody ISEC, MDM oraz nastrzyki substancji nieulegających retencji.

Odwrócona chromatografia wykluczania pozwala w bardzo dokładny sposób określić zarówno V_i jak i V_p oraz przedstawić jakość zapakowanego złoża. Wyznaczany jest wykres zależności objętości elucji (V_R) od promienia kłębka statystycznego (r_G) cząsteczek polistyrenu o znanej masie cząsteczkowej. Mniejsze cząsteczki eluują dłużej z kolumny ze względu na możliwość dostania się do wnętrza mniejszych porów. Im promień cząsteczki polimeru jest większy, tym objętość elucji określona dla danego analitu jest mniejsza, gdyż ograniczona zostaje możliwość wejścia do mniejszych porów. Po przekroczeniu pewnej granicznej wielkości cząsteczki są zbyt duże, aby przedostawać się do wnętrza porów i objętość elucji zaczyna być tylko i wyłącznie objętością międzyziarnową (V_i).

Ekstrapolując prostoliniowe odcinki wykresu, możliwe jest dokładne wyznaczenie objętości martwej (V_M) oraz objętości międzyziarnowej (V_i), a z ich różnicy - objętości porów (V_p). Schemat wyznaczenia objętości martwej został przedstawiony na **Rysunku 3**. Warty podkreślenia jest fakt, iż model ten bierze pod uwagę takie czynniki jak: heterogeniczność powierzchni, kinetykę wejścia i wyjścia z porów oraz polidyspersyjność polimerów. Również gęstość pokrycia w przypadku modyfikowanych faz stacjonarnych może zostać określona, dzięki dokładnemu wyznaczeniu wielkości porów oraz informacji o ich wielkości dla niemodyfikowanej powierzchni. Przyłączone ligandy będą zajmować pewną część poru, co obserwowane jest poprzez redukcję objętości poru. Modyfikacja powierzchni zlokalizowana jest głównie w porach i nie będzie miała wpływu na wartość V_i. Jedyną wadą metody ISEC jest zastosowanie odmiennej fazy ruchomej niż ta, która później stosowana jest w analizach, gdyż pomiary przeprowadza się z zastosowaniem czystego tetrahydrofuranu (THF). Takie warunki są konieczne do zapewnienia elucji polistyrenu bez jego retencji. W fazach ruchomych stosowanych w układach RP LC oraz HILIC ulegałby retencji, co uniemożliwiałoby określenie objętości martwej kolumny chromatograficznej [102,105–108].



Rysunek 3. Schemat wyznaczania objętości martwej (V_M), objętości porów (V_p) oraz objętości międzyziarnowej (V_i) na podstawie wykresu zależności objętości elucji (V_R) od promienia kłębka statystycznego polimeru (r_G) dla kolumny Diol-P-C10.

Metoda zaburzeniowa pozwala na wyznaczenie termodynamicznej objętości martwej. Poprzez dozowanie niewielkich ilości jednego ze składników fazy ruchomej pojawia się zakłócenie w składzie eluentu, co widoczne jest w postaci piku na chromatogramie. Z różnicy pomiędzy termodynamiczną objętością martwą a objętością retencji wyznaczona jest nadmiarowa adsorpcja jednego ze składników eluentu. Pomiary przeprowadza się w stałej temperaturze dla całego zakresu składu rozpuszczalnik organiczny/woda, dzięki czemu otrzymana zostaje izoterma adsorpcji nadmiarowej rozpuszczalnika. Metodę tę stosuje się głównie do określania adsorpcji rozpuszczalników na powierzchni fazy stacjonarnej. Oprócz samego wyniku pomiaru objętości martwej metoda ta, poprzez wyznaczenie izoterm, pozwala na opis procesu adsorpcji zachodzącego pomiędzy fazą stacjonarną a ruchomą. W mieszaninie ACN/H_2O, ujemna część izotermy zawsze pojawi się przy wysokich stężeniach acetonitrylu, nawet dla faz stacjonarnych z wbudowanymi grupami polarnymi,

gdyż acetonitryl jest hydrofobową cząsteczką, która nie ulega adsorpcji na nieprzereagowanych wolnych silanolach.

Zaletą tej metody jest praca w układzie rozpuszczalników odpowiadającym rzeczywistym analizom, dzięki czemu otrzymane wyniki termodynamicznej objętości martwej wprost odnoszą się do badanych układów. Wadą MDM jest fakt, iż otrzymany wynik pomiaru objętości martwej jest uśrednionym pomiarem dla szerokiego zakresu składu fazy ruchomej. Przy fazach stacjonarnych z wbudowanymi grupami polarnymi, gdzie objętość retencji może się zmieniać wraz ze składem fazy ruchomej, uogólniona wartość może odbiegać od rzeczywistej przy późniejszych analizach w danych warunkach elucji izokratycznej [109–117].

Ostatnią stosowaną metodą w pracy były nastrzyki substancji, które nie ulegają retencji, a są powszechnie stosowane do określania objętości martwej. Metoda ta jest szybka i całkowicie nieinwazyjna dla fazy stacjonarnej. Jednakże różne związki ulegają różnym oddziaływaniom, które wpływają na wyznaczenie rzeczywistej objętości martwej. Skład fazy ruchomej oraz ciśnienie będzie zmieniać otrzymaną wartość V_M. W przypadku gdy oddziaływania analitu z fazą ruchomą są silne, natomiast z fazą stacjonarną znikome, badana substancja nie ulegnie retencji. Stosowanie takich związków jak tiomocznik czy uracyl do określenia czasu martwego możliwe jest dla kolumn wypełnionych fazami stacjonarnymi o bardzo małej polarności. Nieorganiczne sole, ulegając całkowitej dysocjacji, powinny dać najbardziej rzeczywiste wartości objętości martwej w przypadku faz hydrofobowych. Badania jednak wykazują, iż zastosowanie samej soli uniemożliwia wejście jonów do porów, co w konsekwencji daje wartość objętości martwej obarczoną dużym błędem. Istotne staje się zastosowanie dodatku soli do fazy ruchomej. Omija się wtedy występowania efektu Donnan'a, a badany marker może przedostać się do wnętrza porów poprzez maskowanie ładunków występujących przy wolnych silanolach poprzez jony pochodzące z soli oraz poprzez wyeliminowanie odpychania elektrostatycznego [118–122].

W trakcie realizacji niniejszej pracy wykonano trzy eksperymenty mające na celu wyznaczenie objętości martwej dla każdej z czterech otrzymanych kolumn wypełnionych fazami: Diol-P-C10, Diol-P-C18, Diol-P-benzyl oraz Diol-P-chol. Zestawienie otrzymanych wyników pozwoliło na określenie, która metoda daje najbliższe rzeczywistości wartości objętości martwej dla faz stacjonarnych o polarno-niepolarnym charakterze. Wyniki te są przedmiotem części badań wykonanych w ramach stażu naukowego na Uniwersytecie w Pecz (Węgry) i będą opublikowane w postaci artykułu naukowego.

2.6.1. Wyniki otrzymane z metody ISEC

Wyznaczenie objętości martwej oraz innych istotnych fizykochemicznie parametrów, przy użyciu stochastycznego modelu ISEC, zostało oparte na założeniach teoretycznych oraz równaniach opisanych w publikacji Bacskay i współpr. [107]. Obliczona została objętość międzyziarnowa, objętość porów, średnica porów ($r_{p,0}$) oraz odchylenie standardowe rozkładu wielkości porów (σ). Wyznaczono zależności V_R od r_G dla każdej z badanych kolumn. Do badań użyto serii wzorców polistyrenu o znanej masie cząsteczkowej, promieniu kłębka statystycznego oraz polidyspersyjności. W **Tabeli 2** zestawiono dane dla zastosowanych wzorców masy cząsteczkowej.

Tabela 2. Dane dla próbek standardów polistyrenu – M_W – masa cząsteczkowa [Da], M_W/M_n – polidyspersyjność oraz wyznaczona wartość promienia żyracji - r_G [nm].

Masa cząsteczkowa M_W [Da]	**Polidyspersyjność M_W/M_n**	**Promień kłębka statystycznego r_G [nm]**
580	1,12	0,678
1480	1,06	1,133
3070	1,04	1,692
3950	1,03	1,943
5120	1,03	2,240
6930	1,03	2,645
10110	1,02	3,254
31480	1,02	6,071
70950	1,03	9,484
170800	1,02	15,363
578500	1,02	30,001
1233000	1,05	45,457
3250000	1,04	77,421

Zastosowany do obliczeń model zakłada, iż cząstki krzemionki są sferyczne, a pory mają cylindryczny kształt. Wyniki obliczeń zestawiono w **Tabeli 3**. Dla wszystkich czterech kolumn wartości objętości międzyziarnowej (Vi) są bardzo zbliżone. Nie ma zatem zależności między właściwościami chemicznymi fazy stacjonarnej a objętością między ziarnami

krzemionki. Takie wyniki potwierdzają również fakt zastosowania tej samej procedury pakowania kolumn charakteryzującej się porównywalną jakością. Wyniki pomiarów objętości (V_p) oraz średnicy porów ($r_{p,0}$) maleją w kolejności: łańcuch decylowy, oktadecylowy, podstawnik benzylowy, cząsteczka cholesterolu. Spowodowane jest to wzrastającą objętością zajmowaną przez cząsteczki ligandów. Rozkład wielkości porów (σ) jest zróżnicowany, co jest oczywiste, biorąc pod uwagę różną gęstość pokrycia oraz zapotrzebowanie przestrzenne przyłączonych cząsteczek ligandów.

Tabela 3. Zestawienie wyników otrzymanych parametrów przy zastosowaniu stochastycznej metody ISEC. $V_{kolumny}$ – geometryczna objętość wewnętrzna kolumny, V_M – objętość martwa, V_i – objętość międzyziarnowa, V_p – objętość zlokalizowana w porach, $r_{p,0}$ – wielkość porów, σ – odchylenie standardowe rozkładu wielkości porów.

	Diol-P-C10	**Diol-P-C18**	**Diol-P-benzyl**	**Diol-P-chol**
$V_{kolumny}$ (cm^3)	2,076	2,076	2,076	2,076
V_M (cm^3)	1,651	1,623	1,610	1,609
V_i (cm^3)	0,811	0,802	0,814	0,814
V_p (cm^3)	0,840	0,822	0,797	0,795
$r_{p,0}$ (nm)	6,213	6,101	5,968	5,841
σ	0,260	0,274	0,308	0,281

2.6.2. Wyniki objętości martwej obliczone za pomocą metody MDM, nadmiarowe izotermy adsorpcji

W trakcie przepływu fazy ruchomej przez kolumnę ustala się równowaga adsorpcji pomiędzy fazą stacjonarną a płynącą mieszaniną rozpuszczalników. Równowagę tę można zaburzyć poprzez dozowanie niewielkiej ilości jednego ze składników fazy ruchomej. Objętość martwa może zostać wyznaczona poprzez scałkowanie objętości retencji pików zaburzenia w przedziale 0-100% zawartości modyfikatora organicznego, zgodnie ze wzorem:

$$V_M = \frac{1}{C_{max}} \int_0^{C_{max}} V_R(C)dc \qquad (10)$$

gdzie C – stężenie badanego rozpuszczalnika [mol/l], V_M – objętość martwa [ml], V_R – objętość retencji [ml].

Znając objętość martwą oraz objętość retencji zaburzonego piku, można obliczyć, w tych samych punktach wykresu, nadmiarową adsorpcję jednego ze składników fazy ruchomej zgodnie z równaniem:

$$\Gamma(C) = \frac{1}{S}\int_0^C (V_R(C) - V_M)dc \qquad (11)$$

gdzie Γ –adsorpcja nadmiarowa [mol/m^2], S – powierzchnia właściwa adsorbentu w kolumnie [m^2] [110].

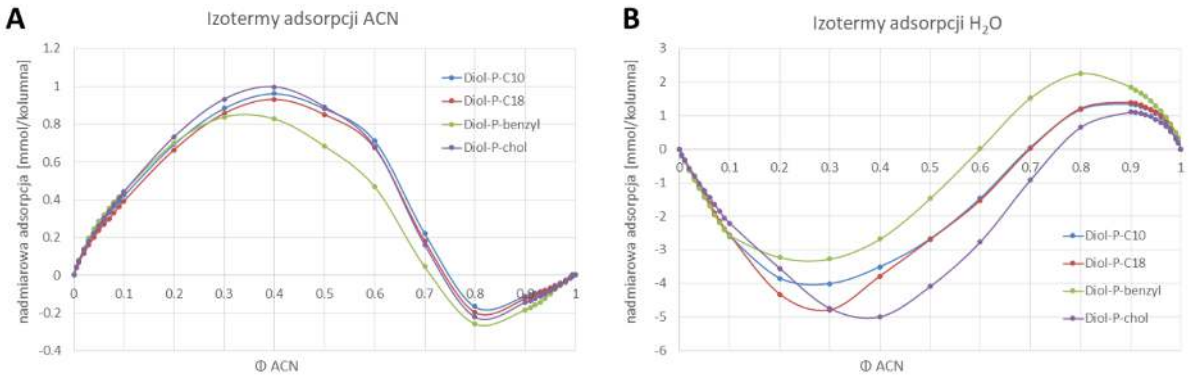
Wartości wyznaczonych objętości martwych są mniejsze w porównaniu do wyników otrzymanych metodą ISEC. Zmiany objętości martwej kolumny w zależności od rodzaju fazy stacjonarnej otrzymane obiema metodami są zgodne i maleją w kolejności: Diol-P-C10, Diol-P-C18, Diol-P-benzyl, Diol-P-chol. Wyniki dla metody MDM zestawiono w **Tabeli 4.**

Tabela 4. Wyniki objętości martwej (V_M) otrzymane za pomocą metody zaburzeniowej (MDM).

	Diol-P-C10	**Diol-P-C18**	**Diol-P-benzyl**	**Diol-P-chol**
V_M (cm^3)	1,589	1,584	1,555	1,573

Wyznaczenie nadmiarowych izoterm adsorpcji modyfikatora organicznego (acetonitryl) oraz wody dla każdej z badanych kolumn pozwala również na wyciągnięcie szeregu wniosków dotyczących charakteru powierzchni badanych materiałów. Wykresy przedstawiające wyniki zestawiono na **Rysunku 4**. Kształt izoterm sugeruje heterogeniczność powierzchni we wszystkich czterech przypadkach. Spowodowane jest to obecnością polarnej grupy fosfodiestrowej, grup hydroksylowych oraz hydrofobowych ligandów w strukturze każdej fazy stacjonarnej. Największa adsorpcja acetonitrylu wystąpiła dla fazy Diol-P-chol, co sugeruje jej największą hydrofobowość. Uszeregowanie faz stacjonarnych według rosnącej adsorpcji ACN jest zgodne z rosnącą hydrofobowością ligandów: podstawnik benzylowy, łańcuch decylowy, łańcuch oktadecylowy, cząsteczka cholesterolu. Wyniki te różnią się w zestawieniu z wynikami otrzymanymi podczas testu Galushko, gdyż charakter oddziaływań w obu przypadkach jest różny. Wyznaczenie wartości H_G opiera się na retencji toluenu i benzenu, które oddziałują głownie z niepolarnymi ligandami fazy stacjonarnej, natomiast pomiary nadmiarowej adsorpcji uwzględniają całość powierzchni adsorbentu, a zatem zarówno grupy niepolarne, polarne, jak i wolne silanole. Biorąc również pod uwagę części izoterm opisujące adsorpcję wody, wyniki układają się w odwrotnej kolejności

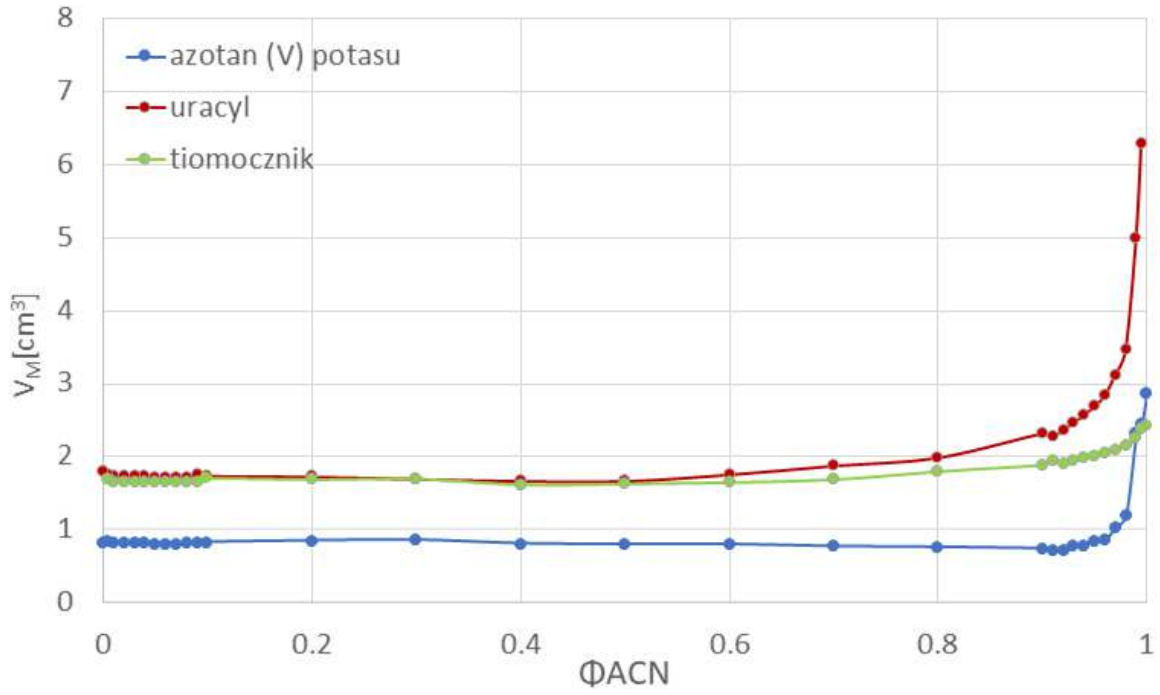
dla każdej z badanych faz stacjonarnych. Wynika to zarówno z oddziaływań z grupami hydroksylowymi ligandów, jak i nieprzereagowanymi grupami silanolowymi. Kolejność jest również zbieżna z gęstością pokrycia faz stacjonarnych, co potwierdza fakt większej dostępności grup znajdujących się bliżej powierzchni krzemionki.



Rysunek 4. Izotermy nadmiarowej adsorpcji acetonitrylu (A) oraz wody (B) dla faz stacjonarnych Diol-P-C10, Diol-P-C18, Diol-P-benzyl, Diol-P-chol.

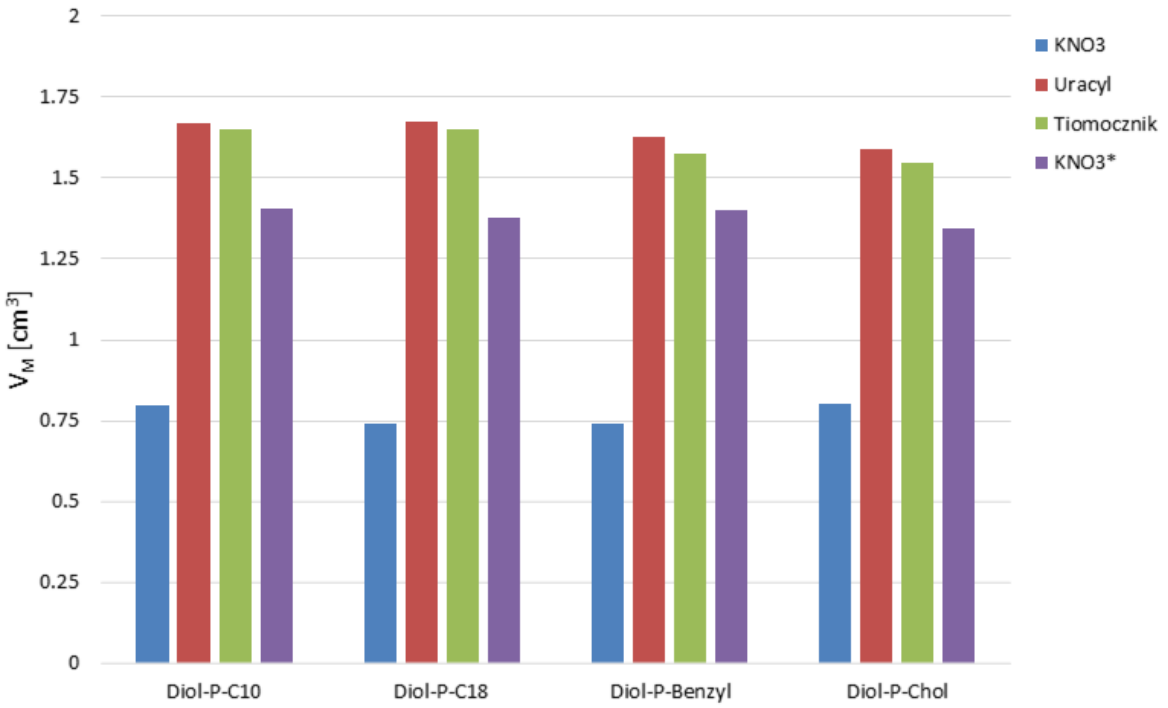
2.6.3. Pomiary za pomocą markerów objętości martwej

Jako standardowe wskaźniki stosowane w RP LC do określania czasu martwego, a co za tym idzie objętości martwej, wykorzystywane są substancje niezatrzymywane na powierzchni fazy stacjonarnej. Należą do nich: azotan(V) potasu (KNO_3), tiomocznik i uracyl. W ramach przeprowadzonych badań substancje te zostały wprowadzone do kolumn dla zakresu ułamka molowego ACN (Φ_{ACN}) w przedziale 0,0-1,0. Otrzymane wyniki potwierdzają wyniki uzyskane podczas pomiaru nadmiarowych izoterm adsorpcji. Przy dużej zawartości ACN w fazie ruchomej woda tworzy tzw. „poduszkę hydratacyjną" poprzez adsorpcję cząsteczek wody na wolnych silanolach, co zwiększa retencję polarnych markerów (mechanizm HILIC). Przy wartościach stężenia ACN między 50%, a 70% nie występuje nadmiarowa adsorpcja wody przy powierzchni fazy stacjonarnej, a zatem objętość martwa stabilizuje się i nie ulega zmianom. Wyniki pomiaru objętości elucji/retencji dla wszystkich trzech markerów zestawiono na **Rysunku 5**.



Rysunek 5. Pomiary zależności objętości martwej dla markerów: KNO_3, uracyl i tiomocznik dla fazy stacjonarnej Diol-P-C10.

Porównując otrzymane wyniki zaobserwowano zbliżone wartości dla uracylu oraz tiomocznika, jednakże zaniżone dla KNO_3. Wartości objętości martwej okazały się być zbliżone do wartości objętości międzyziarnowej V_i wyznaczonej metodą ISEC. Wykluczenie jonów z porów odbywa się w warunkach wysokiej zawartości wody w fazie ruchomej oraz w przypadku powierzchni zawierającej wolne silanole oraz charakter spolaryzowany. Jest to dobrze znany efekt Donnan'a. Temu efektowi można przeciwdziałać poprzez zastosowanie fazy ruchomej w postaci roztworu soli nieorganicznej (KNO_3) o stężeniu 0,025 M . Następuje ekranowanie wolnych silanoli oraz eliminowanie odpychających oddziaływań elektrostatycznych, przez co jon NO_3^- może wnikać do wnętrza porów, gdzie głównie znajdują się cząsteczki ligandów zmodyfikowanej powierzchni krzemionki [103]. Otrzymane w ten sposób wyniki wyznaczonych objętości martwych dla składu 50/50 ACN/H_2O przedstawiono na **Rysunku 6** i są one zbieżne z wynikami otrzymanymi z analiz tiomocznika i uracylu.



Rysunek 6. Zestawienie wyników pomiarów objętości martwej z użyciem markerów. Fazą ruchomą dla KNO_3* była mieszanina 50/50 ACN/H_2O + 0,025M KNO_3.

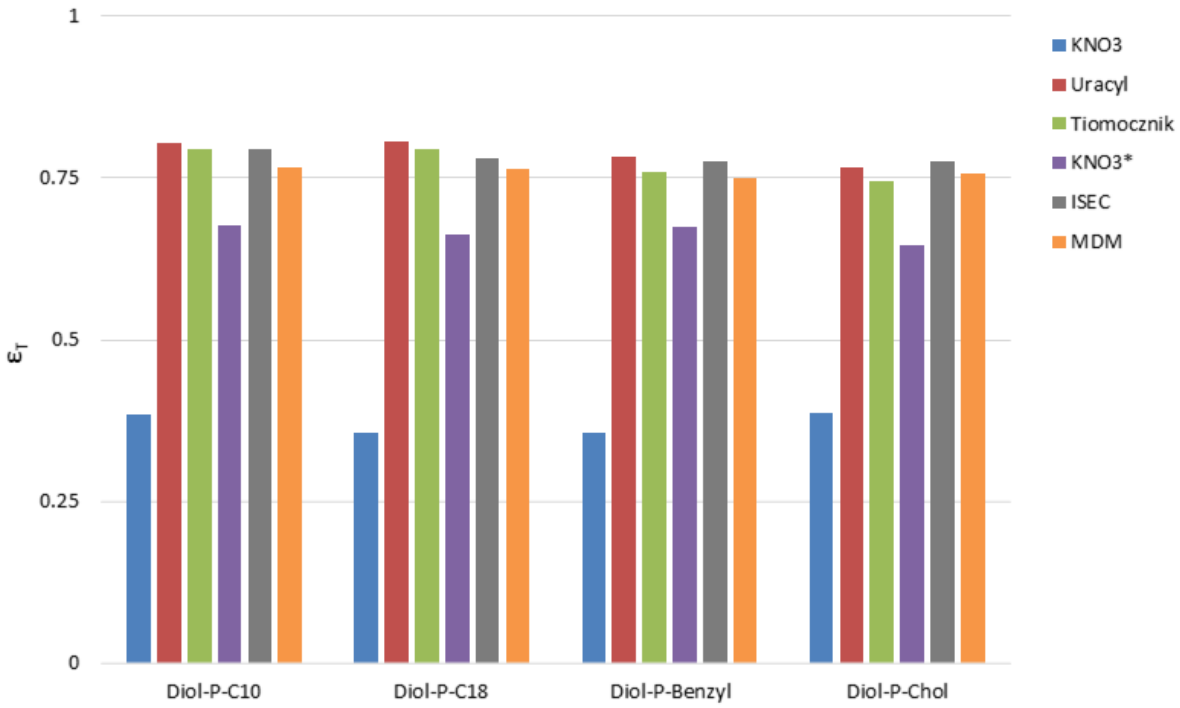
2.6.4. Porównanie zastosowanych metod pomiaru V_M – porowatość całkowita (ε_T)

Porowatość całkowita (ε_T) pozwala na dopełnienie fizykochemicznego opisu powierzchni badanych faz stacjonarnych. Wyznacza się ją w oparciu o wzór:

$$\varepsilon_T = \frac{V_M}{V_G} \tag{12}$$

gdzie ε_T – porowatość całkowita, V_G – objętość geometryczna kolumny [ml].

Dla zastosowanych metod oraz markerów wyniki obliczonej porowatości całkowitej na podstawie wyznaczonej objętości martwej zostały zestawione na **Rysunku 7**. Wykorzystanie azotanu(V) potasu bez dodatku soli do fazy ruchomej świadczy o niewnikaniu jonów do porów i odpowiada to wartości ε_T dla nieporowatej fazy stacjonarnej. Takie wyniki umożliwiają wyznaczenie porowatości zewnętrznej kolumny. Zastosowanie roztworu soli nieorganicznej poprawiło otrzymane wartości, lecz są one nadal niższe o około 15-20% w stosunku do wyników otrzymanych w z pozostałych analiz.



Rysunek 7. Zestawienie wyników obliczonej porowatości całkowitej (ε_T) na podstawie pomiarów objętości martwej różnymi metodami. Wyniki dla KNO_3* zostały wykonane w fazie ruchomej o składzie 50/50 ACN/H_2O + 0,025M KNO_3.

Całość wykonanych badań świadczy o konieczności przemyślanego doboru metody wyznaczenia objętości martwej w szczególności dla nowych faz stacjonarnych o heterogenicznym charakterze powierzchni. Z sukcesem udało się przeprowadzić takie badania na materiałach przygotowanych w niniejszej pracy doktorskiej. Spośród zastosowanych metod ISEC uwzględnia wszystkie wolne przestrzenie znajdujące się w kolumnie chromatograficznej, co pozwala na najbardziej precyzyjne wyznaczenie V_M. Metoda MDM daje zbliżone wyniki i również może być rozpatrywana jako istotna przy wyznaczaniu objętości martwej. Daje ona także obraz charakteru powierzchni faz poprzez możliwość wyznaczenia izotermy nadmiarowej adsorpcji rozpuszczalników wchodzących z skład fazy ruchomej.

Fazy stacjonarne z wbudowanymi grupami fosfodiestrowymi oraz różnymi ligandami hydrofobowymi charakteryzują się nietypowymi właściwościami chromatograficznymi, co umożliwia zastosowanie ich zarówno w trybie RP LC, jak i HILIC. Kształty izoterm sugerują również dominację oddziaływań hydrofobowych (RP LC), jednakże występująca nadmiarowa adsorpcja wody przy składzie fazy ruchomej o wysokim stężeniu ACN sugeruje możliwość zastosowania tych faz stacjonarnych do analizy polarnych analitów w układzie

HILIC. W pracy **D5** wyznaczone parametry chromatograficzne zostały obliczone w oparciu o czas retencji mierzony z wykorzystaniem KNO_3 bez dodatku soli nieorganicznej do fazy ruchomej. W świetle przedstawionych wyników (uzyskanych później) wartość ta nie odzwierciedlała faktycznego czasu martwego. Jednakże biorąc pod uwagę opis wyników w pracy **D5** obejmującej możliwości separacyjne faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi, błąd ten nie miał negatywnego wpływu na wyciągane wnioski dotyczące możliwości rozdzielania związków polarnych oraz hydrofobowych, a także pracy z zastosowaniem czystej wody jako jedynego składnika fazy ruchomej.

2.7. Wstępne badania chromatograficzne faz stacjonarnych z wbudowaną grupą fosfodiestrową

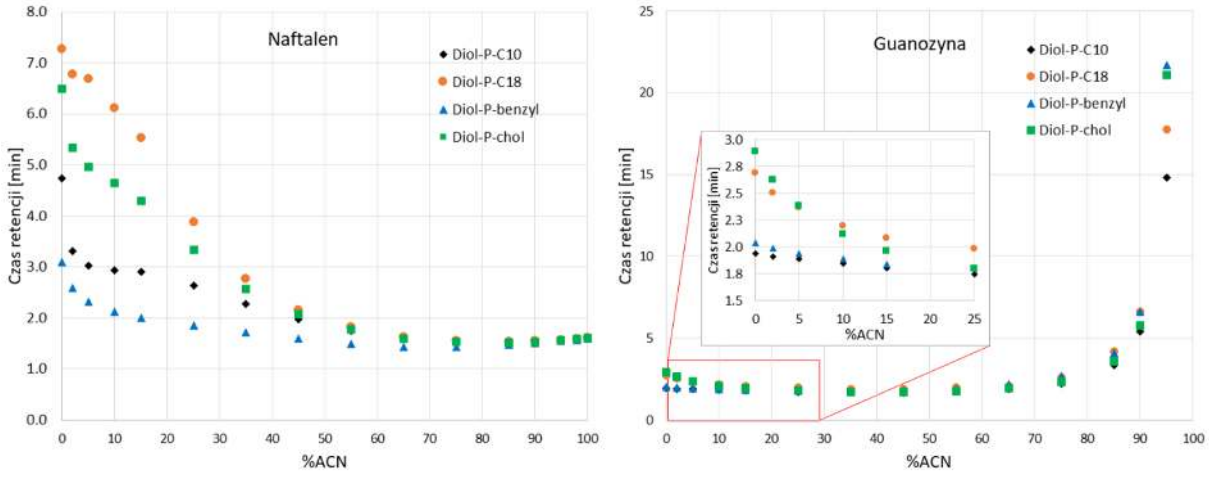
Podczas rozdzielenia chromatograficznego może zachodzić szereg mechanizmów prowadzących do retencji analitów. Należą do nich: mechanizm adsorpcyjny, podziałowy, jonowymienny, wykluczenia i powinowactwa. W większości przypadków kilka mechanizmów zachodzi jednocześnie, jednakże jeden z nich bierze główny udział. W przypadku RP LC główny udział ma mechanizm podziałowy. Podczas przepływu fazy ruchomej wraz z analitami przez kolumnę zachodzi retencja wynikająca z większego powinowactwa niepolarnych analitów do powierzchni hydrofobowej fazy stacjonarnej. Bardziej polarne anality mają większe powinowactwo do polarnej fazy ruchomej, a zatem są krócej zatrzymywane i eluują z kolumny chromatograficznej jako pierwsze. Mechanizm adsorpcyjny pełni w tym układzie mniejszą rolę. Zachodzi adsorpcja poszczególnych substancji wchodzących w skład analizowanej próbki na powierzchni fazy stacjonarnej, a następnie ich desorpcja. Mechanizm podziałowy będzie dominował w układzie RP LC tylko w przypadku, gdy gęstość pokrycia powierzchni fazy stacjonarnej ligandami węglowodorowymi będzie tak duża, że badany analit nie będzie miał możliwości przedostać się do powierzchni krzemionki, a jedynie będzie mógł oddziaływać z końcowymi częściami łańcuchów alkilowych. Kontrolując prędkość przepływu fazy ruchomej, jej siłę elucyjną oraz temperaturę, wpływa się na retencję. W przypadku HILIC faza stacjonarna jest polarna, natomiast faza ruchoma zawiera zazwyczaj niewielką zawartość wody (2-30%). Powoduje to powstanie warstwy zaadsorbowanej wody przy powierzchni krzemionki. W tej technice również zachodzi mechanizm podziałowy. Ustala się równowaga pomiędzy stężeniem analitu w cieczy unieruchomionej przy powierzchni fazy stacjonarnej, a stężeniem analitu w płynącej pod ciśnieniem fazą ruchomą. Konieczne podkreślenia jest iż zarówno w RP LC, jak i HILIC

występuje równolegle mechanizm podziałowy, adsorpcyjny oraz jonowymienny. Ten ostatni zachodzi pomiędzy zjonizowanymi grupami na powierzchni zmodyfikowanej krzemionki a analitami w postaci jonowej [113,123–126].

Zestawiając chromatografię w odwróconym układzie faz oraz chromatografię oddziaływań hydrofilowych, konieczne jest stosowanie faz stacjonarnych o całkowicie różnej polarności oraz faz ruchomych o zazwyczaj innym składzie. Fazy stacjonarne o mieszanym mechanizmie retencji, do których należą między innymi fazy stacjonarne z wbudowanymi grupami polarnymi, pozwalają na rozdzielanie mieszanin związków chemicznych o różnej polarności jednocześnie w obu układach chromatograficznych, otrzymując symetryczne piki rozdzielone do linii bazowej. Zazwyczaj związki hydrofobowe rozdzielane są przy niskiej zawartości modyfikatora organicznego, wykorzystując mechanizm podziałowy pomiędzy fazę ruchomą a fazę stacjonarną zachodzący w układzie RP LC, natomiast związki polarne przy wysokich zawartościach rozpuszczalników organicznych w układzie HILIC, wykorzystując mechanizm podziałowy między fazę ruchomą i zaadsorbowaną warstwę wodną. Fazy stacjonarne umożliwiające rozdzielanie tej samej grupy związków w obu układach chromatograficznych umożliwiają pracę bez konieczności zmiany składu eluentu i płukania aparatury. Możliwa jest zatem ciągła praca w warunkach wysokiej zawartości wody, rozdzielając mieszaniny związków polarnych oraz niepolarnych. Korzystne jest to z punktu ekonomicznego oraz ekologicznego, gdyż krótszy czas pracy, jak i mniejsza zawartość modyfikatora organicznego w fazie ruchomej generuje mniejsze ilości odpadów. [127–130].

W ramach pracy pt. *Phosphodiester stationary phases as universal chromatographic materials for separation in RP LC, HILIC, and pure aqueous mobile phase* (D5) przeprowadzono analizy chromatograficzne mające na celu wykazanie możliwości rozdzielania grup związków o różnej polarności w układzie RP LC oraz HILIC z wykorzystaniem zsyntezowanych faz stacjonarnych. W tym celu przygotowano mieszaniny związków polarnych: alkaloidów purynowych oraz zasad azotowych, a także mieszaninę związków niepolarnych – benzenu i wielopierścieniowych węglowodorów aromatycznych. Wyznaczenie zależności retencji od stężenia procentowego acetonitrylu w organiczno-wodnej fazie ruchomej potwierdziło zatrzymywanie związków polarnych na powierzchni faz stacjonarnych w obu układach chromatograficznych. Wykresy przestawiono na **Rysunku 8**. W przypadku RP LC naftalen jako związek niepolarny wykazywał retencję tylko przy niewielkiej zawartości acetonitrylu w wodzie. Istotne jest zaznaczenie, iż elucja

naftalenu była również możliwa przy zastosowaniu czystej wody. Zależności te zaobserwowano dla każdej z badanych kolumn.



Rysunek 8. Zależności czasu retencji od składu fazy ruchomej (ACN/H_2O) dla naftalenu oraz guanozyny.

Porównując czasy retencji dla poszczególnych kolumn oraz zakresu udziału procentowego ACN odpowiadającego zakresowi RP LC, dla naftalenu zgadza się ona z wyznaczoną hydrofobowością faz stacjonarnych, jednakże dla guanozyny jest zupełnie inna. Polarny analit powinien najsłabiej oddziaływać z powierzchnią najbardziej hydrofobowej fazy stacjonarnej i w konsekwencji jego retencja powinna być najniższa, natomiast faza stacjonarna o najmniejszej hydrofobowości spośród wszystkich testowanych wypełnień kolumn chromatograficznych powinna powodować najwyższą retencję. Ze względu na fakt, iż badania nie wykazały takiej tendencji, spodziewany jest bardziej złożony mechanizm retencji zarówno analitów polarnych, jak i niepolarnych. Z tego powodu w kolejnych etapach pracy zostały wykonane badania mające na celu określenie mechanizmu retencji poprzez wyznaczenie izoterm adsorpcji.

Wyniki analiz grup związków polarnych i niepolarnych przedstawione w pracy **D5**, wykazały możliwość rozdzielania z wysoką sprawnością związków hydrofilowych zarówno w układzie RP LC, jak i HILIC. Po raz pierwszy udało się również rozdzielić mieszaninę związków hydrofobowych, a mianowicie benzen, naftalen i fenantren przy użyciu czystej wody jako jedynego składnika fazy ruchomej. Badane fazy stacjonarne charakteryzują się zatem możliwością rozdzielania małocząsteczkowych związków o różnej polarności w dwóch różnych układach chromatograficznych. Mogą być używane do rozdzielenia związków polarnych i niepolarnych w czystej wodzie, jak i związków polarnych z układzie

HILIC. Znacząco wpływa to na aspekt ekonomiczny i ekologiczny stosowania chromatografii cieczowej, gdyż nie tylko można ograniczyć produkcję toksycznych rozpuszczalników, ale również ograniczyć liczbę wykorzystywanych kolumn, gdyż jeden materiał posiada szerokie możliwości zastosowania.

2.8. Mechanizm retencji – wyznaczenie izoterm adsorpcji

Jeżeli faza stacjonarna umożliwia rozdzielanie zarówno w układzie RP LC, jak i HILIC, rodzi się pytanie, jaki mechanizm odpowiada za retencję w tego typu materiałach. Bardziej wiarygodny opis mechanizmu retencji dają nieliniowe metody chromatograficzne w zestawieniu z analizami chromatograficznymi próbek w dużym rozcieńczeniu. Zatem wyznaczane są izotermy adsorpcji, aby określić i zrozumieć retencję na granicy fazy ruchomej i stacjonarnej. Istnieje wiele metod pozwalających na zbadanie jednoskładnikowych izoterm adsorpcji, jednakże w niniejszej pracy skupiono się na dwóch z nich: metodzie analizy czołowej oraz metodzie inwersyjnej [99,131]. W tym przypadku przez termin adsorpcja opisuje się wszystkie odziaływania sorpcyjne, bez rozróżnienia adsorpcji i absorpcji.

Metoda analizy czołowej po raz pierwszy była użyta w latach 50-tych ubiegłego wieku. Polega ona na użyciu dwóch roztworów do utworzenia eluentu. Jedna z nich to mieszanina wody z modyfikatorem organicznym stanowiąca fazę ruchomą, druga to nasycony roztwór badanego analitu w fazie ruchomej o znanym stężeniu. Zaczynając od minimalnego stężenia roztworu zawierającego analit, wprowadzany jest on przy stałej prędkości przepływu fazy ruchomej przez kolumnę. Następuje adsorpcja analitu i ustalenie się równowagi termodynamicznej. Na chromatogramie widoczne jest to w postaci wypłaszczenia (plateau) odpowiedzi detektora. Następnie stężenie roztworu z analitem jest zwiększane i ponownie następuje adsorpcja i ustalenie się równowagi. Taka krokowa analiza wykonywana jest do osiągnięcia maksymalnego stężenia roztworu zawierającego badaną substancję. Poprzez wyznaczenie pierwszej pochodnej z takiego schodkowego chromatogramu możliwe jest wyznaczenie krzywych przebicia oraz obliczenie stężeń zaadsorbowanego analitu:

$$q_{i+1} = q_i + \frac{(C_{i+1} - C_i)(V_{F,i+1} - V_0 - V_{ex})}{V_a} \tag{13}$$

gdzie q_i oraz q_{i+1} – stężenia zaadsorbowanego analitu [g/dm3], iteracja odpowiada stężeniom analitu płynącego przez kolumnę, określonych jako C_i oraz C_{i+1} [g/dm^3], $V_{F,i+1}$ – objętość retencji dla krzywej przebicia w i+1 kroku [dm^3], V_0 – objętość martwa

wyznaczona na podstawie nadmiarowych izoterm adsorpcji [dm^3], V_{ex} – objętość systemu bez kolumny chromatograficznej [dm^3], V_a – objętość adsorbentu w kolumnie [dm^3].

Metoda analizy czołowej jest najbardziej precyzyjna przy wyznaczaniu właściwości fizykochemicznych powierzchni adsorbatu. Jej wadą jest czasochłonność oraz duże zużycie rozpuszczalników organicznych i badanych substancji. Dodatkowo konieczne jest zastosowanie analitów o wysokiej czystości. Pojawienie się niewielkich zanieczyszczeń, które cechują się podobnymi właściwościami adsorpcyjnymi do badanej substancji, będzie wpływać na błędne wyniki pomiaru adsorpcji. Biorąc pod uwagę, iż jest to metoda krokowa, z każdym kolejny wzrostem stężenia roztworu z analitem, ilość zanieczyszczeń rośnie, zmieniając kształt krzywych przebicia [132–135].

Metoda inwersyjna polega na wyznaczeniu pików przy przeładowaniu kolumny analitem oraz dopasowaniu do profilu eksperymentalnego modelowego profilu teoretycznego. Poprzez iteracyjne dobieranie parametrów modelu tak, aby zminimalizować różnice między zmierzonymi oraz obliczonymi z modelu przeładowanymi profilami, wyznaczana jest izoterma adsorpcji dla danej substancji. Wymaga to zastosowania czterech kroków:

1) wstępne założenie parametrów izotermy adsorpcji,
2) obliczenie przeładowanego profilu poprzez całkowanie równania bilansu mas dla modelu równowagowo-dyspersyjnego,
3) obliczenie sumy kwadratów różnic między zbadanym oraz symulowanym profilem,
4) dostosowanie wstępnie założonych parametrów izotermy oraz minimalizacja sumy kwadratów różnic pomiędzy zarejestrowanym oraz modelowym chromatogramem.

Metoda ta jest alternatywą do wyznaczania izoterm adsorpcji. Jest ona szybsza i wymaga mniejszej ilości rozpuszczalników organicznych w porównaniu do FA. Badania wykonane przez Vajdę i współpr. [136], wykazały prawie identyczne parametry izoterm dla metody IM oraz FA przy badaniu proliny w układzie HILIC. Wadą tej techniki jest konieczność preselekcji modelu izotermy, co może być kłopotliwe dla nietypowych kształtów przeładowanych pików [132,136,137].

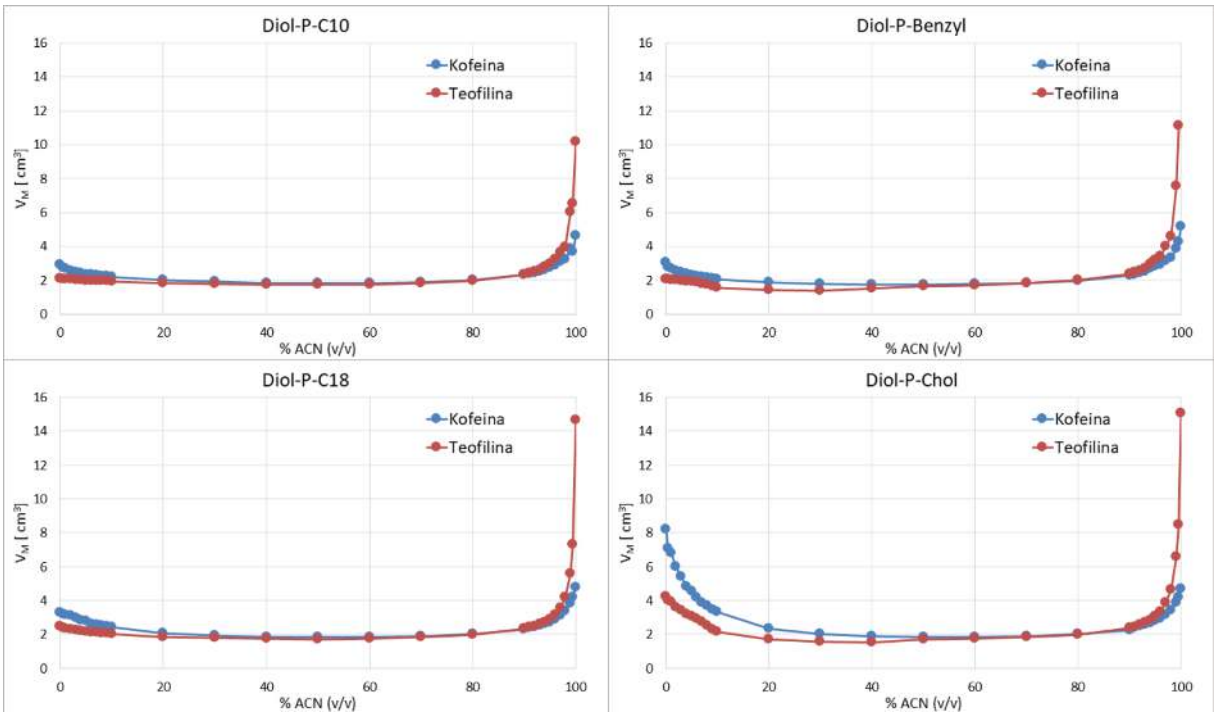
Aby dopełnić zestaw parametrów geometrycznych opisujących kolumny, wyznaczono również stosunek fazy ruchomej do stacjonarnej β zgodnie ze wzorem:

$$\beta = \frac{1-\varepsilon_T}{\varepsilon_T} \tag{14}$$

Tabela 5. Stosunek fazowy dla kolumn wypełnionych fazami stacjonarnymi z wbudowanymi grupami fosfodiestrowymi.

	Diol-P-C10	**Diol-P-C18**	**Diol-P-benzyl**	**Diol-P-chol**
β	0,307	0,311	0,336	0,320

W badaniach pozwalających na wyznaczenie izoterm adsorpcji wykorzystano fakt retencji kofeiny i teofiliny w granicznych wartościach stężeń acetonitrylu w wodzie: w warunkach czystej wody (RP LC) oraz przy 97% ACN w wodzie (HILIC). Na **Rysunku 9** można zaobserwować, iż kolejność retencji tych związków zmienia się w zależności od składu fazy ruchomej. W czystej wodzie jako pierwsza eluuje teofilina, natomiast przy wysokiej zawartości modyfikatora organicznego wykazuje się ona wyższą retencją w porównaniu do kofeiny. Teofilina, posiadając jedną grupę metylową mniej niż kofeina, posiada mniej hydrofobowy charakter, dlatego jej retencja w czystej wodzie jest mniejsza od kofeiny, natomiast przy składzie o dużym stężeniu acetonitrylu wykazuje większą retencję. Potwierdza to zachodzenie mechanizmów podziałowego i adsorpcyjnego charakterystycznych dla układów RP LC i HILIC. Jednocześnie wyniki te dowodzą, iż ta sama mieszanina może być rozdzielona przy użyciu jednej kolumny chromatograficznej zarówno w czystej wodzie, jak i przy wysokim stężeniu ACN. Zmiana układu chromatograficznego z RP LC na HILIC wiąże się z odwrotną kolejnością elucji analitów z kolumny chromatograficznej. Całkowita odwrotność kolejności nie musi zostać zachowana, szczególnie przy bardziej złożonych mieszaninach, ze względu na różny mechanizm retencji w obu stosowanych układach chromatograficznych.



Rysunek 9. Objętości retencji kofeiny i teofiliny dla faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi w czystej wodzie. Temperatura 30ºC, prędkość przepływu 1 ml/min, objętość dozowania 1µl.

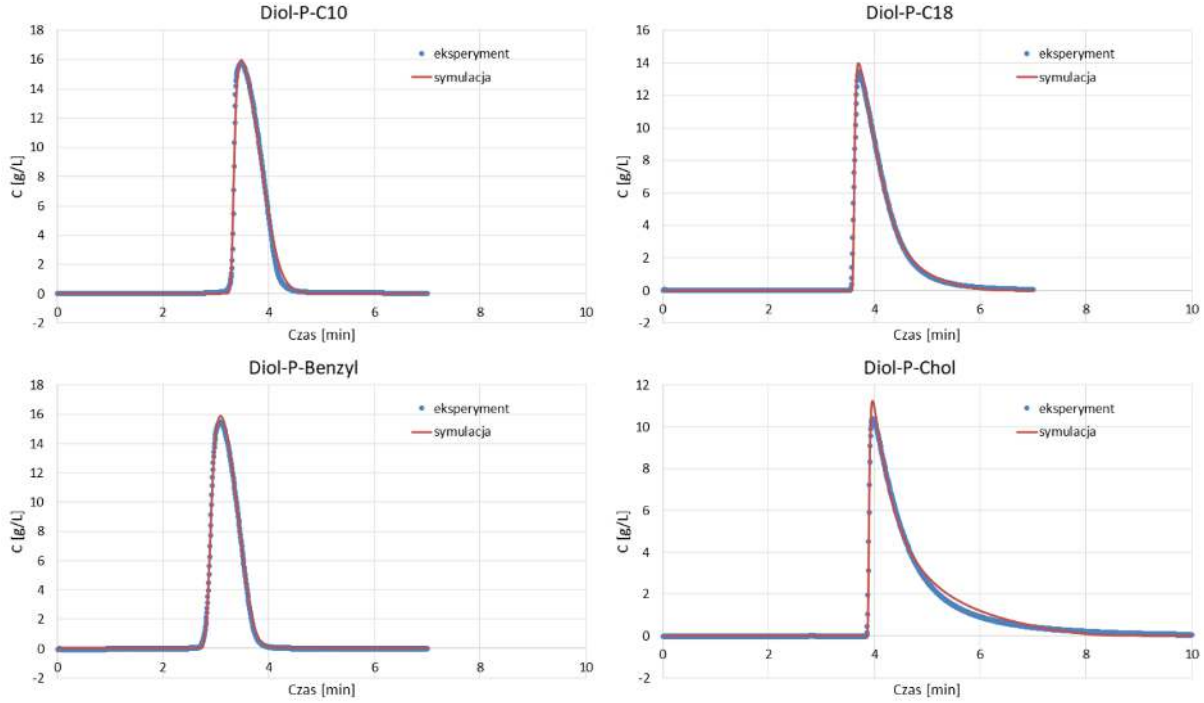
2.8.1. Adsorpcja w czystej wodzie (RP LC)

W strukturze ligandów dołączonych do powierzchni fazy stacjonarnej grupy alkilowe, pierścienie aromatyczne lub dołączona cząsteczka cholesterolu tworzą niepolarne centra adsorpcyjne, natomiast wolne silanole oraz grupy diolowe i fosforanowe tworzą polarne centra adsorpcyjne. Ta heterogeniczność powierzchni fazy stacjonarnej sugeruje zastosowanie modelu bi-Langmuir'a do opisu izotermy w tych warunkach. Model ten zakłada niejednorodność powierzchni, na której występują dwa rodzaje miejsc adsorpcyjnych. W omawianym przypadku są to centra hydrofilowe oraz hydrofobowe. Nie uwzględnia on oddziaływań cząsteczek adsorbatu między sobą. Model bi-Langmuir'a opisany jest wzorem:

$$q = \frac{q_{s1} b_1 C}{1+b_1 C} + \frac{q_{s2} b_2 C}{1+b_2 C} = \frac{a_1 C}{1+b_1 C} + \frac{a_2 C}{1+b_2 C} \qquad (15)$$

gdzie q oraz C to równowagowe stężenia analitu odpowiednio w fazie stacjonarnej i ruchomej, a_1, a_2 – stałe Henry'ego, $q_{S,1}$, $q_{S,2}$ – jednowarstwowe pojemności nasycenia w miejscach adsorpcyjnych 1 i 2, b_1, b_2 – stałe równowagi adsorpcji-desorpcji w miejscach adsorpcyjnych 1 i 2.

Wykorzystując metodę inwersyjną (IM), wyznaczono izotermy adsorpcji kofeiny i teofiliny poprzez przeładowanie kolumn tymi analitami. Otrzymane wyniki dopasowania modelu bi-Langmuir'a do danych doświadczalnych otrzymanych dla kofeiny przedstawiono na **Rysunku 10**, natomiast liczbowe wyniki dla obu badanych związków zestawiono w **Tabeli 6**. Dobrany model dobrze opisuje otrzymane wyniki. Ze względu na znaczące wartości parametrów a_1 oraz a_2, można wnioskować, iż zarówno hydrofobowe, jak i hydrofilowe oddziaływania kontrolują mechanizm retencji w tych warunkach.



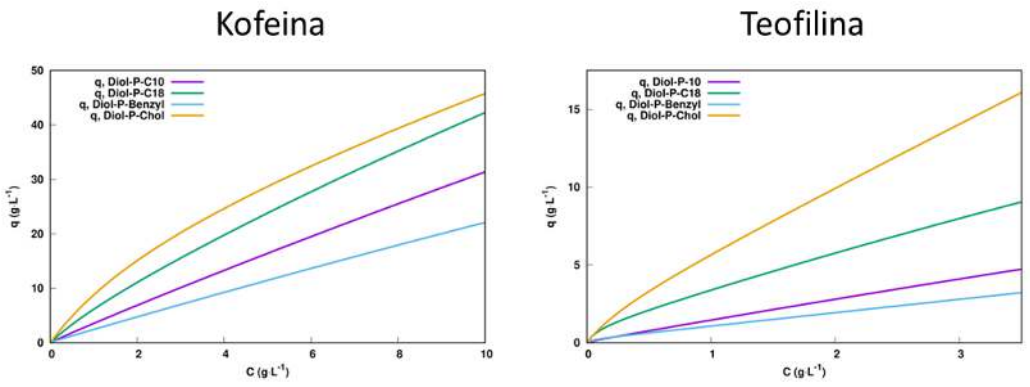
Rysunek 10. Zestawienie pomiędzy modelowymi oraz eksperymentalnymi wynikami dla przeładowanych pików kofeiny dla faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi w czystej wodzie. Temperatura 30ºC, prędkość przepływu 1 ml/min.

Tabela 6. Parametry izoterm adsorpcji dla modelu bi-Langmuir'a w czystej wodzie.

Kolumna	**Kofeina**			
	a_1	b_1 (L/g)	a_2	b_2 (L/g)
Diol-P-C10	3,31898	0,00733	0,5812	1,21323
Diol-P-C18	4,35409	0,01324	3,28889	0,76497
Diol-P-Benzyl	2,34754	0,00684	1,61521	13,11876
Diol-P-Chol	3,16706	0,00346	7,8369	0,38591

	Teofilina			
	a_1	b_1 (L/g)	a_2	b_2 (L/g)
Diol-P-C10	1,40605	0,01638	1,72081	22,68424
Diol-P-C18	2,54964	0,02971	10,6818	10,76853
Diol-P-Benzyl	0,86501	0,00194	5,37742	25,064
Diol-P-Chol	4,07163	0,10141	6,3065	2,98005

Wyznaczone izotermy adsorpcji kofeiny i teofiliny nieznacznie wykraczają poza liniowy zakres. Powodem jest ograniczona rozpuszczalność tych substancji w wodzie, przez co nie można osiągnąć pełnego nasycenia faz stacjonarnych. Zatem fizyczne znaczenie parametrów izoterm jest niepewne. Początkowy fragment izoterm związany jest z hydrofobowością faz stacjonarnych. Kąt nachylenia poszczególnych izoterm jest zgodny z ich hydrofobowością i potwierdza wyniki otrzymane przy badaniu nadmiarowych izoterm adsorpcji. Najbardziej hydrofobową fazą jest Diol-P-chol, natomiast najmniej - Diol-P-benzyl. Otrzymane izotermy przedstawiono na **Rysunku 11**.



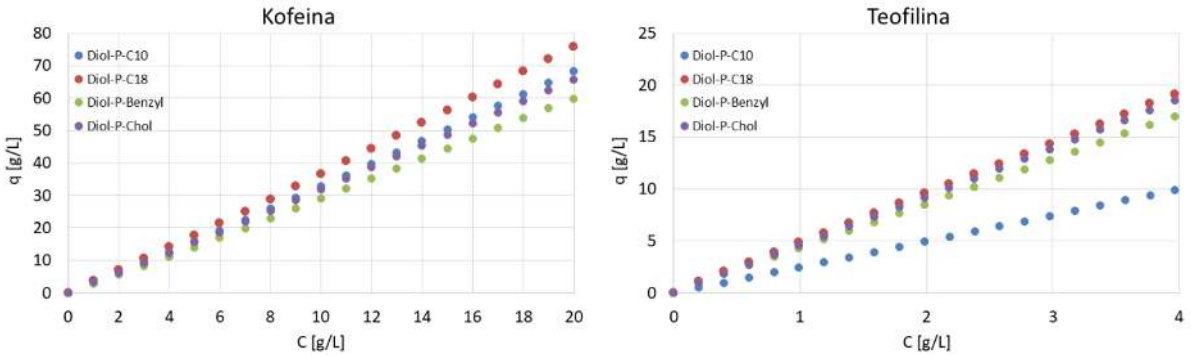
Rysunek 11. Zestawienie izoterm adsorpcji otrzymanych z modelu bi-Langmuir'a wykorzystując metodę inwersyjną (IM).

2.8.2. Adsorpcja w układzie HILIC

Wykorzystując kształty przeładowanych pików, możliwe jest dobranie odpowiedniego modelu do opisu danych eksperymentalnych. W przypadku gdy rozpuszczalność badanej substancji jest niewielka lub wykazuje się ona słabą retencją, otrzymany pik

przy przeładowaniu kolumny może nie być wystarczającą informacją dla doboru odpowiedniego modelu. Podczas badań wyznaczono kształty przeładowanych profili dla kofeiny i teofiliny przy stężeniu acetonitrylu wynoszącego 97% (HILIC). Ze względu, iż ich kształt nie sugerował konkretnego modelu, postanowiono zbadać adsorpcję, wykorzystując analizę czołową (FA).

Podobieństwo wizualne sprawia, iż wyniki otrzymane podczas analizy czołowej nie pozwalają na dostrzeżenie różnic w adsorpcji pomiędzy poszczególnymi kolumnami i analitami. Na podstawie krzywych przebicia zostały wyznaczone punkty izoterm adsorpcji. Zależność stężenia analitu w fazie ruchomej (C) od stężenia w fazie stacjonarnej (q) przyjmuje prawie liniową postać. Zatem ponownie wyznaczone zostały tylko początkowe części izoterm. W celu określenia typu adsorpcji wykreślono również wykresy Scatchard'a przedstawiające zależność q/C od q [99]. Wyniki przedstawiono na **Rysunku 12**. W każdym przypadku wykresy Scatchard'a przy niskich stężeniach analitu w fazie stacjonarnej maleją do wartości granicznej, a następnie krzywa wzrasta. Dla metody inwersyjnej oraz kształtów wykresów Scatchard'a zbliżonych do otrzymanych w niniejszych badaniach dobrym modelem opisującym adsorpcję jest model bi-Moreau [138].



Rysunek 12. Zestawienie izoterm adsorpcji kofeiny oraz teofiliny otrzymanych metodą analizy czołowej (FA). Faza ruchoma: 97/3 (ACN/H_2O).

W przypadku adsorpcji, podczas której zachodzi konkurencyjność i cząsteczki adsorbatu oddziałują na siebie, konieczne jest zastosowanie modelu, który uwzględnia tego typu interakcje. Model izotermy Moreau zakłada występowanie dwóch niezależnych miejsc adsorpcyjnych – hydrofobowego i hydrofilowego – oraz interakcję boczną zaadsorbowanych cząsteczek [138]. Równanie izotermy bi-Moreau przyjmuje następującą postać:

$$q = q_{s,1}\frac{b_1 C + I_1 b_1^2 C}{1 + 2b_1 C + I_1 b_1^2 C^2} + q_{s,2}\frac{b_2 C + I_2 b_2^2 C}{1 + 2b_2 C + I_2 b_2^2 C^2} \quad (16)$$

gdzie I_1, I_2 są parametrami opisującymi interakcje absorbat-absorbat w miejscach adsorpcyjnych 1 oraz 2. Stałe adsorpcji b_1 oraz b_2 związane są odpowiednio z energiami $\varepsilon_{a,1}$ oraz $\varepsilon_{a,1}$ i opisane wzorem:

$$b_i = b_0 e^{\varepsilon_{a,i}/RT} \tag{17}$$

gdzie b0 jest wstępnym współczynnikiem wykładniczym, który można wyprowadzić z podziałów molekularnych w fazie ruchomej i stacjonarnej, R – stała gazowa [$J \cdot mol^{-1} \cdot K^{-1}$], T – temperatura [K]. Parametr opisujący oddziaływania adsorbat-adsorbat (I) może zostać obliczony według wzoru:

$$I = exp\left(\frac{\varepsilon_{AA}}{RT}\right) \tag{18}$$

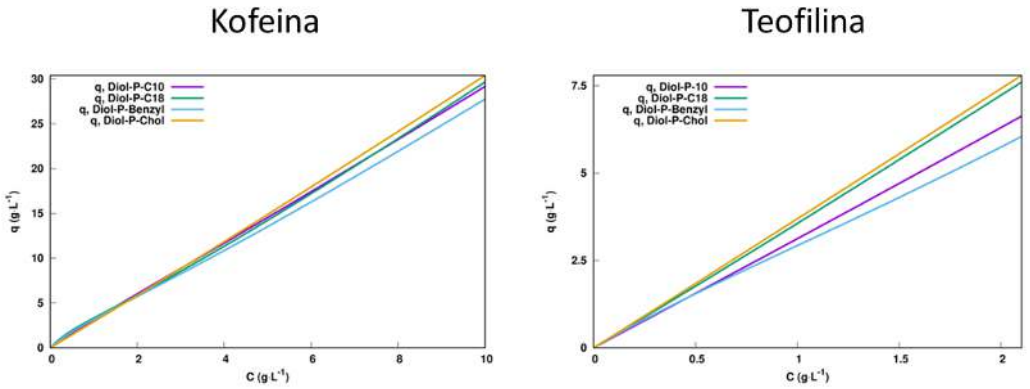
gdzie ε_{AA} jest energią oddziaływań pomiędzy sąsiadującymi cząsteczkami adsorbatu (w niniejszym przypadku kofeiny lub teofiliny).

Kluczowym etapem podczas stosowania metody inwersyjnej jest wprowadzenie parametrów początkowych. Wyznaczając izotermy adsorpcji w układzie HILIC dla kofeiny i teofiliny metodą IM, parametry początkowe zostały wprowadzone w oparciu o obliczenia izoterm wykonane metodą analizy czołowej. Otrzymano dobre dopasowanie modelu do eksperymentalnych chromatogramów uzyskanych podczas przeładowania kolumn. Ponownie izotermy obejmują tylko liniowy fragment, co uniemożliwia przypisanie fizycznego znaczenia obliczonym parametrom. Na **Rysunku 13** przedstawiono otrzymane izotermy adsorpcji. Wartości parametrów obliczone na podstawie modelu bi-Moreau zestawiono w **Tabeli 7**. Biorąc pod uwagę, iż parametr I_1 we wszystkich przypadkach jest większy od I_2, sugeruje to, iż boczne oddziaływania pomiędzy cząsteczkami analitu są bardziej prawdopodobne w hydrofobowych miejscach adsorpcyjnych. Nachylenie izoterm dla poszczególnych faz ułożone jest w kolejności skorelowanej z gęstością pokrycia, co wskazuje na kontrolę mechanizmu retencji przez wolne silanole oraz diole obecne w grupie fosfodiestrowej.

Tabela 7. Parametry izoterm adsorpcji dla modelu bi-Moreau w 97% ACN (HILIC).

Kolumna	**Kofeina**					
	a_1	b_1 (L/g)	I_1	a_2	b_2 (L/g)	I_2
Diol-P-C10	395,7631	0,0065	3,753	0,381	1,1998	0,0002
Diol-P-C18	273,5468	0,0092	4,7118	4,1288	0,6328	0,0007

Diol-P-Benzyl	206,663	0,0101	5,8565	3,338	0,771	0,0956
Diol-P-Chol	421,8935	0,0068	3,209	0,0383	18,4323	0,0004
	Teofilina					
	a_1	b_1 (L/g)	I_1	a_2	b_2 (L/g)	I_2
Diol-P-C10	90,2733	0,0299	3,877	1,2994	0,3263	0,7664
Diol-P-C18	57,65	0,045	5,0287	1,2953	0,6966	1,9387
Diol-P-Benzyl	48,7348	0,0245	21,5988	5,6095	0,3988	2,1696
Diol-P-Chol	101,9431	0,031	3,6941	1,0417	0,4389	2,1992



Rysunek 13. Zestawienie izoterm adsorpcji otrzymanych metodą inwersyjną (IM) przy zastosowaniu modelu bi-Moreau.

Podsumowując wyniki otrzymane podczas badania mechanizmu retencji na podstawie izoterm adsorpcji kofeiny i teofiliny, można stwierdzić, iż w zależności od składu fazy ruchomej występuje inny mechanizm adsorpcji oraz retencja jest kontrolowana przez inne odziaływania. W czystej wodzie (RP LC), przeładowane chromatogramy potwierdzają adsorpcję jednowarstwową dla obu badanych związków. Dobre dopasowanie modelu bi-Langmuir'a z wykorzystaniem metody inwersyjnej wskazuje na heterogeniczność powierzchni każdej z badanych kolumn. W warunkach odpowiadających układowi HILIC konieczne było posłużenie się metodą analizy czołowej w celu dobrania odpowiedniego modelu. Model izotermy bi-Moreau okazał się odpowiedni dla opisu danych, jednakże zbyt wąski zakres uzyskanej izotermy uniemożliwia jednoznaczne określenie fizycznego charakteru otrzymanych parametrów. Biorąc pod uwagę znaczące wartości parametru odpowiadającego za boczne oddziaływania analitu, prawdopodobnie model wielowarstwowy

(np. model Brunauera-Emmetta-Tellera - BET) dobrze opisałby izotermy otrzymane przy pełnym przeładowaniu kolumn. Zmiana mechanizmu retencji dla tego samego związku, przy użyciu tej samej fazy stacjonarnej, sugeruje, iż faza ruchoma kontroluje retencję w fazach stacjonarnych z wbudowanymi grupami polarnymi. Uzyskane wyniki po raz pierwszy dają pełen obraz mechanizmu retencji dla faz stacjonarnych zawierających grupę fosfodiestrową oraz ligandy hydrofobowe w swojej strukturze.

2.9. Aplikacyjność – rozdzielanie β-blokerów

Istotnym etapem podczas przygotowania nowych materiałów jako faz stacjonarnych do analiz chromatograficznych jest ich praktyczne wykorzystanie w przemyśle. Spośród substancji farmakologicznie istotnych beta-blokery stanowią grupę leków wykorzystywanych w kardiologii. Pojawiają się jednak doniesienia, iż długotrwałe przyjmowanie tego typu substancji może powodować stany depresyjne, a w konsekwencji ryzyko samobójstw. Ponadto beta-blokery stosowane są podczas transportu zwierząt w celu zmniejszenia zachorowalności, co w konsekwencji powoduje ich obecność w mięsie oraz produktach pochodzenia zwierzęcego. Konieczne jest zatem przeprowadzenie miarodajnych analiz zarówno dla próbek wzorcowych, jak i rzeczywistych, takich jak mocz, krew czy osocze. Metodyka powinna się skupiać na określaniu czystości preparatów, badaniach nad farmakokinetyką, badaniach metabolizmu czy testach antydopingowych, gdyż niektóre z nich są substancjami zakazanymi w rywalizacji sportowej [139–146].

Badania wykonane w trakcie realizacji pracy pt. *Beta-blockers separation on phosphodiester stationary phases - the application of Intelligent Peak Deconvolution Analysis* [**D6**] skupiały się na wykorzystaniu faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi do rozdzielania grupy beta-blokerów w układzie RP LC oraz HILIC. W ramach pracy przebadano 8 różnych beta-blokerów, wykorzystując cztery wcześniej omówione fazy stacjonarne. Grupa fosforowa wbudowana w strukturę ligandu posiada pKa 1,45±0,5, a zatem przy pH 7,5 jest zjonizowana, co umożliwia zachodzenie takiego mechanizmu. Wartości pKa dla badanych beta-blokerów wskazują, iż w tych warunkach wszystkie z nich występują w postaci jonowej (**D6**). Zatem podczas wszystkich analiz, oprócz oddziaływań hydrofobowych w RP LC oraz hydrofilowych w HILIC, miały miejsce oddziaływania jonowymienne. Zbadanie zależności retencji od składu fazy ruchomej wykazało, że analizowane związki wykazywały retencję zarówno przy wysokiej zawartości wody z dodatkiem soli, jak i wysokiej zawartości acetonitrylu. Zazwyczaj w przedziale

45-55% zawartości ACN badane w niniejszej pracy fazy stacjonarne wykazują minimum retencji, zarówno dla związków polarnych, jak i niepolarnych, a elucja następuje w okolicach czasu martwego. W wykonanych analizach współczynniki retencji były wysokie i znajdowały się w przedziale między 4 a 7 w zależności od fazy stacjonarnej. W szerszym zakresie, między 30% a 70% ACN, wszystkie badane związki wykazywały retencję, jednakże ich zbliżony czas elucji uniemożliwiał ich rozdzielenie. Jest to efekt wymiany kationowej z grupą fosfodiestrową, która zapewnia zatrzymanie analitów, jednakże nie zapewnia odpowiedniej selektywności. W zależności od przyłączonego liganda organicznego fazy stacjonarne charakteryzowały się innymi właściwościami retencyjnymi. Różnice wynikały z rozbieżności w gęstości pokrycia, a w efekcie - dostępności do niezwiązanych silanoli oraz z różnic w hydrofobowości badanych faz stacjonarnych. Ze względu na złożoność budowy strukturalnej beta-blokerów (występowanie zarówno grup polarnych, jak i niepolarnych) kolejność elucji w HILIC nie jest odwrotnością względem kolejności elucji w RP LC, tak jak wynika to z założeń teoretycznych. Wyznaczenie zależności retencji od hydrofobowości badanych beta-blokerów wykazało, iż w analizach w układzie RP LC wyższa hydrofilowość związku powoduje niższą retencję, natomiast w układzie HILIC retencja dla związków hydrofilowych jest największa. Zależności logk od logP w obu przypadkach są nieliniowe. Wykresy te potwierdzają fakt, iż oba rodzaje oddziaływań (hydrofobowe oraz hydrofilowe) mają wpływ na retencję każdego ze związków. W zależności od hydrofobowości związku dany analit oddziałuje silniej z centrami hydrofobowymi lub hydrofilowymi.

Wykonanie analiz chromatograficznych przy składzie fazy ruchomej bez dodatku soli, a zatem w pH w okolicach 6,8, skutkuje całkowitym brakiem elucji. Spowodowane jest to brakiem jonów w fazie ruchomej, które umożliwiałyby wymywanie analitów. Obecność w roztworze przeciwjonów pochodzących z soli umożliwia elucję zjonizowanych beta-blokerów z powierzchni fazy stacjonarnej zgodnie z kationowymiennym mechanizmem retencji. Takie badania potwierdzają występowanie mieszanego mechanizmu retencji w każdym z badanych składów fazy ruchomej oraz fakt, iż badane fazy stacjonarne są słabymi wymieniaczami kationowymi.

Rozdzielanie chromatograficzne mieszanin wymaga odpowiednich czynników, takich jak retencja, selektywność czy sprawność kolumny. Ostatni z nich może być optymalizowany podczas pakowania, jednakże to selektywność jest kluczowa, gdyż zapewnia ona możliwość rozdzielenia mieszaniny na pojedyncze anality. Wykorzystane kolumny nie wykazywały

się wysoką sprawnością podczas analizy beta-blokerów, a dodatkowo mechanizm wymiany kationowej pogłębiał efekt poszerzania pasma chromatograficznego, co doprowadzało do pogorszenia sprawności oraz rozdzielczości. Zestawienie selektywności mieszaniny 8 beta-blokerów wykazało, iż nie ma możliwości rozdzielenia ich w warunkach izokratycznych. Dodatkowo takie związki jak atenolol oraz acebutolol, charakteryzujące się wysoką hydrofilowością, posiadały wysoką retencję w układzie HILIC, co jest zrozumiałe ze względu na występowanie oddziaływań hydrofilowych, a także w układzie RP LC, co potwierdza, iż oprócz hydrofobowych oddziaływań również interakcje polarne oraz jonowymienne mają swój kluczowy udział w procesie retencji. Ponownie potwierdziło to mieszany mechanizm retencji.

Zatem w celu uzyskania rozdzielenia badanych beta-blokerów zastosowano elucję gradientową, która została zoptymalizowana przy użyciu programu ChromSword. Pozwoliło to na ustalenie profilu gradientu pozwalającego na rozdzielenie 6 beta-blokerów, natomiast dwa z nich nie zostały rozdzielone. Wykorzystując analizę dekonwolucji pików (i-PDeA II), cyfrowo udało rozdzielić się nierozdzielone anality. Jest to możliwe dzięki różnicom w widmach UV-Vis badanych związków. Zgodnie z literaturą, zastosowanie takiego algorytmu wiąże się z błędem mniejszym niż 1%. Można zatem stwierdzić, iż w bliski rzeczywistości sposób pozwala na określenie pików pojedynczych substancji z nierozdzielonego sygnału. Przedstawione w pracy **D6** wyniki są badaniami wstępnymi i konieczne jest ich rozwinięcie w kierunku szerszego opisu jonowymiennych właściwości faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi oraz optymalizacji procesu rozdzielania różnych grup związków [147].

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Pure water as a mobile phase in liquid chromatography techniques

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ABSTRACT

Liquid chromatography is a technique that is gaining increasing interest and application, for example in the pharmaceutical industry. At the same time as the increase in the number of analyses performed, the amount of organic waste produced while working with high-performance liquid chromatography (HPLC) apparatus is growing. Therefore, new methods and materials are being searched for to achieve the so-called "green" chromatography. In the following review we describe one of them, specifically the replacement of harmful organic solvents such as acetonitrile, methanol or isopropanol, with pure water used as the sole component of the mobile phase. In order to achieve a single component mobile phase, different methods or materials are used: the use of elevated temperature, the selection of new stationary phases, the utilization of changes in the properties of the stationary phase while using a highly polar eluent.

1. Introduction

Organic solvents and hazardous waste productions is a worldwide problem that has influence not only human health but is also dangerous for our environment. Facing this problem, it is obvious that chemical industry including all laboratories, need to introduce new technologies and innovations that allow to reduce the amount of waste production. That awareness brings us to development of "green" chemistry such as high-performance liquid chromatography (HPLC) using nontoxic mobile phases, which has received increased attention. The idea of elimination of huge amount of organic and hazardous waste produced everyday with environmentally friendly water is beneficial both ecologically and economically [1].

Quick, easy and environmentally friendly methods of HPLC separation are sought for. Due to the harmful effects of organic solvents, affecting not only humans, but also the environment, "green" technologies are being developed. Separation techniques, including chromatographic techniques are based on the consumption of large amounts of organic solvents. Thus, interest and demand for research towards the separation of mixtures under environmentally friendly conditions are increasing. There are many ways to make liquid chromatography "green". These include: decreasing the dimensions of the HPLC column to use smaller flows that reduce the amount of organic waste generated [2]; using mobile phase additives such as cyclodextrins [3] or surfactants; stationary phases with shorter carbon chains [4]; core-shell columns; operating at elevated temperatures [5]; replacing organic solvents with other, more "green" solvents such as water, ethanol or supercritical CO_2•. Among the above-mentioned, separations performed with use pure water as the only eluent in HPLC are the main issue of this paper. For such analyses to be carried out, it is necessary to apply appropriate separation conditions and special columns allowing for selective separation of the substance with such a highly polar solvent [6].

Solvents that are commonly used for reversed phase liquid chromatography, such as acetonitrile, methanol, isopropanol, tetrahydrofuran and additives (i.e. trifluoroacetic acid, which is highly ecotoxic and slowly decompose) are the main product of harmful waste. Considering "green" chromatography they should to be replaced with environmentally friendly alternatives. Therefore, it is important to find HPLC techniques in which the above-mentioned solvents are replaced with less toxic ones, such as ethanol [7] (ethanol is assigned to "green" solvents due to its low toxicity and, possible synthesis from renewable sources, but mainly because its lifecycle has low impact on environment), water [8,9], superheated water [10,11] or liquid carbon dioxide [12]. Reaching totally "green" liquid chromatography which completely eliminates organic solvents, there are only left water and carbon dioxide (supercritical or subcritical) as a mobile phase. This exchange is difficult and need special methods,

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conditions and equipment. For example, eluotropic strength of water can be increased but elevating temperature, reaching even superheated water [11]. Moreover, using of conventional C18 stationary phase results with phase collapse, when water is used as a mobile phase [6].

Replacement of solvents with less harmful ones is one of the Rs of green analytical chemistry (Reduce, Replace, Recycle). Not only reducing of unwanted risks and harmful solvents, but also total elimination of them can be provided by using this solution of greening separation techniques. Recently there is growing interest in greening principles in laboratory work, where minor changes in procedures multiplied by the number of analyses performed in laboratories around the world, can together give a significant impact [13]. High-performance liquid chromatography is one of the most common analytical techniques. Stationary phase columns used for separation usually has 4.6 mm internal diameter (i.d.), 25 cm length, and work at mobile phase flow rate between 1 and 1.5 ml/min [14]. These conditions lead to daily production of over 1 litter of effluent by each HPLC instrument that has to be utilized as a chemical waste. While this amount of solvent waste seems small in comparison to industrial production, however, the ubiquity of HPLC apparatus makes its significant. It is not uncommon that single pharmaceutical company can operate with over 1000 HPLC apparatus. Adding the fact that automation of this technology provide 24-h operation, cause that problem with waste-care, has to be handle out [13].

2. Subcritical water as a mobile phase

Term “high-temperature” appear to be not exactly defined. Usually it is explained as “higher than room temperature” or “higher than the boiling point of the solvent of mobile phase” but also definitions like “higher than 100°C” or “in range between 40°C and 200°C” can be found in the literature [15,16]. Interest related with temperature as a key parameter in liquid chromatography is still inconsiderable and does not appear in routine laboratory work. Lack of commercially available equipment, such as column oven, that can reach and maintain high temperature (up to 200°C) and deficiency in thermally stable stationary phases, may be the limits of this technique. Although in last two decades this topic has grown in interest what can be seen in several reviews that have been published [10,11,15–21]. Large spectrum of the physicochemical parameters affected by the temperature may discourage the analysts. Only influence of temperature on the decrease of viscosity of mobile phase is often mentioned. Other important effects such as decrease of eluent strength, increase in diffusivity and change in selectivity and dissociation rate of ionizable compounds are often not considered. This provides to change of conclusions regarding the manipulation of this parameter, from the fact that real benefits and restrictions of high-temperature analysis are not considered entirely [16].

The change of analysis temperature can replace the change of concentration of organic solvent in mobile phase or eliminate it at all. It can be found that temperature change may have similar effect to change in the solvent concentration. Bowermaster and McNair [22] found that change of 1% in methanol concentration is equivalent to change temperature for 3.75°C. Chen and Horváth [23] found similar effect with acetonitrile. The increase of 1% ACN (acetonitrile) corresponds to temperature increase of 5°C. Similar results were obtained by Tran et al. [24]. Kondo and Yang [25] conducted series of experiments that proved temperature change correspond to change of given organic solvent concentration depends on conditions and column that has been used. For example, 3.5°C rise in temperature is equivalent to approximately 1% increase of methanol concentration in methanol-water mobile phase when polystyrene-divinylbenzene (Hamilton PRP-1) column was used. Temperature change between 5°C and 8°C was similar while change concentration of acetonitrile by 1% in acetonitrile-water mixture was performed. However, usage of Zorbax RX-C18 column give results of 1% increase of methanol concentration equivalent to 2°C rise of the temperature, while same change in acetonitrile concentration is equivalent to 3°C rise of the temperature. The retention time of three compounds: pyrogallol, resorcinol and catechol were compared in above mentioned experiment [25].

Depending on the author and the conditions used, this technique can be named as “Subcritical water chromatography” (SBWC), “Chromatography in very hot water” “Superheated Water Chromatography” (SHWC) [16], “High Temperature Liquid Chromatography” (HTLC) [18,26] or “Pressurized Hot Water Liquid Chromatography” (PHW-LC) [27]. Pure water in elevated temperature can be used as mobile phase in liquid chromatography mainly due to change of dielectric constant. Water dielectric constant is reduced from 85 at 25°C to 35 at 200°C cause that water behave like an organic solvent. Because of that, water can became an extremely effective solvent for low-polarity, organic substances, such us organic pollutants [28]. This allows to conduct experiments in completely “green” conditions for thermally stable solutes and stationary phases [15].

Water in the conditions below critical point, that is 374°C and 218 atm., is considered as subcritical water (Fig. 1). Another terminology for water in these conditions is: superheated water, hot-temperature water, pressurized hot water. In ambient conditions water is too polar to be efficient eluent in chromatographic analysis. However, with increase of temperature it can be observed dramatically decrease of water polarity. Such high critical point of water allows wide spectrum of temperature and pressure values to choose. Therefore, such unique characteristic of subcritical water provides widely tunable parameters such as dielectric constant, surface tension and viscosity, which decrease with increase of water temperature (Fig. 2). However, this does not mean that solvents are optimized for a single parameter, e.g. a dielectric constant, as this single parameter does not affect either the selectivity or the elution strength. It is necessary to take into account all changing parameters with a change in the temperature of the mobile phase, as well as their tendency to change – increase or decrease. Pressure has low influence on those above-mentioned parameters, as long as water remains in liquid phase. Thus, subcritical water can imitate traditional RP (reversed phase) separations with organic solvents as modifiers in mobile phase. Additionally, dissociation constant increases by three orders of magnitude when temperature is elevated from 25°C to 250°C. Further increase of temperature causes decrease in constant. It means that water in high temperature acts as both stronger acid and stronger base compared to water in ambient temperature [10].

Although full range of applications and possibilities has not been recognized for SBWC, this technique should work for substances in the full spectrum of polarity. At low temperatures of analysis this technique works for polar compounds, moderately polar substances in mild temperatures and for non-polar substances in high temperatures, if stationary phase is stable in that range of temperature. This division is related to the influence of temperature on the adsorption of the analyte. Analytes adsorption is reduced with the temperature increase but this change is dependent on their polarity. At the same time, the viscosity of water is also reduced. Lower viscosity of mobile phase causes by elevated temperature gives low backpressure that allows SBWC separation to be performed with much higher flow rate. Thanks to that, shorten separations times can be achieved, analytes will be less exposed to high temperature, so it will minimize the analytes degradation.

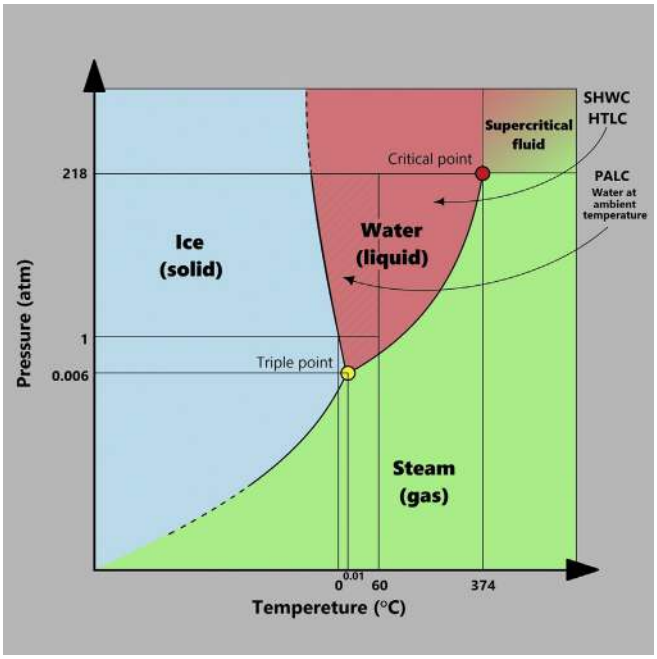

Fig. 1. Schematic water phase diagram. Below the critical point, that is 374 K and 218 atm., the water is in the liquid state and is termed subcritical water, hot-temperature water, pressurized hot water [10].

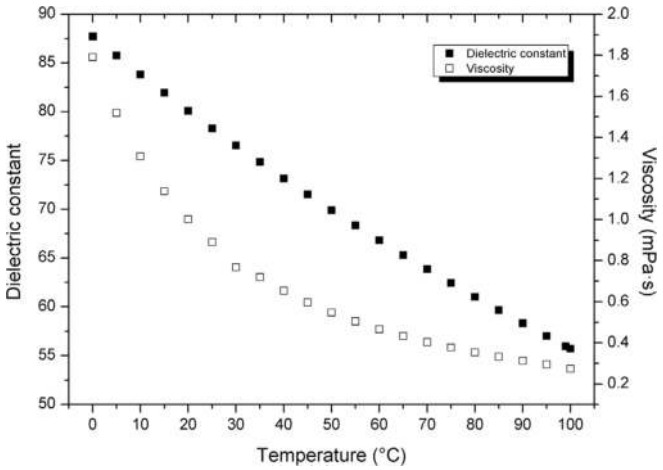

Fig. 2. Changes in dielectric constant [168] and viscosity [169] of water in the temperature range 0–100°C.

Therefore, degradation of analytes is not major problem using separation techniques in SBWC for low molecular weight substances [10].

First reported separation with use of subcritical water as a mobile phase was conducted by Smith and Burgess [29]. Experiment was carried out with superheated water up to 210°C to separate number of phenols, parabens and barbiturates on a polystyrene-divinylbenzene (PS-DVB) column. They showed that retention factors systematically decrease with increase of the temperature. Also, there was performed comparison of superheated water separation with conventional reverse-phase conditions. As approximately equivalent to 180°C water the 20:80 acetonitrile–water mixture was used. Separations were performed to show that superheated water can be used to resolve analytes which are characterize by wide range of hydrophobicity. To separate more hydrophobic propyl and butyl esters a higher temperature of 210°C was required. The oxidization and degradation of solutes or column was not observed as well as hydrolysis of ester functional group. Under these conditions the low back pressure of about 15 bars was noticed, as the vapor pressure of water is relatively low. As a conclusion authors report that comparison of conventional reversed-phase chromatography using water organic eluent and use of superheated water as mobile phase, showed that SHWC have enhanced separation and even shorter analysis time [29].

2.1. Application of subcritical water chromatography

For chromatographic analysis conducted at elevated temperatures, one of the concerns is the instability or degradation of the analyzed compounds. Considering water as a mobile phase, the two most likely reactions are hydrolysis and oxidation [11]. Due to the higher temperature of the chromatographic analysis, the viscosity of the water is reduced and the backpressure is low. This allows a higher mobile phase flow rate to be used. By combining high temperature and high flow rate, the analysis time is shortened. Therefore, SBWC analyses cause less exposure of the sample to hot water, resulting in no degradation of the analytes [10]. With an analysis time of between 5 and 30 min and an elevated temperature, a typical chemical reaction includes dehydration. However, dehydration will not occur due to the entirely aqueous conditions of the separation. The oxidation reaction will also not occur due to the prior degassing of the sample and the mobile phase, or by flushing with nitrogen to avoid rusting of the metal parts of the apparatus [11]. Taking parabens as an example, the alkyl p-hydroxybenzoates can be expected to undergo oxidation or hydrolysis reactions. However, Smith and Burgess [29] demonstrated that even under analysis conditions up to 200°C these compounds are thermally stable and no degradation has been observed. It has also been reported that some pharmaceutical compounds will not decompose when using fast LC (liquid chromatography) analysis. Reduced analysis time results in lower exposure of analytes to the reaction occurring on the column. Thompson and Carr analyzed a number of drugs and alkaloids to determine their thermal stability. They found that using reversed phase liquid chromatography, the stability of the studied compounds is related to the rate of their degradation under ambient conditions and the residence time in the column. Only norpseudoephedrine showed significant degradation. Therefore, it was found that many complex compounds can be determined at high temperatures [11,30]. It is therefore claimed that degradation of analytes is not a major problem during SHWC analyses with most low molecular weight compounds [10].

Several other cases where degradation took a place have also been reported. Thiamine can be analyzed up to 50°C, while at 160°C it breaks down to form a range of degradation products determined by on-line mass-spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy [31]. Nitrobenzene was tested at temperatures above 220°C on the polystyrene divinylbenzene column, where it was degraded. At lower temperatures and with the use of a less retentive column, it showed stability [11]. Research on polycyclic aromatic hydrocarbons in the temperature range from 100°C to 350°C and heating time from 10 to 240 min in pressurized hot water was aimed at demonstrating possible degradation. Most of the compounds disintegrated in a short time of 10 min at a temperature above 300°C. In some cases the losses were observed already at the temperature of 100°C [32].

As mentioned above, SHWC should work for substances with full range of polarity, mainly due to decrease of water viscosity and polarity with increase of analysis temperature. A wide number of low molecular weight compounds has been already separated with

use of superheated water chromatography including alcohols [28,33–38], aldehydes [39], aliphatic aromatic ketones [40] alkanols [26,33,35,41–44], alkyl benzenes/chlorinated benzenes/benzene derivatives [34,37,45–51], amino acids [52–54], anilines [25,45,54,55], aromatic acids [40], aromatic hydrocarbons [56], aromatic standards and alkyl aryl ketones [40,57,58], barbiturates [29,59,60], benzoates [61], caffeine derivatives [28,46,62,63], carbohydrates [52–54,61,64], carboxylic acids [52,65], chlorinated hydrocarbons [38], chlorophenols [66], diethyl phthalates [48,61], flavones [67], model drugs [68–72], nucleobases [61], parabens [29,59,61,63,73], phenols [25,28,29,38,45,47,54,55,65,70,74–76], phosphonic acids [53], polychlorinated biphenyls (PCBs) [77], polycyclic aromatic hydrocarbons (PAHs) [47,61,77], polyethylene glycols [78], polyhydroxybenzenes [25], PTH–amino acids [79–81], pyridines [25,55], steroids [80,82–84], sulphonamides [85,86], triazine herbicides [87], triazole fungicides [88] and water-soluble vitamins [31].

2.2. SHWC instrumentation

The application of the SHWC technique requires the use of suitably adapted apparatus. Additional elements need to be inserted in the conventional HPLC instruments. Although the standard HPLC apparatus has a heating oven, the temperature range does not exceed 80–100°C. In addition, such a heating system does not maintain a constant temperature in the column during the analysis [10,11].

Carr and coworkers developed a high-temperature ultra-fast LC apparatus that uses additional pumps to eliminate thermal mismatch, and avoid band broadening by using a separate pump that allows direct injection of the analyte into the column [76]. Most of the systems operating in SHWC are based on GC ovens, which have the ability to heat up to 350°C, and temperature programming. However, there is a problem with the delay in achieving thermal equilibrium between the oven and the column. In high-temperature chromatography it is important to ensure fast heating of the column. Tautenberg and co-workers [82] developed a special oven that allows for fast heating of the column up to 225°C and efficient cooling with circulating oil, which reduces the cycle time. Another way to avoid such a problem is the idea of direct heating of the column by wrapping it with resistively heated wire was developed, which allows for more rapid and repeatable heating of the column than in the case of common heating methods [33]. Currently, there are several column ovens available on the market, which allow to heat up to 200°C in both isocratic and temperature gradient conditions. The ovens have three separately controlled heating zones, pre-heating, column heating and cooling system.

Another emerging problem is the creation of back pressure at the output of the column to prevent water from boiling. One of the solutions is to use a simple length of PEEK tube with a narrow diameter (typically 3 m × 0.13 mm I.D.), which may be sufficient at a flow rate of 1 ml/min [11].

During the injection, there is a problem with the preparation of the sample. If the sample is water-soluble, the injection will be normal. However, if the solubility of the analyte in water is low, an organic solvent may be used to allow for homogeneous sample preparation, if the solvent has a higher retention rate than the analyte. The problem of band broadening due to the presence of organic solvent is avoided [89].

Due to the temperature difference between the mobile phase and the chromatographic column, there is a problem with the thermal mass entering the column, which can cause an unwanted cooling effect. In the SHWC, it is therefore important to use preheating in the form of a coil inside the oven or heating on an inlet line. This problem is more severe when using high flow rates of the mobile phase. It is therefore suggested that the temperature difference between the mobile phase and the column should not exceed 5°C. Also the length of the preheating coil must be adjusted to the flow rate and parameters of the column. Fields and coworkers [90] investigated that a 15 cm long coil with an internal volume of 3.4 μl yields distorted peaks at flow rates above 0.7 ml/min. However, in the same conditions, when using a 140 cm long coil, good peak shapes were maintained even up to a flow of 1.5 ml/min. Using a metal block in close contact with the inlet tube, causes heat transfer allowing the use of a relatively short tube, 15 cm long, sufficient up to 190°C [11,82,91].

2.3. Packing materials in SHWC

When considering analyses carried out with water in a subcritical state, it is important to pay attention to the thermal stability of the stationary phases. Most of the columns stable under ambient HPLC analysis may show instability under superheated water conditions. In both, ambient temperature HPLC and SHWC, there can be seen a linear van't Hoff relationship between retention factor (k) and inverse of absolute temperature ($1/T$) [57,69]. One of the main examples is the commercially available octadecylsilane (ODS) silica material for column packing, which undergoes degradation in conditions above 80°C [11]. Therefore, there is a demand to search for new materials or modify them in order to find a thermally stable column packing in the SHWC. The elevated temperature lowers the polarity and surface tension of the water, so that superheated water can be used for the separation of both polar and non-polar compounds. Studies on interactions between analytes in superheated water and the normal-phase and reversed-phase packing materials have been studied by Yang et al. [92]. They concluded that the energy, and therefore the temperature needed to elute the compounds when using the reversed phase columns, is higher than when using the normal phase columns. In addition, the presence of aromatic interactions increased this differentiation.

Analyses carried out with ODS silica gel allowed us to observe the phenomenon that occurs at the water concentration in the mobile phase of over 95%. In such conditions a sudden decrease in retention times occurs while maintaining efficiency. It was found that this effect occurred when the columns were not used for some time and were not under pressure. This phenomenon was attributed to the so-called “phase collapse” [93]. It consists in the laying of long, hydrophobic hydrocarbon chains C_{18}, which reduces the adsorption available surface of the stationary phase. Therefore, an attempt was made to eliminate this effect. For this purpose, polar groups between the connections of hydrophobic chains C_{18} with the surface of silica were incorporated. This allowed to obtain a mixed hydrophilic-hydrophobic phase with simultaneous improvement of its wettability. Such phases were named after polar-endcapped phases, polar-embedded phases or aqueous phases [94,95]. Changes in retention using SHWC analysis were observed for columns with built-in polar groups, especially when they cooled down and left without flow for a period of time. The ‘phase collapse’ process is reversible using an eluent with a high content of organic solvent. However, the occurrence of this effect occurs suddenly, even between analyses. The behavior of stationary phases in high aqueous eluent has been described by Walter et al. [96], where attempts were made to predict the above mentioned effects. Currently, the effect of “phase collapse” is explained by the phenomenon of de-wetting of the phase surface, which reduces the effective volume of the column by removing water molecules from the pores [97].

The lack of stability of commercial silica columns with attached octadecyl groups forced the search for new column packings that

are stable in superheated water. Currently, in SHWC and other chromatographic analyses using high percentage of water in the mobile phase, modified silica phases, polymer phases, zirconia-based and other metal oxides phases, carbon phases and hybrid phases are used [10,11].

2.3.1. Silica-based packing materials

Conventional silica columns are used in water conditions. However, their lifetime and the temperature range in which they can operate is low. Most of the C_{18} phases have a low lifetime, although they are used in SHWC analyses [68]. The most common columns with silica filling include Nucleosil C18 AB [45,62], Zorbax [69], Zorbax RX-C8 [62], Zorbax RX-C-18 [25,98], Xterra [68,69,84], Xterra RP 18 [99], Gemini [69], Kromasil-C18 [46], Luna C18 [100] and many more, see Yang [10]. Typical temperature range for silica-based columns is from 50°C up to 150°C. With a small addition of an organic modifier this range can be slightly increased to 200°C, although the recommended range with 100% water is up to 100°C, for the same column material [101].

2.3.2. Polymer-based packing materials

Polymeric phases are commonly used in elevated temperature chromatography, e.g. for size exclusion chromatography. Because of that they are expected to be the most thermally stable material for analysis with superheated water as a mobile phase. The most commonly used columns with polymer filling for use in SHWC include polystyrene divinylbenzene column PLRP-S (PS-DVB) [29,31,46,59,63,68,85,86] and filling with crosslinking polymer PRP-1 [25,28,33,39,52,54,102]. Thermal stability of these columns is in the range from 100°C to 200°C. There are even works with new polymers containing amino acid, which are suitable to work under superheated water conditions up to 150°C for 500 h [103]. However, in comparison to columns filled with silica, they have a lower column efficiency, which may result from the high retention capacity, which causes that elution of moderately polar compounds, such as alkyl aryl ketones, can be achieved only at high temperature.

2.3.3. Zirconia-based packing materials

Thermal instability of silica phases directed the researchers in search of matrices built of oxides of other metals, such as alumina, zirconia or titania oxides. A wide reviews of this compounds used in chromatography were prepared by Nawrocki et al. [104–106]. Zirconia-based materials for chromatographic column packings were first developed by Carr and co-workers [104,107]. However, problems arose during modification of the surface of amphoteric oxides with alkyl carbon chains. This required the use of mobile phase additives in the form of e.g. fluoride ions. Finally, it turned out that only zirconium compounds are used as effective materials for the preparation of chromatographic columns. The most commonly used are encapsulated zirconia by polybutadiene (PDB) known as ZirChrom-PDB [43,46,48,49,67,68,82,88,90,108] and by polystyrene (PS) – ZirChrom-PS [62,67,76]. There are also other zirconia-based materials such as carbon coated zirconia (CARB) – ZirChrom-CARB [49,68,100] or secondary bonded C_{18} zirconia column (Diamondbond) e.g. ZirChrom-DB-C18 [46]. Thermal stability of PDB columns has been determined at 200°C. However, manufacturers recommend using it up to the upper limit of 150°C. Wu et al. [43] discovered that the stability of the PDB column in the recommended conditions is about 200 h, while the upper limit of temperature at which it can actually be used is about 260°C. Kephart et al. [49] showed that capillary PDB and CARB columns are stable up to 370°C and 300°C respectively at the pressure of about 758 bars. They were successfully used for separation of phenol compounds and alkylbenzenes.

2.3.4. Carbon-based packing materials

Carbon based packing materials for chromatographic column were investigated mainly due to its high thermal stability. For this reason, porous graphitized carbon (PGC) was used [109–111]. However, large activity of carbon materials caused that contamination problems appeared, what conclude in problem with asymmetric peak shapes. Even with such high-thermal stability, PGC column efficiency declined with a time, due to difference in thermal expansion of carbon and stainless-steel column material. This resulted in a mechanical stress through which the column loses its separative properties [87].

2.4. Detectors applied in superheated water chromatography

Detectors used in conventional liquid chromatography techniques are dependent on the composition of the mobile phase, in particular the organic modifier concentration. Thus, the detection used in liquid chromatography was dominated by UV–Vis and fluorescence spectroscopy as well as by refractive index detection, mainly due to the small influence of the matrix composition changes on the quality of the determinations. In addition, the high availability, versatility and low cost of UV detection, have led to their use even in analyses using superheated water. However, the use of 100% water as a mobile phase has opened up the possibility to use other, more universal methods for the determination of analytes. These techniques include Flame Ionization Detector (FID), Mass Spectrometry (MS), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Nuclear Magnetic Resonance (NMR), Evaporative Light Scattering Detection (ELSD) and others like amperometric detector or Fourier Transform Infrared Spectroscopy (FTIR) [10,11,18] (Fig. 3).

Using a UV/visible absorption technique, using pure water as the only eluent component has the advantage of no background peaks from organic solvent. This type of liquid chromatography uses a thermal gradient instead of a concentration gradient, making available techniques where an organic modifier causes detection difficulties, e.g. by changing the volatility of the mobile phase and the analytes. Also, it offers the possibility of determination of analytes at short wavelengths, even below 200 nm. Yarita et al. [78] showed that this technique is suitable for determination of low molecular weight polyethylene glycols at wavelength of 190 nm. The use of wavelengths below 200 nm allows for increased

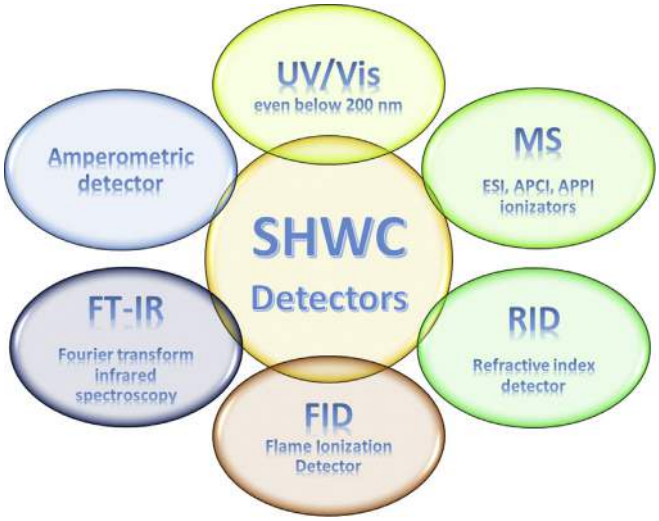

Fig. 3. Diagram illustrating the wide detection spectrum possible through the use of pure water in superheated water chromatography (SHWC) [10,11,18].

sensitivity only for clean samples. Determination of real samples involves limitations due to the presence of matrix compounds that strongly absorb high energy radiation, resulting in the hiding of analytes' peaks [18]. However, there are also other restrictions related to the use of hot water. Due to the use of elevated temperatures, the back-pressure has to be controlled for keeping water at liquid state, so the flow cell of the detector should be able to withstand this pressure [11]. For this reason, a back-pressure regulator or resistor should be placed at the outlet of the UV/Vis detector. Most of UV/Vis detectors are not adapted to use in high temperature, therefore the cooling system has to be applied to introduce liquid analysis at ambient temperature. Decreasing of temperature cause increase of polarity of aqueous mobile phase, thus deposition of moderately and nonpolar analytes in transfer tubes can appear [10]. It can be seen that UV/Vis technique, when elevated temperature is applies, losses its universal application, mainly due to appearance of above-mentioned drawbacks.

The flame ionization detector is a sensitive and universally used detector in gas chromatography. Due to the lack of such a sensitive apparatus in LC techniques, attempts have been made to use it for liquid phase determination. It is well known that the FID is used for non-volatile analytes and for the determination of all substances containing carbon, which makes it a very versatile detector. Unfortunately, the widespread use of organic solvents in both normal and reversed phase systems in LC increases the volatility of the analytes and provides a significant background for FID-based determinations. Water is transparent for FID, so the use of 100% water as a mobile phase increases the possibility of using FID in liquid chromatography determinations. However, pure water at room temperature is not a good eluent for all columns and compounds in LC. For analytes that can be separated at ambient temperature in pure water, Synovec et al. [37,38] have developed a FID for the determination of alcohols and hydrocarbons. Krejčí and coworkers [112] performed first determination using capillary column LC online coupled with modified FID with usage of 100% water as eluent. In later years many FID modifications for liquid chromatography determinations were carried out, but all of them required the elimination of organic modifiers from the mobile phase [37,38,53]. An unmodified FID was used by Miller and Hawthorne [42] to determine groups of polar organic compounds using the SHWC. They concluded that placing the end of the capillary resistor placed at the output of the column at a level below 3 cm from the tip of the FID gave results in which the detector signals were the most stable. These results were also confirmed in later works [36,39,52,54]. Such placement of the resistor allowed to keep the water in liquid state even at temperatures above 100°C. Also thermal gradient was investigated as part of their studies [42].

Mass spectrometry is one of the few detection techniques which, in addition to information on the presence of a product, is able to determine what is the product. The use of MS detection in liquid chromatography requires an ionization technique that allows the liquid to be ionized. The most common techniques are electrospray ionization (ESI) [113–115], atmospheric pressure chemical ionization (APCI) [116–118] and atmospheric pressure photoionization (APPI) [119]. The possibility of coupling MS detection with liquid chromatography gives the possibility to use it also using techniques such as SHWC. The effectiveness of coupling to the MS detector is related to the processes of nebulization, evaporation and desolvatation. As a consequence, the different compositions of the mobile phase in the LC, and therefore its different properties such as surface tension, polarity and viscosity, influence the detection results. Therefore, there are few applications in the combination of APCI-MS detector and HTLC [120]. This is due to the low sensitivity and mass dependence of this type of ionization. The ESI ionizer, which was used in temperature programming, enjoys a higher sensitivity compared to the concentration gradient, as a result of the faster evaporation of the hot moving phase, which reduces noise [121–123].

Although NMR detection is much less sensitive than other analytical methods, the fact that it is non-destructive for the analyte causes a growing interest in it as a detector to LC. Applying NMR analysis to conventional LC coupling requires the use of suitable deuterated solvents such as methanol and acetonitrile, which are expensive. This is necessary due to the presence of too large background signals from protons of non-deuterated solvents. The use of HTLC coupling with NMR detection is therefore beneficial. Deuterated water is easily available in high purity and at an affordable price [18]. NMR spectra have significantly less interference from the mobile phase with clean water than with conventional solvents. In addition, there is a possibility of coupling NMR-MS detection, which allows to simultaneously obtain the spectrum of both 2D and 3D [70]. SWHC-NMR-MS was used to analyze barbiturates [60], drugs [70], sulfonamides [86] and ginger extract [99]. PS-DVB polymer columns [31,60,70,71,84,99] were mainly used, but ODS columns [99] were not excluded, which are characterized by lower thermal stability.

Among other noteworthy detection methods one can distinguish refractive index detection (RID), amperometric detection or Fourier transform infrared spectroscopy (FTIR). The main problem with the use of these techniques is the change in the baseline resulting from the concentration gradient used in conventional LC methods. However, an alternative to this gradient may be temperature programming, which is used in the SHWC. The instability of the base line and the presence of high noise levels compared to other methods is a feature of RID. The use of a single-component mobile phase in SHWC allows to reduce these effects. The temperature change of course influences the physicochemical properties of the eluent, which is also reflected on the detector base line, however, this can be corrected by appropriate software. It has been proven that the use of high mobile phase flows and good cooling of the eluent before entering into the refractive index detector system allows the detection results to be obtained without loss of sensitivity using a temperature gradient [124,125]. Amperometric detector was successfully used for temperature programmed analyses with electrochemically active compounds. The drifts of baseline were eliminated by simple mathematical approach. There were no evidence of influence of temperature gradient on retention nor sensitivity [126]. The coupling LC-MS has a drawback that do not distinguish between isomers. Therefore, the combination of these techniques with infrared spectroscopy will allow full separation and analysis of mixtures. Also, in this case the use of a temperature gradient is more advantageous in relation to the composition gradient. The HTLC coupling with FTIR was performed by Greibrokk et al., where this technique was used to separate complex antioxidants [127].

3. *Per aqueous* liquid chromatography (PALC)

As mentioned above, the elimination of organic modifiers from the mobile phase in the LC makes only water and CO_2 in the sub- or supercritical condition are the only two fluidic media that can be used as a fully “green” solvent. Considering the water, which is the subject of this review work, temperature control allows for changes in the eluotropic strength of water [128]. However, there are still doubts about the stability of both the columns and the solutes at high temperature.

Hydrophilic Chromatography (HILIC) allows the separation of mainly polar compounds. Currently the most common solvent in HILIC analyses is ACN. The influence of water presence in the mobile phase on silica hydride columns using aqueous normal phase

(ANP) technique, has been pioneeringly described by Pesek et al. [129,130]. Bimodal curves form 0–100% water concentration were performed to determinate the changes in retention for separation of epiribicin and analogs, as well as to investigate the mobile phase layer rich in water associated with the surface of silica [131,132]. Bidlingmeyer studies were the first in which pure silica was used to separate amines in HILIC and also the mobile phase with high water content was studied [133]. They have proven that with high water content, the surface of silica becomes non-polar. Siloxane groups contribute to the hydrophobic character of silica [134]. The hydrophilic character is assigned to free silanols, which, depending on the pH, may have an ionic nature.

The use of pure water makes polar silica in HILIC adopt a non-polar character. The use of the term reversed HILIC suggests that the RP-LC technique is used. Therefore, A. dos Santos Pereira et al. introduced a new name, *per aqueous* liquid chromatography (PALC) to distinguish this method from HILIC, RP-LC and ANP LC (aqueous normal phase LC) [135]. A schematic illustration of the mobile phase and the stationary phase used in PALC is shown in Fig. 4. At first, they used the two coupled Zorbax-Rx-SIL columns for the determination of underivatized amino acids in biological liquids. The plot of retention times in function of ACN concentration was determined. It showed that after a dip related with decreasing of ACN content, there can be observed an increase of retention times with major changes in selectivity. They also demonstrated that the separation of catecholamines, which failed in standard HILIC on superficially porous particles [136], was successfully carried out using PALC with excellent peak shapes and high efficiency. In later works aimed at developing a description of the PALC technique, Gritti and A. dos Santos Pereira explain the mechanism of retention and the effect of the use of the water-rich mobile phase on efficiency [137,138]. The adsorption mechanisms were explained by the adsorption isotherms measured by frontal analysis (FA) on core-shell particles of neat porous silica (Halo) column package.

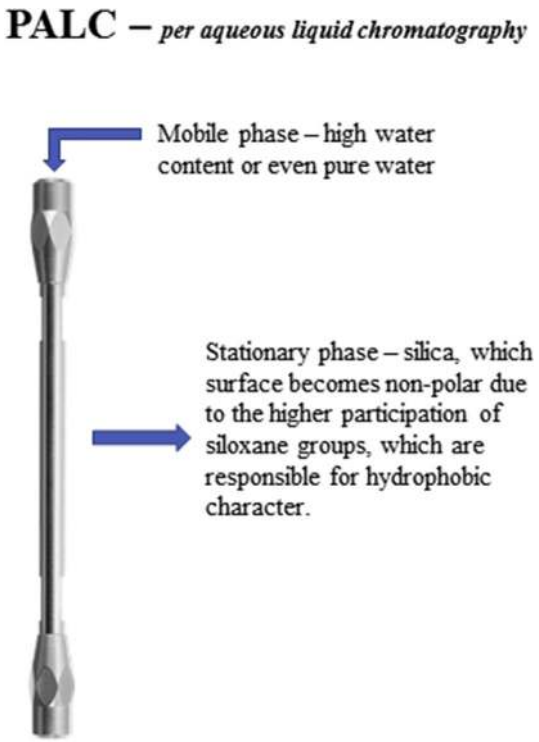

Fig. 4. Diagram showing the chromatographic arrangement used in the PALC technique [135].

The major differences between PALC and HILIC adsorption mechanisms were proven. In both, water-rich and acetonitrile-rich conditions, retention factors were large. Intermediate content of acetonitrile and water concluded with minimum retention factor. The mechanism was determined with use of pyridine in whole scale of acetonitrile concentration. In purely PALC conditions (low acetonitrile content) surface of silica exhibits strongly heterogenous character. High density hydrophobic siloxane groups take part in less than 10% of sample retention. Basically, pyridine retention is controlled by the active adsorption sites present on the surface of low-density silica. They can be attributed to hydrophobic micropores, which adsorb the pyridine molecules. In purely HILIC conditions (high acetonitrile content) active sites are occupied by ACN molecules, which are excess in the mobile phase. This prevents their hydrophobic interactions with pyridine. Therefore, single silanol groups (constituting 75%) and geminal and/or vicinal hydroxyl groups (constituting the remaining 25%) are responsible for the retention [137]. Efficiency measurements were carried out for the same Halo column, using caffeine and pure water–acetonitrile mobile phase, without ionogenic substance added. Retention factors for both mechanisms were determined in the range from 0.5 to 2.5 at room temperature. For such retention factors efficiency measurements were carried out. PALC mechanism is the most efficient at low retention factors $k' < 2$, and should be avoided for strongly retained samples. For $k' > 2.5$ height equivalent to a theoretical plate (HEPT) is lower for HILIC mechanism in compare to PALC [138].

In the next years the PALC technique was studied for various columns, polar analytes and ionic additives to the mobile phase. First, Li et al. used the polysaccharide-modified stationary phase (PMSP) column to determine six polar substances: melamine, vitamin B2, vitamin B6, caffeine, benzoic acid and hydroquinone [139]. Further studies confirm that the use of the PALC method has a comparable retention with HILIC for the determination of polar compounds. The connection of functionalized carbon nanoparticles (CNPs) obtained from corn stalk soot to the silica surface allows to obtain polar stationary phases working with both water-rich and acetonitrile-rich mobile phase [140]. The effect of the buffer or salt presence in the mobile phase (ammonium formate, pH = 5) on the separation of ionized catecholamines using a bare silica column (Zorbax-Rx-SIL) was then investigated. The poor peak shapes were obtained mainly due to overloading of few, but strong adsorption sites [141]. The use of 1.7-μm ethylene bridged hybrid silica stationary phase to determinate twelve imidazolium-based ionic liquid cations showed that PALC mode gives both hydrophobic and ion-exchange mechanism [142]. Fungicides containing polar groups are reluctant to partition into the water-rich layer at a high ACN content in the bulk phase. Therefore, the application of the PALC method allows to increase the retention at reduced ACN content in the mobile phase [143]. Attempts have also been made to use combined techniques. Matos et al. combined PALC with size exclusion chromatography (SEC) and diode array detector (PALCx-SEC-DAD) to determine water-soluble organic matter (WSOM) in atmospheric aerosols collected at different seasons of the year from urban area [144]. PALC analyses are also used as a complement to RP-LC analyses, mainly due to their positive environmental and economic aspects. A silica-based column with attached Congo red molecules (Sil-CR) was used to determine four types of biogenic amines and five bases and nucleotides [145]. The results showed that PALC analysis in this case gives better separation than the use of acetonitrile-rich HILIC mode. Also, such a result gives useful information for exploration of the PALC chromatographic column materials. The most recent publications report on subsequent syntheses of materials for chromatographic columns packings, such as polysaccharide-modified stationary phase [146] and porous

organic cage embedded C18 amide silica stationary phase [147] and separation and determination of groups of polar and hydrophilic compounds such as protein A [148], to develop the PALC mode.

The PALC method is a relatively new technique, covering the last decade. However, its development is observed mainly in the context of the analytical technique competing with HILIC, as well as due to its economic and ecological advantages. Research into the synthesis of new stationary phases used in PALC and separation of groups of polar and hydrophilic compounds will be the main issue of the development of this method.

4. Water as a mobile phase at ambient temperature

The use of a fully aqueous mobile phase at room temperature is the most advantageous form when considering environmentally friendly liquid chromatography. Approximately ambient conditions are understood as analyses performed at temperatures below 60°C. PALC is also considered as a type of these techniques (Fig. 4). A new subcategory name for such analyses has been proposed – water-only reversed phase liquid chromatography (WRP-LC) [50]. Studies under such conditions were performed more than three decades ago [149]. Satisfactory results were achieved primarily due to the application of silica surface modification, e.g. by bonding hydrophobic alkyl chains (C-8). However, in later studies it was confirmed that the highly polar mobile phase favors a large proportion of surface groups of silanols, which influence the separation mechanism. The use of short alkyl chains (up to 4 carbon atoms) allows to obtain stable stationary phases due to their high surface coverage density. Connecting longer chains causes a decrease in coverage density, and at low content of organic solvent (less than 5–10%) there is a decrease in phase solvation and causes the phase tend to collapse [94,150].

In order to reduce the influence of free silanols on retention, polar-endcapped and polar-embedded stationary phases were used. Polar-endcapped stationary phases are obtained in two stages. In the first stage long chains, e.g. alkyl chains, are attached. In the second stage, unreacted silanols are endcapped by a specific reagent. Polar-embedded stationary phases are obtained by incorporating a polar functional group into the hydrophobic ligand of the stationary phase, which allows for better water solvation under highly aqueous conditions (Fig. 5). The advantage of these phases is that they can be used in RP-LC, different selectivity of polar compounds compared to standard alkyl stationary phases, the ability to work in highly aqueous conditions, even up to 100% water content and limited influence of surface bound silanols, which favors the lack of peaks tailing of basic compounds [94,151–162].

There were many works showing the possibility of separation of substances with a wide range of polarity, using pure water at ambient temperature as a mobile phase. Buszewski et al., in 1994 demonstrated the possibility of separating the group of alkylanilines using a chemically bound phase (CBP) in particular alkylamide (AA) phase [163]. The use of ODS-packed column modified with strongly positive/negative charges surfactant (ODS coated with zwitteragent) for separation of nucleosides and their bases as a representative of typical polar organic compounds, were proposed several times [164–166]. Separation of compounds with different hydrophobicity was carried out using Poly(N-isopropylcrylamide)-Modified Silica (PIPAAm-modified silica). The influence of temperature on resolution was investigated. It was shown that already at room temperature there is a possibility of effective separation of steroids [167]. Another polymeric stationary phase were used by Šatínský et al. were analytes of different polarities were determinated [6]. Their main objective was to obtain a "green" method, so they used a column that would allow separation using a low-toxic mobile phase that is pure water. Kiridena et al. used their developed

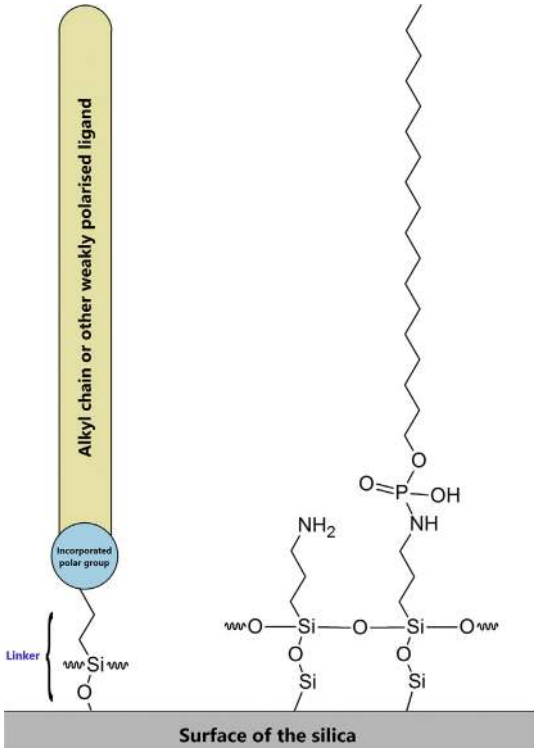

Fig. 5. Schematic structure of the polar-embedded stationary phase [155–162]. Linker is connected to the silica surface and to the incorporated polar group. A hydrophobic alkyl chain of different number of carbon atoms or another non-polar molecule (e.g. cholesterol) is connected to the polar group. The figure shows an example Amino-P-C18 phase [159].

polar-endcapped chromatographic column (Synergi™ Hydro-RP) at room temperature and elevated temperature (not exceeding 65°C) [95]. The most recent studies confirm the use of polar-embedded stationary phases for the separation of polar compounds in both HILIC and RP-LC. By selecting the appropriate polar groups, it is possible to obtain stationary phases that will be effective at room temperature, giving reasonable retention times. It is important that the stationary phase meets three basic requirements. The first one is the presence of polar and hydrophobic groups allowing to obtain specific properties of the phase surface. The second is to allow the adsorption of the analyte molecules, although it should be selective. The last requirement is to allow the water molecule to elute the analytes at different times with retention factors between 1 and 10 and reasonable retention times [158,159]. Bocian and Krzemińska in their latest work published the results, which present the successful separation of the series of nucleic bases, nucleosides and purine alkaloids, using a completely aqueous mobile phase. They used a standard HPLC apparatus equipped with a diode-array UV–Vis detector. The temperature of each measurement was 30°C. The N,O-dialkylphosphoramidate phase [152] and ester bonded phases [159] were used as a stationary phases. Because these are further studies demonstrating successful separation in pure water, such results confirm that the use of stationary phases from the polar-embedded group allows the separation of mixtures in a single component, aqueous mobile phase, at room temperature [158]. Exemplary results are presented in Fig. 6.

Most of the research using pure water as the only component of the mobile phase is directed towards the use of polar-embedded and polar-endcapped stationary phases. They work not only in RP and HILIC systems, but also with the use of pure water at room

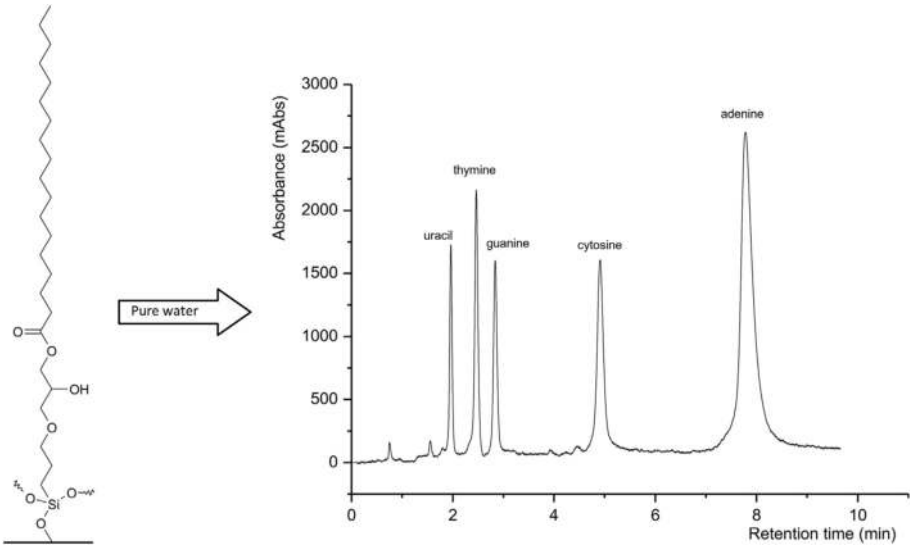

Fig. 6. Separation of five nucleosides on Ester-18 stationary phase using pure water as a mobile phase basis on [158].

temperature as the only eluent. Research on the development of subsequent phases of this type will certainly allow for the best possible optimization of the separation in water-only liquid chromatography.

5. Summary

The analytical methods presented in this review work prove that separation using high-performance liquid chromatography uses pure water as a mobile phase, it is achievable. There are different approaches, but all based on the selection of analysis conditions, stationary phase or possible additives to the mobile phase. The change in conditions is based on an increase in temperature, thanks to which water has different physical and chemical properties. There are changes in its polarity, dielectric constant, viscosity, surface tension and many other properties. The solubility of analytes, which at room temperature may be insoluble in such a strongly polar solvent, also changes. The stationary phase must have appropriate properties, in particular aqueous stability, thermal stability (using SHWC) and selectivity in relation to mixtures of compounds of different polarity. Thus, a number of stationary phases based on silica, carbon, polymers or metal oxides are obtained. Additions to the mobile phase in the form of acids or pH-regulating compounds (buffers) enable more selective separation and often favor shorter analysis times. The main objective of this review work was to encourage the search for HPLC analytical methods that will allow for greater environmental friendliness.

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Review

Stationary Phases for Green Liquid Chromatography

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Abstract: Industrial research, including pharmaceutical research, is increasingly using liquid chromatography techniques. This involves the production of large quantities of hazardous and toxic organic waste. Therefore, it is essential at this point to focus interest on solutions proposed by so-called "green chemistry". One such solution is the search for new methods or the use of new materials that will reduce waste. One of the most promising ideas is to perform chromatographic separation using pure water, without organic solvents, as a mobile phase. Such an approach requires novel stationary phases or specific chromatographic conditions, such as an elevated separation temperature. The following review paper aims to gather information on stationary phases used for separation under purely aqueous conditions at various temperatures.

Keywords: pure water; liquid chromatography; chemically bonded stationary phases; polar-embedded stationary phases; subcritical water chromatography; SBWC; superheated water chromatography; SHWC; per aqueous liquid chromatography; PALC

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1. Introduction

Liquid chromatography is a common separation technique that is commonly used in laboratories and various industries. Unfortunately, the liquid chromatographic separation process typically uses a significant number of organic solvents, and produces considerable quantities of harmful waste. This is a global problem that directly affects human life and the functioning of the environment. Thus, many ideas have been proposed to make chromatographic separation more environmentally friendly in the past decade. Solutions proposed by "green chemistry" are also increasingly used in high-performance liquid chromatography (HPLC). Replacing toxic solvents with "green" alternatives has attracted more and more interest, since it reduces the amount of waste products, which has a positive ecological effect, reduces costs, and improves the economic aspect. [1,2]. However, it must be emphasized that a purely aqueous mobile phase must have special requirements; thus, it is likely to increase costs in other areas, such as stationary phases or instrument operation at elevated temperatures.

To achieve utterly green chromatography, it is necessary to completely eliminate organic solvents and replace them with pure water [3–7] or supercritical carbon dioxide [8], while still being able to separate groups of compounds; this is only possible with the use of suitable phases. Currently, there are more and more references in the literature to commercially available or homemade stationary phases that allow working under such conditions. Knowledge of such phases is incredibly important for researchers involved in environmental and ecological fields in industries using chromatographic analysis.

This work collects information on the current state of knowledge of stationary phases used under purely aqueous conditions, describing the advantages as well as the requirements associated with the use of pure aqueous conditions; above all, it collects information about already-used, commercially available stationary phases working in 100% water in a structured and systematic manner.

Separation techniques commonly applied in various laboratories and industries, including chromatographic techniques, consume large amounts of organic solvents. Acetonitrile, methanol, isopropanol, tetrahydrofuran, and additives (e.g., trifluoroacetic acid) are some of the toxic solvents commonly used in reversed-phase liquid chromatography (RP-LC). Considering "green" chromatography and thinking about our environment, these should be replaced with more environmentally friendly alternatives, e.g., water. A similar situation is observed in normal-phase liquid chromatography (NP-LC) and hydrophilic interactions liquid chromatography (HILIC), and the latter has gained more popularity in recent years. The general assumptions of these modes are shown in Figure 1.

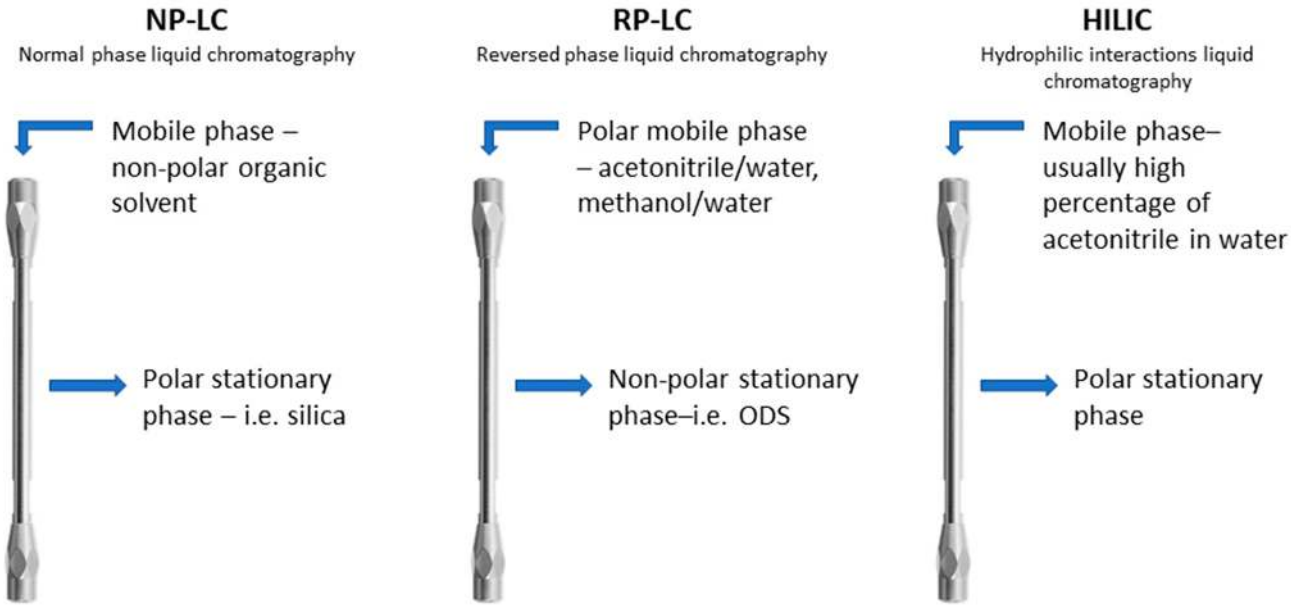

Figure 1. Characterization of the stationary phase and mobile phase in the NP-LC, RP-LC, and HILIC modes.

Numerous "greener" ways to perform liquid chromatography were reviewed in our previous work [9]. The most common are downsizing the dimensions of the HPLC column [10]; the application of mobile phase additives, e.g., cyclodextrins [11] or surfactants; replacing organic solvents with water [3–7], ethanol [12], or supercritical CO_2 [8]; and changes in the used stationary phases. The first solution involves the use of stationary phases modified with shorter carbon chains [13], materials based on core–shell particles [10], and the application of polar-embedded stationary phases [14]. Traditional stationary phases may also be operated at elevated temperatures [15]. Of those previously mentioned, this paper's major topic is the application of stationary phases for HPLC enabling analysis in pure water. For such an approach, the proper selection of stationary phases is necessary.

A second approach to achieve more "green" analytical chemistry is to use mathematical data analysis methods. Using multivariate and multiway chemometric methods makes it possible to calculate separation conditions where the resolution will be increased, especially for more complex mixtures of analytes. Multiway methods in chemometrics are based on the analysis of higher order data than second-order data (matrix, second-order array)—for example, third-, fourth-, or higher order data. Such occurrence of data is common in chemistry, e.g., liquid chromatography coupled with mass spectrometry (LC–MS) [16,17]. This approach saves time, experimental work, consumption of organic solvents and analytes, and energy use [18–20]. Nevertheless, it will not always be a completely "green" solution, as organic solvents are used during the work, and must be treated as waste after the experiments; however, their consumption can be drastically reduced, so it is a more environmentally friendly alternative compared to commercially used methods.

A very "green" solution for liquid chromatography is to use software such as DryLab or ChromSword, among others [21,22]. These programs enable computer simulations of analyses, providing data on the optimal analysis conditions for a given mixture of analytes and chromatographic column. Thus, the analysis stage needed in order to optimize the separation method of a given group of compounds is omitted; hence, there is a significant reduction in organic solvents, energy costs, and working time.

When applying pure water as a mobile phase, there are two options for elution and separation: The first is to use specific stationary phases that increase the relative elution strength of water. The second option is to increase the temperature of the separation significantly. In both cases, the stationary phase properties are fundamental in the case of proper retention, elution, and thermal stability.

2. Purely Aqueous Conditions: Features and Requirements

As mentioned above, the use of pure water allows chromatographic analyses to be performed in a completely "green" manner. With industry and science growing faster and faster, the amount of waste generated is increasing. Therefore, we have no choice but to look for ways to ensure that modern and future-oriented analytical work does not add to environmental pollution. An extensive description of the problem and solutions was given by Welch et al. [23]. The past two decades have seen a growing interest in the application of pure water as a mobile phase. One can find many experimental works and several published review papers related to this issue [6,7,9,24–31]. Changes to standard liquid chromatography approaches are needed in order to make the separation in pure water possible. Two paths are possible: the first includes elevated temperatures, while the second remains at ambient temperatures but focuses on selecting appropriate stationary phases.

The magnitude of the temperature increase can vary between applications. However, the often-used phrase "high-temperature" is not exactly precise. Less precise terms such as "higher than room temperature" or "higher than 100 °C" and more precise terms such as "higher than the boiling point of the mobile phase solvent" or "in the range between 40 °C and 200 °C" appear in the literature [25,26]. Changes in water temperature affect a large spectrum of physicochemical parameters, which may dishearten analysts. Usually, only the influence on the viscosity is frequently discussed in the literature. Increases in eluent strength, increases in diffusivity, and changes in the dissociation rates of ionizable compounds are equally significant, but less frequently considered. Therefore, the overall benefits and difficulties associated with high-temperature analysis are not considered exhaustively [26]. Our previous paper described the discussion about temperature-dependent changes in water properties and their effects on analyses in pure water [9].

In RP-LC, increasing the temperature produces an effect analogous to that of increasing the organic solvent concentration in the mobile phase. The studies carried out by Bowermaster and McNair [32], and Chen and Horváth [33] show that a 1% increase in ACN concentration corresponds to a 5 °C increase in temperature. In contrast, an equivalent increase in methanol concentration corresponds to a 3.75 °C increase in temperature. Of course, the precise effect depends on the stationary phases used [34,35]. For example, in the methanol–water mobile phase, a temperature increase of 3.5 °C corresponds to a 1% increase in the concentration of the organic component, while a temperature increase of between 5 °C and 8 °C corresponds to a 1% increase in the concentration of acetonitrile in an acetonitrile–water mixture on polystyrene–divinylbenzene stationary phase (Hamilton PRP-1). Changing the stationary phase to Zorbax RX-C18 gives results of 1% of organic solvent concentration being equivalent to 2 °C for methanol–water and 3 °C for acetonitrile–water mobile phases [35]. The dielectric constant of water is reduced from 85 at 25 °C to 35 at 200 °C. Elevated temperatures make the water behave like an organic solvent; hence, it becomes a very effective solvent for separating weakly polar compounds [36]. Application of water at elevated temperatures allows experiments to be conducted under

completely "green" conditions if the separated substances and the stationary phase are thermally stable [25].

If the properties of water depend on the temperature, changing the temperature makes it possible to separate substances with a full spectrum of polarity. High temperatures enable elution of non-polar compounds, moderate thermal conditions enable elution of weakly polar compounds, and low temperatures are sufficient for non-polar substances. Of course, this is limited by the thermal stability of the stationary phase. A higher temperature of the water changes the polarity and reduces the viscosity, but also reduces the analyte adsorption—which is exothermal—on the stationary phase. Depending on the conditions used and the authors, chromatography with water at elevated and ambient temperatures can be referred to by different terms. The modes with the description of the conditions of pure water as a mobile phase and features of stationary phases are shown in Figure 2.

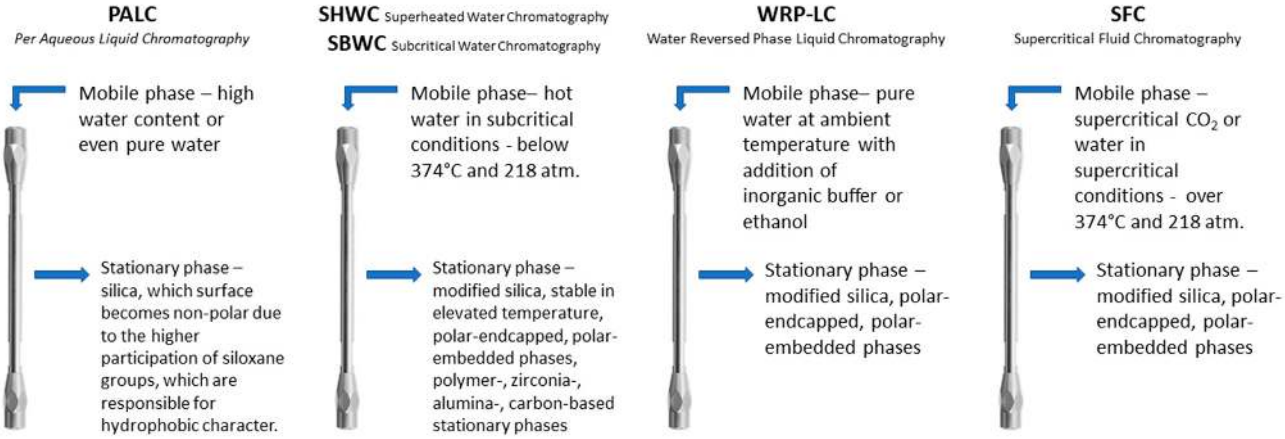

Figure 2. Characterization of stationary phases and conditions for using pure water as a mobile phase in different liquid chromatography modes.

More than 30 years ago, a study was conducted on separation in pure water [37]; sadly, it did not gain popularity. Satisfactory results were obtained when the surface of the stationary phase was modified with shorter hydrophobic alkyl chains not exceeding eight carbon atoms. Later studies confirmed that free silanols at the surface of silica particles play the main part in retention when such highly polar eluents as pure water are used; however, their excessive activity adversely affects the shape of the peaks and, therefore, the separation results. The use of even shorter carbon chains (up to C4) allowed us to obtain stable stationary phases with high coverage density. The problems associated with the attachment of longer alkyl chains are then avoided, since these phases have lower coverage densities and, at low contents of organic modifier in the mobile phase, tend to form chain bonds and decrease the stationary phase solvation [38,39].

The adverse effect of silanols on retention led to the search for new solutions, one of which is the use of polar-endcapped and polar-embedded stationary phases [40–43]. More details related to the structure, capabilities, and requirements of such phases will be described in the next section of this work.

An increase in water temperature causes changes in its eluotropic strength [44]. Unfortunately, the thermal stability of both column packing and analytes is a problem. In such a case, the next option is to change the mode of liquid chromatography. This issue can be overcome by using more polar stationary phases that allow for higher eluotropic strength of water.

Applying a purely aqueous mobile phase at room temperature is the most environmentally friendly mode of liquid chromatography, with advantages such as lack of toxic waste and low energy consumption. Usually, ambient conditions in liquid chromatography are interpreted as analyses performed below 60 °C. The literature suggests a new subcategory of

this type of analysis—water-only reversed-phase liquid chromatography (WRP-LC)—and per aqueous liquid chromatography (PALC) is also among these techniques [45].

High elution strength of water is observed in hydrophilic interaction liquid chromatography (HILIC). Hydrophilic interaction chromatography is mainly used for the separation of polar compounds. The water content in HILIC is usually low, and it is impossible to carry out HILIC with pure water, due to the elution strength being too high.

An alternative to HILIC may be a silica hydride stationary phase applied in an aqueous normal-phase (ANP) system. ANP was named and described for the first time by Pesek et al. [46,47]. The changes in the retention of polar compounds from 0 to 100% water concentration present bimodal curves. The highest retention is observed at both ends—for high organic solvent content similarly to HILIC, and for pure water or high-water content [48–50].

The surface properties of silica gel in the purely water mobile phase are somewhat surprising. The hydrophilic character of silica is attributed to free silanol groups. Silanols, depending on the pH of the solution, may ionize. Bidlingmeyer et al. separated amines in HILIC on pure silica using pure water as a mobile phase [51]; the authors demonstrated that at high water content, the silica surface shows non-polar behavior. This is due to non-polarized siloxane groups that give the silica surface a hydrophobic nature [52]. Thus, the use of pure water makes the polar nature of the silica surface in HILIC change to non-polar. Therefore, A. dos Santos Pereira et al. proposed a new name for this reversed-HILIC mode—per aqueous liquid chromatography (PALC)—to distinguish this method from HILIC, RP-LC, and ANP-LC [53]. PALC requires the application of specific stationary phases.

The application of pure water as a mobile phase causes several problems. If the stationary phase is highly hydrophobic, such as C18, the bonded ligands may collapse to the support surface. This usually results in a decrease in retention, and reduces the reproducibility of the separations [3]. The second problem is that in RP-LC the elution strength of water may be too low to perform the elution. The opposite problem is observed in HILIC, where the elution strength is too high and prevents retention.

When using water at elevated temperatures, further constraints arise. Although the elution strength, viscosity, surface tension, and many other properties of water are favorably altered, high temperature places some limitations. A stationary phase operating under these conditions must be thermally stable. The substances to be analyzed must also exhibit thermal stability over the temperature range used. Degradation of all or some of the analytes of the tested mixture will completely disturb the results obtained. The thermal instability of analytes can be circumvented in several ways. The lower viscosity of the purely aqueous mobile phase causes low backpressure, enabling the use of much higher flow rates for SBWC separation; as a result, the degradation of analytes is reduced, because the analysis time and, consequently, their exposure to high temperatures, is reduced. Thus, analyte degradation is no longer a significant problem when using the SBWC technique for low-molecular-weight compounds [6].

The use of elevated temperatures is also associated with the use of appropriate instrumentation. It is necessary to ensure a stable temperature throughout the analysis, pre-heating of the mobile phase, and a suitable thermostat for the column [6,54].

3. Stationary Phases Used in Pure Water Conditions

The first reported separation using subcritical water as a mobile phase was carried out by Smith and Burgess in 1996 [55]. The separations of several phenols, parabens, and barbiturates on a polystyrene–divinylbenzene (PS–DVB) stationary phase were carried out at 210 °C. The authors demonstrated that retention factors of separated substances decrease with increasing temperature. Compared to conventional reversed-phase conditions, superheated water separation at 180 °C provides a similar result to a 20:80 acetonitrile–water mobile phase at ambient temperature. No degradation or oxidation of any analytes or the stationary phase structure was observed in the experiments performed. Thus, the

study concluded that SHWC, compared to the conventional RP-LC technique, gives better separation and allows shorter analysis times [55].

As discussed above, the thermal stability of analytes can be an obstacle to using water at elevated temperatures. The thermal stability of analytes was investigated by Thompson and Carr [7,56] in the case of drugs and alkaloids; they concluded that in order to not affect the analytes' stability, it is necessary to reduce the analysis time. Doing so will allow even compounds that degrade at high temperatures to be separated, as the short residence time in the elevated-temperature column will not affect their stability [6]. The observed rule also has some exceptions; several other cases have been reported in which degradation has occurred. Thiamine can only be analyzed below 50 °C, while a temperature of 160 °C results in many degradation products [57]. Nitrobenzene was degraded above 220 °C using a PS–DVB column; however, a reduced temperature and a less retentive phase allowed the decomposition to be prevented [7]. Even polycyclic aromatic hydrocarbons can degrade; analyses in the range of 100–350 °C and an extensive range of heating times from 10 to 240 min in pressurized hot water showed that even at the shortest time interval, they degraded above 300 °C; at longer times, damage to the structure of the compounds occurred even at 100 °C [58].

Despite the examples of degradation described above, SHWC can work for substances with a broad polarity range, mainly due to decreased water viscosity and polarity with increasing temperature. Several commercial chromatographic columns may be used to separate low-molecular-weight compounds using supercritical water. By selecting a suitable stationary phase or suitable analysis conditions, working with pure water at room temperature is also possible. These materials are described in the literature and listed in Tables 1–4.

3.1. Instability of Stationary Phases—A Motivation to Search for Solutions

In most cases, the thermal stability of analytes is not a significant problem, and a wide range of substances may be analyzed. Another serious problem is the thermal stability of the stationary phase used. Most of the materials used as stationary phases that are stable under ambient temperature HPLC may not be stable under superheated water conditions. Despite the temperature range, the van 't Hoff relationship remains linear for both ambient temperature HPLC and SHWC [59,60].

One of the leading examples of a thermally unstable stationary phase is the most commonly used chromatographic material—octadecyl-modified silica (ODS)—which undergoes degradation at temperatures above 80 °C [7]. For this reason, it is essential to develop new materials or modify existing ones in order to ensure their thermal stability when using pure water as the only eluent. When analyses are carried out with ODS silica materials at water contents higher than 95%, a sudden decrease in retention times occurs while maintaining efficiency. This is due to a phenomenon called the "phase collapse" effect, which is associated with the stacking of long-chain alkyl ligands [61]. In this case, the adsorptive surface area of the stationary phase is strongly reduced. However, the "phase collapse" process is reversible, and this can be achieved by using a mobile phase with a high organic modifier content. This effect is explained by the de-wetting of the phase surface, which lowers the effective volume of the column by removing water molecules from the pores [62].

Many attempts have been made to prevent this effect. For example, polar groups were incorporated between the hydrophobic octadecyl chains and the silica surface; this led to the obtaining of mixed hydrophilic–hydrophobic phases. Such materials have improved wettability in high-water-content or even in purely aqueous mobile phases. Novel materials were named polar-endcapped, polar-embedded, or aqueous stationary phases [39,63].

With SHWC, and using phases with embedded polar groups, changes in retention were also observed. This was achieved by cooling the column and leaving it without flow for a period of time; however, this effect can occur suddenly—even between performed analyses. The overall behavior of stationary phases under aqueous conditions was described in detail by Walter et al. [64].

Consequently, the inability to successfully use commercial octadecyl phases has forced the search for other stationary phases that are thermally stable under pure water conditions. Currently, several chromatographic phases are used in SHWC and other techniques using pure water as the only eluent. The most common are: modified silica phases, polymer phases, zirconia-based phases, other metal oxide phases, carbon phases, and hybrid phases [6,7].

3.1.1. Silica-Based Packing Materials

Despite the problems mentioned above, conventional silica stationary phases are applied in water conditions. Unfortunately, the temperature range in which they can be operated is low; additionally, in most cases, their lifetime is reduced. This is mainly observed in the case of ODS materials.

Problems that arise during analyses at elevated temperatures mainly focus on the degradation of analytes. The occurrence of such a phenomenon is not necessarily due to the use of pure water or elevated temperatures. For example, using a Zorbax RRHD Eclipse Plus column for the separation of coumarin, vanillin, and ethyl vanillin gave ethyl vanillin as a degradation product. However, the use of other columns under the same conditions did not cause degradation of the analyte [65]. This confirms that the degradation resulted from analyte–stationary phase interactions, rather than from factors associated with the application of pure water or elevated temperature. In addition, it can be concluded that by having a wide range of stationary phases commercially available, the reasons for degradation can be checked efficiently and avoided.

The thermal stability of the column can be measured—simply perform a mixture separation analysis and then repeat the analysis after a specified volume has passed through the column. An example study was performed by He and Yang [66], investigating the change in retention of a mixture of caffeine, benzene, and methyl benzoate. Nucleosil C18 AB phase degradation occurred when passing more than 8000 column volumes at 100 °C. The reason for increasing the retention of the polar compound and decreasing the retention of the non-polar compound is the gradual removal of the C18 chains from the silica surface, causing a decrease in its non-polar character.

Studies also confirm that stationary phases operating over a wide pH range have greater thermal stability [67]. In addition, those based on ethylene-bridged hybrid (BEH) technology are currently the most thermally stable and pH-stable silica-based columns [68].

Using elevated temperatures can be problematic, so performing analyses at room temperature using pure water is highly desirable. The PALC technique mentioned above allows operation under highly aqueous or fully aqueous conditions while maintaining ambient temperature; it has become competitive with HILIC and, combined with the environmentally beneficial aspect, interest around it has increased. Initially, in the literature, one may encounter the term reversed HILIC; however, this suggests that the retention mechanism occurs according to RP-LC, for which reason the term PALC has been introduced.

The application of Zorbax-Rx-SIL to separate catecholamines in standard HILIC was unsuccessful on superficially porous particles [69]; however, it was successful using PALC, obtaining excellent peak shapes and high efficiency. In a later work, Gritti and dos Santos Pereira explained the retention mechanism occurring in PALC, and described the effect of the water-rich mobile phase on the separation efficiency [70,71]. The adsorption mechanism was explained by determining adsorption isotherms using frontal analysis (FA) on pure porous silica (HALO) core–shell structures; this enabled determination of the differences in mechanisms between HILIC and PALC. The low acetonitrile content in PALC makes the silica surface strongly heterogeneous; in contrast, in HILIC, the high acetonitrile content makes the free silanols responsible for the retention: single silanol groups and geminal and/or vicinal hydroxyl groups [70]. HALO column efficiency measurements showed a lower value of height equivalent to a theoretical plate (HETP) for PALC compared to HILIC [71].

PALC gained wider popularity in the following years, and various materials were tested as a stationary phase. At first, Li et al. used a polysaccharide-modified stationary phase (PMSP) to separate six polar substances: melamine, vitamin B2, vitamin B6, caffeine, benzoic acid, and hydroquinone [72]. Subsequent detailed studies proved that PALC could obtain retention factors as good as HILIC for separating polar compounds. Modifying silica particles with the functionalized carbon nanoparticles (CNPs) obtained from corn stalk soot allows polar stationary phases to be obtained. Such materials can work with both water-rich and acetonitrile-rich mobile phases [73].

PALC also enables the use of hybrid silica materials such as a 1.7 μm ethylene-bridged hybrid silica stationary phase (BEH HILIC); its application to the separation of 12 imidazole-based ionic liquids' cations showed that the PALC system could enable retention with both hydrophobic and ion-exchange mechanisms [74]. Another application of PALC was used by Matos et al., combining it with size-exclusion chromatography (SEC) to determine the water-soluble organic matter (WSOM) content in atmospheric aerosols collected from urban areas during different seasons [75].

Due to its positive ecological and economic aspects, PALC is sometimes used as a complementary technique to RP-LC. Detection of four types of biogenic amines and five nucleic bases and nucleotides was performed on a silica-based column with Congo red molecules attached (Sil-CR) using PALC [76]. The results obtained showed better separation with PALC than using HILIC, which consumes large amounts of organic modifiers.

In the RP-LC technique, applying pure water as a mobile phase requires surface modification of the silica-based stationary phase. These phases have embedded polar groups to provide a mixed retention mechanism and allow water to interact with the silica surface. The polar-embedded and polar-endcapped phases will be discussed in subsequent sections of this paper. Commercially available silica-based stationary phases and the groups of compounds separated on them are summarized in Table 1.

Table 1. Literature dataset of silica-based chromatographic columns and groups of chemical compounds separated by them using pure water.

Chromatographic Column	Group of Chemical Compounds																					
	Alcohols	Aliphatic and Aromatic Ketones	Alkyl Benzenes/Chlorinated Benzenes/Benzene Derivatives	Anilines	Aromatic Acids	Aromatic Hydrocarbons	Barbiturates	Benzoates	Caffeine Derivatives	Carbohydrates	Chlorophenols	Diethyl Phthalates	Model Drugs	Nucleobases	Parabens	Phenols	Polychlorinated Biphenyls (PCBs)	Polycyclic Aromatic Hydrocarbons (PAHs)	Polyhydroxybenzenes	Pyridines	Steroids	Water-Soluble Vitamins	Literature
Akzo Nobel Kromasil Eternity-2.5-C18								x		x		x		x	x				x					[68]
Chromatorex C-18														x		x			x	x				[35]
Daiso (ODS-BP)										x														[77]
Develosil C30-UG-5	x																							[78]
Fuji Silysia Chromatorex 10 μm											x													[79]
Hyperprep C18			x																					[80]
Hypersil BDS C18									x				x											[66,81,82]
Interchim Uptisphere Strategy C18-2 and C18-3								x		x		x		x	x				x					[68]
Kromasil-C18			x						x															[83]
L-column ODS Chemicals Evaluations and Research Institute, Japan															x									[84]
Nanoporous glass modified with TFPS and ethyl acetate			x																					[80]
Novapak C18													x			x							x	[57,85]
Nucleodur-C18			x																					[83]
Nucleosil C18 AB			x	x					x							x								[66,86]
ODS Chrompack	x																							[87]
Partisil ODS2 ES			x													x		x						[88]

Table 1. *Cont.*

Chromatographic Column	Group of Chemical Compounds																					
	Alcohols	**Aliphatic and Aromatic Ketones**	**Alkyl Benzenes/Chlorinated Benzenes/Benzene Derivatives**	**Anilines**	**Aromatic Acids**	**Aromatic Hydrocarbons**	**Barbiturates**	**Benzoates**	**Caffeine Derivatives**	**Carbohydrates**	**Chlorophenols**	**Diethyl Phthalates**	**Model Drugs**	**Nucleobases**	**Parabens**	**Phenols**	**Polychlorinated Biphenyls (PCBs)**	**Polycyclic Aromatic Hydrocarbons (PAHs)**	**Polyhydroxybenzenes**	**Pyridines**	**Steroids**	**Water-Soluble Vitamins**	**Literature**
Silica-silicon-based ethyl-bridged hybrid C18 5 µm	x		x																				[89]
Spherisorb octadecylsilane (ODS)-bonded silica						x	x								x								[90,91]
Spherosil XOA (200, 600, 800 mesh)-C18	x					x										x							[92]
Supelco Ascentis Express C18 (fused-core)								x		x		x		x	x			x					[68]
Xselect CSH C18								x		x		x		x	x			x					[68]
Xbridge Amide								x		x		x		x	x			x					[68]
Xbridge C18		x			x								x										[81,93,94]
XTerra MS C18 and Xterra phenyl organic/inorganic hybride		x	x						x														[83,95]
Xterra RP C8		x			x								x								x		[93,96,97]
Xterra RP C18		x	x		x				x												x		[83,93,97]
YMC Triart C18								x		x		x		x	x			x					[68]
Zorbax RX-C-18	x													x		x			x	x			[35]
Zorbax RX-C-8									x							x							[66,82]
Zorbax-ODS																	x	x					[98]

3.1.2. Polymer-Based Stationary Phases

Polymeric materials usually exhibit higher thermal stability. Such fillings are successfully used in elevated-temperature chromatography, e.g., for size-exclusion chromatography; thus, it is reasonable to expect them to be the most thermally stable chromatographic materials for analyses using superheated water as a mobile phase. Among polymer materials, two of them are most commonly used in SHWC: polystyrene divinylbenzene PLRP-S (PS–DVB) [55,57,81,83,90,99–101], and crosslinked polymer PRP-1 by Hamilton [35,36,77,87,89,102,103]. All polymeric stationary phases and the groups of compounds separated on them are summarized in Table 2. The thermal stability of these phases is between 100 °C and 200 °C. Polymer-based phases are more thermally stable than silica-based phases; they are therefore used in conditions of prolonged operation at temperatures higher than 200 °C. However, when comparing retention times and temperature, using the silica phase is sometimes more advantageous, because the analysis time is shorter and does not require such high temperatures. Of course, the limiting parameter for using these phases is their lifetime, so for long-term use, polymeric phases are mostly used [90]. The literature also mentions new polymeric phases that contain attached amino acids that allow them to operate in heated water up to 150 °C for 500 h [104]. Despite better thermal stability, polymeric stationary phases also have some disadvantages compared to silica-based materials, e.g., lower column efficiency; this is likely related to the high retention capacity; thus, groups of moderately polar compounds such as alkyl- and aryl ketones can be eluted only at elevated temperatures.

Table 2. Polymer-based stationary phases used to separate groups of compounds in pure water.

Chromatographic Column	Group of Chemical Compounds																											
	Alcohols	Aldehydes	Aliphatic and Aromatic Ketones	Alkyl Benzenes/Chlorinated Benzenes/Benzene Derivatives	Amino Acids	Anilines	Aromatic Acids	Barbiturates	Benzoates	Caffeine Derivatives	Carbohydrates	Carboxylic Acids	Chlorophenols	Diethyl Phthalates	Model Drugs	Nucleobases	Parabens	Phenols	Polycyclic Aromatic Hydrocarbons (PAHs)	Polyethylene Glycols	Polyhydroxybenzenes	PTH–Amino Acids	Pyridines	Sulfonamides	Water-Soluble Vitamins	Literature
Aminex HPX 87-strong cationic resin	x										x															[105]
Oasis polymer															x											[81]
P(NIPAAm-co-BMA-co-DMAPAAm) modified silica																						x				[106]
P(NIPAAm-co-tBAAm-co-AAc) modified silica																						x				[106]
PL HiPlex 8µm H			x				x																			[93]
PLRP-S PS–DVB Polymer Laboratories			x	x			x	x		x					x		x	x						x	x	[56,60,63,81,83,85,90,93,99,100,107,108]
Poly(GMA-co-EDMA) particles						x												x					x			[109]
Polystyrene-Coated Zirconia (PS-ZrO_2)																		x								[54]
PRP-1 Hamilton	x	x			x	x				x	x	x	x			x		x		x	x		x			[35,66,77,79,82,86,87,102,110,111]
Showa Denko Shodex ET-RP1 4D									x		x			x		x	x		x							[68]

3.1.3. Zirconia-Based Stationary Phases

Silica, due to the low pH of the point of zero charge (pH_{pzc}), allows only cation exchange under neutral pH conditions. The dissociation of silanols then occurs, and the silica surface acquires a negative charge. Therefore, chromatography of basic compounds on silica is troublesome, because it requires a significant change in pH—which adversely affects the attached ligands or the silica itself—or the use of ion-exchange displacers or anionic ion-pairing agents [112–114].

Metal oxides—especially zirconium, aluminum, and titanium oxides—have much higher pH_{pzc}, which means they do not have a negative surface charge, so no electrostatic interactions will occur; they behave as amphoteric ion exchangers, so anionic or cationic exchange can occur depending on the pH. Due to the presence of Lewis acid sites, the additional ligand exchangeability works to the advantage of using these supports [112,113].

Carr et al. were the first research group to examine the use of zirconium oxide as a potential packing material for chromatography columns [113,115]. This raised issues about modifying its surface with alkyl ligands. The modification required special conditions and additives. Finally, it turned out that among metal oxides, only zirconia could be used as effective chromatographic material.

Zirconium oxide has many adsorption sites, so it can be easily modified. This action favors a wide adaptation of the use of zirconium-based stationary phases. In general, there are three types of zirconium oxide modification: (1) dynamic, where the mobile phase contains a strongly interacting Lewis base; (2) permanent, where the zirconium oxide surface is permanently silylated as a result of binding to adsorption sites; and (3) physical screening, e.g., coating the zirconium surface with polymer or carbon. The most commonly used approaches are zirconia encapsulated by polybutadiene (PDB)—which is also known as a ZirChrom-PDB [81,83,106,116–122]—and zirconia encapsulated by polystyrene (PS), called ZirChrom-PS [54,66,120]. Other zirconia-based packings include carbon-coated zirconia (CARB)—ZirChrom-CARB [81,117,123]—or a secondary bonded C_{18} zirconia-based stationary phase (DiamondBond) e.g., ZirChrom-DB-C18 [83]. All zirconium- and aluminum-based stationary phases are listed in Table 3.

Zirconium oxide used as a support in chromatographic analyses has good thermal stability and is stable under pure water conditions. For ZirChrom-PDB, the manufacturer recommends an upper temperature limit of 150 °C; however, the thermal stability of this material has been observed up to 200 °C. ZirChrom-PDB was tested by Wu et al. [116], who showed that the upper limit of the column's thermal stability reaches 260 °C. The ZirChrom-PDB and ZirChrom-CARB columns tested by Kephart et al. [117] reached thermal stability of 370 °C and 300 °C, respectively, using a pressure of 75.8 MPa. Compounds from the phenol and alkylbenzene groups were successfully separated on both stationary phases.

It is also important to note that complete removal of CO_2 from the mobile phase is necessary before working in pure water, as it binds to the Lewis acids sites, completely changing the surface character of the stationary phase. Therefore, it is necessary to boil the water before use as a mobile phase, degas it, or use a scrubber or pre-column [113].

Table 3. Collection of zirconium- and aluminum-based stationary phases used to separate the listed groups of compounds in pure water.

Chromatographic Column	Group of Chemical Compounds									
	Alcohols	Alkyl benzenes/Chlorinated Benzenes/Benzene Derivatives	Caffeine Derivatives	Diethyl Phthalates	Flavones	Model Drugs	Phenols	Steroids	Triazole Fungicides	Literature
Alumina Keystone Scientific		x					x			[86]
PBD-encapsulated zirconia	x									[116]
ZirChrom–Carb		x				x				[81,117]
ZirChrom-DB-C18		x	x							[83]
ZirChrom-PBD	x	x	x	x	x	x	x	x	x	[81–83,106,118,119,124]
ZirChrom-PS			x		x					[66,120]

3.1.4. Carbon-Based Packing Materials

Of the carbonaceous materials, only porous graphitized carbon (PGC) has found application in liquid chromatography. This material is obtained by immersing silica grains in a phenol–formaldehyde mixture. Next, gradual heating results in the formation of a phenol–formaldehyde resin, which is then carbonized at 900 °C in a nitrogen atmosphere. Then, the silica template is removed using KOH. The final carbonaceous material is heated at 2500 °C in an oxygen–argon atmosphere. The resulting material consists of uniform porous carbon, with a known pore structure and a surface oxidized under oxygen conditions.

Some chromatographically relevant features characterize carbon support—it is highly thermally resistant, with a well-reproducible structure and a surface that does not show the presence of charge during chromatographic operation. The specific surface of this material is suitable to provide retention, and to maintain linear capacity over a wide range of analyte concentrations. The porous structure does not exhibit the presence of micropores, and the pore size is larger than 10 nm, which ensures efficient mass transfer to and from the solution from the particles. PGC is resistant to highly interfering solvents; thus, no swelling, shrinkage, or dissolution occurs. This material is stable over the entire pH range and at high salt concentrations; it exhibits a unique retention mechanism and selectivity. Unlike common ODS phases, it has stereoselective properties that allow it to separate isomers and compounds with very similar structures [125].

The first PGC-packed columns were made by Hypersil—a division of Thermoquest Corporation; the columns are now sold under the Hypercarb name. In 1991, two Japanese groups—one from Tonen Corporation and the other from Nippon Carbon Company and Tosoh Corporation—released porous graphites obtained via a completely different procedure. Despite the differences in how these materials are obtained, their chromatographic properties do not differ. The type of chromatographic columns filled with PGC depends on the preparation procedure and the silica used for templating. Thus, several versions of such columns are commercially available, although their chromatographic properties coincide [125].

A more detailed study of Hypercarb shows that it is a very high-thermal-strength column. The dependence of $\log k$ on $1/T$ for this column, in the temperature range 20–180 °C,

is linear, with a very good fit, which proves the independence of ΔH and ΔS from temperature over a wide range, regardless of the mobile phase composition—even in pure water. Although zirconium-based stationary phases exhibit nonlinear Van 't Hoff plot relationships, for all of the substances studied by Guillarme, the Hypercarb relationships remained linear, indicating no effect of temperature on the eventual surface modification of the carbon material and excellent regularity and rigidity [83].

The disadvantage of PCG is the contaminants that form due to the high activity of the carbon materials, resulting in a problem with the formation of asymmetric peak shapes in the chromatograms. The second disadvantage of PGC columns is that their efficiency declines with time. Although carbon materials have high thermal stability, the significant difference in thermal expansion compared to a stainless steel column causes high mechanical stresses, resulting in a loss of separation properties of the bed [126]. All carbon-based stationary phases are listed in Table 4.

Table 4. Overview of carbon-based stationary phases and groups of compounds that were separated using pure water as a mobile phase.

Chromatographic Column	Group of Chemical Compounds														
	Alcohols	Aldehydes	Aliphatic and Aromatic Ketones	Alkyl Benzenes/Chlorinated Benzenes/Benzene Derivatives	Amino Acids	Caffeine Derivatives	Carbohydrates	Carboxylic Acids	Model Drugs	Nucleobases	Peptides	Steroids	Triazine Herbicides	Morphine-Based Opiates	Literature
Hypercarb 5 µm	x			x	x	x	x	x					x		[83,87,102,124,126]
Hypercarb PH porous graphitic carbon									x						[81]
Porous graphitized carbon (PGC)		x	x		x						x	x		x	[125]

4. Stationary Phases with Integrated Polar Groups

Surface modifications of silica by placing both polar and non-polar parts on the phase surfaces appeared in the early 1990s. This was due to the search for materials that allow for effective and selective operation in a wide range of organic modifier concentrations. This phase structure allows a mixed retention mechanism, among other things. Both polar and non-polar compounds are retained through interactions with hydrophobic and hydrophilic parts of the stationary phase [41–43].

Among the stationary phases possessing a polar group and a hydrophobic ligand, we can distinguish three types: Polar-embedded stationary phases are obtained by attaching a polar group to the silica surface in place of a silanol, and then a hydrophobic part is attached to it; this construction of the phase with an incorporated polar group and an attached non-polar component allows for better water solvation at high-water mobile phase conditions. Polar-endcapped stationary phases are obtained in a two-step process. First, the surface of the stationary phase is modified by the attachment of non-polar parts, such as alkyl chains; in the second step, polar groups are endcapped by a specific reagent that possesses a polar group. The methodology is analogous to typical hydrophobic endcapping. Polar-headed stationary phases have a non-polar part attached to the silica surface, and the hydrophilic group is located at the end [40]. A schematic representation of these phases is shown in Figure 3.

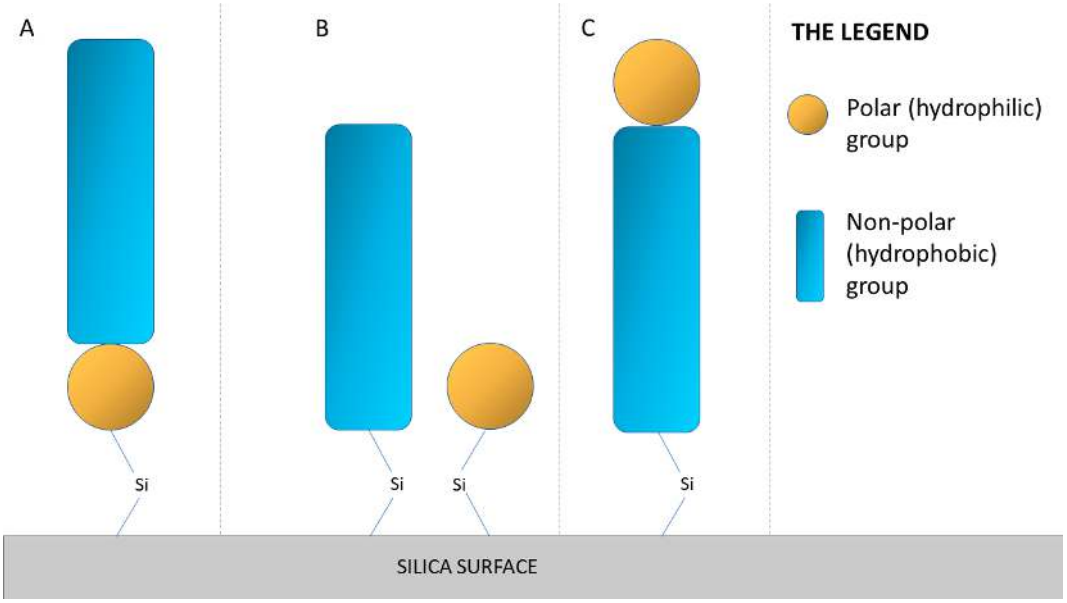

Figure 3. Schematic representation of the possibility of incorporating a polar group into the structure of a non-polar phase. (**A**): polar–embedded; (**B**): polar–endcapped; (**C**): polar–headed [40].

Such materials have a series of advantages; they can be used in an RP-LC system and, compared to typical alkyl-bonded stationary phases, they provide a different selectivity for polar compounds. Polar-embedded stationary phases also allow operation using highly polar mobile phases, so they can successfully be used for separations using 100% water. Polar groups reduce the influence of residual silanols that result in the absence of tailing of basic chemical compound peaks [14,39,127–138].

Many papers have now been published demonstrating the separation of substances with a wide range of polarities using pure water at room temperature. Successful separations of alkylanilines using an alkylamide chemically bonded phase were described in 1994 by Buszewski et al. [139]. Some attempts have been made to apply octadecyl stationary phases modified with strongly positively/negatively charged surfactants; such stationary phases were used for the separation of nucleosides and nucleic bases [140–142]. Compounds of different hydrophobicity were separated using poly(N-isopropylacrylamide)-modified silica [143]; it was shown that it is possible to separate steroids efficiently at ambient temperature; the authors also investigated the influence of the temperature on the resolution. It must be remembered that in a single-component mobile phase (pure water), the separation temperature is the only parameter that can influence the retention and separation; thus, the proper choice of the stationary phase for a given mixture to be separated is essential. Another polymeric material used in pure water separation was a polyethylene glycol stationary phase (Supelco Discovery HS PEG), which was used by Šatínský et al. for the separation of analytes of different polarities [3]. Kiridena et al. applied a polar-endcapped chromatographic column (Synergi^{TM} Hydro-RP) at room temperature and elevated temperatures, but below 65 °C [63]. Recent work indicates the possibility of using polar-embedded phases in both RP-LC and HILIC systems to separate polar compounds [14].

The ability to freely select the polar group results in stationary phases giving an efficient performance at room temperature of the mobile phase, with reasonable retention times. The polar-embedded stationary phase to be applied in the pure water mobile phase has to meet three basic requirements: First, it must provide retention of analytes, and this should be selective for different substances. Second, pure water must elute analytes in a reasonable time. Finally, it must provide unique selectivity and specific surface properties due to the presence of a polar group and a hydrophobic ligand [14,127]. The successful separation of a series of nucleic bases, nucleosides, and purine alkaloids is described in a

recent paper by Bocian and Krzemińska. Pure water was the only mobile phase component at 30 °C, and a standard HPLC system was used for the analyses. As a stationary phase, the N,O-dialkylphosphoramidate phase [131] and a series of ester-bonded phases [127] were used. The obtained results confirm that applying polar-embedded stationary phases may enable water-only separation at ambient temperature conditions [14]. Exemplary structures of stationary phases used for purely aqueous separation are presented in Figure 4.

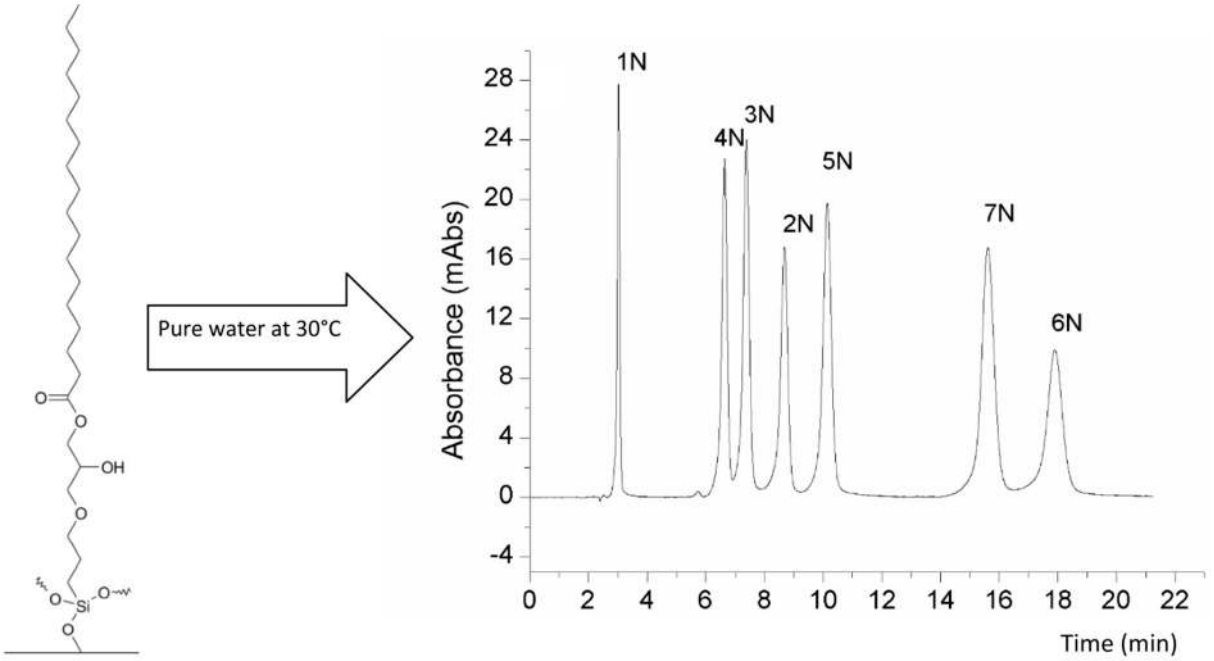

Figure 4. Separation of seven nucleosides on a polar-embedded stationary phase (Ester-C18) using pure water at low temperatures as a mobile phase. Description of the compounds—1N: uridine; 2N: guanosine; 3N: 1-methylinosine; 4N: thymidine; 5N: 1-methylguanosine; 6N:N2-methyloguanosine; 7N: adenosine. Adapted from ref. [14].

Another advantage of such stationary phases is that they may also be operated in RP-LC and HILIC systems. Nevertheless, the separation using pure water at room temperature as the only eluent is the most exciting and most "green" application. Unfortunately, in such a case, the selectivity of the separation results only from the stationary phase. However, there is an option to modify the temperature. Thus, designing and synthesizing new stationary phases with a different selection of polar and non-polar parts will certainly allow different selectivity, and will also lead to the optimization of techniques using pure water in liquid chromatography. These are options that can be implemented in the low-temperature range; broader possibilities appear when the water temperature is raised significantly.

5. Summary

The separation in liquid chromatography can be performed using pure water as a mobile phase. The application of pure water is environmentally friendly, and it is the best option for "green" chromatography.

Separations in purely aqueous conditions may be carried out at room temperature using specific polar-embedded stationary phases; however, in such a case, the selectivity depends almost entirely on the stationary phase. The second option is separation at elevated temperatures—superheated water chromatography. The change in water temperature changes its polarity, dielectric constant, viscosity, surface tension, and many other properties; as a result, the elution strength of water increases. Unfortunately, another problem may arise in this case—the thermal stability of both separated substances and stationary phases. Thus, several stationary phases based on silica gel, carbon, polymers,

or metal oxides were obtained for application in purely aqueous separation. More new stationary phases will likely be developed soon, and "green" liquid chromatography will gain popularity in the coming years.

For completely "green" chromatography, it is necessary to use only nontoxic, environmentally friendly solvents; such solutions include carbon dioxide, ethanol, and water. In this review paper, we focus on water; its use as the sole component of the mobile phase presents many challenges to chromatographers, including viscosity, high elution force, dielectric constant, degradation of stationary phases, hydrolysis, and decomposition of analytes. Thus, it is necessary to select either an appropriate analysis method—as we discussed in our previous work [9]—or an appropriate choice of stationary phase, as described in this work; often, the two can be combined. The most convenient option is to use a stationary phase that allows the use of water without increasing the temperature or using additives in the form of salts or buffers; such solutions include phases with silica, polymer, zirconium or aluminum oxide, or carbon supports. In recent years, silica-based phases with embedded polar groups have proven to be very promising; they are thermally stable and, through the mixed hydrophilic–hydrophobic nature of the surface, allow operation under pure water conditions without the use of additives or elevated temperatures. The goal is to find phases that allow us to simultaneously understand retention mechanisms, control selectivity (e.g., by selecting appropriate groups at the functionalization stage of the stationary phase), and obtain good resolution and efficiency. The phases mentioned above were found by attempting to change the support; in contrast, polar-embedded and polar-endcapped stationary phases open possibilities to manipulate the parameters via a good selection of polar and non-polar groups attached to the silica surface at the synthesis stage; this provides an extensive range of possibilities for the preparation of different phases. In our opinion, it is on these phases that research aimed at finding stationary phases to work under pure water conditions should focus.

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Solvent Influence on Zeta Potential of Stationary Phase—Mobile Phase Interface

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Abstract: Zeta potential is a surface characteristic formed on the solid surface and liquid interface. It is an interesting way to describe the surface properties of materials; thus, a series of four homemade polar embedded stationary phases that contain phosphate groups incorporated into hydrophobic ligands were investigated according to surface zeta potential. Measurements were carried out using Zetasizer Nano ZS for the stationary phases suspensions prepared in various solvent and solvent binary mixtures. The negative zeta potential values were obtained for most cases due to negatively charged residual silanols and phosphate groups. However, in some solvents: tetrahydrofuran, isopropanol, and toluene zeta potential are positive. Additionally, it was observed that the zeta potential seems to be independent of the type of silica gel used for the stationary phase synthesis.

Keywords: zeta potential; stationary phase; solvent; surface

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1. Introduction

Silica gel is the most common support of stationary phases synthesis. Despite the modification procedure, some portions of residual silanols remain unreacted at all times. The bonding of hydrophobic or polar groups to the silica surface influences the surface properties, changing their polarity. It also influences the zeta potential of such a surface when it is in contact with a liquid or a liquid mixture [1–3].

The zeta potential is the potential of the electric field created by surface charges in the point from which the liquid phase can move either by pressure gradient or by the action of the outer electric field [1,4]. From the practical point of view, the zeta potential is the critical characteristic of the electric double layer. Thus, this parameter is essential in describing the mechanisms occurring at the surface where the stationary phase is in contact with the liquid mobile phase. The creation of an electric double layer takes place in each liquid chromatographic separation and influences the retention and selectivity of the separation.

From the stationary phase point of view, the zeta potential corresponds to the surface charge of the stationary phase. It is evident for ion chromatography where stationary phases possess charges [5]; however, it is observed even for octadecyl materials [6] as a result of ionized silanol that was not reacted due to the steric hindrance [7]. For polar embedded stationary phases, the presence of electronegative atoms, such as nitrogen and oxygen, the polarization, and donor-acceptor interactions, also influence the zeta potential values [8]. Incorporating polar functional groups into the nonpolar ligands induces their different polarization and changes the ionization of the silanols on silica particles. This action affects the results obtained from zeta potential measurements and varies depending on the type of surface modification performed [8–10].

The zeta potential is affected by the type of liquid in which the silica is immersed. Various solvents solvate the surface differently; they differ in polarization and other physicochemical properties. A significant amount of solvent (e.g., water) may adsorb on the surface of the silica gel support [11]. For example, adsorbed water molecules support the ionization of surface silanols. It leads to negative charging of the silica gel surface. A

similar situation may be observed in other solvents that may solvates protons. In such a case, solvent properties may strongly influence the zeta potential of given materials. Additionally, it is postulated that zeta potential may be affected by the adsorption of ions from the solution, especially hydroxide ions [12].

According to DVLO theory, the main energy affecting the stability of a particle in solution is the potential energy consisting of the force of attraction and repulsion of particles between each other [13,14]. In a stable suspension, the attractive van der Waals forces and repulsive forces between the double adsorption layers of the particles are in equilibrium, and there is no coagulation of the particles. If the repulsive forces are insignificant, the particles will aggregate, and the suspension will not be stable. So, to ensure the stability of the suspension, it is necessary to take care of the dominant effect of the repulsion energy of the double adsorption layers between the particles. It is possible to provide by steric or electrostatic repulsion [15]. In the case of silica grains of 5 um size, where the particle surface is modified with the phosphodiester group and attached hydrophobic groups, it is difficult to speak about steric repulsion. Therefore, electrostatic repulsion will play an important role. It is connected with the silica surface's physicochemical character and the solvent properties in which the suspension is prepared.

The performance of a chromatography column depends on many determinants. One of them is the quality of packing of the stationary phase bed in the column. This quality is affected by several different factors. Among them, one of the more important is the proper choice of slurry and packing solvent. In their work, Vissers et al. [16–18] confirm that the suspension's stability is not necessarily related to the complete absence of aggregation. Various techniques to ensure stability can be found in the literature but mainly involve balancing the suspension density, chemical, and mechanical stabilization, or the use of high or low viscosity solvents [18]. Research confirms that the presence of particle aggregation in suspension while ensuring its stability is favorable [17,19]. Among other things, zeta potential measurements allow one to determine the stability of a suspension without determining whether or not aggregation occurs in solution [15,17].

During the last years, zeta potential measurements became one of the methods to characterize chemically bonded stationary phases [6,20]. The easiest way is to determine the zeta potential based on electrophoretic mobility measurement (μ). The calculation can be done using the Smoluchowski, Hückel, or Henry equation [4,21,22]. They differ by considering the ratio of the radius of the test particle to the thickness of the electric double layer (EDL)-κa. Henry's equation is expressed as:

$$\mu = \frac{2}{3}\frac{\varepsilon_r \varepsilon_0 \zeta}{\eta} \cdot F(\kappa a)$$

where μ is electrophoretic mobility, η is the viscosity of the solution, ε_r is the relative permittivity of the medium, ε_0 is the absolute permittivity of vacuum, ζ is zeta potential, and F(κa) is Henry's function. If the value of the Henry function F(κa) is 1.0, this means that κa is much smaller than 1, and therefore, the thickness of the electric double layer is much larger than the radius of the test particle. In this case, the Hückel equation is used.

$$\mu = \frac{2}{3}\frac{\varepsilon_r \varepsilon_0 \zeta}{\eta}$$

On the opposite side of the calculation, we have a situation in which the particle's radius is much larger than the thickness of the electric double layer so that κa is much larger than 1.0, and the Henry function F(κa) is equal to 1.5. The Smoluchowski mathematical approximation is then used.

$$\mu = \frac{\varepsilon_r \varepsilon_0 \zeta}{\eta}$$

In an intermediate situation, i.e., a kappa value slightly greater or less than 1, calculations are used to precisely determine the Henry function. One of them could be the

Ohshima approximation or the O'Brien calculation [23–25]. The size of the test particle in the approximation is known and constant. At the same time, the thickness of the electrical double layer depends on the type of solvent and mainly on the presence of ions in it, that is, the ionic strength. A higher concentration of ions decreases the thickness of EDL, while a low concentration makes this layer thicker. Of course, when ions are present in the solution, the pH of the suspension solution will also play a significant role [4,23,26].

In our study, particles of 5 μm size are used, suspended mostly in organic solvents. Therefore, it is difficult to talk about the presence of ions in solution and ionic strength. The main role of charge distribution near the surface and in bulk is performed by functional groups or atoms of molecules with free electron pairs. With such large particles as 5-micron silica grains and poorly ionized solvents, the use of the Smoluchowski equation for zeta potential calculations is justified [21,27]. The zeta potential value is calculated according to the formula:

$$\zeta = \frac{\mu\eta}{\varepsilon_r \varepsilon_0}$$

Various chromatographic packings were investigated according to their zeta potential in chromatographic conditions. Materials with chemically bonded alkyl groups, with and without end-capping, and other novel stationary phases were tested. Pure silica gels were compared with silica-hydride materials. [5,6,8–10,28,29]. In addition to chemically bonded ligands, which has the most crucial influence on zeta potential, other studies were performed: (i) the coverage density of bonded groups [6], (ii) the ionization of chemically bonded functionalities [5,6], (iii) the influence of electronegative atoms (polar groups) in the structure of bonded moieties [8], (iv) the impact of the mobile solution composition, pH of the solution, and ionic strength [2,9,10], (v) the formation of water enriched layer [30–32], and (vi) hydroxide ion adsorption on the stationary phase surface [33–35].

The stationary phase zeta potential was usually tested in methanol, acetonitrile, water, and its mixture in the previous works. However, it seems reasonable to check how different solvents influence the zeta potential of stationary phases. Thus, the goal of our study was to determine the zeta potential of four polar embedded stationary phases with different organic moieties in the presence of 16 different solvents and solvent mixtures. It allows obtaining information about slurry properties used for column packing. Although, as reported in the literature [36], theoretically, the zeta potential results are ideal at infinite sample dilution. In reality, there is a limit to the ratio of solvent molecules to measured particles, which depends on the particle size. There is also an upper limit of sample concentration which, if exceeded, makes the measurement of zeta potential more complicated; however, it is individual and depends on the type of sample, the size of grains, the light transmission through the sample, or the polydispersity of the particle [37]. In our study, the concentration was increased. It is known that the suspension's aggregation and stability depend on the concentration of solute and particles [26,38]. So, increasing the stationary phase concentration in our study is due to practical aspect as high-concentration suspension is used for packing chromatography columns. Hence, the results obtained more adequately relate to the existing solutions used for column packing.

2. Results and Discussion

The stationary phases tested in the study contain a relatively low carbon load compared to typical reversed-phase materials. The first reason was that we wanted to obtain a relatively weakly hydrophobic stationary phase to allow the elution in purely aqueous conditions. The second reason is that ligand binding with an ionized (or at least highly polar) phosphate group does not allow high coverage due to electrostatic repulsion.

Sixteen different solvent and solvent mixtures were used in the study. Solvent and its mixtures, viscosities, dielectric constants, and refractive indexes are listed in Table 1. Data from the table were used for zeta potential determination using Zetasizer Nano ZS. The research aims, among other things, to determine the stability of stationary phase suspensions in solvents of different polarities and viscosity. These results will allow the

appropriate selection of solvents for slurry preparation and the choice of packing solvent when packing chromatographic columns.

Table 1. Characteristic of solvents used in the study.

Solvent/ Mixture Number	Solvent/Solvent Mixture (1/1 *v*/*v*)	Viscosity μ [cP] (20 °C)	Dielectric Constant ε_r (20 °C)	Refractive Index RI	Literature
1	Dichloromethane	0.43	9.08	1.424	[39,40]
2	Tetrahydrofuran	0.55	7.60	1.407	[40]
3	Hexane	0.31	1.89	1.375	[40]
4	Isopropanol	2.86 (15 °C)	18.3 (25 °C)	1.377	[40]
5	Chloroform	0.85	4.81	1.446	[40]
6	Acetone	0.32	20.7 (25 °C)	1.359	[40]
7	Toluene	0.59	2.4 (25 °C)	1.496	[40]
8	Methanol	0.55	32.6 (25 °C)	1.329	[40]
9	Acetonitrile	0.37	37.5	1.344	[40]
10	Water	1.00	78.54	1.333	[40]
11	Acetonitrile/Water	0.81	58.02	1.336	[40,41]
12	Isopropanol/Water	2.58	48.42	1.355	[40,42]
13	Isopropanol/Chloroform	0.64	11.6	1.411	[40,43]
14	Methanol/Isopropanol	0.97	25.5	1.353	[40,44]
15	Methanol/Chloroform	0.65	18.7	1.383	[40,45]
16	Methanol/Water	1.54	78.5	1.340	[40,41]

The stationary phases zeta potential measurement results in different solvents and solvent mixtures are presented in Figure 1. It has to be emphasized that there is no data on the plot for some solvents, mostly hexane (3) and water (10). It is a result of suspension instability that disallows the zeta potential measurement.

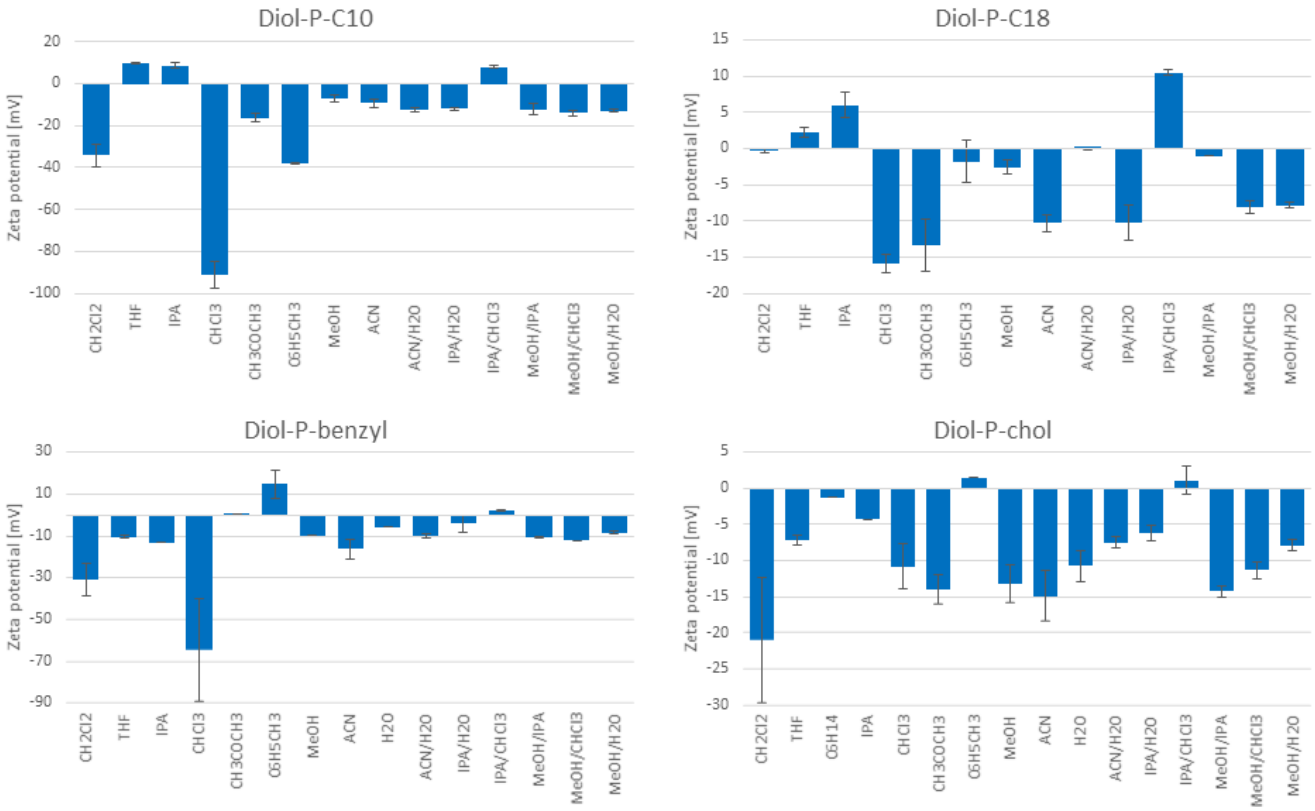

Figure 1. The zeta potential of stationary phases in different solvents with standard deviations (mV).

At first look, it can be seen that both positive and negative values of zeta potential were observed. In the case of octadecyl stationary phases (results published earlier [6]), independent of coverage densities, usually negative values were obtained regardless of the type of organic modifier (methanol or acetonitrile), or the water content in aqueous-organic solvent mixtures.

Negative zeta potential values indicate the accumulation of positive charges near the particle's surface and, therefore, the electrically negative nature of the particle itself. In the case of phosphate embedded stationary phases tested in the study, negative zeta potential values may be caused by the partially ionized residual silanols and an ionized phosphate group. Residual silanol groups can possess different acidities [7,46]. More acidic with a pKa value between 3.5 and 4.6 are vicinal silanols. The single silanols are less acidic than vicinal silanols, with a pKa between 6.2 and 6.8 [46,47].

Since all tested stationary phases were synthesized on a silica support, a negatively charged surface will influence the zeta potential of all stationary phases. From the initial silanols on silica gel surface equaling 7.7 μmol/m^2 determined in the previous study [48], around half are reacted or shielded by bonded ligands. Nevertheless, at least 3 μmol/m^2 of silanols are available for interactions, and some of them can ionize while affecting the negative zeta potential values. Additionally, this effect may be enhanced by the presence of phosphates. However, different functional groups used for silica modification may weaken the influence of silanols on the silica support on the zeta potential due to different interactions with solvent molecules. In some solvents, tetrahydrofuran (2), hexane (3), isopropanol (4), and toluene (7), zeta potential values are positive. Positive values were also observed for all stationary phases in the isopropanol/chloroform mixture (13). It is observed mainly for the Diol-P-C10 and Diol-P-benzyl stationary phases. Also, the highest negative values were observed for chloroform for these materials.

From a suspension stability point of view, the criterium of zeta potential is higher than ±30 mV. If the value is lower than ±30 mV, the suspension may not be stable. In the data obtained, the values are usually lower than ±30 mV. Most of the results are in the range of ±20 mV. The most stable suspensions were obtained in chloroform, reaching up to −91 mV. As determined by zeta potential measurements, suspension stability does not imply a complete absence of aggregation. Suspended solids may aggregate into small aggregates while maintaining suspension stability. Therefore, the interpretation of the obtained results should lean toward the evaluation of suspension stability rather than statements determining the presence or absence of aggregates. Suspension stability can also be obtained by balancing the solvent and dispersed phase density or using a high viscosity solvent. Determining the stability of the suspension allows for a good choice of slurry solvent, which is one of the essential parameters when efficiently packaging chromatography columns.

Although it is known that zeta potential is not a direct measure of a surface charge, it may give some information, such as which surface attracts ions. However, we can explain the negative zeta potential values by negative charges on the surface. In theory, the highest negative charge should be observed in proton-acceptor solvents, e.g., water, which solvates protons and enhances the ionization of silanols and phosphates. The highest negative values are observed for chloroform, which does not meet these conditions. This case may be related to the non-zero dipole moment of the chloroform and its significant number of free electron pairs present at the chlorine atoms. Adsorption of chloroform molecules on the surface of the phase can cause high negative charge accumulation, which is manifested by high negative zeta potential values. It shows that values of zeta potential are difficult to predict without measurements.

Indirect correlation between surface charge and zeta potential explains the positive values obtained in measurement. Based on the stationary phases' surface properties, there are no positive charges on them. Additionally, no acceptor atoms can accept protons such as nitrogen and provide a positive surface charge [8]. Thus, the positive values at the stationary phase with an embedded phosphodiester group and a nonionic solvent must be

due to the accumulation of excess positively charged molecules on the particle surface. This occurs with reduced dissociation of free silanols and phosphate groups, which depends on the type of solvent. In this case, positive zeta potential values are caused by more complicated phenomena, such as solvation, accumulation of some ions from solution, etc. Positive zeta potential values were also obtained in our previous work [6]. The use of a hydrophobic phase (C18) and a protic solvent, such as methanol, nevertheless gave a positive zeta potential value. This confirms that some kind of phenomena occurring on the surface of stationary phase particles are more complicated and attempts to predict and describe them are only theoretical assumptions.

Another problem in comparison may be caused by stationary phase wettability that is somewhat different in various solvents. It has to be emphasized that the silica gel used in the study is porous, with a specific surface area of 320 m^2/g. This surface is mainly located in pores, and the accessibility to these pores may be different, changing the solvents. Porosity causes energy inhomogeneity of the surface. According to Ståhlberg [49], the distance from the surface at which the electrostatic potential in the electric double-layer approaches zero may be as large as 30 nm. It means that the potential may be present in the total inner pore volume of particles commonly used in packed columns for HPLC with a pore diameter around 10 nm. When measuring electrophoretic mobility, the double electrical layer that forms at individual pore walls can overlap, causing "clogging" of the pore. Therefore, much of the charge can be compensated inside the pores of the particle or in the empty spaces between particles in aggregates. Only the part coming from ionized groups on the outer particle surface will be responsible for the electrophoretic mobility. Of course, it changes depending on the solution composition. Nevertheless, according to Smoluchowski's theory, the zeta potential is a global value, referring to whole particles or aggregates.

To interpret obtained results, the corresponding stationary phase was synthesized using a different support, Luna 100 Å instead of Kromasil 100 Å. The comparison of these two phases is presented in Figure 2. The shape of the radar plot for both stationary phases is very similar. The most crucial difference was that for Kromasil there was not possible to measure the zeta potential of the material in hexane and water suspensions (solvent no. 3 and 10 are omitted in the plot). It shows that zeta potential is reproducible if the surface physicochemical properties are similar. The convergence of the result trends is associated with the same surface modification. However, the different initial properties between Kromasil and Luna result in slight differences. The pure Kromasil and Luna silica zeta potential results on a DTS1070 capillary cell in pure water gave -45.8 ± 2.23 mV and -46.9 ± 0.78 mV, respectively. Numerically, these results cannot be compared with those obtained from measurements using a dip cell. On the other hand, comparing them indicates that the type of silica support used does not significantly affect the zeta potential value, so the surface modification influences the differences between the values obtained for the Diol-P-C10 phase on Kromasil and Luna.

It has to be emphasized that the similarities observed are in character and value. A significant difference was observed only for tetrahydrofuran (2) and toluene (7). In the case of tetrahydrofuran, a positive value was observed on Kromasil Diol-P-C-10, and on Luna Diol-P-C-10, it was negative. Kromasil Diol-P-C-10 exhibits a significant negative value for toluene, whereas on Luna Diol-P-C-10, the value was almost 0. The slight differences between the results may be due to differences in the coverage density of the two base materials. The different amounts and activity of free silanols may affect solvent solvation processes at the surface of silica grains.

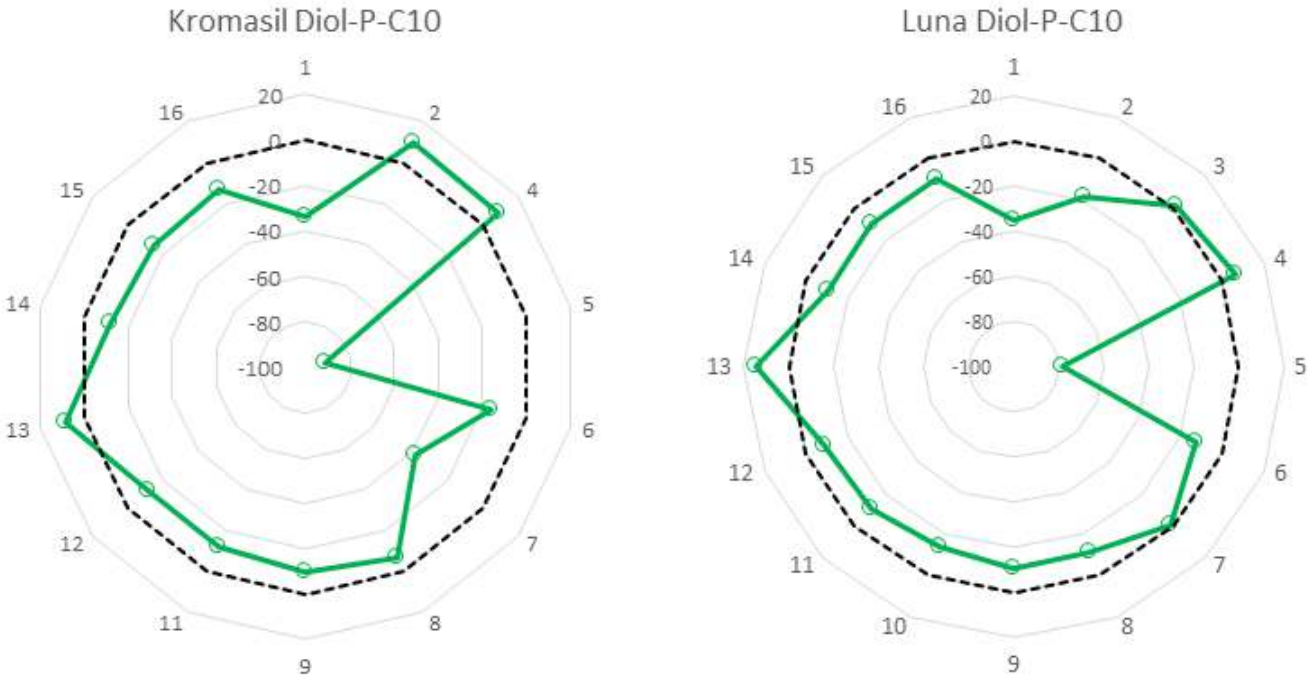

Figure 2. Comparison of zeta potential for stationary phases synthesized on a different support.

3. Materials and Methods

3.1. Materials

A series of polar embedded stationary phases that contain phosphate and hydrophobic functional groups were tested. As a support for the synthesis, the Kromasil 100 silica gel (Akzo Nobel, Bohus, Sweden) was used. For the comparison, some stationary phases were also synthesized on Luna (Phenomenex, Torrance, CA, USA). Detailed characteristic of silica gels used for synthesis is presented in Table 2.

Table 2. Characteristic of silica gels used in the study.

Parameter	Kromasil 100	Luna
Particle size [µm]	5	5
Specific surface area [m^2/g]	320	400
Average pore size [nm]	11	10
Pore volume [cm^3/g]	0.9	1.0

Four phosphodiester bonded stationary phases were tested. Structures of the materials are shown in Figure 3.

The properties of the stationary phases are listed in Table 3. Two Diol-P-C10 were synthesized on two different supports: Kromasil and Luna.

Reagents for the stationary phase synthesis: (3-glicidoxypropyl)trimethoxysilane, decanol, octadecanol, cholesterol, benzyl alcohol, and phosphoryl chloride were purchased from Alfa Aesar (Karlsruhe, Germany). Organic solvents used during synthesis: toluene, methanol, and hexane were ACS grade, purchased from Avantor Performance Materials (Center Valley, PA, USA).

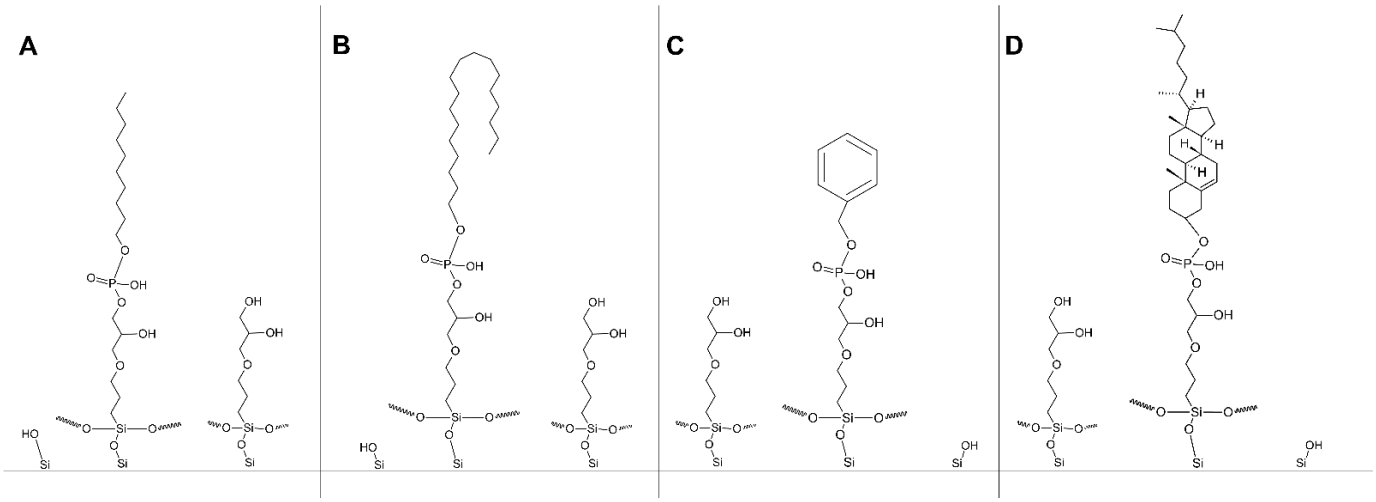

Figure 3. Schematic representation of stationary phase structures with embedded phosphodiester groups. (**A**) Diol-P-C10, (**B**) Diol-P-C18, (**C**) Diol-P-Benzyl, and (**D**) Diol-P-Chol. Each diagram also presents the structures of unreacted diol and residual silanols.

Table 3. Characteristic of stationary phases used in the study.

Stationary Phase	Carbon Load [%]	Coverage Density [μmol/m^2]
Diol-P-C10	3.43	0.56
Diol-P-C18	4.18	0.42
Diol-P-Benzyl	2.86	0.56
Diol-P-Chol	9.31	0.87
Luna-P-C10	4.58	0.51

3.2. Stationary Phase Synthesis

Before the chemical modification of silica gel, a sample of adsorbent was placed in a glass reactor protecting against the contact of the reagents with the external environment. Silica gel was dried at 180 °C under vacuum for 12 h. Then, the temperature was decreased to 90 °C, and (3-glicidoxypropyl)trimethoxysilane was added. After 12 h, the reaction products were washed out with toluene, methanol, and hexane and dried.

The obtained material was treated with 1% sulfuric acid to hydrolyze the epoxide ring in the second step. After the hydrolysis, the diol-bonded stationary phase was washed in water and methanol and dried under a vacuum.

Further, the diol-bonded silica was placed in a glass reactor and heated up to 100 °C for 10 h. Next, dried material was modified using phosphoryl chloride and proper alcohol: decanol, octadecanol, cholesterol, or benzyl alcohol to obtain Diol-P-C10, Diol-P-C18, Diol-P-Chol, and Diol-P-benzyl, respectively. Dried diol-modified silica was suspended in dry toluene. Next, the solution of phosphoryl chloride and alcohol was added. Reactions were carried out with the addition of triethylamine at 65 °C during 12 h under reflux. The reaction products were washed out with toluene, methanol, and hexane. Synthesized material was dried under a vacuum.

3.3. Instruments

The zeta potential measurement was performed using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with a dip cell. Dip cell was equipped with a quartz cuvette. A Malvern DTS1070 capillary cell was used to measure suspensions of pure silica in water.

3.4. Methods

For each measurement, around 5 mg of the stationary bonded phase was suspended in 1 mL of solvent or solvent mixture using an ultrasonic bath for 10 min to help to obtain a

stable suspension due to removing air from pores. Sample concentrations are increased over standard zeta potential measurements to make the results more relevant for the practical use of slurries in packing chromatography columns where the slurry concentration is high. The zeta potential measurement was done immediately after removing the suspension from the ultrasonic bath. The zeta potential measurement temperature was 25 °C. Each sample was measured three times, and the standard deviation was calculated from the results.

Before measurement, the stability of the suspension was routinely tested by Zetasizer. In the case of good suspension, the stationary bonded phase's zeta potential in solutions was automatically calculated using Smoluchowski's equation by Zetasizer from electrophoretic mobility. A detailed description was provided in the previous studies [5,6].

4. Conclusions

Five phosphate embedded stationary phases were tested according to the zeta potential in various solvents and solvent mixtures. The negative zeta potential values were obtained for most cases due to negatively charged residual silanols and phosphate groups. However, in some solvents, tetrahydrofuran, isopropanol, and toluene zeta potential get positive values, which may result from solvation phenomena and adsorption of ions from the solution. Additionally, it was observed that the zeta potential seems to be independent of the type of silica gel used for the stationary phase synthesis.

The results obtained will allow the appropriate selection of solvents to prepare stationary phase slurries used in the chromatography column packing procedure. As mentioned in the text, the appropriate choice of solvent is based on the formation of a stable slurry and the stationary phase. According to the work of Vissers [16–18], packing efficiency depends on both the aggregation of the stationary phase particles in the suspension and the stability of the slurry. For the most suitable choice to be made, research must continue in terms of aggregation evaluation using optical microscopy and chromatographic studies performed on the efficiency of columns packed with stationary phases from different slurry solvents. Such studies are planned to be performed by our team. The results will be able to more precisely answer the question regarding the proper solvent for packing columns with stationary phases with embedded phosphodiester groups.

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RESEARCH ARTICLE

Optimization of the packing process of microcolumns with the embedded phosphodiester stationary phases

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The development of new home-made stationary phases involves their packaging procedure and is crucial to obtain satisfactory working parameters. The parameter that illustrates the quality of the packed bed is its efficiency measured as the height equivalent to the theoretical plate. According to the Van Deemetr's equation, it depends on three factors, but only one of them, eddy diffusion, does not depend on the linear flow velocity. Therefore, in order to obtain it as low as possible, it is necessary to focus on a good filling of the column. Among many parameters affecting the quality of column packing, in our work we have focused on the choice of slurry solvent. Novel stationary phases with an embedded phosphodiester group were investigated. The suspensions in 16 solvents and solvent mixtures were studied for their stability, aggregation, sedimentation, and viscosity comparison. The efficiency of the packed microcolumns and its comparison was determined by chromatographic analyses using a polar (thymidine) and a nonpolar compound (naphthalene). The results obtained led to the conclusion that for these stationary phases, the best slurry solvent is the one that aggregates the phase while maintaining stability and having high viscosity.

KEYWORDS
efficiency, liquid chromatography, microcolumn packing, polar-embedded stationary phase, slurry solvent

1 | INTRODUCTION

The separation of compounds is what chromatography is all about and the chromatographic system's specific "heart" is a chromatographic column filled with a stationary phase. The parameter that describes the "quality" of the separation is column's resolution which, according to Purnell equation, depends on the selectivity and efficiency. On the one hand, selectivity, being a thermodynamic parameter, depends on the interactions of the solutes with the stationary phase surface in the presence of a given mobile phase. On the other hand, the efficiency can be connected with the physical properties of the stationary phase particles themselves (size, size distribution, shape, sphericity, porosity) and their arrangement (favorably dense and homogeneous) along the column and in its cross-section [1, 2]. Thus, for the chromatographer, the height equivalent to the theoretical plate is an important indicator of the quality of the packed bed. According to Van Deemeter's equation (Equation 1), three factors influence the height equivalent to a theoretical plate: multiple analyte paths term (eddy diffusion – parameter A), longitudinal diffusion term (B), and resistance to mass transfer term (C) [3]. While the last two can be influenced during the chromatographic analysis itself (mobile phase com-

Article Related Abbreviation: RPLC, reversed phase liquid chromatography.

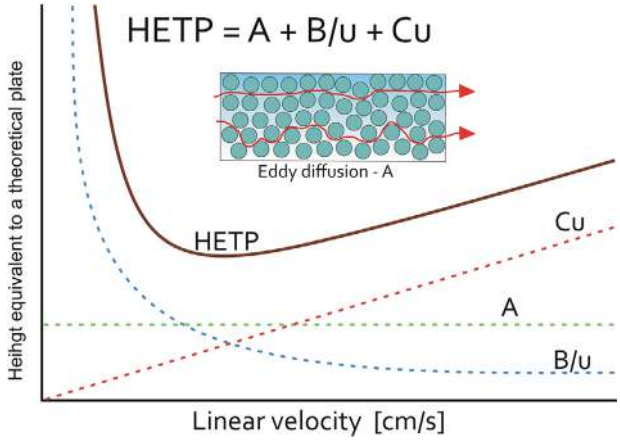

FIGURE 1 Presentation of the individual components of the Van Deemter equation and a schematic illustration of the eddy diffusion process occurring in a chromatographic column packed with particle grain filling

position, flow rate), the *A*-term is constant and depends on the quality of the stationary phase bed, which directly depends on the column filling process (Figure 1).

$$\mathrm{HETP} = A + \frac{B}{u} + \mathrm{Cu}, \tag{1}$$

where u is linear velocity of mobile phase ($\mathrm{cm \cdot s^{-1}}$).

In order to compare the efficiencies of columns of different lengths and particle diameters, it is necessary to implement testing and data description procedures so that the compared values can be unified. Bristow and Knox [4] already developed such a procedure based on reduced variables in the 1970s. These include reduced height equivalent to the theoretical plate (h), reduced mobile phase velocity (ν), dimensionless flow resistance parameter (ϕ), column capacity factor (k'), reduced column length (l) or a value allowing general column efficiency comparison, which is dimensionless separation impedance (E) (Equation 2; [2, 5–7]).

$$E = h^2 \phi. \tag{2}$$

As early as the 1970s, Unger described factors affecting column quality during filling it with stationary phase particles [8]. The following have been regarded as essential: (1) packing the particles as densely as possible, (2) the interparticle spaces should be as uniform in size as possible to ensure homogeneity of the flow, (3) avoidance of uneven packing density across and along with the column—homogeneity of the cross-section and longitudinal section. During the development of liquid chromatographic columns, two main filling techniques were used: dry filling procedure and wet filling using stationary phase slurry. The former included packing methods by tamping the bed or rotating the dry matter in the column. Dry filling technique can be used to particle size down to ca. 20 μm. For smaller particles, slurry filling method was developed. It was important for the solvent to limit intermolecular interactions (no coagulation occurring) and sedimentation. The general selection of a suitable solvent depended on the type of silica. Pure silica, a hydrophilic material, was prepared in more polar solvents, while silica modified for the reversed-phase LC was prepared in nonpolar solvents [9].

Essential points during the filling procedure were also highlighted in the literature. Preparing the material by cleaning it with fines and smaller particles helps prevent the frit from being clogged during packing. The use of a balanced-density slurry helped avoid sedimentation of the stationary phase and its aggregation. There are also emerging methods that use stirring of the slurry during the packing process to make the particles float in the liquid. The solvent used should wet the silica surface to remove air bubbles from the pores, the presence of which could lead to inhomogeneity of the packed bed. Another parameter to select the slurry solvent can be its high viscosity to reduce the settling velocity of the spherical particles. However, in this case the packing process will be longer. The suspension should also have an appropriate concentration. Initially, a concentration of 1%–30% (w/w) was recommended, and a concentration of 10% was optimal for packing at pressures of 400–500 bar. Nevertheless, using too high pressure with modern stationary phases can lead to the formation of fine particles from the broken stationary phase beads [9, 10]. The last and crucial step in packing is the exchange of solvents in the column and conditioning under the conditions in which it will operate [8].

Extensive research on different aspects of filling methods for capillary LC columns of various diameters was performed by Vissers [11–14]. His research tended to avoid coagulation; however, both slurry packing techniques from balanced and unbalanced density, chemically and mechanically stabilized, high and low viscosity solutions were used. Initially, his team obtained results showing a predominance of noncoagulating suspensions. The results were consistent with zeta potential measurements, and the conclusions drawn were aimed at individually optimizing the suspensions and packing conditions of each stationary phase [11]. It was not until subsequent years that studies by both the Vissers [11, 12] group and the Shelly and Edkins group [15] showed results favoring solvents coagulating the stationary phase. The studies also confirmed the relevance of bed compaction and, therefore, bed conditioning under operating conditions with a connected reservoir to fill any spaces that may arise due to slight bed rearrangements [14].

In the following years, it was explained why it is important to use solvents that provide coagulation while avoiding too rapid sedimentation to achieve high bed

homogeneity. Cross-sectional heterogeneity makes the analyte flow paths through the bed diverse, leading to high eddy diffusion and differences in local flow rates between the bed near the column walls and the center. Particles suspended in low viscosity solvent at high pressures can forcefully exchange particles already arranged in the bed, leading to a more packed bed. The high aggregation of particles in the solvent promotes reduced selective segregation of smaller diameter particles near the column walls and larger particles in the center of the bed. In addition, high pressure, which is an acceleration of the first packing step, allows particles to settle faster and not exchange already packed particles. Large aggregates ensure the randomness of particle size arrangement and reduce particle rearrangement leading to radial homogeneity. Comparing measurements of sedimentation velocity, zeta potential and optical microscopy, the latter gives results that are most consistent with measurements of column efficiency relative to particle aggregation [16, 17].

Other factors that increase the performance of the packing process as measured by the efficiency of the columns obtained are the sonication of the suspension and the use of a high concentration slurry [18, 19]. Suspension at a 200 mg/ml concentration allows for a reduction of the interparticle spaces. It, therefore, enables a reduction of the eddy diffusion in the transchannel and short-range intrachannel ranges. Nevertheless, maintaining all these conditions for preparing the suspension for packing in the Resing study [20] showed different column efficiencies at the column's inlet, center, and outlet. This is due to a decrease in particle–particle interactions as the packing process progresses. The outlet part of the bed has the best efficiency since there are most interactions at the beginning of the process.

In our group, we have been working on developing a wide range of new stationary phases for liquid chromatography so the column filling process is of high importance in our laboratory practice. Some of the newly developed chromatographic materials containing polar and nonpolar groups do not follow general indications for slurry solvent selection. Hence, this work aimed to study slurry solvents that allow efficient filling of polar-embedded stationary phases. Solvent selection is based on zeta potential measurements, microscopic observations, and viscosity. A comparison of packed capillary columns was performed based on chromatographic analyses under standardized conditions [21]. The material was packed into capillaries of the same diameter. Furthermore, the analyses were performed on the same liquid chromatograph, so there is no need to determine the separation impedance since comparing the height equivalent to the theoretical plate (H) or the reduced plate (h) already allows to compare the quality of the packed bed.

2 | MATERIALS AND METHODS

Four stationary phases with embedded phosphodiester groups were used in this study. The silica support was Kromasil 100Å silica (Akzo Nobel, Bohus, Sweden), with a diameter 5 μm, a specific surface area of 320 m^2/g, an average pore size of 11 nm, and a pore volume of 0.9 cm^3/g. Each stationary phase has a polar phosphodiester group attached to it and an organic molecule: a decyl chain (Diol-P-C10), an octadecyl chain (Diol-P-C18), an aromatic ring (Diol-P-benzyl), and a cholesterol molecule (Diol-P-chol) (Figure 2). Our previous publication has included the detailed synthesis procedure and a complete characterization of the above-mentioned phases [22]. Some of the more important phase parameters for this work are summarized in Table 1.

Nine organic solvents were used to prepare stationary phase slurries: dichloromethane, tetrahydrofuran, hexane, isopropanol, chloroform, acetone, toluene, methanol, and ACN (J.T. Baker, Deventer, The Netherlands). In addition, a slurry was also prepared in water that was purified using Milli-Q system (Millipore, El Paso, TX, USA) in our laboratory. From the mentioned solvents, six binary mixtures were prepared: ACN/water, isopropanol/water, methanol/water, isopropanol/chloroform, isopropanol/methanol, and chloroform/methanol to prepare further suspensions. A total of 16 slurries were prepared: 10 with single solvents and six from binary mixtures.

High-purity ACN "for HPLC" purchased from J.T. Baker (Deventer, The Netherlands) was used for chromatographic analyses. Water was purified using the Milli-Q system (Millipore, El Paso, TX, USA) in our laboratory. Before analyses, all solvents for sample preparation and chromatographic analyses were degassed using an ultrasonic bath. For chromatographic analyses, naphthalene (Sigma-Aldrich, St. Louis, MO, USA) and 2′-deoxythymidine (Applichem, Darmstadt, Germany) were used as analytes.

Microscopic observations were made using a fluorescence stereomicroscope (Olympus, SZX16, Tokyo, Japan) equipped with a charged-coupled device camera and AppliChem a personal computer with the CELL software for data collection.

Capillary columns were filled using a slurry method. Fused silica capillaries of 400 μm of inner diameter and 794 μm (1/32″) of outer diameter were purchased from Polymicro representative CM Scientific Ryefield (EU) (Dublin, Ireland). The capillary columns were slurry-packed using a set-up consisting of a DSF-122 high-pressure air-driven pump (Haskel, Burbank, USA), a slurry reservoir (100 × 2 mm), a manometer, and a cut-off valve.

All chromatographic measurements were performed on a capillary LC system consisting of a pump delivering the

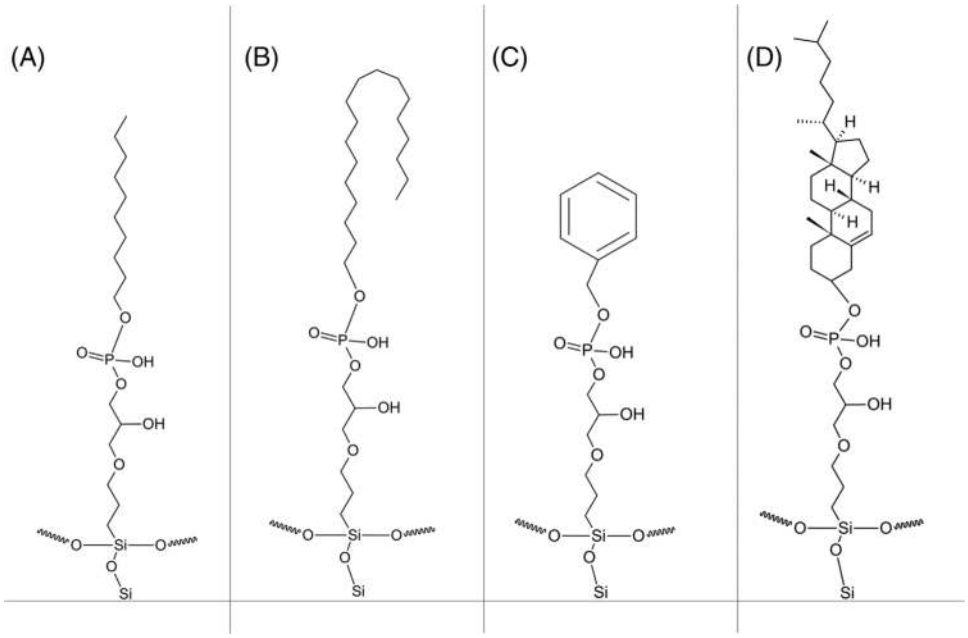

FIGURE 2 Structures of stationary phases with embedded phosphodiester groups. Diol-P-C10 (A), Diol-P-C18 (B), Diol-P-benzyl (C), and Diol-P-chol (D)

TABLE 1 Characteristics of stationary phases with embedded phosphodiester groups used for the study

Stationary phase	Carbon load (%)	Coverage density ($\mu mol/m^2$)
Diol-P-C10	3.43	0.56
Diol-P-C18	4.18	0.42
Diol-P-benzyl	2.86	0.56
Diol-P-chol	9.31	0.87

mobile phase (Agilent 1260 cap pump with degasser, Agilent Technologies, USA), 10-port valve with a microelectric actuator model C72MX-4694EH (Vici Valco Instruments Inc. Co., Houston, TX, USA) with a 50 nl capillary loop, and a set of connecting capillaries (TSP capillaries of various diameters from Polymicro Technologies). The detection was performed using the Spectra-100 (Thermo Separations Products, San Jose, CA, USA) detector equipped with a removable cell designed for in-capillary detection. The chromatographic system was controlled, and the data were collected by the Clarity software (Data Apex, Prague, Czech Republic).

2.1 | Methods

2.1.1 | Microscopic observations

Slurries of the stationary phases were prepared with 5 mg of material and 1 ml of solvent or solvent mixture in 1.5-ml glass vessels. Samples were sonicated for 10 min before microscopic observations were made. Then 30 μl of the suspension was immediately placed on a primary slide and covered with a coverslip. Some of the observation photographs could not be taken because of the rapid solvent evaporation or the coincident refractive index of the silica and the liquid phase.

2.1.2 | The capillary column packing procedure

To prepare the capillary, a long section (ca. 3 m) of the capillary was washed thoroughly with (consecutively): dichloromethane, acetone, water, acetone, and dichloromethane, and after that, it was dried in the stream of nitrogen for 30 min. This long section was then cut into smaller, ca. 20 cm long pieces: the column blanks. The outlet internal frit was prepared in each empty column according to the procedure described in Ref. [23] with a slight modification consisting that the sodium water glass solution was used instead of potassium water glass. The initial length of the frit was 2.5 cm, and after the packing procedure was complete, it was cut to the final length of 0.5 cm. The slurry was prepared by suspending 50 mg of the stationary phase in 0.47 ml of the proper solvent and sonicating it for 15 min. Then the slurry was transferred to the slurry reservoir with the capillary column blank attached to its outlet. The columns were packed for 120 min at 40 MPa. After that time, the air pressure was

released, and the column with the reservoir was left for slow depressurization. The packed capillary and the connected reservoir were then connected to the μHPLC system for column conditioning. The flushing was carried out at different mobile phase concentrations of ACN/water, starting with a 90/10 (v/v) composition, through 50/50, and ending with 10/90. Each flushing step lasted 60 min at a pressure of 100 bar. Finally, the column was left in 100% ACN. The capillary inlet after the packaging process was not closed with a frit. The conditioning step was intentionally carried out with the reservoir connected and over a wide range of concentrations of the ACN–water mixture. If the bed settled, the excess stationary phase held in the reservoir would fill in any potential gaps in the capillary. The conditioning helped to avoid the possible settlement of the bed during chromatographic analyses.

2.1.3 | Chromatographic analysis

Chromatographic analyses were performed in the solvent solution of ACN/H_2O. For polar substances, where retention on stationary phases occurs according to the HILIC (hydrophilic interaction liquid chromatography) mechanism, the mobile phase composition was 80/20 (v/v, ACN/H_2O), while for nonpolar substances, where retention occurred according to the reversed-phase liquid chromatography (RPLC) mechanism, the mobile phase composition was 30/70 (v/v, ACN/H_2O). The dead time was determined using potassium nitrate. All analyses were performed at a flow rate of 2 μl/min and a valve opening time of 0.005 min. The detection wavelength of naphthalene was 220 nm, while that of thymidine and potassium nitrate was 200 nm. All chromatographic analyses were performed in triplicate.

3 | RESULTS AND DISCUSSION

In order to properly select solvents for column packing, measurements of zeta potential, published and discussed in a previous publication, observations and descriptions of suspensions using optical microscopy, and solvent viscosity calculations were taken into account.

Based on the literature that aggregation is a desirable process, slurry solvents were selected that yielded aggregating and nonaggregating suspensions based on microscopic observations and zeta potential measurements. This criterion was chosen to compare aggregation when packing stationary phases with embedded phosphodiester groups. The zeta potential results are presented as radar plots (Figure 3), allowing the determination of a stable solvent according to the criterion accepted in the literature that a value of ± 30 mV indicates a stable slurry [24]. A detailed description of the results obtained with zeta potential measurements, their description, and analysis have been published in a previous paper by our team [22], so they are not discussed in detail here [25].

Complete results from the microscopic observations of the slurry aggregation and visual assessment of sedimentation are included in Table S1. For the Diol-P-C10 phase, chloroform and methanol solvents were chosen based on zeta potential measurements, while isopropanol and ACN were chosen based on microscopic measurements. Initially, both an aggregation-inducing solvent and a nonaggregation-inducing solvent were chosen to confirm the validity of this factor based on more recent literature reports [17] when using new LC stationary phases. The optical microscope images showing the aggregation of modified silica grains are presented in Figure 4. A significant aggregation is observed for the slurry for which isopropanol and methanol were used. The lack of aggregation was observed when ACN was used. It was impossible to make a microscopic picture of the suspension with chloroform due to the rapid evaporation of the solvent; however, direct observation showed the lack of aggregation of the particles.

Capillary columns filled using the above suspensions were chromatographically tested. For this purpose, naphthalene was analyzed using a 30/70 ACN/H_2O mobile phase and thymidine using an 80/20 ACN/H_2O mobile phase. Preliminary results gave the best efficiency for the column packed with isopropanol.

Isopropanol is the solvent that gave results showing instability according to the zeta potential measurement. However, it caused aggregation of the stationary phase. Additionally, it has the highest viscosity of all the solvents and mixtures (2.86 cP). Therefore, another capillary column was packed using a mixture of solvents to prepare the suspension. Isopropanol/water mixture was chosen as it has similarly high viscosity (2.58 cP); however, it did not show phase aggregation ability in microscopic observation. The results of the chromatographic testing showed the column efficiency comparable to the column packed using ACN. Based on the results obtained, it was preliminary concluded that the best solvent for packing this type of stationary phase is a high viscosity solvent, which causes aggregation. Also, our observations are consistent with the previous literature data that microscopic observation compared to zeta potential measurements proved to be a better indicator for determining stationary phase aggregation. The results of reduced plate height measurements for naphthalene and thymidine are shown in Figure 5.

For filling the column with Diol-P-C18 phase, chloroform was chosen as the solvent with the highest stability based on zeta potential measurements, isopropanol as the

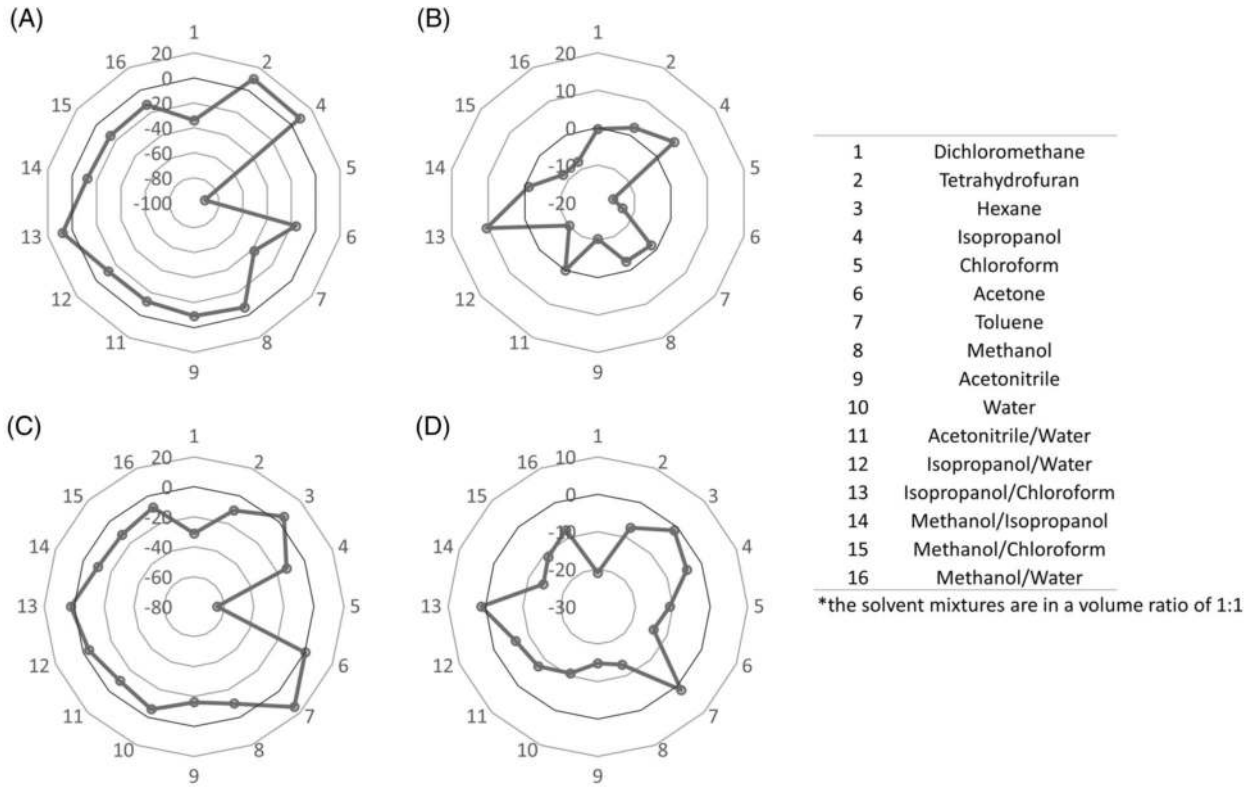

FIGURE 3 The zeta potential results for slurries of stationary phases (A) Diol-P-C10, (B) Diol-P-C18, (C) Diol-P-benzyl, and (D) Diol-P-chol. Some results were not obtained, mainly for hexane (3) and water (10) due to the high instability of the suspensions making it difficult to obtain a result. The solvents used are listed in the table beside

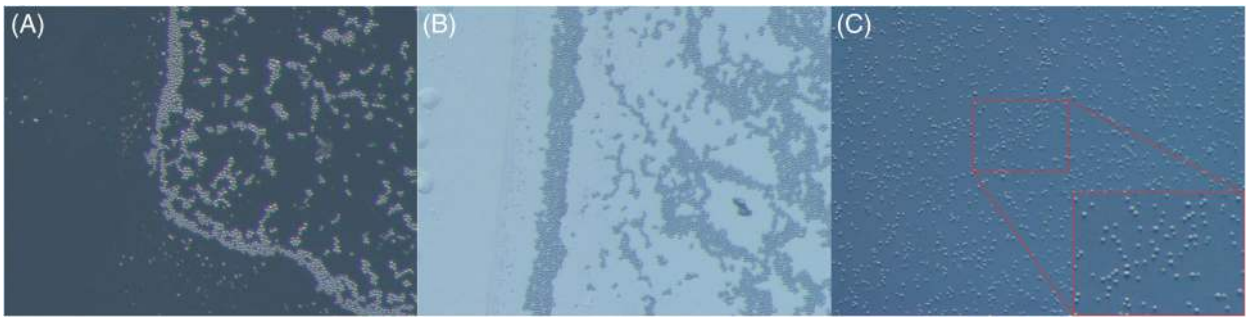

FIGURE 4 Optical microscope images for stationary phase slurries of Diol-P-C10 in methanol (A), isopropanol (B), and acetonitrile (C). The differences in the shading of the images are due to differences in the refractive indices of the solvents, so the light intensity during the observations varied. The absence of a photo for the chloroform slurry is due to the fast evaporation of the solvent

aggregating solvent based on microscopic observations, and water as the aggregating solvent with lower viscosity than isopropanol. The chromatographic results carried out in the same out analogously to the Diol-P-C10 phase confirmed that the solvent aggregating the stationary phase and having high viscosity, which in this case is isopropanol, gave the best results. The reduced plate heights h for naphthalene using chloroform, isopropanol, or water as the slurry solvent were 11.42, 2.99, and 4.26, respectively, and for thymidine the values were 85.81, 3.09, and 4.60, respectively. Thus, using a 15-cm-long column with a particle size of 5 μm, an efficiency of 9500 theoretical plates for naphthalene could be obtained. This value indicates a good filling quality of the column using isopropanol as a slurry solvent. In our study, we used the same particle size, so the lower the h values, the higher the column efficiency, indicating a better filling procedure.

Subsequently, a solvent was selected to pack the Diol-P-benzyl phase. Based on microscopic images and viscosity, again isopropanol was found to be the best solvent.

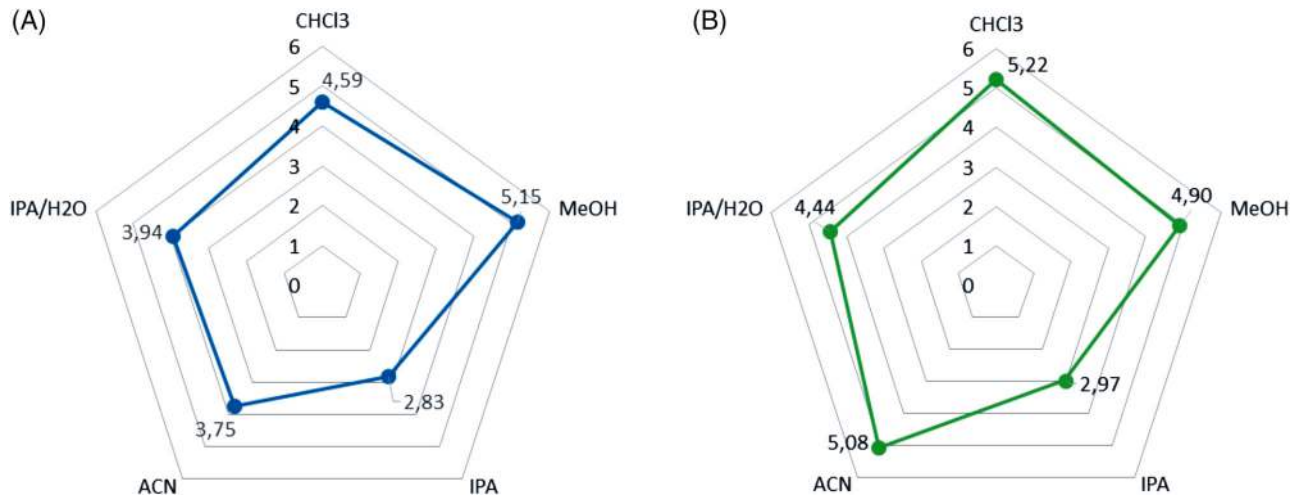

FIGURE 5 Results of a reduced height equivalent to the theoretical plate h measurements for the Diol-P-C10 stationary phase using different slurry solvents. Analyses were performed at a flow rate F = 2 µl/min with spectrophotometric detection at $\lambda = 220$ nm. (A) Naphthalene, mobile phase 30/70 ACN/H_2O; (B) thymidine, mobile phase 80/20 ACN/H_2O

Chromatographic studies showed a reduced plate height of 2.85 for naphthalene and 2.33 for thymidine. Such results confirmed the appropriate choice of slurry solvent, which was again isopropanol. To eliminate possible error in interpretation, the last phase of Diol-P-chol was also initially packed with isopropanol. This phase is the only one that did not aggregate in isopropanol. Thus, the result obtained allowed us to determine whether only viscosity is the most relevant parameter for selecting the suspension solvent, or whether it is aggregation that plays a key role. For the Diol-P-chol column filled with isopropanol-based slurry, the reduced plate height for naphthalene was 5.55, while for thymidine, it was 5.67, which confirms that aggregation of the stationary phase in the solvent seems to be a crucial parameter. When combined with the use of high viscosity solvent, it gives the best results. The properties of the slurries used to package all columns are summarized in Table 2.

Using a 150-mm-long capillary column and a stationary phase of $d_p = 5$ µm, the reduced plate height between two to three corresponds to 10,000–15,000 theoretical plates [14]. The results obtained show a satisfactory column performance. The fact that high efficiency was obtained for both nonpolar and polar analytes, naphthalene and thymidine confirm the increased range of application of stationary phases with embedded polar groups as compared to columns dedicated for RPLC or HILIC operation [26–28]. All results of the reduced plate height and retention factor for each of the packed columns are summarized in Table 3. The differences in retention factor values for the same stationary phase but with different packing quality are due to the ratio of the number of particles, and therefore functional groups responsible for analyte retention, to the volume of empty intergrain spaces. A less compact stationary phase affects the increase of void time (t_0) due to a larger void volume. At the same time, a smaller amount of stationary phase in the capillary results in a shorter retention time (t_r).

4 | CONCLUDING REMARKS

When developing and using new materials for liquid chromatographic analyses, it is important to optimize the column filling procedure. This step is crucial in achieving good column performance, and its omission can lead to unsatisfactory results that cannot be optimized during the chromatographic analysis itself. Many factors contribute to the filling process optimization, including the choice of pressure, filling time, slurry solvent, post-packing (conditioning) solvent, sonication, etc. The stationary phases we used with embedded phosphodiester groups were optimized with respect to the choice of slurry solvent. The results obtained confirm that phase aggregation in suspension is crucial to obtain well-packed capillary columns. A side parameter that positively affects the packing process is the high viscosity of the solvent. For the phases Diol-P-C10, Diol-P-C18, and Diol-P-benzyl, the best solvent is isopropanol. For the Diol-P-chol phase, isopropanol did not prove to be as effective solvent as for the previous phases. This is related to the absence of aggregation of this phase in isopropanol. Therefore, it is necessary to select a solvent that will both aggregate the Diol-P-chol phase and have a high viscosity. By optimizing the packing process, we obtained columns with efficiencies up to two times better than when packing with standard solvents

TABLE 2 Stability based on zeta potential; aggregation based on microscopic observations and viscosity of solvents for stationary phase slurries

Slurry solvent	Aggregation (microscope observation)	Observed stability (zeta potential [mV])	Viscosity μ [cP] (20°C)
	Diol-P-C10		
Chloroform	No	Stable (−91.1)	0.85
Methanol	Occurs	Unstable (−7.0)	0.55
Isopropanol	Occurs	Unstable (8.6)	2.86 (15°C)
Acetonitrile	No	Unstable (−9.4)	0.37
Isopropanol/water	No	Unstable (−12.0)	2.58
	Diol-P-C18		
Chloroform	No	Unstable (−15.8)	0.85
Isopropanol	Occurs	Unstable (6.0)	2.86 (15°C)
Water	Occurs	No result	1.00
	Diol-P-benzyl		
Isopropanol	Occurs	Unstable (−13.2)	2.86 (15°C)
	Diol-P-chol		
Isopropanol	No	Unstable (−4.3)	2.86 (15°C)

TABLE 3 Reduced plate heights and retention factors of naphthalene and thymidine analysis for columns with embedded phosphodiester groups

Column	Slurry solvent	Analyte	Mobile phase ACN/H_2O (%/%) v/v	Reduced plate height *h*	Retention factor *k*
Diol-P-C10	Chloroform	Naphthalene	30/70	4.59	1.54
		Thymidine	80/20	5.22	0.89
	Methanol	Naphthalene	30/70	5.15	1.41
		Thymidine	80/20	4.90	0.93
	Isopropanol	Naphthalene	30/70	2.83	1.92
		Thymidine	80/20	2.97	0.88
	Acetonitrile	Naphthalene	30/70	3.75	1.76
		Thymidine	80/20	5.08	0.94
	Isopropanol/water 1:1 v/v	Naphthalene	30/70	3.94	1.48
		Thymidine	80/20	4.44	0.89
Diol-P-C18	Chloroform	Naphthalene	30/70	11.42	1.72
		Thymidine	80/20	85.81	1.00
	Isopropanol	Naphthalene	30/70	2.99	1.65
		Thymidine	80/20	3.09	0.93
Diol-P-benzyl	Isopropanol	Naphthalene	30/70	2.85	0.76
		Thymidine	80/20	2.33	1.09
Diol-P-chol	Isopropanol	Naphthalene	30/70	5.55	1.75
		Thymidine	80/20	5.67	0.96

(chloroform) for packing columns designed for RPLC operation. Unfortunately, these results are not universal for every type of stationary phase, even polar-embedded, and must be optimized each time a new stationary phase is developed.

Nevertheless, the results obtained and presented here can lead to a better and faster solvent selection. However, as the polar-embedded chromatographic materials are, by their nature, hydrophilic, it is very likely that different water-based solvents (buffers/salts/bases) used as constituents of slurry, packing, or conditioning liquids may have the influence on the aggregation and zeta potential values and affect column quality. This will be a field of our future research.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Phosphodiester Stationary Phases as Universal Chromatographic Materials for Separation in RP LC, HILIC, and Pure Aqueous Mobile Phase

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Abstract: Modern analytical chemistry techniques meet the need for greater attention to ecological and economic aspects. It is becoming necessary to seek solutions to reduce harmful waste production, especially in large quantities. High-performance liquid chromatography is a technique widely used in many industries, including mainly pharmaceuticals, and requires an approach to reduce the significant amount of organic solvent waste. One of the green chemistry solutions is using environmentally benign substitutes, such as pure water, supercritical dioxide, and ethanol. Our work focuses on the preparation and application of new stationary phases with embedded hydrophilic groups for separations using pure water in liquid chromatography. Polar-embedded stationary phases are obtained by attaching a phosphodiester group and 4 different hydrophobic molecules. The studies consisted of hydrophobicity measurements, concentration dependence of retention of the organic additive to the mobile phase, and chromatographic separations of polar and non-polar substance mixtures in RP-LC and HILIC systems. Three mixtures were studied: purine alkaloids, benzene, and polycyclic aromatic hydrocarbons and nucleosides. The stationary phases interact differently with the analytes depending on the attached hydrophobic group. It is possible to use pure water to separate each mixture under study. It is also significant that it has been possible to separate a mixture of completely non-polar compounds using pure water for the first time. The research being carried out is crucial in synthesizing new polar-embedded stationary phases, providing work versatility and high environmental performance.

Keywords: polar-embedded stationary phases; pure water; nucleosides; liquid chromatography

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1. Introduction

Modern high-performance liquid chromatography is based on two retention modes that differ in the mechanism—reversed-phase system (RP) and hydrophilic interaction chromatography (HILIC). Nowadays, the normal-phase system (NP) is rarely used due to a large amount of harmful organic solvents [1]. The essence of chromatographic separation is provided by interactions occurring at the surface of the stationary phase between the analyte and the solvent, the analyte and the stationary phase, and the solvent and the stationary phase [2–4].

A reversed-phase system (RP LC) is used to analyze non-polar compounds. A non-polar stationary phase in the form of silica, polymers, or modified silica with alkyl chains (e.g., C8, C18) is rinsed with a polar mobile phase usually consisting of water and methanol or acetonitrile. The analytes are separated in terms of their hydrophobicity. An adsorption/partition mechanism predominates here, in which the non-polar parts of the analytes are attracted to the hydrophobic ligands of the stationary phase, which induces retention. Elution of the analytes follows their decreasing polarity. Polar stationary phases and a mobile phase with a high content of organic modifiers and buffers are used in HILIC. This reduces hydrophobic interactions between the sample and the stationary phase, allowing

more polar substances to be separated and eluted. The mechanism is based on partitioning the analyte between adsorbed water layer on the stationary and mobile phases. In the normal phase system, the stationary phase is polar—neat silica—while the mobile phase is non-polar—hexane, heptane, cyclohexane. The mechanism is based on the adsorption of polar analytes on the silica surface, where silanol and siloxane groups are located. The more polar the analyte, the stronger the interaction; thus, the elution of a particular compound will be later. When using bonded stationary phases, the primary mechanism is partitioning the analyte between the bulk of the mobile phase and the groups chemically bonded to the silica surface [5–7].

When the stationary phase has bonded hydrophobic groups, it is possible to retain non-polar compounds whose interactions with the organic-water mobile phase are weaker than those with non-polar groups. We are then dealing with an inverted phase system (RP). Increasing organic water content increases retention because non-polar substances have a higher affinity for bound hydrophobic groups. The mechanism can either partition when the analyte gets between the alkyl chains attached to the silica or adsorption when they adsorb onto the alkyl-silica surface [5]. Analysis of polar compounds requires modifying silica with polar groups. We then deal with hydrophilic interaction chromatography (HILIC) [8–10]. Using a high content of an organic modifier in the mobile phase causes a small water content to form a so-called "hydrophilic pillow" at the silica surface. It allows analyte partitioning between the bulk of the mobile phase and the water adsorbed near the surface. Increasing the water content of the mobile phase decreases retention as the mobile phase becomes more polar, so the affinity of the hydrophilic analyte increases [6,7,11,12].

The separation of polar and non-polar compounds requires both different stationary phases and different mobile phase conditions. It raises the question: is it possible to prepare such a stationary phase that allows simultaneous operation in RP and HILIC? Phases with embedded polar groups, in which hydrophilic groups and hydrophobic parts in the form of alkyl chains, aromatic rings, or whole weakly polar molecules are simultaneously present, are of increasing interest. There are several ways to attach polar groups. They can be bonded at the stage of secondary silanization (polar-end-capped), embedded between the silica surface and the hydrophobic group (polar-embedded), or attached to the end of the non-polar group (polar-headed) [13–16]. They exhibit a mixed retention mechanism and can work at high organic modifier content separating polar compounds in HILIC and at high water content separating non-polar compounds in RP [17–22].

Modern industries using liquid chromatography produce significant amounts of harmful organic solvent waste. The unit waste generation by a chromatograph is insignificant; however, multiplying this by the number of instruments working in laboratories and the amount of time they operate, the values increase significantly [23]. Therefore, looking for solutions to reduce harmful and sometimes even toxic residues is crucial. The basis is to follow the principle of green chemistry—reduce, replace, recycle. So, the solution may be to replace harmful solvents with greener alternatives. Among them are ethanol, supercritical carbon dioxide, and water. In order to use such replacements, it is necessary to introduce a suitable stationary phase to work under these conditions [1,13,24]. Thus, there is a requirement in liquid chromatography for the synthesis and broad characterization of new materials that allow work under conditions that provide ecological benefits. There are many reports in the Literature regarding stationary phases operating in mixed retention mode [25–28]. Recent solutions that provide such operation are polar-embedded and polar-end-capped stationary phases. They have both polar and non-polar parts attached. The synthesis method as well as the appropriate selection of attached groups provides the possibility to moderate the selectivity as well as the working range of the prepared material. This opens up possibilities for the preparation of an incredible number of new stationary phases with diverse properties and applications. It is also possible to obtain such materials that will provide efficient and selective work in pure water conditions.

In our work, we consider pure water the only mobile phase component. New stationary phases with embedded polar groups provide the possibility of such analyses. By appropriate selection of the polar group and the attached hydrophobic group, selectivity can be controlled [17,18,20,29,30]. Thus, four stationary phases have been prepared in which a phosphodiester group fulfills the role of the hydrophilic group. In contrast, the non-polar group consists of four different substituents: an octadecyl chain, a decyl chain, an aromatic ring, and a cholesterol molecule. This work focuses on the presentation of new stationary phases and a broad description of their chromatographic properties. The prepared materials were characterized in terms of operation in both RP and HILIC modes, which allowed the separation of polar and non-polar groups of compounds, as well as effective work in "green chromatography" conditions, that is, in pure water.

2. Materials and Methods

2.1. Equipment and Chemicals

All chromatographic analyses were performed using the Shimadzu Nexera UHPLC system (Kioto, Japan). This chromatograph is equipped with a binary solvent delivery system (LC-30AD), an autosampler with a 20 µL volume loop (SIL-20AC), a column thermostat (CTO20AC), and a diode-array UC-detector (SPD-M20A). LabSolution LC/GC 5.65 software (Shimadzu, Kioto, Japan) was used to collect and process data and control the apparatus. Acetonitrile (ACN) (HPLC Grade) and methanol (MeOH) (HPLC Grade) were purchased from Sigma–Aldrich (Steinheim, Germany). Packaging solvents, isopropanol, and methanol were purchased from J.T. Baker, Deventer, the Netherlands. Water was prepared with a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA, USA). Standards of nucleosides—adenosine, guanosine, uridine—were obtained from Applichem (Darmstadt, Germany). Non-polar compounds—naphthalene, benzene, phenanthrene, caffeine, theophylline, and theobromine—were obtained from Sigma–Aldrich (St. Louis, MO, USA). For the synthesis of stationary phases (3-glycidoxypropyl) trimethoxysilane, decanol, octadecanol, cholesterol, benzyl alcohol, and phosphorus chloride were used, which were purchased from Alfa Aesar (Karlsruhe, Germany). Haskel (Burbank, CA, USA) laboratory equipment and packing pump were used to pack the stationary phases into 125 × 4.6 mm long empty columns.

2.2. Materials

Four stationary phases with embedded phosphodiester groups were synthesized for the study. They were prepared on Kromasil 100 Å silica (Akzo Nobel, Bohus, Sweden), with a diameter of 5 µm, a specific surface area of 320 m^2/g, an average pore size of 11 nm, and a pore volume of 0.9 cm^3/g. The phases were prepared by attaching a short linker in the form of a propyl chain and then a phosphodiester group to the surface silanols. Each of the stationary phases has a different hydrophobic molecule attached to a phosphodiester group: Diol-P-C10, a decyl chain; Diol-P-C18, an octadecyl chain; Diol-P-benzyl, a benzyl group; and Diol-P-chol, a cholesterol molecule. A detailed procedure for synthesizing and characterizing these materials has been described in previous works [31,32], while the structures are shown in Figure 1.

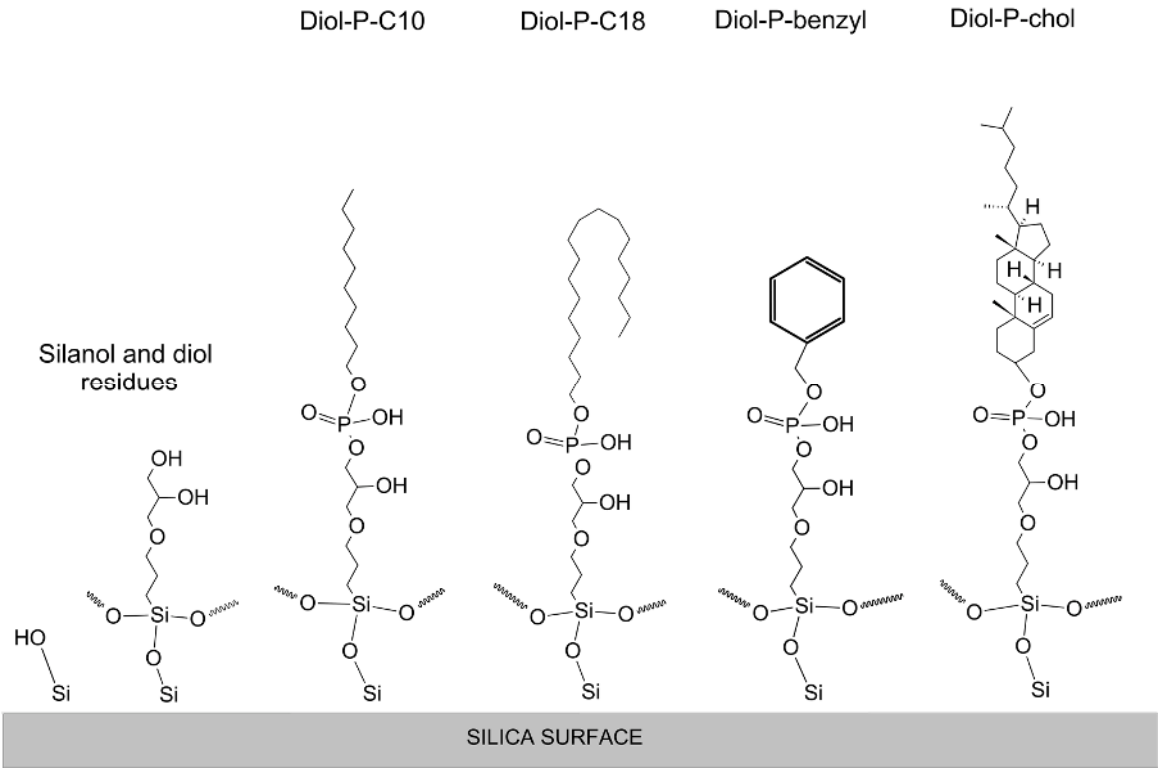

Figure 1. Schematic representation of four different stationary phases with embedded phosphodiester groups. On the left side of the figure, residual silanol and diol structures are shown as possible functional groups occurring in every phase.

2.3. Methods

The stationary phases were packed using the slurry method. 1.7 g of each phase was suspended in 15 mL of isopropanol, then shaken for 1 min and placed in an ultrasonic bath for 10 min before packing itself. Packing was performed at 400 bar for 2 h, and methanol was used as the pressing solvent. After this time, the pressure was reduced to 100 bar, and the column was flushed with 90/10, 50/50, and 10/90 ACN/H_2O solutions for 1 h each. The final packed column was left in pure methanol. The packaging optimization was described in a previously published paper.

The hydrophobicity of stationary phases was performed based on a modified Galushko test [33]. For this purpose, toluene and benzene samples prepared in a 40:60 MeOH:H_2O solution were used. Instead of a mobile phase of 60:40 MeOH:H_2O composition, the same solvents of 40:60 MeOH:H_2O composition were used. This was due to the low retention of analytes. Hydrophobicity (H_G) was calculated based on Equation (1). The dead volume was measured for an excess amount of one of the mobile phase components: acetonitrile.

$$H_G = (k\_toluene + k\_benzene)/2, \quad (1)$$

where H_G is hydrophobicity, k_toluene is the retention factor of toluene, and k_benzene is the retention factor of benzene. The dependence of retention on the mobile phase composition was determined for a polar compound: guanosine, and a non-polar compound: naphthalene. Each sample was analyzed at increasing concentrations (% v/v) of acetonitrile in water (0, 2, 5, 10, 15, 25, 35, 45, 55, 65, 75, 85, 90, 95, 98, 100) by injecting 0.1 µL of guanosine solution and 1 µL of naphthalene solution. The mobile phase was prepared by an on-line mixer. Analyses were performed at 30 °C. The signal was detected with a UV-Vis detector at 254 nm.

Single compound analyses were performed to determine retention and select mixtures' substances. The analysis conditions were the same as those for determining the

dependence of retention on the mobile phase composition. Analyses were performed for nucleosides, purine alkaloids (caffeine and its derivatives), benzene, and polycyclic aromatic hydrocarbons. All samples were prepared in a 50/50 (v/v) acetonitrile-water solution. Dead time was determined for acetonitrile at a mobile phase composition of 60/40 ACN/H_2O. For each mixture, analyses were carried out in the RP LC system—100% water and HILIC—acetonitrile concentration selected for best separation.

All chromatographic analyses, both for hydrophobicity measurements, retention-mobile phase concentration relationships, as well as separation analyses, were performed at least three times. In the results, it did not make sense to include error bars on the graphs, as they were not visible at this scale.

3. Results and Discussion

3.1. Hydrophobicity

Novel chromatographic materials have to be characterized. In order to describe the surface properties of the stationary phases used, a Galushko test [33] was performed to determine the hydrophobicity and silanol activity. The first property is presented in Table 1. The silanol activity could not be determined due to ion-exchange interactions of aniline. On the other hand, determining silanols on such polar embedded stationary phase provides information about all polar groups, e.g., diol, not only silanols. The phase with an attached benzyl group proved to be the least hydrophobic. It may be due to a delocalized electron cloud in the aromatic ring and a small number of carbon atoms compared to the other phases. The phase with an attached octadecyl chain proved to be the most hydrophobic. Less polar than Diol-P-C18 is the Diol-P-chol phase, most likely due to its size and steric efficiency it shields less well the polar effect of the phosphodiester group.

Table 1. Characteristics of bonded polar-embedded stationary phases. Hydrophobicity was determined based on the Galushko test.

Stationary Phase	Carbon Load [%]	Coverage Density [μmol/m^2]	Hydrophobicity (Hg)
Diol-P-C10	3.43	0.56	0.149
Diol-P-C18	4.18	0.42	0.303
Diol-P-Benzyl	2.86	0.56	0.044
Diol-P-Chol	9.31	0.87	0.221

3.2. Retention Analyses

In the next stage of the study, analyses were conducted to determine retention's dependence on the acetonitrile concentration in the acetonitrile-water mobile phase. For this purpose, naphthalene was chosen as a non-polar compound and guanosine as a polar compound. The retention trends are shown in Figure 2. The non-polar compound showed increased retention with increasing water content in the mobile phase (Figure 2A). This typical mechanism occurs in RP-LC. A difference in retention can be seen depending on the stationary phase used. The attachment of the benzyl group exhibited the lowest retention, while the cholesterol molecule showed the highest retention. The appearance of naphthalene retention in pure water for each of the studied stationary phases confirms the mixed retention mechanism. Both hydrophilic and hydrophobic interactions as well as secondary interactions are involved in retention. The varying retention time on each phase is due to their differences in hydrophobicity. An increase in retention is observed with an increase in hydrophobicity.

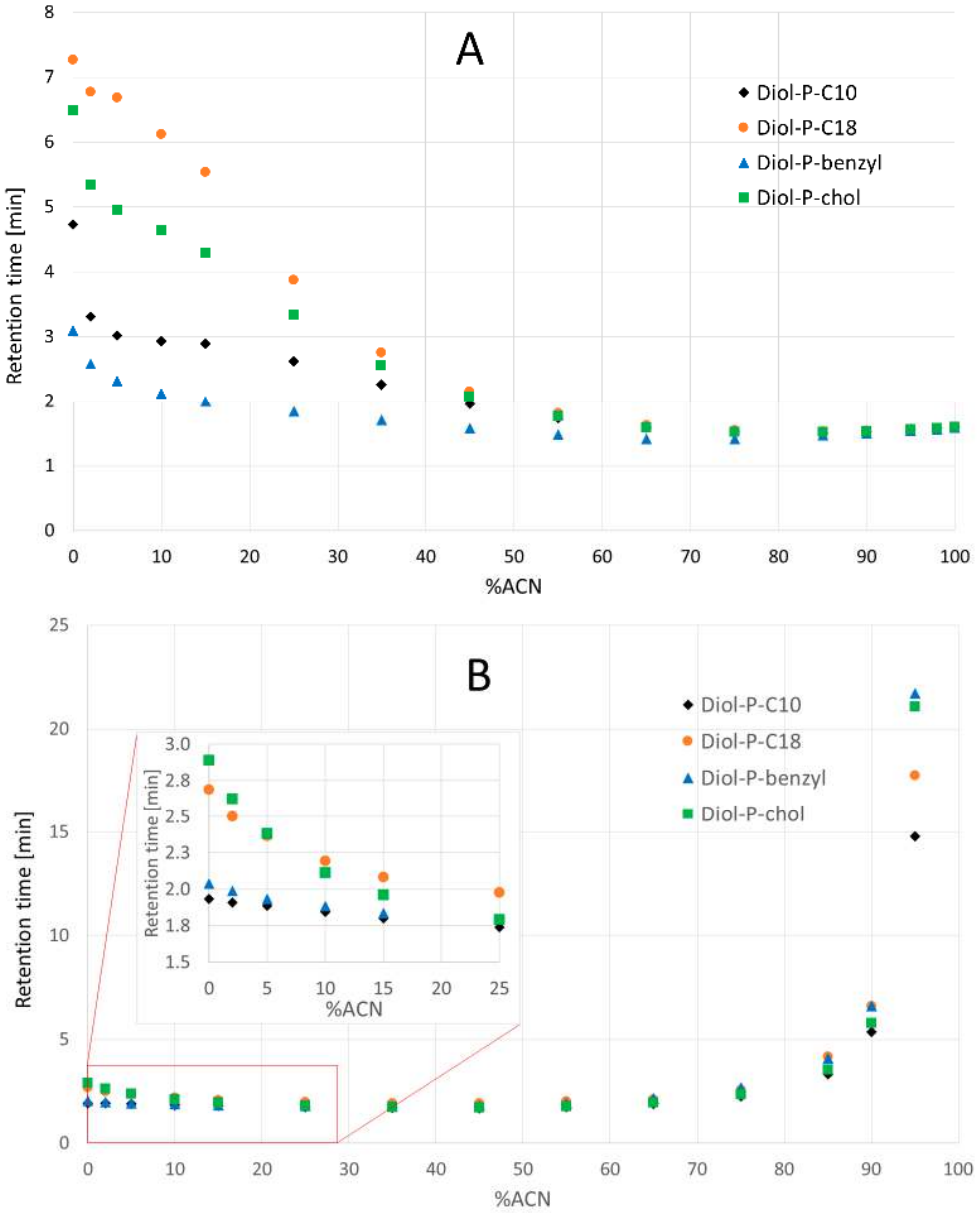

Figure 2. Dependence of naphthalene (**A**) and guanosine (**B**) retention time on the percentage of acetonitrile in the ACN/H2O mobile phase for stationary phases with incorporated phosphodiester groups.

Guanosine retention occurs at both ends of the percentage of water content in the mobile phase. Under highly aqueous conditions, the retention is small compared to the high acetonitrile concentration. It is important to note that all tested phases allow retention in RP LC and HILIC. The differences between the retention in each phase are due to their structure and the interactions involved in the mechanism. It is also evident that on the RP LC side, the order of the phases with increasing retention differs from the arrangement when analyzing naphthalene. This is because other types of interactions contribute more to the retention mechanism. In the case of high water content, a so-called "hydrophilic pillow" is formed, which allows polar compounds to be retained near the surface of the mobile phase [6]. The presence of phosphodiester groups at the surface of each phase promotes the formation of this "pillow" and its attainment of a greater height than if it were formed only with the participation of free silanols presented on the silica surface. The high acetonitrile content provides retention according to the well-known HILIC mechanism. The low hydrophobicity of the Diol-P-benzyl column indicates the high amount of free silanols and unfunctionalized diols. This makes hydrophilic interactions the strongest,

and retention of guanosine is the highest on Diol-P-benzyl among the columns tested. However, it has to be emphasized that other analytes in HILIC, at different mobile phase compositions, may create other retention orders.

3.3. Mixture Separation

To confirm the ability to separate polar and non-polar compounds in pure water and to separate polar mixtures in HILIC mode, the following mixtures were prepared: (A) caffeine, theophylline, and theobromine; (B) adenosine, guanosine, and uridine; (C) benzene, naphthalene, and phenanthrene.

3.3.1. Purine Alkaloids

The results of the mixture (A) separation in pure water are shown in Figure 3.

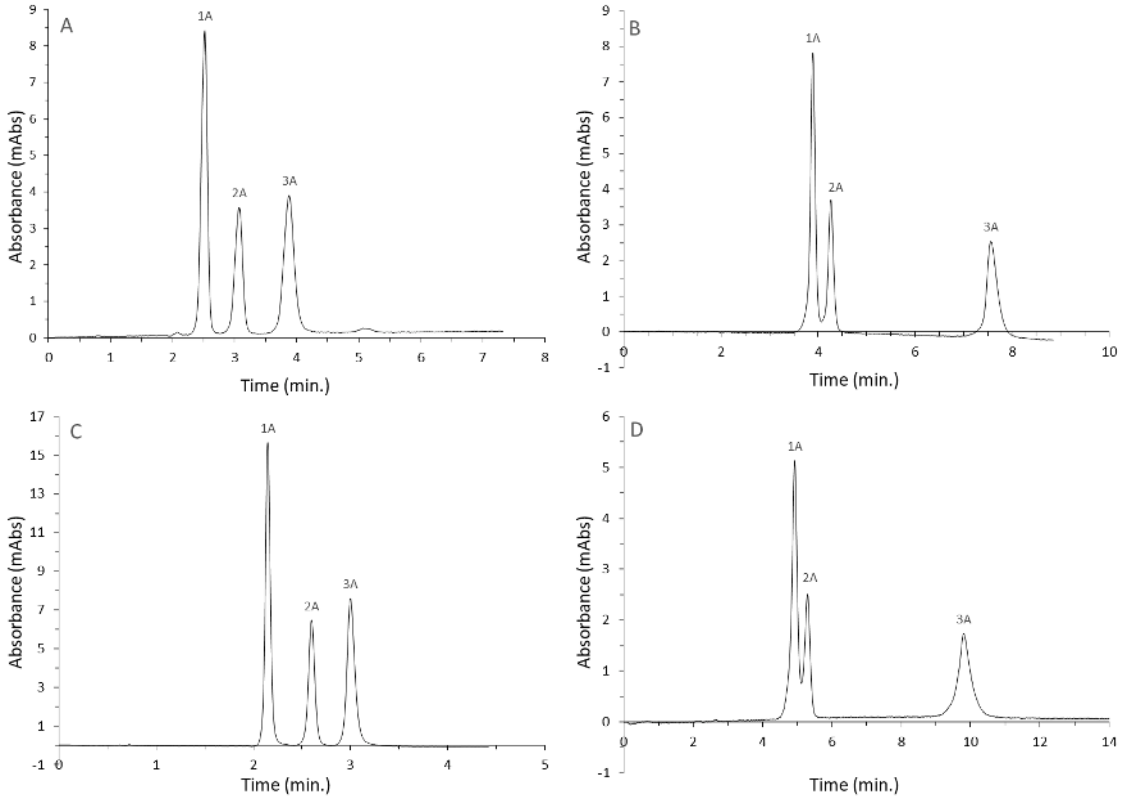

Figure 3. Separation of purine alkaloid in pure water on a series of chromatography columns: (**A**)—Diol-P-C10, (**B**)—Diol-P-C18, (**C**)—Diol-P-benzyl, (**D**)—Diol-P-chol, compounds: 1A—theophylline, 2A—theobromine, 3A—caffeine.

Applying pure water as a mobile phase allows the complete elimination of organic solvents, making it the "greenest" solution in chromatography. Despite using a single-component mobile phase, it is possible to separate the mixture's components in each tested stationary phase. The separation to the baseline was possible on the Diol-P-C10 and Diol-P-benzyl columns. They also had the shortest retention times, making separation possible in less than 4 min. It is due to their lower hydrophobicity. At the same time, Diol-P-C18 and Diol-P-chol phases provided longer retention times. There was also no separation of theophylline from theobromine to the baseline. It confirms the effect of the hydrophobic group on separation selectivity.

Using the same columns and the purine alkaloid mixture, separating them in HILIC mode was possible. The optimal concentration of ACN/H_2O was selected for each column. Chromatograms are shown in Figure 4.

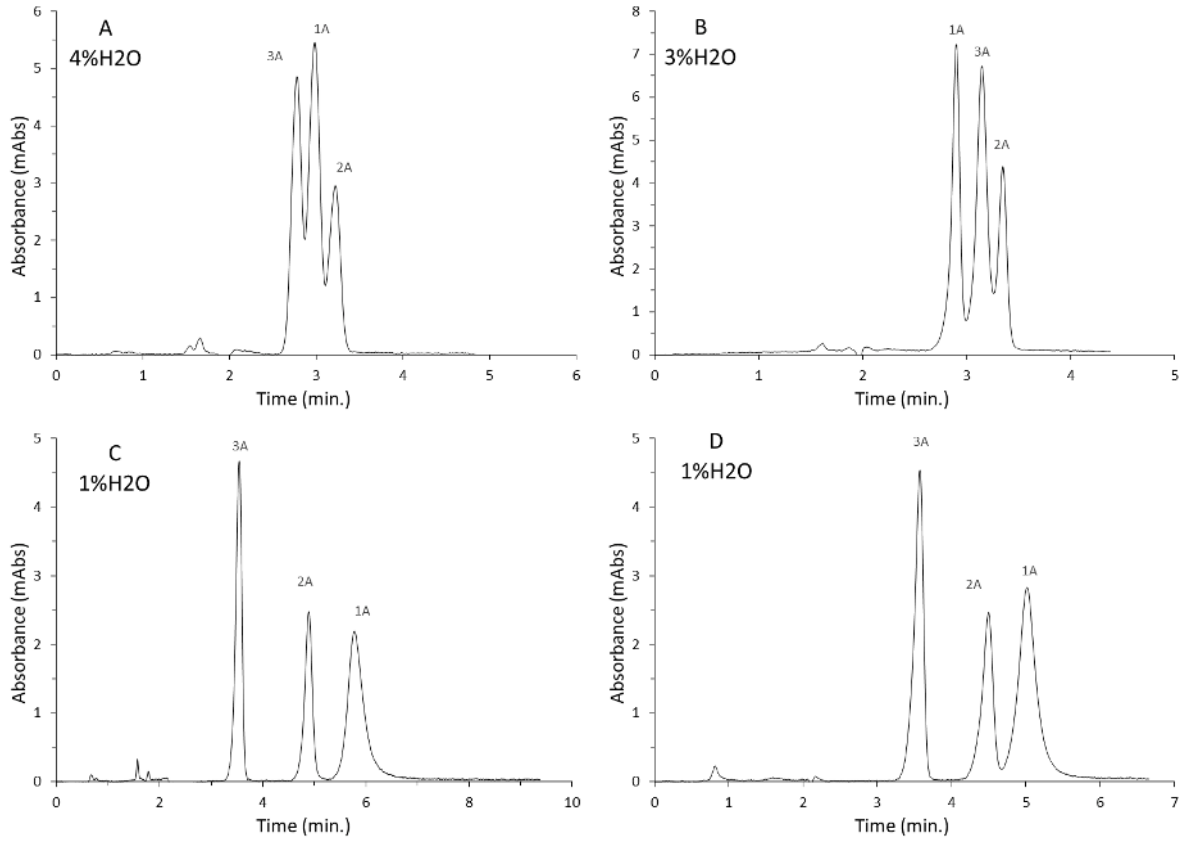

Figure 4. Separation of purine alkaloid in HILIC on a series of chromatography columns: (**A**)—Diol-P-C10, (**B**)—Diol-P-C18, (**C**)—Diol-P-benzyl, (**D**)—Diol-P-chol, compounds: 1A—theophylline, 2A—theobromine, 3A—caffeine.

Only the Diol-P-benzyl column allowed the separation of the mixture to the baseline. It is the stationary phase with the lowest hydrophobicity. The differences in the structure of the caffeine derivatives include the different amounts and locations of the methyl group in the imidazolopyrimidine ring. Using phases with C10 and C18 chains attached did not allow the separation of either component to the baseline. In these two cases, it was necessary to use a higher water content than in the case of the Diol-P-benzyl and Diol-P-chol phases. This was due to the fact that the retention of the mixture's components increased differently with a change in ACN concentration. At lower concentrations, the first two components eluted together, while at higher concentrations, the second and third components eluted together, making separation impossible. Using a buffer to stabilize the pH and ionic strength would likely allow narrower peaks and separation to the baseline in each case.

3.3.2. Nucleosides

The second group of tested polar compounds was nucleosides. The mixture included uridine, guanosine, and adenosine. Each of the columns analyzed allowed separation of the mixture components using pure water as the only mobile phase component. The Diol-P-benzyl column performed the worst, as it failed to separate uridine and guanosine to the baseline. The Diol-P-chol column yielded more than 14,000 theoretical plates for the least retained component of the mixture (uridine), which is comparable to commercial columns operating in pure water. The chromatograms for this phase are shown in Figure 5. The elution order of the compounds in each case was the same when using pure water and in HILIC. This is untypical behavior because the retention order in HILIC and RP conditions is usually the opposite. This may indicate the strong influence of the phosphodiester group, present in each phase, on elution.

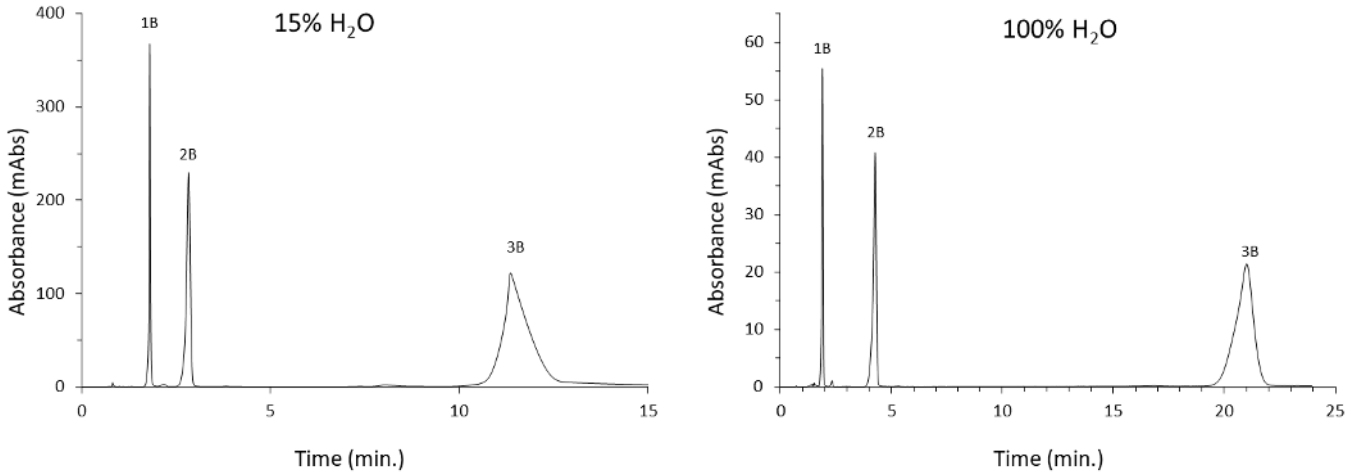

Figure 5. The separation of nucleosides in pure water and 15% of water on a Diol-P-C18 column. 1B—uridine, 2B—guanosine, 3B—adenosine.

Applying a high acetonitrile content mobile phase also had the desired effect of separating the mixture. The worst phase was Diol-P-C10, where elution times were very long, and the tailing of peaks affected lengthy analysis times reaching up to 60 min. The Diol-P-C18 phase yielded a reduced plate height (h) within 3, which is considered outstanding efficiency [34]. The Diol-P-benzyl column in this phase system proved to perform much better. It succeeded in separating the mixture in less than 5 min using it. All results for the nucleoside analyses are summarized in Table 2.

Table 2. Summary of the results of separation of nucleosides in pure water and in HILIC on a series of columns with embedded phosphodiester groups. 1B—uridine, 2B—guanosine, 3B—adenosine.

Stationary Phase	Mobile Phase	Compound	R_t	k	NTP	R_s	$As^{0.1}$
Diol-P-C10	100% H_2O	1B	1.720	0.025	2737	9.383	0.981
		2B	1.907	0.136	2467	1.311	0.939
		3B	2.655	0.582	2324	4.004	0.916
	4% H_2O	1B	5.774	2.441	62	-	2.141
		2B	14.079	7.390	102	1.952	3.022
		3B	25.476	14.182	255	1.904	2.155
Diol-P-C18	100% H_2O	1B	1.782	0.087	9210	-	0.944
		2B	2.722	0.661	2667	6.591	1.061
		3B	20.373	11.430	1586	15.641	1.845
	15% H_2O	1B	2.126	0.297	8197	-	0.879
		2B	4.357	1.658	8099	15.511	0.838
		3B	10.951	5.681	5292	16.572	1.397
Diol-P-benzyl	100% H_2O	1B	2.727	0.606	659	2.668	2.36
		2B	4.949	1.914	366	3.042	5.398
		3B	8.487	3.998	102	1.612	7.274
	15% H_2O	1B	2.466	0.452	5638	-	1.203
		2B	3.427	1.018	5064	5.933	1.249
		3B	4.204	1.476	3306	3.204	1.232
Diol-P-Chol	100% H_2O	1B	1.800	0.068	14,503	15.136	0.921
		2B	2.828	0.679	3804	8.458	0.810
		3B	11.355	5.739	1403	12.216	1.478
	25% H_2O	1B	1.908	0.133	6656	-	0.821
		2B	4.283	1.542	5240	14.382	0.730
		3B	21.023	11.477	2824	18.403	0.807

R_t—retention time, k—retention factor, R_s—resolution, NTP—number of theoretical plates, $As^{0.1}$—asymmetry factor.

3.3.3. Benzene and Polycyclic Aromatic Hydrocarbons

In RP LC, the eluotropic array of solvents for separating non-polar compounds is reversed, so organic solvents such as acetonitrile and methanol have greater elution strength than water. Thus, at high organic solvent content, separation is not possible, as all substances will elute at a void time. Only by increasing the water content can retention be increased, and with proper column selectivity, a given mixture of compounds can be separated. In classical RP LC, a small presence of an organic modifier is necessary to enable elution, as using pure water to separate non-polar compounds would result in a lack of elution and the permanent retention of analytes in the silica bed. However, the use of polar-embedded stationary phases enables a mixed retention mechanism. As a result, it was possible to separate a mixture of completely non-polar compounds in pure water for the first time. Of course, the separation efficiency, retention time, or peak symmetry are unsatisfactory and compare inferiorly with the separation in RP LC by adding an organic modifier. However, the sole fact of using pure water without organic additives from an ecological and economic point of view is significant for modern liquid chromatography. An example chromatogram for the Diol-P-C10 column is shown in Figure 6, while all analysis parameters are summarized in Table 3.

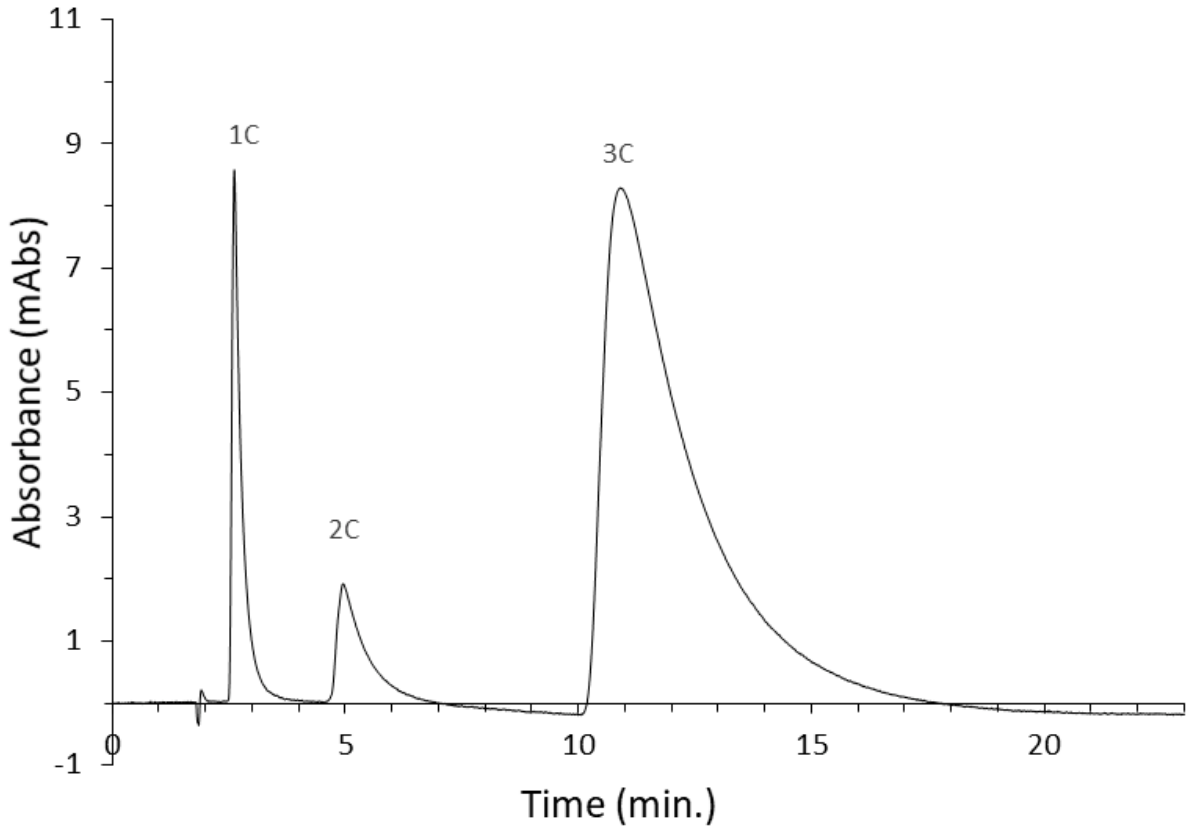

Figure 6. The separation of benzene and polycyclic aromatic hydrocarbons in pure water on a Diol-P-C10 column. 1C—benzene, 2C—naphthalene, 3C—phenanthrene.

All separations have been made to the baseline. Due to the very weak interactions of the analytes with the mobile phase, there was significant peak tailing in each case. On the less hydrophobic phases—Diol-P-C10 and Diol-P-benzyl—the separation was completed in about 10 min; however, using the more hydrophobic phases—with an octadecyl chain or cholesterol molecule attached—significantly increases the analysis time.

Table 3. Summary of the results of benzene and polycyclic aromatic hydrocarbons separation in pure water on a series of columns with embedded phosphodiester groups. 1C—benzene, 2C—naphthalene, 3C—phenanthrene.

Stationary Phase	Compound	R_t	k	NTP	R_s	$As^{0.1}$
Diol-P-C10	1C	2.610	0.555	1168	2.949	2.624
	2C	4.942	1.945	434	3.718	3.440
	3C	10.906	5.499	174	2.799	3.687
Diol-P-C18	1C	2.632	0.606	1842	4.083	4.082
	2C	6.519	2.978	714	6.367	4.894
	3C	33.867	19.663	1261	11.417	3.524
Diol-P-Benzyl	1C	2.727	0.606	659	2.668	2.360
	2C	4.949	1.914	366	3.042	5.398
	3C	8.487	3.998	102	1.612	7.274
Diol-P-Chol	1C	3.088	0.832	1447	4.706	1.943
	2C	7.111	3.220	1725	7.971	1.941
	3C	42.533	24.242	998	11.671	2.908

R_t—retention time, k—retention factor, R_s—resolution, NTP—number of theoretical plates, $As^{0.1}$—asymmetry factor.

Analyzing all the results of the chromatographic analyses, it is apparent that stationary phases with embedded phosphodiester groups can work effectively to separate both polar and non-polar compounds in water. In addition, these phases operate on both sides of the organic modifier content range in combination with water in the mobile phase (RP LC and HILIC). This paper presents the possibilities of separating simple mixtures of polar and non-polar compounds. Preliminary studies for this work have shown that more complex mixtures of compounds of closer polarity may not be completely separated. In such a case, in order not to give up purely aqueous conditions, it is possible to use a temperature gradient already published many times [35–37]. The possibility of improving the results by, for example, stabilizing the pH or achieving a better-packed bed in the column arises. These phases offer great potential for modern chromatography, bearing in mind the ecology and economics of this branch of science and analytics. Research is currently underway to analyze β-blockers under both isocratic and gradient conditions. There is a plan to publish the obtained results in the near future.

4. Conclusions

This work focused on the preparation and description of chromatographic properties of stationary phases of the polar-embedded type, where the polar part was a phosphodiester group. An important part of the research was the chromatographic analysis allowing the separation of compounds of different polarities. The possibility of using each studied phase to separate a group of caffeine derivatives, nucleosides, benzene, and polycyclic aromatic hydrocarbons in pure water was confirmed. These results are groundbreaking, as they are the first stationary phases working in pure water with the ability to separate polar and non-polar compounds. In addition, their simultaneous ability to operate in a HILIC system with high efficiency makes these materials an incredible potential for today's high-performance liquid chromatography. The use of such materials will significantly improve the current unfavorable environmental aspect of HPLC associated with the production of large quantities of organic solvents while at the same time opening the way for the use of a single material for multiple chromatographic applications. The considerable number of combinations of different polar groups with attached non-polar groups represents the immense opportunities posed by polar-embedded stationary phases. The results require further research to improve efficiency, selectivity, and peak symmetry.

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Beta-Blocker Separation on Phosphodiester Stationary Phases—The Application of Intelligent Peak Deconvolution Analysis

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Abstract: Beta-blockers are a class of medications predominantly used to manage abnormal heart rhythms. They are also widely used to treat high blood pressure. From the liquid chromatography separation point of view, beta-blockers are interesting molecules due to their hydrophobic–hydrophilic properties. Thus, the study aimed to investigate the beta-blocker separation selectivity on four phosphodiester stationary phases in reversed-phase liquid chromatography (RP LC) and hydrophilic interactions liquid chromatography (HILIC). On tested stationary phases, beta-blockers provide retention in both chromatographic systems, RP LC and HILIC. Additionally, it was found that cation-exchange mechanisms have a significant contribution to retention. Separations were enhanced by applying ChromSword software for gradient optimization and Intelligent Peak Deconvolution Analysis to separate unseparated peaks digitally.

Keywords: liquid chromatography; beta-blocker; separation; stationary phases; ChromSword; peak deconvolution analysis; i-PDeA II

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1. Introduction

Beta-adrenergic blockers (β-blockers) represent an important class of drugs used to treat cardiac diseases, which are a problem in approximately one-third of the worldwide population [1–3]. At least 20 β-blockers are now commonly used, e.g., metoprolol, atenolol, propranolol, alprenolol, carvedilol, etc. [4–10]. On the other hand, long-term treatment with β-blockers might induce depression and consequently the risk of suicide [8]. Reliable methods of their analysis are indispensable, especially for determining their purity, for pharmacokinetic and pharmacodynamic studies, metabolism studies (several metabolites are pharmacologically active and harmful), or even for doping control, since some β-blockers are prohibited in athletic competitions [1,4,6,7,9]. Moreover, β-blockers are used to reduce morbidity in animals during their transportation; consequently, these drugs are present in meat or milk [11]. Appropriate methods of analysis of these compounds are needed for standard substances, drug-active substances, and biological samples (blood, plasma, urine) [1,4,6,7,9,12,13]. Their analysis may provide useful information for clinical studies. For these reasons, β-blocker drug testing requires methods of high efficiency and selectivity in a short time.

Reversed-phase high-performance liquid chromatography with UV, fluorescence, or mass spectrometry detection has become the so-called 'gold standard' technique for the separation, qualification, and quantification of various β-blockers. However, some hydrophilic interaction liquid chromatography methods also showed excellent separation efficiency and selectivity [9,14]. In most cases, separation has been performed on alkyl stationary phases, such as C18 and C8. The cyano stationary phases and unmodified silica-based columns have also been used [4–6,9,11,12], as well as monolithic ones [7]. Mobile phases applied to β-blocker analysis usually consist of different combinations of acetonitrile

or methanol with buffers. Phosphate buffers have been used most frequently (5 mM to 50 mM) [5–7,10]. However, sodium chloride, sodium perchloride, ammonium formate, and ammonium acetate are used. The ion-pair reagents (sodium dodecyl sulfate with tetrabutylammonium dihydrogen phosphate) were also utilized [4,6,7,9,12]. Additionally, mixtures of water and acetonitrile with the addition of acetic or formic acid have been applied, providing satisfactory β-blocker resolution when MS detection was applied [9,11].

As usual, the optimization of RP HPLC analysis is the key to obtaining complete separation of β-blocker mixtures. The literature shows that these compounds' most influential chromatographic parameters are the mobile phase composition (especially in buffer selection), pH, flow rate, and temperature [1,7–9,12]. β-blockers are protonated at low pH of the mobile phase [7]. Usually, the increase in β-blocker retention is observed when the pH of the mobile phase is increased to 6.5. The pH increase reduces the protonation, increasing the hydrophobicity [7,9]. Another critical parameter is the type of stationary phase and its particle size. Four compounds were separated in 25 min using 5 µm C18; however, changing the column to monolithic allowed the time to be reduced to 5 min (with an increased flow rate) [4,7]. Reducing the particle size of the C18 stationary phase to 3.5 µm allows the separation of a 5–8 component mixture of β-blockers in 10 min [6], while a further reduction to 1.7 µm provides separation of up to a dozen compounds in the same time [12].

Stationary phases with incorporated polar groups mixed with the hydrophobic alkyl ligands, so-called polar-embedded stationary phases, are promising chromatographic materials [15,16]. Such materials containing both hydrophobic and hydrophilic ligands can be applied in reversed-phase liquid chromatography (RP LC). They can also be used in hydrophilic interaction liquid chromatography (HILIC). Depending on the mobile phase pH, mixed-mode stationary phases may be ionized. [17,18]. They may separate both polar and nonpolar analytes [19]. It was recently proven that polar-embedded stationary phases allow chromatographic elution and separation using pure water as a mobile phase [20]. These stationary phases are called mixed-mode stationary phases.

In many cases, the complete separation of the mixture is difficult, especially when separated compounds have similar structures. It is a common problem in the pharmaceutical industry. For this reason, many solutions are being developed to facilitate method optimization and data analysis. One is peak deconvolution analysis with photodiode array (PDA) detectors that allow using 3D PDA data [21]. Using a unique software function, it can separate peaks that are not resolved on-column. It offers better detection results and minimizes method development and analysis time [22].

The study aimed to characterize the selectivity of four phosphodiester stationary phases for separating beta-blockers. The research was enhanced by applying Peak Deconvolution Analysis and gradient optimization software.

2. Results and Discussion

Phosphodiester stationary phases represent a group of polar-embedded materials. Structures are presented in Figure 1. The presence of a phosphate group and hydrophobic ligand allows for the retention of compounds in RP LC and HILIC. Thus, β-blockers are interesting compounds due to their various polarities and variety of functional groups. The presence of hydrophobic and polar groups allows the investigation of the selectivity of phosphodiester stationary phases in different liquid chromatography modes. The phosphate group in the ligand structure has pKa around 1.45 ± 0.5. Thus, phosphate groups are ionized in the mobile phase pH equal to 7.5 and constitute cation exchange sites. The ionized groups are presented in Figure 1. Thus, despite polar and hydrophobic properties, phosphodiester stationary phases are also weak cation exchangers.

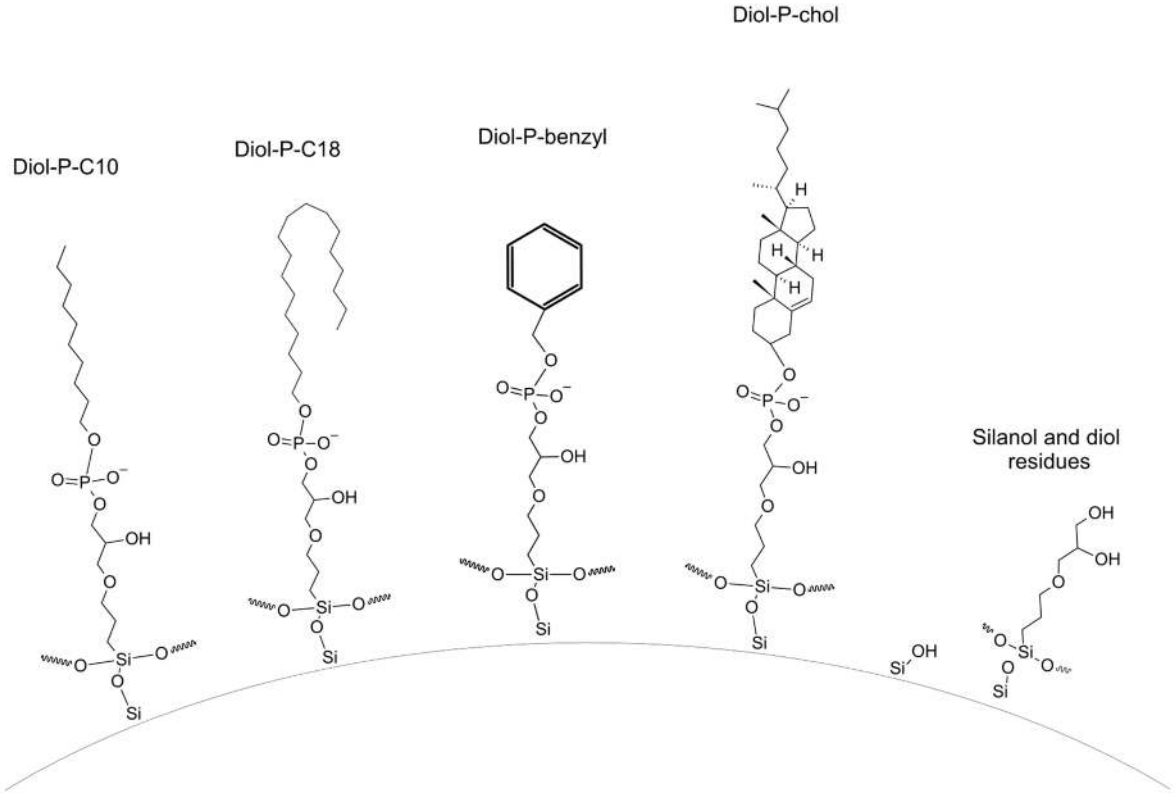

Figure 1. Structures of chemically bonded stationary phases used in the study at pH = 7.5.

Tested β-blockers exhibit pKa values in the range of 9.2–9.7 (details are listed in Table 1). The pH of the mobile phase was 7.5. It means that the hydrogen cation concentration was two orders of magnitude higher. Thus, in the analysis conditions, all compounds are protonated (see Figure 2). As a result, despite hydrophobic interactions in RP LC and hydrophilic interactions in HILIC, β-blockers can ion-exchange with the stationary phase surface. Anytime, in RP LC and HILIC, we observe a mixed-mode retention mechanism. Various types of interactions complicate the optimization of separation conditions but offer different separation possibilities.

Table 1. Characteristics of compounds used in the study.

Abbreviation	Beta-Blocker	Number of Hydrogen Bonds Donor	Number of Hydrogen Bonds Acceptor	Log P *	pK_a *
A	metoprolol	2	4	2.15	9.56–9.70
B	propranolol	2	3	3.48	9.53–9.45
C	cicloprolol	2	5	2.40	9.2
D	mexiletine	1	2	2.15	9.14–9.15
E	atenolol	3	5	0.16	9.54–9.60
F	acebutolol	3	6	1.71	9.52–9.67
G	oxprenolol	2	4	2.10	9.57
H	pindolol	3	4	1.75	9.25–9.54

* Data according to [23].

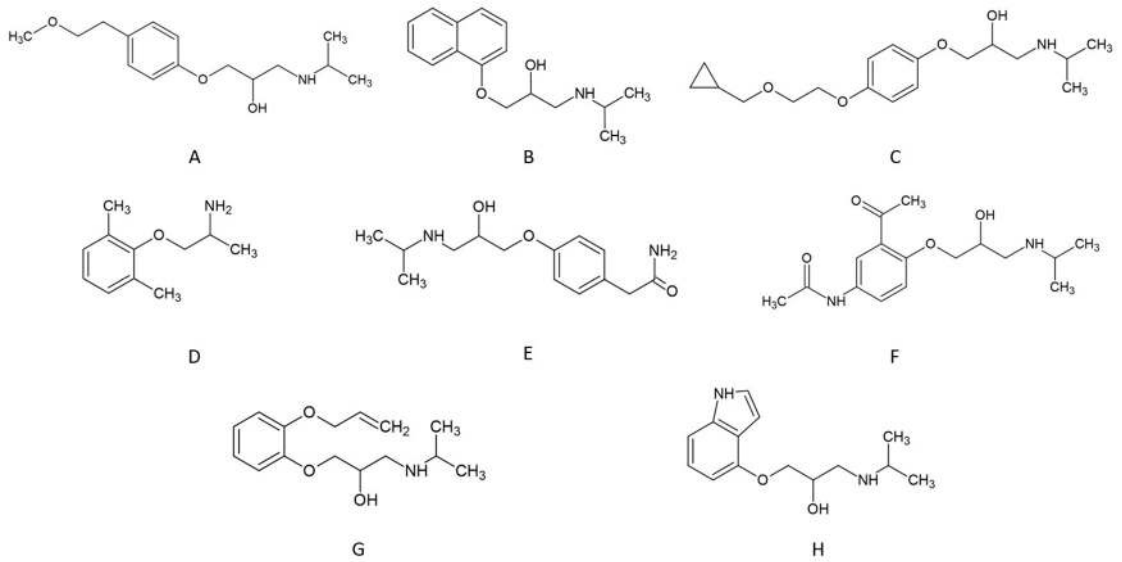

Figure 2. Structure of tested β-blockers at pH equal 7.5: (**A**)–metoprolol, (**B**)–propranolol, (**C**)–ciciloprolol, (**D**)–mexiletine, (**E**)–atenolol, (**F**)–acebutolol, (**G**)–oxprenolol, and (**H**)–pindolol.

2.1. Retention Investigation

All β-blockers exhibit a U-shape of retention behavior over the mobile phase composition. The plots of retention factor (k) are presented in Figure 3. First, on all columns, all β-blockers exhibit significant retention, independent of the mobile phase composition. Usually, for polar-embedded stationary phases, for mobile phase composition in the range of 45–55% of organic modifier, there is a minimum of retention. In many cases, compounds in such conditions are eluted in or near the column void volume. Here, we can observe a retention factor of around five (for Diol-P-C-Benzyl and Diol-P-C18). It increases to around k = 7 for Diol-P-Chol (for a wide range of 30–70% of ACN). The lowest retention (around k = 4) is observed for Diol-P-C10. Detailed data on retention factors are listed in Table S1.

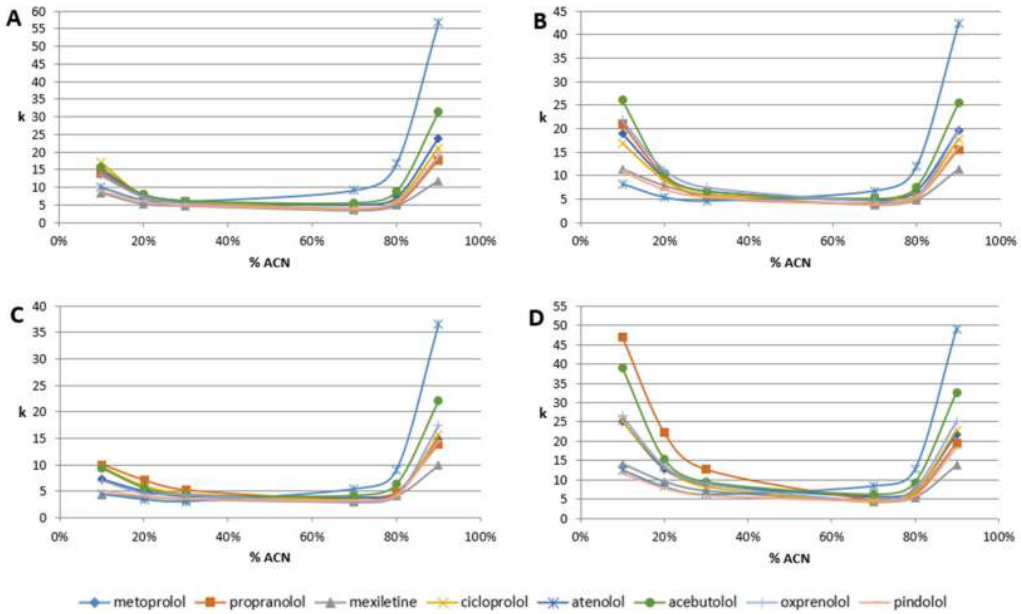

Figure 3. Retention factor (k) dependence for different mobile phase composition (%ACN in 10 mM ammonium acetate in water, pH equal 7.5); (**A**)-Diol-P-Benzyl, (**B**)-Diol-P-C18, (**C**)-Diol-P-C10, and (**D**)-Diol-P-Chol.

In the ACN concentration range of 30–70% in ammonium acetate, the retention of all β-blockers is similar, which makes separation impossible (Figure 3). It is a result of the cation-exchange mechanism. Protonated molecules interact with ionized phosphate groups that result in retention but do not offer significant selectivity.

The decreasing or increasing ACN concentration out of this range enormously increases retention. The retention varies between RP LC and HILIC depending on the stationary phase. Diol-P-Benzyl and Diol-C10 stationary phases provide higher retention in HILIC compared to the RP LC. In contrast, the retention on Diol-P-Chol and Diol-P-C18 exhibits comparable retention properties in both RP LC and HILIC (Figure 3). Generally, the highest retention factor was observed for Diol-P-Chol on both sides, RP and HILIC; however, atenolol in HILIC has the highest retention on Diol-P-Benzyl. It results from higher carbon load and surface coverage density of Diol-P-Chol stationary phases (see Table 2). Lower surface coverage and resulting higher accessibility to the silanol group on Diol-P-Benzyl and Diol-PC10 probably cause the domination of the HILIC mechanism.

Table 2. Characteristics of stationary phases used in the study.

Stationary Phase	Carbon Load [%]	Coverage Density [μmol/m^2]
Diol-P-C10	3.43	0.56
Diol-P-C18	4.18	0.42
Diol-P-Benzyl	2.86	0.56
Diol-P-Chol	9.31	0.87

Specific retention of atenolol on Diol-P-Benzyl in HILC is a result of two factors. First, the Diol-P-Benzyl stationary phase has the lowest coverage of hydrophobic groups and higher accessibility to hydroxyl groups that increase the HILIC retention mechanism. Second, atenolol has the lowest hydrophobicity (see Table 1) and the highest hydrophilicity, which allows strong retention in HILIC.

Changes in the particular β-blockers' retention depend on their structure. Usually, more polar compounds exhibit higher retention in the HILIC range than in RP LC. On the other hand, more hydrophobic molecules exhibit higher retention in RP LC and lower in HILC. Thus, the retention order is usually the opposite between RP LC and HILIC. In the case of β-blockers, molecules possess both hydrophobic and polar groups (see Figure 2). It causes some of them to have similar retention in RP LC and HILIC, for example, acebutolol. On the other hand, atenolol significantly changes the retention order between HILIC and RP LC.

In RP LC, retention is governed mainly by hydrophobic interactions between the stationary phase and the solute. As evidence, the dependence of log k of particular compounds plotted against its log P value is linear. In the case of the polar-embedded stationary phases, the surface is heterogenous and possesses hydrophobic and polar adsorption sites. If the solute is polar, the retention mechanism is governed mainly by polar interactions (e.g., hydrogen bonds). The number of hydrogen bond donors and acceptors for each β-blocker is listed in Table 1. However, hydrophobic interactions may also occur but to a lower extent. These polar (hydrophilic) interactions are responsible for retention in HILIC. However, they cannot be omitted in reversed-phase conditions. As a result, the dependence of log k vs. log P is nonlinear for β-blockers on tested stationary phases at 10% ACN in the mobile phase. Detailed results are presented in Figure 4. Nevertheless, the linear dependence log k vs. log P slope confirms the hydrophobicity of the stationary phases. Higher hydrophobicity of the stationary phases provides a greater value of the curve slope. The most hydrophobic, in order, are Diol-P-Chol and Diol-P-C18, and the weakest hydrophobicity is exhibited by Diol-P-Benzyl.

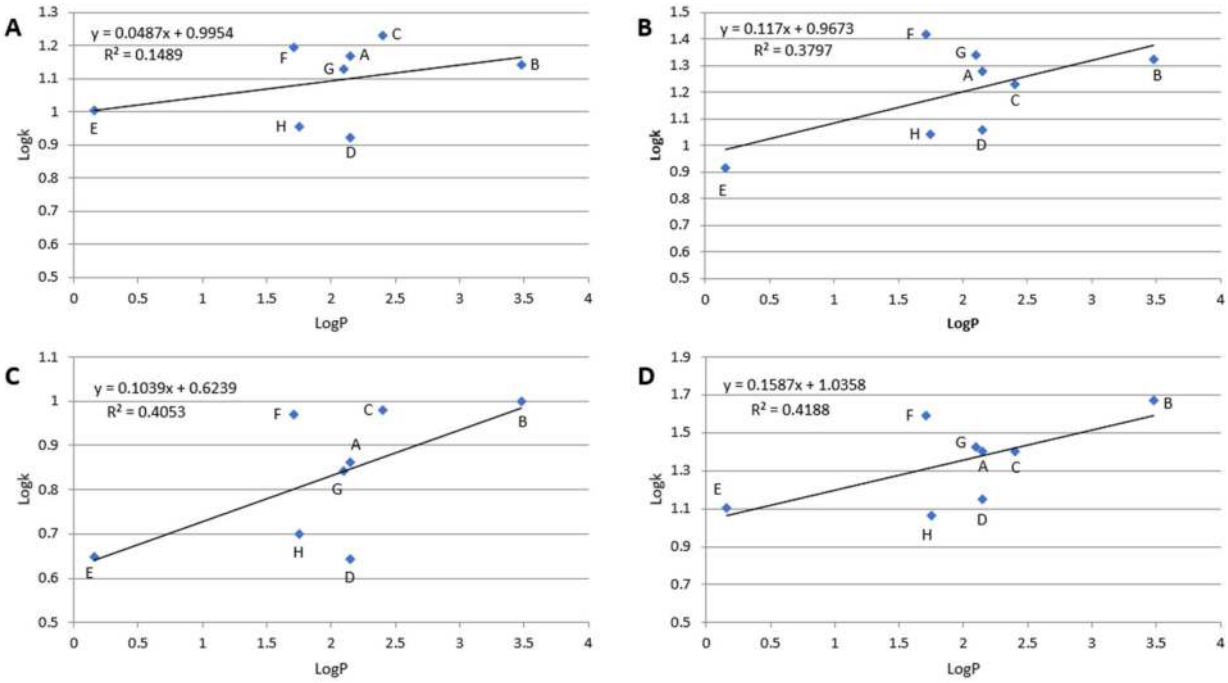

Figure 4. Dependences of log k vs. log P; (**A**)–Diol-P-Benzyl, (**B**)–Diol-P-C18, (**C**)–Diol-P-C10, and (**D**)–Diol-P-Chol measured for 10% ACN in 10 mM ammonium acetate in water. (A)–metoprolol, (B)–propranolol, (C)–ciciloprolol, (D)–mexiletine, (E)–atenolol, (F)–acebutolol, (G)–oxprenolol, and (H)–pindolol.

Deviations from the trend line in Figure 4 result from specific interactions between the solute and the stationary phases. For example, acebutolol (compound F in Figure 4) exhibits retention significantly higher than other compounds resulting from its hydrophobicity. Comparing the chemical structure, acebutolol possesses the highest ability for hydrogen bond creation with the stationary phase (see Table 1), significantly impacting its retention (Figure 2). On the other hand, mexiletine (compound D in Figure 4) provides lower retention than predicted from the trend, which may result from the weak ability for polar interaction compared with other tested β-blockers. Mexiletine has the lowest hydrogen bond donor and acceptor groups (see Table 1)

The opposite situation is observed in Figure 5. The dependence of retention (Log k) on hydrophobicity is declining. This is very logical because, in HILIC, the retention increases with the hydrophilicity of the molecule and decreases when the hydrophobicity increases.

Nevertheless, it should be noted that despite hydrophilic interaction in HILIC and hydrophobic interactions in RP LC, β-blockers are retained mainly by the cation-exchange mechanism.

The presence of cation-exchange properties was confirmed through attempts to elute β-blockers in the ACN-water mobile phase without salt addition at an apparent pH of around 6.8. This pH does not change the form of molecules' protonation nor the ionization of the stationary phase. However, any of the tested compounds were eluted from the stationary phase. Salt addition provides counterions that allow the protonated β-blockers' elution from the phosphodiester stationary bonded phases according to the cation-exchange mechanism. It confirms that tested mixed-mode stationary phases exhibit weak cation-exchange properties. The detailed investigation of the cation-exchange mechanism was not the topic of this study. It will be continued in future work.

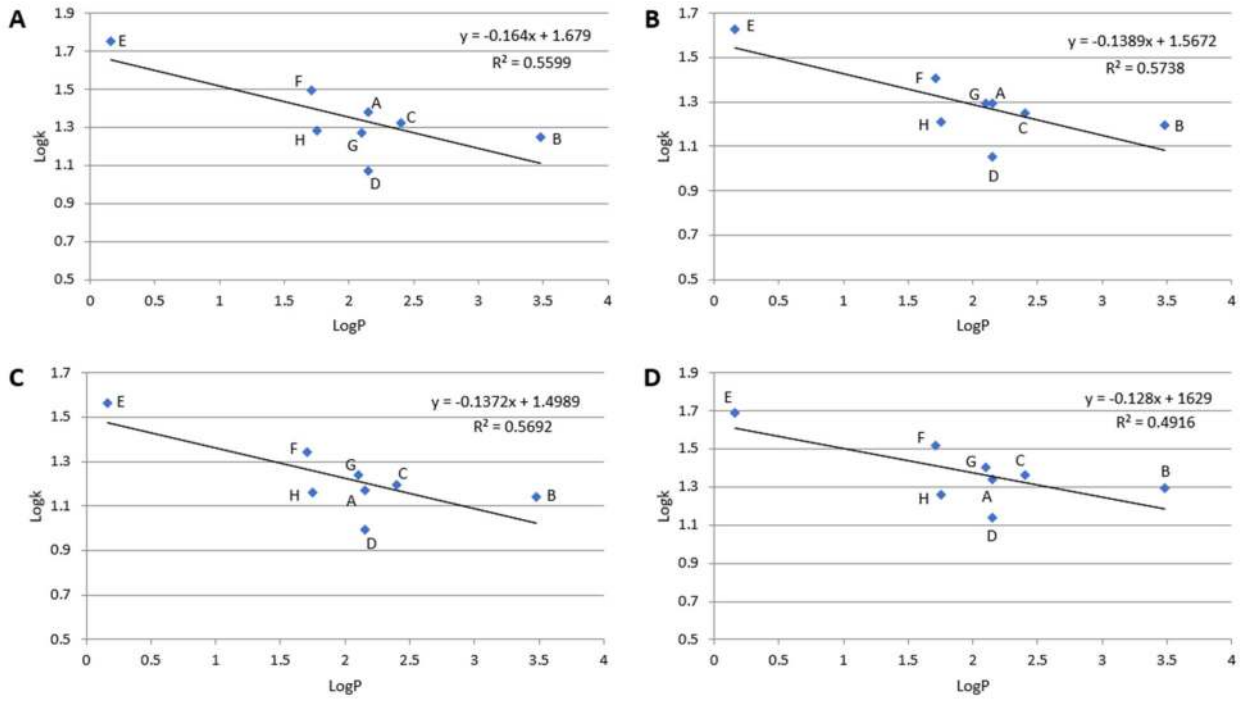

Figure 5. Dependences of log k vs. log P; (**A**)–Diol–P–Benzyl, (**B**)–Diol–P–C18, (**C**)–Diol–P–C10, and (**D**)–Diol–P–Chol measured for 90% ACN in 10 mM ammonium acetate in water. (A)–metoprolol, (B)–propranolol, (C)–ciciloprolol, (D)–mexiletine, (E)–atenolol, (F)–acebutolol, (G)–oxprenolol, and (H)–pindolol.

2.2. Separation

The separation of very similar compounds may be a difficult task. Chromatographic resolution depends on three factors: retention, selectivity parameter, and column (system) efficiency, which is measured as a number of theoretical plates. Two of them are crucial, the separation factor and efficiency. High column efficiency in modern UHPLC systems enormously improves chromatographic resolution. However, the selectivity offered by the stationary phase (or mobile phase) is critical.

The present study tested four homemade columns according to chromatographic selectivity in RP LC and HILIC modes. Unfortunately, the efficiency of tested columns was up to 70,000 theoretical plates per meter. Cation-exchange properties cause peak broadening. It reduces efficiency, which results in the loss of resolution. However, the obtained result compares the selectivity of various functionalities bonded as a stationary phase. The selectivity of the separation in RP LC (10% of ACN in ammonium acetate) and for HILIC (90% of ACN in 10 mM ammonium acetate) are presented in Table 3.

Comparing the selectivity in RP (10% ACN) and HILIC (90% ACN), it is easy to conclude that overall selectivity is higher in HILIC. It is observed for all stationary phases, even for more hydrophobic ones such as Diol-P-Chol.

The surprise is that for Diol-C-10 and Diol-P-Chol, atenolol and acebutolol provide the highest retention in both 10% and 90% of ACN. These compounds behave with relatively low hydrophobicity, so the highest retention in RP LC must be governed not only by hydrophobic interactions typical for RP but also due to some polar interactions with the stationary phase surface and cation-exchange mechanism. It confirms the mixed-mode retention model on the tested stationary phase.

Table 3. Selectivity in RP (10% ACN) and HILIC (90% ACN).

Column	Pair	10% ACN	Pair	90% ACN
Diol-P-C10	mexiletine/atenolol	1.01	mexiletine/propranolol	1.41
	atenolol/pindolol	1.13	propranolol/pindolol	1.04
	pindolol/metoprolol	1.39	pindolol/metoprolol	1.02
	metoprolol/cicloprolol	1.05	metoprolol/cicloprolol	1.06
	cicloprolol/oxprenolol	1.28	cicloprolol/oxprenolol	1.11
	oxprenolol/acebutolol	1.02	oxprenolol/acebutolol	1.27
	acebutolol/atenolol	1.04	acebutolol/atenolol	1.65
Diol-P-C18	atenolol/pindolol	1.34	mexiletine/propranolol	1.38
	pindolol/mexiletine	1.04	propranolol/pindolol	1.03
	mexiletine/cicloprolol	1.48	pindolol/cicloprolol	1.10
	cicloprolol/metoprolol	1.12	cicloprolol/metoprolol	1.11
	metoprolol/propranolol	1.10	metoprolol/oxprenolol	1.00
	propranolol/oxprenolol	1.05	oxprenolol/acebutolol	1.29
	oxprenolol/acebutolol	1.19	acebutolol/atenolol	1.66
Diol-P-Benzyl	mexiletine/pindolol	1.07	mexiletine/propranolol	1.51
	pindolol/atenolol	1.12	propranolol/oxprenolol	1.06
	atenolol/oxprenolol	1.33	oxprenolol/pindolol	1.01
	oxprenolol/propranolol	1.04	pindolol/cicloprolol	1.11
	propranolol/metoprolol	1.06	cicloprolol/metoprolol	1.13
	metoprolol/acebutolol	1.06	metoprolol/acebutolol	1.31
	acebutolol/cicloprolol	1.09	acebutolol/atenolol	1.81
Diol-P-Chol	pindolol/atenolol	1.09	mexiletine/pindolol	1.33
	atenolol/mexiletine	1.12	pindolol/propranolol	1.07
	mexiletine/metoprolol	1.78	propranolol/metoprolol	1.12
	metoprolol/cicloprolol	1.00	metoprolol/cicloprolol	1.05
	cicloprolol/oxprenolol	1.05	cicloprolol/oxprenolol	1.10
	oxprenolol/acebutolol	1.47	oxprenolol/acebutolol	1.29
	acebutolol/atenolol	1.20	acebutolol/atenolol	1.50

2.3. Intelligent Peak Deconvolution Analysis

Insufficient separation of target compounds resulting from insufficient selectivity or low efficiency is a significant problem for chromatography. However, if it is possible to collect 3D data, for example, from a PDA detector, data analysis allows separating signals of unseparated peaks. One of them is Intelligent Peak Deconvolution Analysis. Details on the algorithm were described in [24]. According to the literature [24], the algorithm provided less than ±1.0% error between true and separated peak area values.

During the study, i-PDeA II was applied to keep track of unseparated peaks during gradient separation. The exemplary results are listed in Figure 6. In such conditions, oxprenolol and pindolol elute together and provide one symmetrical peak. After performing the deconvolution, two peaks may be determined. According to Table 1, the selectivity of these two compounds equals only 1.01.

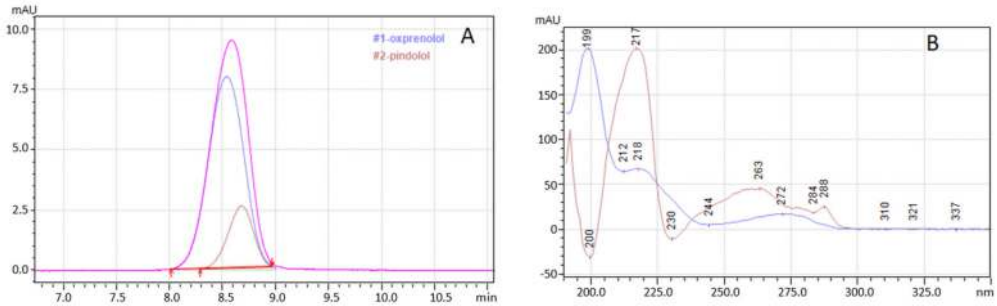

Figure 6. Deconvolution of unseparated oxprenolol and pindolol on Diol-P-Benzyl column in 90% of ACN (**A**) and corresponding UV spectra (**B**). Pink line represent signal obtained from detector.

The most significant advantage of the deconvolution function is that peaks that are not physically separated can be digitally separated. It reduces the time needed for the analysis of peak tracking in method development and column characterization procedures.

Deconvolution is not limited to two unseparated peaks. In Figure 7, a part of the chromatogram is presented where the blue line presents a measured signal that contains two bands (A and C). Part C is a zoom of part A. After deconvolution, in two signals, five chromatographic peaks were found. Their corresponding spectra are shown in Figure 7B,D. The effect of deconvolution is reduced if the unseparated substances do not differ in the spectra. As seen in Figure 7, if the spectra are different, several peaks may be digitally separated.

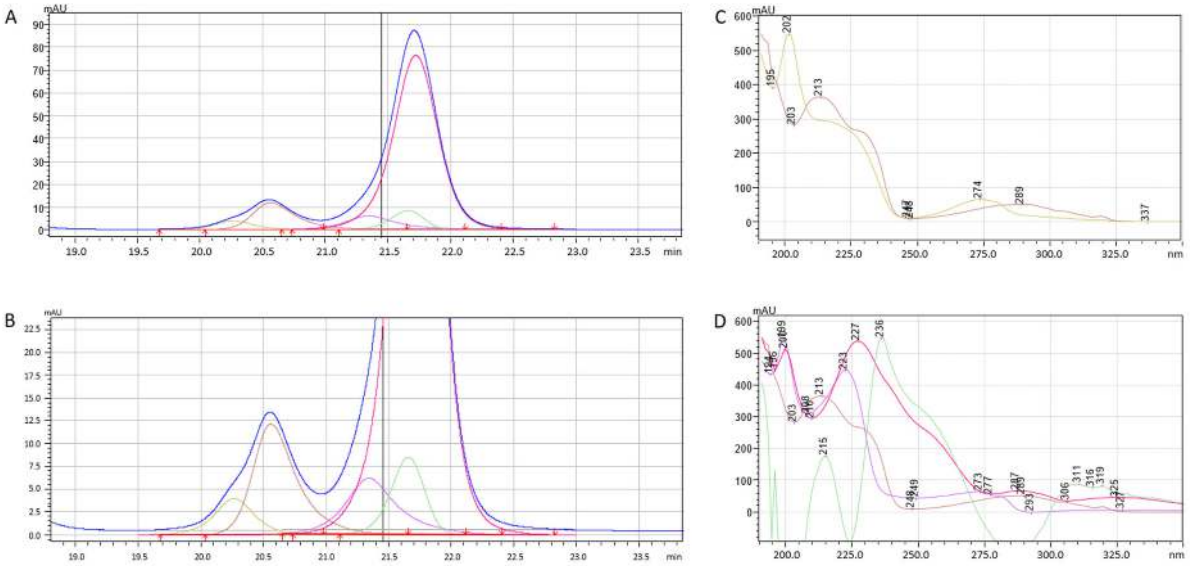

Figure 7. Deconvolution of unseparated compounds (**A**,**B**) and corresponding UV spectra (**C**,**D**). Deconvoluted signal of various compounds are plotted in different colors whereas navy-blue line represents the signal from detector.

2.4. Gradient Optimization

For all columns, gradient analyses were optimized in RP LC and HILIC. The best separation was obtained on the Diol-P-C18 stationary phase in RP LC conditions. The resulting chromatogram is presented in Figure 8. Only two compounds, mexiletine and pindolol, were not fully separated. However, it was overcome by deconvolution analysis. The result shows that β-blockers may be separated on homemade columns using a mixed-mode retention mechanism. The further increase in column efficiency may significantly improve the resolution. It confirms that phosphodiester stationary phases are unique and promising chromatographic materials.

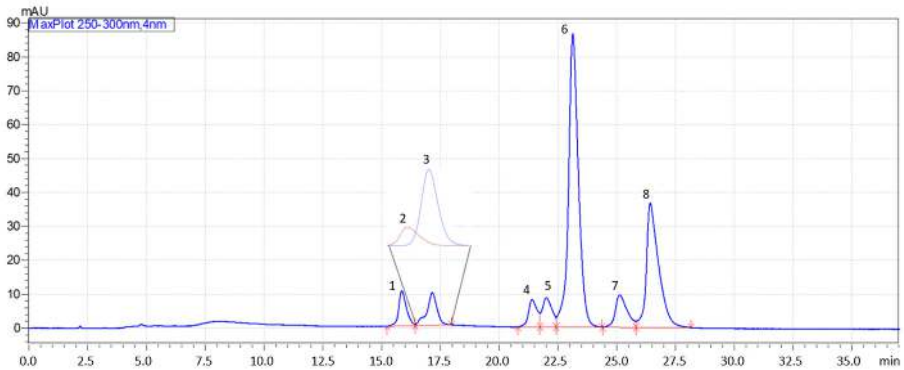

Figure 8. Exemplary chromatogram of optimized gradient separation on Diol–P–C18 stationary phase; linear gradient from 0% to 50% of ACN in 10 mM ammonium acetate; 1–atenolol, 2–mexiletine, 3–pindolol, 4–metoprolol, 5–oxprenolol, 6–acebutolol, 7–cicloprolol, 8–propranolol; compounds 2 and 3 were separated using peak deconvolution.

3. Materials and Methods

3.1. Materials and Reagents

Four house-made stationary phases were tested during the study. These materials contain different hydrophobic groups bonded to diol-silica by a phosphate group. The structures of chemically bonded phases are presented in Figure 1.

Detailed characteristics of these materials are presented in the previous studies [25,26]. Kromasil 100 silica gel (Akzo Nobel, Bohus, Sweden) was used as a support for the stationary phase synthesis. The properties of the stationary phases are listed in Table 2. Stationary phases were packed into 125 × 4.6 mm i.d. stainless steel columns using laboratory-made equipment and a Haskel (Burbank, CA, USA) packing pump using the slurry method.

Acetonitrile was high-purity "for HPLC" gradient grade, and ammonium acetate was "for HPLC" from Sigma-Aldrich (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, El Paso, TX, USA) in our laboratory. Mobile phases consisted of acetonitrile and 10 mM ammonium acetate in water, with pH equal to 7.5 (adjusted with 1M ammonium hydroxide solution).

3.2. Compounds

Eight beta-adrenergic blockers were used in the study. The chemical structures are presented in Figure 2. The hydrophobicity of the compounds, measured as log P value and pK_a values, are listed in Table 2. Compounds were dissolved in HPLC water. The concentration of the samples was 1 mg/mL.

3.3. Instruments

All the experiments were conducted on the Shimadzu Prominence system (Kioto, Japan). This instrument includes a quaternary solvent delivery pump (LC-20AD) with an online degasser, an autosampler (SIL-20A), a column thermostat (CTO-10 AS VP), a spectrophotometric diode-array UV-Vis detector (SPD-M20A), and a data acquisition station. The data were collected in LabSolutions software.

Peak Deconvolution Analysis (Intelligent Peak Deconvolution Analysis i-PDeA II) was applied to track unseparated peaks. i-PDeA II is a part of LabSolutions software by Shimadzu Corporation (Kioto, Japan) [21,22,24].

ChromSword (Riga, Latvia) software was used for optimizing the gradient conditions [27,28].

3.4. Methods

All the measurements were undertaken with the mobile phase's 1 mL/min flow rate. The column thermostat was set to 30 °C, while the autosampler temperature was set to 5 °C. The injection volume was 1 µL for the analysis of single components and 10 µL for mixtures. Measurements were made in triplicate.

The gradient profiles were modeled in an off-line mode basis on the retention data. As an input, the parameters (retention time, peak area, and peak width at 50% high) of two linear gradients from 0 to 50% of a given solvent (acetonitrile in RP LC and 10 mM ammonium acetate in HILIC) in different times were used.

4. Conclusions

Using phosphodiester stationary phases, β-blockers exhibit retention in the RP LC and HILIC range of mobile phase composition. Their retention plot over the mobile phase composition demonstrates a characteristic U shape. The retention mechanism in RP LC is based mainly on the compound hydrophobicity; however, polar interactions play a significant role. It was also confirmed that phosphodiester stationary phases exhibit weak cation-exchange properties. Eluting β-blockers without salt added to the mobile phase is impossible. Unfortunately, cation-exchange properties cause band broadening and reduce the separation resolution, which may be overcome by applying gradient elution.

Applying Intelligent Peak Deconvolution Analysis may solve the problem with the co-elution of particular compounds. Deconvolution allows the digital separation of peaks based on their spectra using a PDA detector. Intelligent Peak Deconvolution Analysis is a promising tool facilitating the optimization of chromatographic methods.

Analyses of β-blockers allow describing the mixed-mode and cation-exchange properties of phosphodiester stationary phases. The best separation was obtained on Diol-P-C18 in gradient elution. Obtained results show the versatility of tested stationary phases for their application in RP LC, HILIC, and as a weak cation exchanger.

This article presents a series of preliminary studies on applying novel materials in RP LC and HILIC. Further investigation will focus on the cation-exchange mechanism, description, and optimization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules28073249/s1, Table S1: Retention factors of beta-blockers on tested phosphodiester statioanry phases.

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5. Wnioski

Przeprowadzone w ramach niniejszej pracy badania skoncentrowane były na przygotowaniu nowych faz stacjonarnych jako materiałów pracujących zarówno w układzie RP LC, jak i HILIC, w celu rozdzielania związków o szerokim spektrum polarności. Założenia zostały zrealizowane poprzez wykonanie następujących etapów pracy:

- synteza czterech faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi oraz dołączonymi czterema różnymi ligandami organicznymi: łańcuchem decylowym, łańcuchem oktadecylowym, podstawnikiem benzylowym oraz cząsteczką cholesterolu,
- charakterystyka otrzymanych faz stacjonarnych wybranymi analizami instrumentalnymi, a w tym: analiza elementarna, pomiary potencjału zeta, chromatograficzne wyznaczenie hydrofobowości,
- optymalizacja procesu pakowania poprzez dobór odpowiedniego rozpuszczalnika zawiesinowego oraz procedury pakowania,
- charakterystyka właściwości fizykochemicznych powierzchni faz stacjonarnych poprzez badania nad najbardziej precyzyjną metodą wyznaczenia objętości martwej oraz wyznaczeniem izoterm adsorpcji nadmiarowej, a także izoterm adsorpcji pozwalających na określenie mechanizmu retencji,
- badania chromatograficzne wykazujące możliwości pracy otrzymanych faz stacjonarnych w czystej wodzie (RP LC) oraz układzie HILIC z możliwością rozdzielania mieszanin związków o szerokim zakresie polarności, a także aplikacyjność badanych materiałów do rozdzielania mieszanin związków z grupy beta-blokerów.

Zrealizowane eksperymenty oraz otrzymane wyniki prowadzą do poniższych wniosków.

- Fazy stacjonarne z wbudowanymi grupami fosfodiestrowymi umożliwiają pracę zarówno w układzie RP LC, jak i HILIC, dzięki czemu nie ma konieczności zakupu i wykorzystania wielu kolumn chromatograficznych.
- Przebadane materiały umożliwiają rozdzielanie mieszanin zasad azotowych oraz alkaloidów purynowych zarówno w czystej wodzie, niewielkiej zawartości rozpuszczalnika organicznego w fazie ruchomej (RP LC), jak i układzie HILIC.

- Po raz pierwszy udało się rozdzielić mieszaninę wielopierścieniowych węglowodorów aromatycznych w czystej wodzie. Przebadane fazy stacjonarne umożliwiają również rozdzielenie tych związków w układzie RP LC.
- Możliwe jest rozdzielenie 8 beta-blokerów na każdej z badanych faz stacjonarnych, wykorzystując analizę gradientową w układzie RP LC, a także analizę dekonwolucji pików.
- Wykonane analizy chromatograficzne potwierdzają, iż zastosowanie przygotowanych faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi wpisuje się w założenia „zielonej" chemii. Możliwe jest przeprowadzenie analiz chromatograficznych, w których zredukowana jest ilość wytworzonych odpadów rozpuszczalników organicznych, a w przypadku wykorzystania tylko wody jako jedynego eluentu fazy ruchomej ilość ta jest maksymalnie ograniczona.
- W przypadku faz stacjonarnych z wbudowanymi grupami polarnymi konieczne jest precyzyjne wyznaczenie objętości martwej, gdyż materiały te charakteryzują się retencją w szerokim zakresie stężeń modyfikatora organicznego w fazie ruchomej.
- Wyznaczenie izoterm adsorpcji kofeiny i teofiliny oraz nadmiarowych izoterm adsorpcji acetonitrylu i wody potwierdziło, iż każda z badanych faz stacjonarnych posiada heterogeniczny charakter powierzchni oraz fakt, iż w układzie RP LC mechanizm retencji opiera się na adsorpcji jednowarstwowej (model bi-Langmuir), natomiast w układzie HILIC - na adsorpcji wielowarstwowej (model bi-Moreau).
- Analizy chromatograficzne potwierdzają, iż wszystkie z badanych faz stacjonarnych wykazują słaby charakter wymieniacza kationowego, a zatem również mechanizm jonowymienny wpływa na retencję, w szczególności podczas analizowania związków polarnych.
- Odpowiednio dobrany rozpuszczalnik zawiesinowy wykorzystany podczas procesu pakowania oraz kondycjonowanie kolumny z podłączonym rezerwuarem w warunkach prowadzonych analiz znacznie poprawia jakość zapakowanego złoża w kolumnie chromatograficznej, co przekłada się na sprawność kolumny.
- Przy doborze rozpuszczalnika zawiesinowego istotne jest znalezienie rozpuszczalnika zapewniającego jednocześnie agregację cząstek stałych w zawiesinie, jak i jej największą stabilność. Najlepszą metodą doboru rozpuszczalnika zawiesinowego dla przebadanych faz stacjonarnych były obserwacje zawiesin pod mikroskopem optycznym.

6. Streszczenie

W ramach niniejszej pracy zsyntezowano i scharakteryzowano cztery fazy stacjonarne z wbudowanymi grupami polarnymi oraz zbadano ich właściwości adsorpcyjne, a także opisano mechanizm retencji. Zsyntezowano fazy stacjonarne zawierające grupę fosfodiestrową jako grupę polarną oraz cztery różne ligandy organiczne: łańcuch decylowy, łańcuch oktadecylowy, podstawnik benzylowy oraz cząsteczka cholesterolu. Uzyskano dzięki temu dwie fazy stacjonarne, które wcześniej opracowano w naszym zespole badawczym (Diol-P-C10, Diol-P-C18) oraz dwie nowe fazy stacjonarne (Diol-P-benzyl, Diol-P-chol). Charakterystykę rozpoczęto od wyznaczenia gęstości pokrycia powierzchni faz stacjonarnych modyfikowanymi ligandami oraz określenia ich hydrofobowości, wykorzystując test Galushko. Optymalizacja procesu pakowania polegała na doborze rozpuszczalnika zawiesinowego, który wybierano w oparciu o wyniki potencjału zeta, badania mikroskopowe oraz lepkość. Finalnie otrzymano kolumny o sprawności zbliżonej do komercyjnie pakowanych kolumn chromatograficznych. W dalszej części pracy skupiono się na opisie właściwości adsorpcyjnych przygotowanych materiałów. Wykorzystano odwrotną chromatografię wykluczenia, metodę zaburzeniową oraz analizy markerów objętości martwej, spośród których ta pierwsza okazała się najbardziej odpowiednią. Wyznaczenie nadmiarowych izoterm adsorpcji acetonitrylu oraz wody potwierdziło heterogeniczność powierzchni każdej z badanych faz stacjonarnych. Wykonano badania chromatograficzne mające na celu potwierdzenie pracy badanych materiałów w warunkach czystej wody z zachowaniem zasad „zielonej chromatografii". Potwierdzono również możliwość rozdzielania polarnych i niepolarnych związków małocząsteczkowych zarówno w układzie HILIC, jak i RP LC. Z powodzeniem udało się rozdzielić mieszaninę alkaloidów purynowych, zasad azotowych oraz wielopierścieniowych węglowodorów aromatycznych. Ze względu na obecność polarnych i niepolarnych grup na powierzchni faz stacjonarnych zbadano mechanizm retencji poprzez wyznaczenie i modelowanie izoterm adsorpcji. Wykorzystano do tego metodę analizy czołowej oraz metodę inwersyjną. Aplikacyjność otrzymanych materiałów potwierdzono, analizując grupę leków beta-adrenolitycznych, gdzie wykorzystano również komputerowe rozdzielenie pików poprzez inteligentną analizę dekonwolucji pików.

Wyniki niniejszych badań pozwoliły na pełną charakterystykę faz stacjonarnych z wbudowanymi grupami fosfodiestrowymi, co poszerza ilość materiałów wykorzystywanych w wysokosprawnej chromatografii cieczowej oraz rozwija nurt „zielonej chromatografii".

7. Abstract

Within the scope of this thesis, four polar-embedded stationary phases were synthesized, characterized and their adsorption properties were investigated as well as their retention mechanism was described.

The stationary phases containing a phosphodiester group as a polar group and four different organic ligands - a decyl chain, an octadecyl chain, a benzyl substituent and a cholesterol molecule - were synthesized. This yielded two stationary phases previously developed by our research team (Diol-P-C10, Diol-P-C18) and two new stationary phases (Diol-P-benzyl, Diol-P-chol). The characterization began by determining the surface coverage density of the stationary phases with the modified ligands and determining their hydrophobicity using the Galushko test. The optimization of the packing process consisted of the selection of the slurry solvent, which was chosen one the basis of zeta potential results, microscopic studies and solvent viscosity. The final result was columns with efficiency similar to commercially packaged chromatography columns. In the following part of the work, the emphasis was on describing the adsorption properties of the prepared materials. Inverse size exclusion chromatography, the minor disturbance method and void volume marker analyses were used, of which the former proved to be the most suitable. The determination of the excess adsorption isotherms of acetonitrile and water confirmed the surface heterogeneity of each of the studied stationary phases. Chromatographic tests were performed to confirm the operation of the tested materials under pure water conditions with the principles of "green chromatography". The ability to separate polar and non-polar small-molecule compounds in both HILIC and RP LC systems was also confirmed. A mixture of purine alkaloids and nucleobases, as well as polycyclic aromatic hydrocarbons, were successfully separated. Due to the presence of polar and non-polar groups on the surface of the stationary phases, the retention mechanism was investigated by determining and modeling adsorption isotherms. For this purpose, the frontal analysis method and the inverse method were used. The applicability of the obtained materials was confirmed by analyzing a group of beta-blocker drugs, where computerized peak separation by intelligent peak deconvolution analysis was also used.

The results of the present study authorized a full characterization of stationary phases with embedded phosphodiester groups, which expands the range of materials used in high-performance liquid chromatography and develops the "green chromatography" trend.

8. Dorobek naukowy

a) Publikacje z listy Journal of Citation Reports:

1. Katarzyna Krzemińska, **Mikołaj Dembek**, Szymon Bocian, The competitiveness of solvent adsorption on polar-embedded stationary phases, *Journal of Separation Scicience*, 41 (2018) 4296–4303;
2. **Mikołaj Dembek**, Szymon Bocian, Pure water as a mobile phase in liquid chromatography techniques, *TrAC – Trends in Analytical Chememistry*, 123 (2020) 115793;
3. **Mikołaj Dembek**, Szymon Bocian, Stationary Phases for Green Liquid Chromatography, *Materials (Basel)*. 15 (2022) 419;
4. **Mikołaj Dembek**, Szymon Bocian, Bogusław Buszewski, Solvent Influence on Zeta Potential of Stationary Phase—Mobile Phase Interface, *Molecules*, 27 (2022) 968;
5. **Mikołaj Dembek**, Michał Szumski, Szymon Bocian, Bogusław Buszewski, Optimization of the packing process of microcolumns with the embedded phosphodiester stationary phases, *Journal of Separation Scicience*, 45 (2022) 3310–3318;
6. Oktawia Kalisz, **Mikołaj Dembek**, Sylwia Studzińska, Szymon Bocian, Beta-Blocker Separation on Phosphodiester Stationary Phases-The Application of Intelligent Peak Deconvolution Analysis, *Molecules*, 28 (2023) 3249;
7. **Mikołaj Dembek**, Szymon Bocian, Phosphodiester Stationary Phases as Universal Chromatographic Materials for Separation in RP LC, HILIC, and Pure Aqueous Mobile Phase, *Materials (Basel)*, 16 (2023) 3539;

b) Publikacje w czasopismach spoza listy Journal of Citation Reports:

1. **Mikołaj Dembek**, Szymon Bocian, Woda jako faza ruchoma w chromatografii cieczowej, *Analityka*, 20 (2019) 18-23;
2. **Mikołaj Dembek**, Szymon Bocian, Różne oblicza wodnej fazy ruchomej w chromatografii cieczowej, *Wiadomości chemiczne*, 74 (2020), 89-106.

Udział w konferencjach

a) Komunikaty ustne

1. **Mikołaj Dembek**, Szymon Bocian, Bogusław Buszewski, Potencjał zeta faz stacjonarnych z wbudowanymi grupami polarnymi, e-Zjazd Wiosenny Sekcji Studenckiej PTChem, 27-29 maja 2021 roku;

2. **Mikołaj Dembek**, Michał Szumski, Szymona Bocian, Bogusław Buszewski, Optymalizacja procesu pakowania mikrokolumn fazami stacjonarnymi do HPLC, II Międzynarodowa Multidyscyplinarna Konferencja Doktorantów Uniwersytetu Szczecińskiego, 22-23.06.2022.

b) Prezentacje posterowe

1. **Mikołaj Dembek**, Szymon Bocian, Katarzyna Krzemińska, Konkurencyjność adsorpcji rozpuszczalników w chromatografii cieczowej: ujęcie objętościowe, XIII Kopernikańskie Seminarium Doktoranckie, Bachotek, 16-18 czerwca 2019.

c) Współautorstwo wykładów, komunikatów i prezentacji posterowych

1. Szymon Bocian, Katarzyna Krzemińska, **Mikołaj Dembek**, Magdalena Skoczylas, Bogusław Buszewski, Fazy stacjonarne z wbudowanymi grupami polarnymi, X Polska Konferencja Chemii Analitycznej, Lublin, 1-5.07.2018, wykład;
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5. Bogusław Buszewski, Hussam Al Saoud, Michał Szumski, **Mikołaj Dembek**, Szymon Bocian, Teofil Jesionowski, Marek Potrzebowski, Myroslav Sprynskyy, Biosilika jako nowej generacji faza stacjonarna do technik separacyjnych : synteza, charakteryzacja i zastosowania, 64. Zjazd Naukowy PTChem. Lublin, 11-16 września 2022, prezentacja posterowa;
6. **Mikołaj Dembek**, Oktawia Kalisz, Katarzyna Krzemińska, Sylwia Studzińska, Szymon Bocian, Let's make chromatography more green, 13th Balaton Symposium on High-Performance Separation Methods, Siofok (Węgry) 4-6 września 2023, wykład w języku angielskim;

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Działalność popularno-naukowa

1. Przygotowanie oraz realizacja pokazów chemicznych pt. „Czas na chemię!" podczas XVIII Toruńskiego Festiwalu Nauki i Sztuki, 20-24.04.2018 r.
2. Przygotowanie i przeprowadzenie pokazów chemicznych podczas XIX. Toruńskiego Festiwalu Nauki i Sztuki, pt. „Oblicza komunikacji", 24-28.04.2019

9. Oświadczenia współautorów

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1. Kalisz Oktawia, Dembek Mikołaj, Studzińska Sylwia, Bocian Szymon, *Beta-blockers separation on phosphodiester stationary phases - the application of Intelligent Peak Deconvolution Analysis*, Molecules. 28 (2023) 3249

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1. Dembek Mikołaj, Bocian Szymon, Pure water as a mobile phase in liquid chromatography techniques, TrAC - Trends Anal. Chem. 123 (2020) 115793

   nadzorze tworzenia pracy, opracowaniu koncepcji, wizualizacji oraz korekcie i edycji całości pracy.

2. Dembek Mikołaj, Bocian Szymon, *Stationary Phases for Green Liquid Chromatography*, Materials (Basel). 15 (2022) 419

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3. Dembek Mikołaj, Bocian Szymon, Buszewski Bogusław, *Solvent Influence on Zeta Potential of Stationary Phase—Mobile Phase Interface*, Molecules. 27 (2022) 968

4. Dembek Mikołaj, Szumski Michał, Bocian Szymon, Buszewski Bogusław, *Optimization of the packing process of microcolumns with the embedded phosphodiester stationary phases*, J. Sep. Sci. 45 (2022) 3310–3318

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5. Dembek Mikołaj, Bocian Szymon, *Phosphodiester stationary phases as universal chromatographic materials for separation in RP LC, HILIC, and pure aqueous mobile phase*, Materials (Basel). 16 (2023) 3539

6. Kalisz Oktawia, Dembek Mikołaj, Studzińska Sylwia, Bocian Szymon, *Beta-blockers separation on phosphodiester stationary phases - the application of Intelligent Peak Deconvolution Analysis*, Molecules. 28 (2023) 3249

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1. Dembek Mikołaj, Szumski Michał, Bocian Szymon, Buszewski Bogusław, *Optimization of the packing process of microcolumns with the embedded phosphodiester stationary phases*, J. Sep. Sci. 45 (2022) 3310–3318

   opracowaniu koncepcji pracy, nadzorze nad prowadzonymi analizami chromatograficznymi oraz interpretacją wyników, dyskusji eksperymentu naukowego, pakowaniu kolumn kapilarnych , ocenie pracy pod względem merytorycznym oraz na korekcie i edycji manuskryptu.

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1. Dembek Mikołaj, Bocian Szymon, Buszewski Bogusław, *Solvent Influence on Zeta Potential of Stationary Phase—Mobile Phase Interface*, Molecules. 27 (2022) 968
2. Dembek Mikołaj, Szumski Michał, Bocian Szymon, Buszewski Bogusław, *Optimization of the packing process of microcolumns with the embedded phosphodiester stationary phases*, J. Sep. Sci. 45 (2022) 3310–3318

nadzorze merytorycznym przy pisaniu pracy, dyskusji eksperymentu naukowego oraz na korekcie i edycji finalnej wersji manuskryptu.

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1. **Dembek Mikołaj**, Bocian Szymon, Pure water as a mobile phase in liquid chromatography techniques, TrAC - Trends Anal. Chem. 123 (2020) 115793
2. **Dembek Mikołaj**, Bocian Szymon, *Stationary Phases for Green Liquid Chromatography*, Materials (Basel). 15 (2022) 419

współopracowaniu koncepcji pracy, przeprowadzeniu przeglądu literatury, napisaniu pierwszej wersji manuskryptu oraz dokonaniu niezbędnych korekt po recenzjach, a także odpowiedzi na recenzje;

3. **Dembek Mikołaj**, Bocian Szymon, Buszewski Bogusław, *Solvent Influence on Zeta Potential of Stationary Phase—Mobile Phase Interface*, Molecules. 27 (2022) 968
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zaplanowaniu oraz przeprowadzeniu kolejnych etapów badań, analizie i interpretacji otrzymanych wyników, utworzeniu wstępnej wersji manuskryptu oraz odpowiedzi na recenzje;

6. Kalisz Oktawia, **Dembek Mikołaj**, Studzińska Sylwia, Bocian Szymon, *Beta-blockers separation on phosphodiester stationary phases - the application of Intelligent Peak Deconvolution Analysis*, Molecules. 28 (2023) 3249

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