
**GDAŃSKI UNIWERSYTET MEDYCZNY**

**Wydział Farmaceutyczny**

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# Wpływ cieczy jonowych na efektywność metod chromatograficznych stosowanych do oznaczania leków cytostatycznych

Rozprawa doktorska

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# SPIS TREŚCI

**WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ**

1. Treder, N., Bączek, T., Wychodnik, K., Rogowska, J., Wolska, L., Plenis, A. The influence of ionic liquids on the effectiveness of analytical methods used in the monitoring of human and veterinary pharmaceuticals in biological and environmental samples - trends and perspectives. *Molecules* 25: (2020) 286.
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**WYKAZ POZOSTAŁYCH PUBLIKACJI W DOROBKU NAUKOWYM**

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## WYKAZ STRESZCZEŃ ZJAZDOWYCH

- ZWIĄZANYCH Z TEMATEM ROZPRAWY DOKTORSKIEJ

1. Treder, N.*, Olędzka, I., Roszkowska, A., Bączek, T., Plenis, A. Ciecze jonowe jako modyfikatory faz ruchomych w analizach farmaceutycznych techniką chromatografii cieczowej. XXVI Konferencja Naukowa Wydziału Farmaceutycznego Gdańskiego Uniwersytetu Medycznego, Gdańsk, Polska, 28-29 stycznia 2021, wystąpienie ustne.

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## WYKAZ SKRÓTÓW

| | | |
|---|---|---|
| **ADME** | Adsorpcja, dystrybucja, metabolizm i ekstrakcja | *Adsorption, Distribution, Metabolism and Extraction* |
| **ATP** | Ekstrakcja w wodnych układach dwufazowych | *Aqueous Two-Phase System* |
| **CE** | Elektroforeza kapilarna | *Capillary Electrophoresis* |
| **DLLME** | Dyspersyjna mikroekstrakcja w układzie ciecz-ciecz | *Dispersive Liquid Liquid Microextraction* |
| **EDX** | Spektroskopia rozpraszania energii | *Energy-dispersive X-ray spectroscopy* |
| **FT-IR** | Spektroskopia furierowska | *Fourier Transform Infrared Spectroscopy* |
| **GC** | Chromatografia gazowa | *Gas Chromatography* |
| **HCA** | Hierarchiczna analiza skupień | *Hierarchical Cluster Analysis* |
| **HF-LPME** | Mikroekstrakcja poprzez membranę do fazy ciekłej | *Hollow fibre liquid phase microextraction* |
| **ILs** | Ciecze jonowe | *Ionic Liquids* |
| **K-means CA** | Analiza skupień metodą *k-średnich* | *K-Means Clustering Analysis* |
| **LC** | Chromatografia cieczowa | *Liquid Chromatography* |
| **LC-FL** | Chromatografia cieczowa połączona z detekcją fluorescencyjną | *Liquid Chromatography with Fluorescence Detection* |
| **LLE** | Ekstrakcja w układzie ciecz-ciecz | *Liquid Liquid Extraction* |
| **LOQ** | Granica oznaczalności | *Limit of Quantification* |
| **MECK** | Micelarna chromatografia elektrokinetyczna | *Micellar Electrokinetic Chromatography* |
| **MIL** | Magnetyczna ciecz jonowa | *Magnetic ionic liquid* |
| **MIP** | Polimer z odciskiem molekularnym | *Molecularly imprinted polymer* |
| **MSPE** | Magnetyczna ekstrakcja do fazy stałej | *Magnetic Solid Phase Extraction* |
| **NACE** | Niewodna elektroforeza kapilarna | *Non-Aqueous Capillary Electrophoresis* |
| **NLPZ** | Niesteroidowe leki przeciwzapalne | *Nonsteroidal Anti-Inflammatory Drugs* |
| **NPs** | Nanocząstki | *Nanoparticles* |
| **PASSIL** | Pasywna ekstrakcja z zastosowaniem ILs | *Passive Sampling with ILs* |
| **RP-LC** | Chromatografia w odwróconym układzie faz | *Reversed Phase Liquid Chromatography* |
| **SBSE** | Ekstrakcja sorpcyjna na mieszadle | *Stir Bar Sorptive Extraction* |
| **SDME** | Mikroekstrakcja do pojedynczej kropli | *Single-drop microextraction* |
| **SFC** | Chromatografia w stanie nadkrytycznym | *Supercritical Fluid Chromatography* |
| **SPE** | Ekstrakcja do fazy stałej | *Solid Phase Extraction* |
| **SPME** | Mikroekstrakcja do fazy stałej | *Solid Phase Microexraction* |
| **TDM** | Terapia monitorowana stężeniem leku | *Therapeutic Drug Monitoring* |
| **TEM** | Transmisyjna mikroskopia elektronowa | *Transmission Electron Microscope* |
| **TGA** | Analiza termograwimetryczna | *Thermogravimetric Analysis* |
| **TLC** | Chromatografia cienkowarstwowa | *Thin-Layer Chromatography* |
| **XRD** | Dyfrakcja rentgenowska | *X-Ray Diffraction* |

## WSTĘP

Ciecze jonowe (ang. *Ionic Liquids*, ILs) zgodnie z powszechnie przyjętą definicją są to kationowo-anionowe połączenia, których temperatura topnienia nie przekracza 100°C. Pomimo, iż pierwsza IL została zsyntezowana w 1914 roku przez Paula Waldena, to dopiero w ciągu ostatnich dwóch dekadach związki te stały się obiektem wzmożonego zainteresowania [1–3]. Niewątpliwie ich popularność wynika z dużej różnorodności strukturalnej warunkującej posiadany profil fizykochemicznych właściwości. Zatem, wybierając odpowiednią kationowo-anionową kombinację istnieje możliwość zastosowania w warunkach doświadczalnych IL o określonej gęstości, lepkości i stanie skupienia, jak również o określonych hydrofilowych lub też hydrofobowych właściwościach. To sprawia, że w literaturze ILs określane są także mianem związków „projektowalnych" [4–11]. Istotnym aspektem jest również ich „zielony" charakter, gdyż ze względu na znikomą prężność par, wysoką stabilność termiczną lub niepalność są uznawane za substancje spełniające kryteria „*Zielonej Chemii*". Choć w ostatnich latach zaczęto kwestionować brak toksyczności niektórych ILs [12], to jednak w większości przypadków ich aplikacja jest bardziej optymalnym rozwiązaniem niż używanie powszechnie stosowanych rozpuszczalników organicznych o potwierdzonej szkodliwości [13–18]. Zatem, stosując ILs istnieje możliwość nie tylko modyfikowania warunków eksperymentalnych, ale także projektowania procedur bardziej przyjaznych środowisku.

Jednym z obszarów potencjalnego użycia ILs jest opracowanie metod analitycznych, które pomimo istotnych osiągnięć w tym zakresie, nadal pozostają ogromnym wyzwaniem dla analityków. Jest to szczególnie trudne wówczas, gdy konieczne jest oznaczanie analitów na bardzo niskich poziomach stężeń występujących w próbce o skomplikowanej matrycy. Przykładem tego typu oznaczeń są analizy jakościowe i ilościowe substancji leczniczych w materiale biologicznym, ponieważ leki są często przyjmowanie przez pacjentów w niskich dawkach, a ponadto podlegają w organizmie procesom adsorpcji, dystrybucji, metabolizmu i wydalania (ang. *Adsorption, Dystribution, Metabolism and Extraction*, ADME) prowadząc do obniżenia poziomu stężeń substancji macierzystej i powstania produktów ich metabolizmu. To sprawia, że monitorowanie substancji leczniczych w matrycach biologicznych wymaga opracowania dokładnych, precyzyjnych i czułych metod analitycznych, które umożliwiają oznaczanie substancji leczniczych na wymaganym poziomie stężeń [19]. Wyzwaniem jest także konieczność ich oznaczania w matrycach zawierających wiele endogennych związków, jak i wspomnianych powyżej metabolitów, mogących utrudniać analizę. Składniki matrycowe mogą bowiem wchodzić w interakcje z analitami i/lub konkurować o miejsce kontaktu z rozpuszczalnikiem ekstrahującym/sorbentem prowadząc do znacznego obniżenia wydajności

ekstrakcji (niska wartość odzysku), a na etapie detekcji prowadzić do nieprawidłowych wskazań urządzeń pomiarowych. Na przykład, jeżeli czas retencji składnika matrycowego w trakcie przebiegu analizy chromatograficznej jest taki sam jak czas retencji związku oznaczanego, to można błędnie wnioskować o obecności analitu (brak selektywności). Ponadto, zbyt wysoki sygnał względem rzeczywistej ilości analitu w próbce może znacznie obniżyć wiarygodność wyników ilościowych (wyniki analiz są zawyżone). W opracowaniu metody analitycznej można zatem wyróżnić dwa kluczowe etapy decydujące o jakości końcowych wyników oznaczeń. Pierwszy dotyczy odpowiedniego przygotowanie próbek do analizy, zaś drugi jest związany z wyborem warunków oznaczania wybranych analitów daną techniką analityczną. Zarówno pierwszy jak i drugi etap jest obszarem intensywnych badań prowadzonych w zakresie poszukiwania nowych rozwiązań pozwalających na poprawę efektywności opracowanych metod analitycznych.

Rozważając etap przygotowania próbek należy podkreślić, że aktualnie dostępnych jest wiele procedur ekstrakcyjnych. Do najpopularniejszych nadal można zaliczyć deproteinizację, ekstrakcję w układzie ciecz-ciecz (ang. *Liquid Liquid Extraction*, LLE) oraz ekstrakcję do fazy stałej (ang. *Solid Phase Extraction*, SPE). Jednakże te klasyczne formy przygotowania próbek wiążą się ze znacznymi ograniczeniami związanymi przede wszystkim z koniecznością stosowania dużych ilości szkodliwych rozpuszczalników organicznych (np. acetonitrylu, metanolu, dichlorometanu czy chloroformu). Dodatkowo, w przypadku ekstrakcji SPE, wymagane jest stosowanie kolumienek ekstrakcyjnych o odpowiednim sorbencie, których żywotność może być znacząco ograniczona w przypadku bogatych matryc biologicznych. Odpowiedzią na te wyzwania są techniki mikroekstrakcyjne, spośród których na szczególnie wyróżnienie zasługuje najszerzej stosowana dyspersyjna mikroekstrakcja w układzie ciecz-ciecz (ang. *Dispersive Liquid Liquid Microextraction*, DLLME) oraz mikroekstrakcja do fazy stałej (ang. *Solid Phase Microextraction*, SPME). DLLME jest zminiaturyzowaną wersją ekstrakcji LLE, w której stosowanie mniejszych objętości rozpuszczalników organicznych jest możliwe ze względu na wykorzystanie ekstrahenta w połączeniu z dyspergatorem pozwalających na osiągnięcie dużej powierzchni kontaktu pomiędzy dwiema niemieszającymi się fazami, co może znacząco podwyższyć efektywność ekstrakcji [20]. W przypadku SPME, stosowane są zminiaturyzowane włókna pokryte odpowiednim materiałem sorpcyjnym, które po umieszczeniu w analizowanych próbkach/nad próbką pozwalają na izolację badanych związków z danej matrycy, np. biologicznej [21,22]. Jednakże, mimo znacznych korzyści w porównaniu do tradycyjnych technik LLE i SPE, wymienione metody mikroekstrakcji posiadają pewne ograniczenia, jak choćby niższa wydajność ekstrakcji wynikająca ze

zmniejszonej ilości użytego rozpuszczalnika ekstrahującego/sorbentu oraz trudnościami z oczyszczeniem włókien ekstrakcyjnych w przypadku SPME [23]. Należy jednak podkreślić, że opracowano także inne techniki ekstrakcji, w tym m.in. mikroekstrakcja do pojedynczej kropli (ang. *Single-Drop Microextraction*, SDME), mikroekstrakcja poprzez membranę do fazy ciekłej (ang. *Hollow Fibre Liquid Phase Microextraction*, HF-LPME), magnetyczna ekstrakcja do fazy stałej (ang. *Magnetic Solid Phase Extraction*, MSPE) lub ekstrakcja z zastosowaniem wirującego elementu sorpcyjnego (ang. *Stir Bar Sorptive Extraction*, SBSE) [24–27]. Spośród nich, interesującym podejściem jest wykorzystanie sorbentów o właściwościach magnetycznych, które dzięki przyłożeniu zewnętrznego pola magnetycznego mogą być szybko oddzielone od matrycy [26]. Jednak, wyzwaniem w aplikacji MSPE jest właściwe przygotowanie sorbentów, obejmujące zarówno procedurę syntezy decydującą o wielkości ich powierzchni właściwej, jak również procedurę funkcjonalizacji, która może determinować powinowactwo analitu do sorbentu [28]. Obecnie, do najczęściej stosowanych materiałów powlekających można zaliczyć krzemionkę, surfaktanty lub polimery [29–32], ale poszukiwanie odpowiednich struktur materiałów powlekających nadal stanowi przedmiot zainteresowania wielu naukowców [29–32]. Należy jednak podkreślić, że MSPE jest mniej popularną techniką, szczególnie w przypadku analiz farmaceutycznych, gdyż dotychczas opisano jej zastosowanie jedynie w przypadku oznaczania środków psychoaktywnych, niesteroidowych leków przeciwzapalnych (ang. *Nonsteroidal Anti-Inflammatory Drugs*, NLPZ), bisfosfonianów, fluorochinolonów lub sildenafilu [26]. Zatem, dowodzi to, iż kierunek badań związany z aplikacją MSPE w obszarze oznaczeń substancji leczniczych powinien być nadal rozwijany.

Należy także podkreślić, że w przypadku metod opartych na chromatografii cieczowej, rozdzielanie substancji leczniczych najczęściej prowadzi się w odwróconym układzie faz (ang. *Reversed-Phase Liquid Chromatography*, RP-LC), jednakże uzyskany wynik analizy nie zawsze jest zadowalający. Jedną z przyczyn mogą być interakcje pomiędzy występującymi w formie zdysocjowanej analitami oraz składnikami matrycy a wolnymi grupami silanolowymi występującymi na powierzchni niepolarnych faz stacjonarnych, co może negatywie wpływać na kształt piku (ogonowanie) i prowadzić do zmniejszenia zdolności rozdzielczych układu chromatograficznego. Aby przezwyciężyć te ograniczenia, analitycy poszukują nowych rozwiązań do kontroli separacji analitów, które obejmują m.in. stosowanie dodatków do fazy ruchomej [33]. Dzięki wprowadzeniu tej modyfikacji dochodzi do dynamicznego powlekania powierzchni fazy stacjonarnej przez składniki fazy ruchomej, co może znacząco zwiększyć efektywność rozdzieleń chromatograficznych [34]. Jednak w tej stosunkowo szybkiej i prostej

procedurze usprawnienia separacji chromatograficznych ograniczeniem jest niewielka liczba dostępnych związków, które można stosować jako dodatki do faz ruchomych. Do najlepiej poznanych zaliczamy nieorganiczne sole, aminy (trietyloamina i dimetylooctylamina) lub anionowe surfaktanty (np. laurylosiarczan sodu) [35–37]. Wcześniejsze doniesienia pokazują także, iż jako dodatki do fazy ruchomej mogą być wykorzystane ILs [38–40]. Jednakże, duża różnorodność tej grupy sprawia, że ich wpływ nie został w pełni poznany, a to z kolei ogranicza rutynowe zastosowanie ILs w opracowaniu metod analitycznych, w tym także dotyczących oznaczania różnorodnych substancji leczniczych.

Innym rozwiązaniem problemów separacyjnych w chromatografii może być zmiana składu fazy stacjonarnej i wykorzystanie faz z ugrupowaniem fenylowym zamiast powszechnie stosowanych faz alkilowych [41]. Niemniej jednak zastosowanie faz fenylowych i ich pochodnych jest znacząco ograniczone w analizach farmaceutycznych, gdyż obecność ugrupowania fenylowego na powierzchni fazy stacjonarnej prowadzi do wystąpienia dodatkowego, silnego oddziaływania typu π-π z analitami posiadających wiązania nienasycone. W konsekwencji, szczególnie dla związków zawierających ugrupowania fenylowe, może dochodzić do znacznej retencji analitów, co wymaga przedłużenia czasu analizy oraz może prowadzić do asymetrii kształtu pików. Ponadto, dla silnie hydrofobowych związków mogą wystąpić problemy z przywróceniem warunków początkowych analizy oraz z relatywnie szybkim obniżeniem sprawności kolumny chromatograficznej, co może wynikać z niepełnej elucji silnie hydrofobowych związków.

Zatem, stale poszukiwane są nowe rozwiązania analityczne w zakresie technik przygotowania prób do analizy oraz warunków prowadzenia rozdzieleń chromatograficznych. W powyższy nurt wpisuje się użycie ILs, gdyż związki te mogą być wykorzystane w obu wyżej wymienionych obszarach opracowania metody analitycznej. Dotyczy to także opracowania metod analitycznych stosowanych do oznaczania leków cytostatatycznych, które zasługują na szczególną uwagę, gdyż charakteryzują się wąskim indeksem terapeutycznym. Sprawia to, że ustalenie właściwego dawkowania jest trudne, a przekroczenie zakresu terapeutycznego może skutkować brakiem efektu farmakologicznego (zbyt niski poziom leku w organizmie) bądź poważnymi działaniami niepożądanymi (zbyt wysokie stężenie). Dodatkowo, biorąc pod uwagę między- i wewnątrzosobnicze różnice farmakokinetyczne u pacjentów onkologicznych oraz sposób prowadzenia terapii (często są to protokoły wymagające podania wielu substancji czynnych o różnorodnych mechanizmach działania przeciwnowotworowego, jak i wspomagającego działanie cytostatyczne), ustalenie właściwego dawkowania jest znacząco utrudnione dla lekarzy i farmaceutów. Z tego powodu, zalecane jest prowadzenie terapii

monitorowanej stężeniem leku (ang. *Therapeutic Drug Monitoring*, TDM), która poprzez oznaczenie poziomu leku w płynach biologicznych u indywidualnego pacjenta pozwala odpowiednio dostosować dawkowanie [42]. Jednak, wymaga to opracowania odpowiednio dokładnych, precyzyjnych i czułych metod analitycznych.

## CEL PRACY

*Cel główny:*

Celem niniejszej rozprawy doktorskiej było zbadanie wpływu ILs na efektywność metod chromatograficznych stosowanych do oznaczania wybranych leków cytostatycznych.

*Cele szczegółowe:*

- weryfikacja dotychczasowych osiągnięć w zakresie zastosowania ILs w zakresie opracowania metod analitycznych stosowanych do oznaczania różnorodnych substancji leczniczych,
- zastosowanie ILs jako dodatków do fazy ruchomej i ocena ich wpływu na efektywność rozdzieleń chromatograficznych wybranych cytostatyków (antybiotyków antracyklinowych) techniką chromatografii cieczowej z detekcją fluorescencyjną (ang. *Liquid Chromatography with Fluorescence Detection*, LC-FL),
- kontynuacja badań w zakresie modyfikacji przebiegu rozdzielań chromatograficznych cytostatyków z użyciem alkilowych i fenylowych faz stacjonarnych w obecności ILs o zróżnicowanych strukturach chemicznych, dodanych do faz ruchomych na różnym poziomie stężeń przy zmiennych warunkach chromatograficznych,
- opracowanie procedury otrzymywania i funkcjonalizacji za pomocą ILs magnetycznych nanocząstek (ang. *Nanoparticles*, NPs) wykorzystywanych na etapie przygotowania próbek do analizy chromatograficznej,
- zbadanie wpływu magnetycznych sorbentów funkcjonalizowanych różnymi strukturami ILs na wydajność ekstrakcji epirubicyny z próbek biologicznych oraz oszacowanie potencjalnych możliwości ich aplikacji w opracowaniu metod chromatograficznych oznaczania innych substancji leczniczych.

## OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

W skład cyklu publikacji będących przedmiotem niniejszej rozprawy doktorskiej wchodzą cztery prace opublikowane w czasopismach ujętych w *Journal Citation Reports* (JCR), przy czym pierwsza jest manuskryptem przeglądowym a trzy pozostałe to publikacje oryginalne opisujące wyniki badań uzyskane w trakcie realizacji pracy doktorskiej.

### Publikacja 1

Treder, N., Bączek, T., Wychodnik, K., Rogowska, J., Wolska, L., Plenis, A. The influence of ionic liquids on the effectiveness of analytical methods used in the monitoring of human and veterinary pharmaceuticals in biological and environmental samples – trends and perspectives. *Molecules* 25: (2020) 286.

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#### *Wprowadzenie i cel pracy*

Jak wspomniano we wstępie, intensywny rozwój badań z udziałem ILs nastąpił w ostatnich dwóch dekadach XXI wieku. Aby określić, jak istotny naukowo i technologicznie postęp w tym zakresie nastąpił oraz jakie problemy należy jeszcze rozwiązać, zdecydowano się szczegółowo prześledzić literaturę naukową. Na bazie uzyskanych danych przygotowano pracę przeglądową podsumowującą dotychczasowe aplikacje ILs w opracowywaniu metod analitycznych stosowanych do oznaczania różnorodnych substancji leczniczych w próbkach biologicznych i środowiskowych. Do niniejszej pracy przeglądowej włączono 238 artykułów, które opublikowano przed ukazaniem się niniejszego przeglądu tj. do końca 2019 roku. Przedstawiono historię, klasyfikację oraz właściwości ILs, pozwalające na pełne zrozumienie przyczyn ich rosnącej popularności w obszarze analitycznym. Zwrócono uwagę na kilka kluczowych momentów w rozwoju ILs, dzięki którym stały się one przedmiotem wzmożonego zainteresowania. Była to m.in.. synteza ILs na bazie 1-etylo-3-metyloimidazolu, który w porównaniu do wcześniej otrzymanych struktur wykazywał dużo wyższą stabilność w wodzie i powietrzu, prowadząc tym samym do otrzymania wielu nowych związków jak również nowych kierunków ich zastosowania [43]. Istotne znaczenie miało także wprowadzenie ILs do komercyjnej sprzedaży pozwalającej na szybki i łatwy dostęp dla szerokiego grona odbiorców [1]. Ponadto, rosnąca popularność ILs wynikała z doniesień na temat ich wyjątkowych właściwości fizykochemicznych odróżniających tę grupę od innych

powszechnie stosowanych odczynników chemicznych. Jak wspomniano we wstępie, dzięki asymetrycznej strukturze, ich temperatura topnienia nie przekracza 100°C, a w wielu przypadkach jest zbliżona do temperatury pokojowej, co stanowi znaczną różnicę w porównaniu do typowych soli nieorganicznych mających temperaturę topnienia często powyżej 400°C. Inną, nietypową właściwością jest znacznie większa lepkość ILs w porównaniu do szeroko stosowanych rozpuszczalników organicznych. Rozważając potencjalne wykorzystanie ILs w opracowaniu metod analitycznych istotny jest także ich hydrofilowy lub hydrofobowy charakter w zależności od rodzaju użytego kationu i anionu [44]. Z uwagi na bardzo duże zróżnicowanie tej grupy związków, istotne znaczenie miało także wprowadzenie klasyfikacji ILs. Przynależność ILs do jednej z trzech generacji (podział ze względu na właściwości fizyczne, chemiczne lub biologiczne), ich klasyfikacja ze względu na strukturę (np. IL z kationem imidazoliowym) lub też wskazanie właściwości w nazwie ILs, jak ma to miejsce w przypadku magnetycznych ILs (ang. *Magnetic Ionic Liquids,* MILs) ułatwiało poszukiwanie właściwych struktur do określonych aplikacji, w których miały być zastosowane.

W dalszym etapie niniejszego przeglądu wyodrębniono główne kierunki zastosowań ILs w opracowaniu metod analitycznych. Jeden z nich obejmował użycie ILs na etapie przygotowania próbek i uwzględniał zarówno popularne techniki ekstrakcji/mikroekstracji do fazy ciekłej (LLE, DLLME) bądź do fazy stałej (SPE, SPME) jak również mniej znane techniki, do których można zaliczyć ekstrakcję w wodnych układach dwufazowych (ang. *Aqueous Two-Phase System*, ATP), SBSE lub też pasywną ekstrakcję z zastosowaniem ILs (ang. *PASsive Sampling with Ionic Liquids,* PASSIL). Wśród opisanych siedemdziesięciu jeden prac badawczych z tej tematyki, pięćdziesiąt publikacji dotyczyło ekstrakcji do fazy ciekłej, przy czym najczęściej ILs wykorzystywano w typowych procedurach DLLME lub jej modyfikacjach (34 publikacje). Spośród nich na uwagę zasługują prace, w których ze względu na ograniczenia ILs wynikające z większej lepkości tych związków w porównaniu do rozpuszczalników organicznych, do osiągnięcia odpowiedniego rozproszenia w próbce zastosowano ultradźwięki, intensywne mieszanie lub zmiany temperatury [45–47]. Należy również podkreślić, iż wprowadzenie ILs w DLLME pozwalało znacznie ograniczyć zużycie szkodliwych rozpuszczalników organicznych, i sprawiało, że opracowana metoda spełniała kryteria „*Zielonej Chemii*”. Przykładem takiego podejścia, było choćby zastosowanie dwóch rodzajów ILs, spośród których jedna ze względu na swoje właściwości hydrofilowe pełniła rolę dyspergatora, zaś druga jako związek hydrofobowy była ekstrahentem. Takie podejście pozwoliło na osiągnięcie wysokich wyników wydajności (88-111%) przy niewielkim zużyciu rozpuszczalnika organicznego (50 µl), który był stosowany do rozpuszczenia pobranej kropli

IL [45]. Mniej popularne było wykorzystanie ILs w procedurach opartych na ekstrakcji/mikroekstrakcji do fazy stałej. Do momentu ukazania się niniejszej pracy przeglądowej, wyniki badań w tym zakresie przedstawiono w dwudziestu jeden publikacjach, które skupiały się głównie na ich aplikacji jako sorbenty w kolumnach monolitycznych do ekstrakcji SPE [46,47] lub jak monomery w syntezie różnorodnych polimerów z odciskiem molekularnym (ang. *Molecularly Imprinted Polymer*, MIP) [48]. Doniesienia literaturowe potwierdziły, że związki te mogą także poprawić wydajność ekstrakcji poprzez zastosowanie ILs jako materiały powlekające włókna SPME [49] lub na etapie desorpcji analitu, jako modyfikatory rozpuszczalnika organicznego [24]. Rozważając wcześniejsze badania związane z użyciem ILs w technikach ekstrakcji/mikroekstracji należy także zwrócić uwagę na rodzaj analitów izolowanych z matrycy za pomocą ILs. Zebrane dane literaturowe dowiodły, że ILs były wykorzystywane najczęściej do ekstrakcji niesteroidowych leków przeciwzapalnych [50], przeciwdepresyjnych [51–53], przeciwpsychotycznych [54] oraz antybiotyków [55,56], podczas gdy żadna praca nie dotyczyła leków cytostatycznych. W pracach zaobserwowano także pewną regularność dotyczącą wyboru struktur ILs testowanych w trakcie optymalizacji metody. Większość badań obejmowała związki oparte na kationie imidazoliowym z łańcuchem alkilowym C2, C4, C6 lub C8, które łączono z anionem chlorkowym [Cl] [57], tetrafluoroboranowym [$BF_4$] [58], heksafluorofosforanowym [$PF_6$] [59] lub rzadziej bis(trifluorometylosulfonylo)imidkowym [$N(SO_2CF_3)_2$] [60] czy metylosiarczanowym [$CH_3SO_4$] [61]. Najwyższą wydajność ekstrakcji wybranych analitów techniką DLLME w większości prac zapewniała IL z anionem [$PF_6$] oraz kationem imidazoliowym z łańcuchem alkilowych C8. Te same struktury były również testowane w ekstrakcji/mikroekstrakcji do fazy stałej, jednakże w przypadku tych technik, pojawiały się także doniesienia dla struktur z dłuższym łańcuchem alkilowym tj. C12 lub C16 oraz dla ILs samodzielnie syntezowanych przed ich aplikacją np. ILs z podstawnikiem winylowym lub winylobenzylowym.

Drugim kierunkiem badań związanym z zastosowaniem ILs w metodach analitycznych była ich aplikacja na etapie separacji analitów technikami chromatograficznymi. Największa liczba prac dotyczyła wykorzystania ILs w chromatografii cieczowej (ang. *Liquid Chromatography*, LC), w której były one stosowane jako dodatki do fazy ruchomej [39,62,63]. Wskazano, że ILs w znaczący sposób mogą wpływać na rozdzielenie chromatograficzne analitów, a szczególnym czynnikiem, który decyduje o uzyskanych wynikach jest rodzaj kationu i anionu tworzącego ich strukturę [64]. W przeciwieństwie do innych modyfikatorów fazy ruchomej, takich jak choćby trietyloamina, oba jony IL mogą być zaangażowane w proces separacyjny analitów, dzięki czemu mogą w większym stopniu być zaangażowane w interakcje

na powierzchni fazy stacjonarnej [65]. Podobnie, jak w przypadku prac dotyczących zastosowania ILs na etapie przygotowania próbek, również jako dodatki do faz ruchomych najczęściej testowano ILs z kationem imidazoliowym i łańcuchem alkilowym o długości C2, C4, C6, C8 oraz anionem [Cl], [$BF_4$] i [$PF_6$]. Należy przy tym podkreślić, że jeżeli badania obejmowały etap wyboru optymalnej struktury IL, to w większości badań przeprowadzano analizy dla trzech lub pięciu związków różniących się długością łańcucha alkilowego lub rodzajem anionu [40],[66]. Efektem takiego podejścia był różnorodny wybór ILs do dalszych badań – w niektórych pracach najbardziej optymalne wyniki zapewniały ILs z anionem [$BF_4$], w innych z kolei związki z anionem [Cl]. Ponadto, autorzy wcześniejszych prac sugerowali, iż efekt ILs na separację analitów może zależeć od innych czynników, w tym stężenia ILs, pH lub rodzaju fazy stacjonarnej [39,66,67], a także to, że ILs były immobilizowane na powierzchni faz stacjonarnych [68]. Wykazano m.in., że dzięki obecności IL z kationem imidazoliowym i anionem bis(trifluorometylosulfonylo)imidkowym [$N(SO_2CF_3)_2$] możliwe było opracowanie faz stacjonarnych do separacji w trybie mieszanym, które pozwalają zarówno na analizy chromatograficzne w układzie faz odwróconych jak i chromatografii oddziaływań hydrofilowych [69]. Dodatkowo, ILs były stosowanie w chromatografii gazowej (ang. *Gas Chromatography*, GC), chromatografii cienkowarstwowej (ang. *Thin-Layer Chromatography*, TLC) oraz chromatografii w stanie nadkrytycznym (ang. *Supercritical Fluid Chromatography*, SFC) [70]–[72]. Szczególnie interesującym był przykład wykorzystania ILs jako wypełnienia kolumn kapilarnych do GC, co wynika z wysokiej stabilności termicznej tych związków w wymaganych warunkach analizy. Wyniki udowodniły, że kolumny pokryte ILs zapewniały m.in. wyższą rozdzielczość i odpowiedni kształt pików [73]. Innym obszarem aplikacji ILs były także techniki elektroforetyczne, w których dominującym kierunkiem było użycie ILs jako modyfikatorów buforu separacyjnego, zarówno w metodach opartych na elektroforezie kapilarnej (ang. *Capillary Electrophoresis*, CE), jak i micelarnej chromatografii elektrokinetycznej (ang. *Micellar Electrokinetic Chromatography*, MECK), ale także niewodnej elektroforezie kapilarnej (ang. *Non-Aqueous Capillary Electrophoresis*, NACE) [74]–[76]. Dzięki dodaniu ILs do buforu separacyjnego dochodziło do dynamicznego powlekania ścian kapilary krzemionkowej przez jony tworzące strukturę IL, co z kolei powodowało zmiany w przepływie elektroosmotycznych i w konsekwencji wzrost intensywności sygnału, lepszą selektywność i bardziej optymalny kształt pików [77,78].

Podsumowując, dotychczasowe dane literaturowe wykazały duży potencjał ILs w poprawie wyników analiz jakościowych i ilościowych różnorodnych substancji leczniczych

w próbkach biologicznych i środowiskowych. Najczęstszym kierunkiem aplikacji ILs w opracowaniu metod analitycznych było ich zastosowanie na etapie przygotowania próbek oraz jako dodatków do faz ruchomych w chromatografii cieczowej. Jednakże, zarówno w procesach ekstrakcji jak i separacji analitów różnorodnymi technikami analitycznymi, zakres testowanych ILs ograniczał się do podobnych chemicznie struktur tzn. ILs z kationem, którym był pierścień imidazoliowy z łańcuchem alkilowym C2-C8, oraz anionem [Cl], [$BF_4$], [$PF_6$] lub [$CH_3SO_4$] [40,79–82]. Ponadto, w przypadku LC, analizy chromatograficzne prowadzone głównie z użyciem oktadecylokrzemionkowych faz stacjonarnych, rzadziej faz stacjonarnych o krótszym łańcuchu alkilowym [39,83], podczas gdy fenylowe fazy stacjonarne w badaniach były uwzględniane sporadycznie [84].

***Wnioski***

Przegląd literatury potwierdził, że ILs mają ogromny potencjał w opracowaniu metod analitycznych stosowanych do analizy substancji leczniczych w próbkach biologicznych i środowiskowych. Jednakże wskazał także „schematyczny" sposób ich testowania (te same struktury ILs używano w badaniach dotyczących przygotowania próbek do analizy, jak i rozdzieleń chromatograficznych oraz elektroforetycznych). W dominującej skali eksperymenty prowadzono przy użyciu oktadecylokrzemionkowych faz stacjonarnych (LC)/niemodyfikowanych kapilar krzemionkowych (CE, MEKC), a zakres analizowanych substancji leczniczych w matrycach biologicznych i środowiskowych był ograniczony.

Wnioski uzyskane na podstawie wykonanego przeglądu literatury potwierdziły słuszność podjęcia niniejszej tematyki rozprawy doktorskiej i miały wpływ na dalszy kierunek prowadzonych badań.

**Publikacja 2**

Treder, N., Olędzka, I., Roszkowska, A., Bączek, T., Plenis, A. Control of retention mechanisms on an octadecyl-bonded silica column using ionic liquid-based mobile phase in analysis of cytostatic drugs by liquid chromatography. *J. Chromatogr. A* 1651: (2021) 462257.

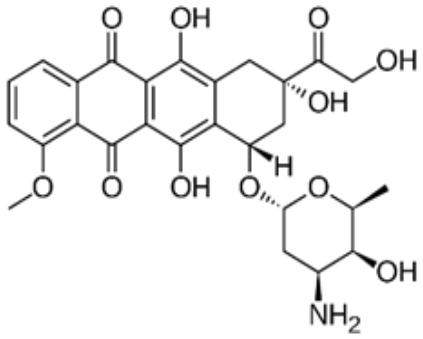
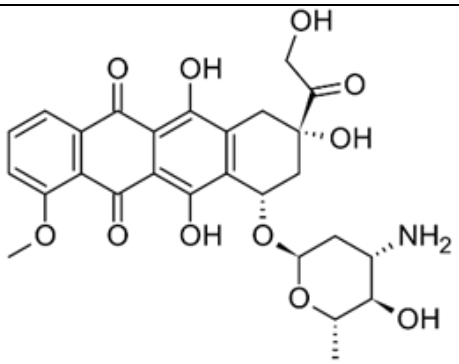
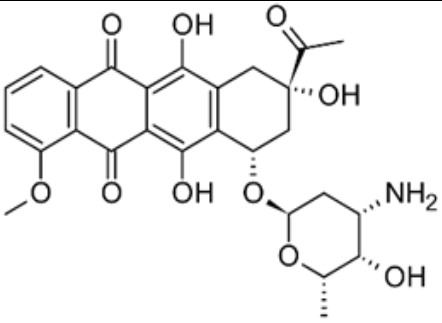
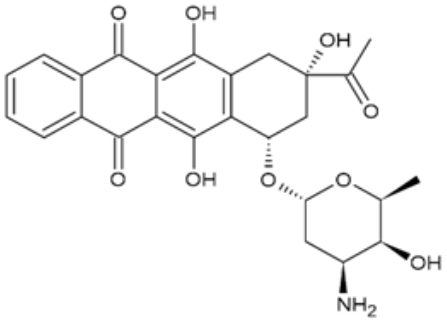
**IF: 4.759**

**MEiN: 100.00**

***Wprowadzenie i cel pracy***

Pierwszym problemem badawczym, jakim zajęto się w ramach niniejszej rozprawy doktorskiej było zbadanie mechanizmów separacyjnych występujących na powierzchni

oktadecylokrzemionkowej fazy stacjonarnej w obecności zarówno już przebadanych, jak i jeszcze nierozpatrywanych ILs, które użyto jako modyfikatory faz ruchomych podczas analizy czterech antybiotyków antracyklinowych (doksorubicyny, epirubicyny, daunorubicyny i idarubicyny) techniką LC-FL. Antybiotyki antracyklinowe to substancje należące do grupy leków cytostatycznych, które pod względem chemicznym są tetracyklicznymi cząsteczkami ze szkieletem antrachinonowym połączonym z ugrupowaniem cukrowym wiązaniem glikozydowym, nadającym charakter zasadowy tym związkom (Rycina 1).

| DOKSORUBICYNA | EPIRUBICYNA |
|---|---|
|  |  |
| DAUNORUBICYNA | IDARUBICYNA |
|  |  |

*Rycina 1. Budowa strukturalna antybiotyków antracyklinowych.*

Powyższa struktura chemiczna warunkuje mechanizm ich działania przeciwnowotworowego, który polega na wbudowywaniu cząsteczek w podwójną helisę DNA oraz hamowaniu aktywności topoizomerazy II. Ponadto, leki biorą udział w tworzeniu wolnych rodników także prowadząc w ten sposób do uszkodzenia DNA. Ten wielokierunkowy mechanizm działania sprawia, że są jednymi z najczęściej stosowanych leków cytostatycznych zarówno w protokołach opartych na monoterapii, jak i terapii wielolekowej [85]. Jednakże, antybiotyki antracyklinowe wykazują także szereg działań niepożądanych, spośród których najbardziej niebezpieczna jest kardiotoksyczność, która może powodować wysokie ryzyko zagrożenia życia pacjenta. Z uwagi na fakt, iż powyższe cytostatyki charakteryzują się wąskim

indeksem terapeutycznym, są traktowane jako potencjalne leki ze wskazaniem do prowadzenia TDM.

Popularną metodą oznaczania antybiotyków antracyklinowych w materiale biologicznym jest RP-LC z detekcją FL, co wynika z naturalnej zdolności do fluorescencji tej grupy związków [86]. Ta właściwość sprawia, iż substancje te mogą być monitorowane w próbkach biologicznych na niskim poziomie stężeń bez konieczności ich derywatyzacji. Z drugiej strony, antracykliny poprzez grupę aminową w trakcie rozdzieleń RP-LC mogą ulegać także interakcjom z grupami silanolowymi występującymi na powierzchni faz stacjonarnych, w tym na fazie octadecylokrzemionkowej, co w konsekwencji może prowadzić do nieprawidłowego kształtu pików tych związków i może skutkować obniżeniem efektywności rozdzieleń chromatograficznych. Dotychczas jednak nie oceniono, czy istnieje możliwość poprawy jakości rozdzieleń chromatograficznych antracyklin poprzez modyfikację układu RP-LC za pomocą ILs.

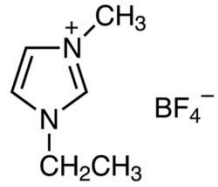
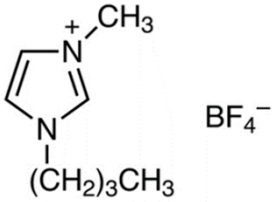
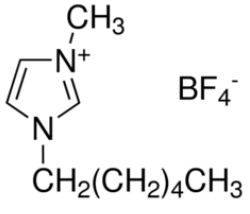
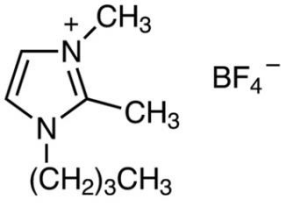
Celem prowadzonych badań była ocena możliwości aplikacyjnych ILs w kierunku poprawienia efektywności metod chromatograficznych stosowanych do oznaczenia antybiotyków antracyklinowych w płynach biologicznych techniką RP-LC-FL. Przy przyjęciu hipotezy, iż jest to możliwe, nowo opracowane metody LC-FL wspomagane ILs mogłyby być stosowane w praktyce klinicznej. Ich zastosowanie w TDM umożliwiłoby bardziej optymalne dostosowanie dawki leku do indywidualnych potrzeb danego pacjenta, a to w dalszej perspektywie mogłoby zwiększać efektywność terapii przeciwnowotworowej u pacjentów onkologicznych.

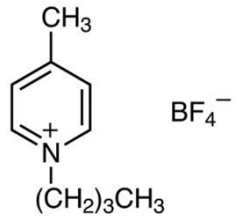
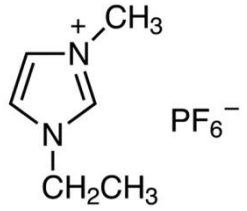
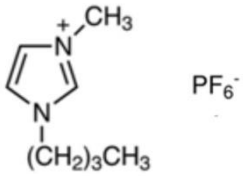
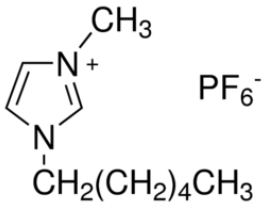
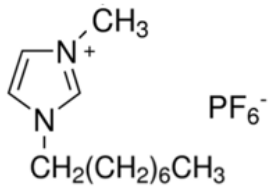
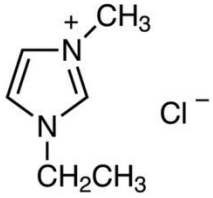
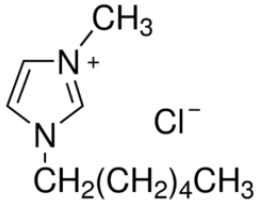
***Materiały i metody***

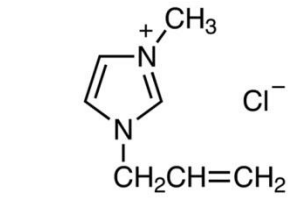
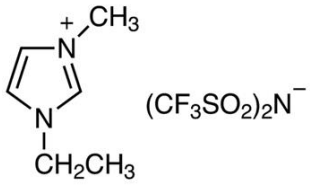
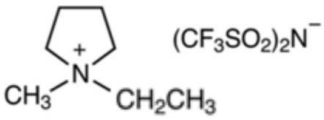
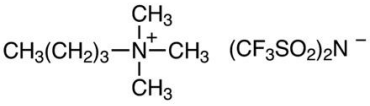
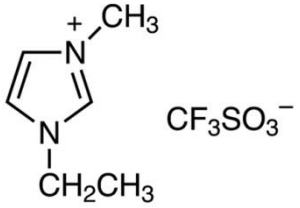
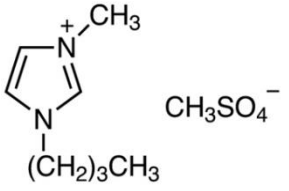
Do badań wytypowano siedemnaście ILs składających się z różnych kationów i anionów, których struktury chemiczne przedstawiono w Tabeli 1. Były to: 1-etylo-3-metyloimidazoliowy tetrafluoroboran [$C_2$MIM][$BF_4$], 1-butylo-3-metyloimidazoliowy tetrafluoroboran [$C_4$MIM][$BF_4$], 1-heksylo-3-metyloimidazoliowy tetrafluoroboran [$C_6$MIM][$BF_4$], 1-butylo-2,3-dimetyloimidazoliowy tetrafluoroboran [$C_4$MMIM][$BF_4$], 1-butylo-4-metylopirydyniowy tetrafluoroboran [$C_4$MPyr][$BF_4$], 1-etylo-3-metyloimidazoliowy heksafluorofosforan [$C_2$MIM][$PF_6$], 1-butylo-3-metyloimidazoliowy heksafluorofosforan [$C_4$MIM][$PF_6$], 1-heksylo-3-metyloimidazoliowy heksafluorofosforan [$C_6$MIM][$PF_6$], 1-octylo-3-metyloimidazoliowy heksafluorofosforan [$C_8$MIM][$PF_6$], 1-etylo-3-metyloimidazoliowy chlorek [$C_2$MIM][Cl], 1-heksylo-3-metyloimidazoliowy chlorek [$C_6$MIM][Cl], 1-allilo-3-metylimidazoliowy chlorek [AllylMIM][Cl], 1-etylo-3-

metyloimidazoliowy bis(trifluorometylosulfonylo)imidek, [$C_2$MIM][$N(SO_2CF_3)_2$], 1-etylo-3-metylopirolidyniowy bis(trifluorometylosulfonylo)imidek, [$C_2$MPyrr][$N(SO_2CF_3)_2$], 1-butylo-3-metyloamoniowy bis(trifluorometylosulfonylo)imidek [$C_4$MAmm][$N(SO_2CF_3)_2$], 1-etylo-3-metyloimidazoliowy trifluorometylosulfonian [$C_2$MIM][$CF_3SO_4$] oraz 1-butylo-3-metyloimidazoliowy metylosiarczan [$C_4$MIM][$CH_3SO_4$]. Analizy chromatograficzne roztworów wzorcowych doksorubicyny, epirubicyny, daunorubicyny i idarubicyny o stężeniu 2,5 μg/ml i 1 ng/ml wykonano stosując kolumnę analityczną Discovery HS C18 (150 × 4,6 mm, 5 μm). Faza ruchoma składała się z acetonitrylu i fazy wodnej, które zmieszano w stosunku objętościowym (25:75, *v/v*). Przepływ fazy ruchomej utrzymywano na poziomie 1 ml/min, a temperatura kolumny analitycznej wynosiła 30°C. Detekcję analitów przeprowadzano przy długości fali wzbudzania i emisji wynoszącej odpowiednio 487 nm i 555 nm.

*Tabela 1. Struktury chemiczne ILs włączone do badań w ramach realizacji pracy doktorskiej*

| **CIECZE JONOWE** | | **SKRÓT** | **STRUKTURA** |
|---|---|---|---|
| **Kation** | **Anion** | | |
| 1-etylo-3-metylo-imidazoliowy | tetrafluoroboran | [$C_2$MIM][$BF_4$] |  |
| 1-butylo-3-metylo-imidazoliowy | tetrafluoroboran | [$C_4$MIM][$BF_4$] |  |
| 1-heksylo-3-metylo-imidazoliowy | tetrafluoroboran | [$C_6$MIM][$BF_4$] |  |
| 1-butylo-2,3-dimetylo-imidazoliowy | tetrafluoroboran | [$C_4$MMIM][$BF_4$] |  |

| | | | |
|---|---|---|---|
| 1-butylo-4-metylopirydyniowy | tetrafluoroboran | $[C_4MPyr][BF_4]$ |  |
| 1-etylo-3-metylo-imidazoliowy | heksafluorofosforan | $[C_2MIM][PF_6]$ |  |
| 1-butylo-3-metylo-imidazoliowy | heksafluorofosforan | $[C_4MIM][PF_6]$ |  |
| 1-heksylo-3-metylo-imidazoliowy | heksafluorofosforan | $[C_6MIM][PF_6]$ |  |
| 1-octylo-3-metylo-imidazoliowy | heksafluorofosforan | $[C_8MIM][PF_6]$ |  |
| 1-etylo-3-metylo-imidazoliowy | chlorek | $[C_2MIM][Cl]$ |  |
| 1-heksylo-3-metylo-imidazoliowy | chlorek | $[C_6MIM][Cl]$ |  |

| | | | |
|---|---|---|---|
| 1-allilo-3-metylo-imidazoliowy | chlorek | [AllylMIM][Cl] |  |
| 1-etylo-3-metylo-imidazoliowy | bis(trifluorometyl-sulfonylo)imidek | [$C_2$MIM][$N(SO_2CF_3)_2$] |  |
| 1-etylo-1-metylo-pirrolidyniowy | bis(trifluorometyl-sulfonylo)imidek | [$C_2$MPyrr][$N(SO_2CF_3)_2$] |  |
| 1–butylo–3-metylo-ammoniowy | bis(trifluorometyl-sulfonylo)imidek | [$C_4$Mamm][$N(SO_2CF_3)_2$] |  |
| 1-etylo-3-metylo-imidazoliowy | Trifluorometylo-sulfonian | [$C_2$MIM][$CF_3SO_4$] |  |
| 1-butylo-3-metylo-imidazoliowy | metylosiarczan | [$C_4$MIM][$CH_3SO_4$] |  |

Modyfikacja warunków separacji polegała na dodaniu wybranej IL do wodnego składnika fazy ruchomej, które stanowiły 0,1% roztwór kwasu mrówkowego w wodzie bądź 10 lub 40 mM bufor fosforanowy w wodzie o zróżnicowanych wartościach pH (3, 5 lub 7), zaś IL była dodawana do tych składników fazy ruchomej na różnych poziomach stężeń (1,25 mM w przypadku ILs z anionem [$PF_6$], 20 mM w przypadku IL z kationem 1-allilo-3-metyloimidazoliowym [AllylMIM] oraz 2,5, 5,0 lub 10,0 mM w przypadku pozostałych ILs). Wpływ dodatku ILs na przebieg rozdzieleń chromatograficznych oceniono na postawie zmiany

wartości następujących parametrów chromatograficznych: czas retencji ($t_R$), pole powierzchni piku (A), liczba półek teoretycznych ($N_A$) oraz współczynnik asymetrii piku ($T_f$), które wyznaczono dla wybranych antracyklin.

***Wyniki***

Badania dotyczące oceny wpływu struktury IL na retencję antybiotyków antracyklinowych na oktadecylokrzemionkowej fazie potwierdziły, że ich obecność w fazie ruchomej pozwala kontrolować retencję analitów. Stosując struktury IL z krótkimi łańcuchami alkilowymi 1-etylo-3-metyloimidazoliowym [$C_2$MIM]) lub z anionami silnie wiążącymi się z fazą stacjonarną (np. anionem [$PF_6$]), możliwe było wzmocnienie retencji. Z kolei po użyciu ILs z długimi łańcuchami alkilowymi i nieadsorbującymi anionami np. 1-heksylo-3-metyloimidazoliowego chlorku [$C_6$MIM][Cl], występowała słabsza retencja analitów. Wynika to z zaangażowania zarówno kationu jak i anionu IL w interakcje na powierzchni fazy stacjonarnej, które mogą przeciwstawnie wpływać na retencję analitów. W przypadku anionów ich zaangażowanie w interakcje na powierzchni fazy stacjonarnej są zależne od pozycji w szeregu Hofmeistera tzn. w przypadku anionów chaotropowych (np. [$PF_6$]) dochodzi do tworzenia par jonowych pomiędzy anionami IL a dodatnio naładowanymi analitami, co sprawia, iż powstałe pary jonowe mogą silniej oddziaływać z ligandami fazy stacjonarnej, bądź aniony IL bezpośrednio oddziaływają z ligandami fazy stacjonarnej, czego następstwem jest tworzenie ujemnie naładowanej strefy nad powierzchnią fazy stacjonarnej i przyciąganie dodatnio naładowanych analitów. Oba efekty wzmacniają retencję antracyklin. Z kolei w przypadku anionów kosmotropowych (np. [Cl]), które nie są adsorbowane na powierzchni fazy stacjonarnej, retencja analitów jest uzależniona od drugiego składnika IL, czyli od kationu. Obecność alkilowych podstawników w strukturze kationu IL sprawia, że mogą być zaangażowane w oddziaływania hydrofobowe z oktadecylowymi łańcuchami alkilowymi na powierzchni fazy stacjonarnej, prowadząc do zwiększenia dodatniego ładunku fazy stacjonarnej i odpychania analitów o charakterze zasadowym (w tej postaci występowały antracykliny w zastosowanych warunkach analizy chromatograficznej). Im dłuższy łańcuch alkilowy przy kationie, tym silniejsze odziaływania hydrofobowe, a tym samym silniejsze odpychanie dodatnio naładowanych analitów. Powyższy efekt obniża retencję antracyklin. Przedstawione mechanizmy pokazują zatem konkurencyjny wpływ anionów i kationów IL na retencję badanych związków, co oznacza, że ILs mogą być stosowane do kontroli ich retencji. Wyniki tych badań są zgodne z danymi literaturowymi [67,87].

W niniejszym badaniu, oprócz powszechnie testowanych struktur ILs takich jak związki z kationem imidazoliowym i łańcuchem alkilowym C2-C6 lub anionami [$PF_6$], [$BF_4$], [Cl] [38,40], wykorzystano jako modyfikatory faz ruchomych także mało poznane ILs z anionem [$CH_3SO_4$] lub trifluorometylosulfonowym [$CF_3SO_4$]. Ponadto, po raz pierwszy oceniono wpływ ILs z anionem [$N(SO_2CF_3)_2$]. Wyniki tych badań udowodniły m.in., że łańcuch allilowy i łańcuch alkilowy o tej samej długości mają taki sam wpływ na retencję analitów. To oznacza, że obecność wiązania nienasyconego nie wpływa na zmianę siły i kierunku działania IL. Udowodniono ponadto, że ILs z anionem [$N(SO_2CF_3)_2$] powodują problemy z przywróceniem początkowych warunków analizy chromatograficznej (prawdopodobnie wynika to ze zbyt silnej retencji anionu IL na oktadecylokrzemionkowej fazie stacjonarnej), zaś korzystnym rozwiązaniem było zastosowanie ILs z kationem 1-butylo-2,3-dimetyloimidazoliowym [$C_4$MMIM] zamiast 1-butylo-3-metyloimidazoliowym [$C_4$MIM]. Wspomniany powyżej [$C_4$MMIM] z uwagi na dodatkowy podstawnik metylowy w strukturze kationu jest zdolny do silniejszych interakcji z fazą stacjonarną, a to prowadzi do bardziej skutecznego odpychania dodatnio naładowanych analitów, co skutkuje większym skróceniem czasów retencji testowanych antracyklin. Oceniono także wpływ struktur ILs na intensywność sygnału i kształt pików badanych analitów. Wykazano, że stosując odpowiednią IL można zwiększyć intensywność sygnału analitycznego, a tym samym obniżyć granicę oznaczalności (ang. *Limit of Quantification,* LOQ) dla badanych substancji czynnych. W niniejszym badaniu udowodniono, że zastosowanie IL z anionem chlorkowym i kationem 1-heksylo-3-metyloimidazoliowym [$C_6$MIM][Cl] pozwoliło na oznaczenie antybiotyków antracyklinowych na poziomie 1 ng/mL, podczas gdy w przypadku użycia fazy ruchomej bez tego dodatku, LOQ analitów wynosiła 2,5 ng/ml. Badania pokazały także, że w zależności od struktury IL może występować zarówno frontowanie jak i ogonowanie pików oznaczanych antracyklin, przy czym dominującym efektem było frontowanie (zmniejszenie wartości $T_f$). Prawdopodobnie wynikało to z obecności na powierzchni fazy stacjonarnej wysoko- (grupy silanolowe) i niskoenergetycznych (łańcuchy alkilowe) miejsc, które charakteryzuje różna szybkość kinetyki zachodzących tam procesów. Z powodu obecności jonów IL wiążących się w różny sposób i z różną siłą z kationami antracyklin znajdującymi się w fazie ruchomej oraz ligandami i grupami silanolowymi znajdującymi się na powierzchni fazy stacjonarnej może dochodzić do zmiany kinetyki tych procesów. Efektem tego zjawiska może być m.in. obniżenie współczynnika $T_f$. Przykładowo, ILs z chaotropowym anionem [$BF_4$] na skutek tworzenia par jonowych z kationami antracyklin znajdującymi się w fazie ruchomej mogą silniej oddziaływać z niskoenergetycznymi łańcuchami oktadecylowymi fazy stacjonarnej, co może prowadzić do

obniżenia wartości współczynnika $T_f$. To obniżenie wartości $T_f$ było większe niż obserwowane po dodaniu do fazy ruchomej ILs z anionem [Cl], który nie tworzy par jonowych z kationami antracyklin, a zatem nie zmienia siły ich oddziaływania z ligandami fazy stacjonarnej. Ponadto, większy spadek wartości współczynnika $T_f$ był zauważalny dla IL z łańcuchem etylowym C2 niż łańcuchem heksylowym C6, co można tłumaczyć mniejszym sferycznym blokowaniem możliwości wystąpienia interakcji pomiędzy parą jonową anion IL-kation antracykliny a łańcuchami alkilowymi na powierzchni fazy stacjonarnej. To zaś sprzyja silniejszemu wpływowi na kinetykę sorpcji i desorpcji analitu ze złoża kolumny chromatograficznej. Dane te są zgodne z doniesieniami literaturowymi [88]. Odnotowano także zmianę wartości parametru $N_A$. Przykładowo, dodanie 2,5 mM 1-heksylo-3-metyloimidazoliowego chlorku [$C_6$MIM][Cl] do fazy ruchomej podwyższało wartość $N_A$ z 8090 do 9010 dla idarubicyny – analitu o najsilniejszej retencji na kolumnie Discovery HS C18.

Dodatkowo, analizy chromatograficzne prowadzono przy zastosowaniu różnych stężeń ILs Wyniki badań udowodniły, że zmiana stężenia IL jest dodatkowym narzędziem w kontroli retencji analitów, gdyż jej zwiększenie powoduje wzmacnianie efektu wynikającego z obecności IL w trakcie przebiegu rozdzielenia chromatograficznego antracyklin. Przykładowo, wzrost stężenia 1-etylo-3-metyloimidazoliowego tetrafluoroboranu [$C_2$MIM][$BF_4$] w fazie ruchomej z 2,5 do 10 mM powodowało wydłużenie czasu retencji idarubicyny z 22.97 min do 27.87 min.

Istotnym etapem badań było także zastosowanie 1-butylo-3-metyloimidazoliowego tetrafluoroboranu [$C_4$MIM][$BF_4$] oraz 1-allilo-3-metyloimidazoliowego chlorku [AllylMIM][Cl] jako dodatków do 10 lub 40 mM buforu fosforanowego. W tych badaniach, kluczowe okazało się stężenie buforu fosforanowego, gdyż przy jego wyższych wartościach efekt IL był tłumiony. Prawdopodobnie wynikało to z obecności jonów fosforanowych, które efektywnie konkurowały z anionem [$BF_4$] o interakcje ze złożem kolumny chromatograficznej, co sprawiało, że jony fosforanowe uniemożliwiały zachodzenie tego typu interakcji dla anionu [$BF_4$]. W efekcie, przyciąganie dodatnio naładowanych analitów na powierzchni fazy stacjonarnej były w niewielkim stopniu zależne od [$BF_4$] i nie prowadziło do zwiększenia retencji analitów przy wyższych stężeniach jonów fosforanowych. W przypadku [AllylMIM][Cl], który posiada kosmotropowy anion chlorkowy, jego użycie jako modyfikatora fazy ruchomej zawierającej bufor fosforanowy także prowadziło do zmniejszenia wpływu tej IL na retencję analitów przy wyższych stężeniach jonów fosforanowych. Zjawisko to było prawdopodobnie także związane z wystąpieniem silniejszych interakcji anionów fosforanowych z ligandami fazy stacjonarnej, co w konsekwencji powodowało wzrost siły

oddziaływania pomiędzy nimi a dodatnio naładowanymi antracyklinami i prowadziło do zwiększonej retencji tych analitów. Powyższe interakcje jednocześnie obniżały możliwość wystąpienia hydrofobowych oddziaływań pomiędzy łańcuchami oktadecylowymi fazy stacjonarnej a łańcuchami allilowymi kationu IL, które przeciwstawnie wpływają na retencję analitów i prowadzą do zmniejszenia tego parametru z uwagi na wzrost ładunku dodatniego na powierzchni fazy stacjonarnej i odpychanie antracyklin [81]. Z kolei analizy przeprowadzone dla 10 i 40 mM buforu fosforanowego o pH 3, 5, 7 pokazały, iż w niniejszym eksperymencie pH było mniej istotnym parametrem niż stężenie IL i buforu fosforanowego. Po dodaniu [$C_4$MIM][$BF_4$] do fazy ruchomej o różnych wartościach pH występowało nieznaczne zwiększenie retencji analitów, co prawdopodobnie wskazywało na niewielką ilość reszt silanolowych występujących na powierzchni fazy stacjonarnej używanej w trakcie tych doświadczeń. W efekcie, zaangażowanie katonów IL w interakcje z ujemnie naładowanymi grupami silanolowymi występującymi na powierzchni fazy stacjonarnej było niewielki.

***Wnioski***

Przeprowadzone badania pozwoliły bardziej szczegółowo opisać mechanizmy retencji czterech wybranych antracyklin przebiegających w odwróconym układzie faz przy użyciu oktadecylokrzemionkowej fazy stacjonarnej w obecności szerokiego panelu ILs dodanych do fazy ruchomej. Zastosowanie różnorodnych modyfikacji warunków chromatograficznych pozwoliło wiarygodnie ocenić wpływ ILs na zachowanie analitów zależnie od testowanych wariantów prowadzenia tego procesu. Tym samym, w szerszym zakresie niż dotychczas opisanym w literaturze, możliwe było rzetelne określenie potencjalnych możliwości aplikacyjnych ILs w opracowaniu nowych metod analitycznych oznaczania wybranych leków cytostatycznych techniką RP-LC.

**Publikacja 3**

Treder, N., Olędzka, I., Roszkowska, A., Kowalski, P., Bączek, T., Plenis, A., Practical and theoretical considerations of the effects of ionic liquids on the separation properties of phenyl-based stationary phases in reversed-phase liquid chromatography. *Microchem. J.* 178: (2022) 107396.

**IF: 4.821**

**MEiN: 70.000**

***Wprowadzenie i cel pracy***

Celem niniejszej pracy było zbadanie interakcji występujących w trakcie przebiegu analiz chromatograficznych antybiotyków antracyklinowych realizowanych w odwróconym układzie faz na kolumnach analitycznych z ligandami fenylowymi lub ich pochodnymi w obecności ILs zastosowanych jako modyfikatory fazy ruchomej. Jak wcześniej wspomniano, fenylowe fazy stacjonarne są rzadko stosowane w analizach farmaceutycznych, co prawdopodobnie wynika z silniejszej retencji substancji leczniczych względem tej występującej na kolumnach alkilowych. W efekcie czas rozdzielenia chromatograficznego jest przedłużony, a dla jego zoptymalizowania wymagane jest zastosowanie faz ruchomych o większej sile elucji (np. wyższy udział modyfikatora organicznego w fazie ruchomej bądź elucja gradientowa). W konsekwencji, zwiększa to koszt analizy oraz utylizacji rozpuszczalników organicznych stosowanych jako składniki faz ruchomych. Jednakże, aplikacja faz stacjonarnych z ugrupowaniem fenylowym lub jego pochodnymi jest cenną alternatywną w przypadku problemów separacyjnych na stacjonarnych fazach alkilowych. Z tego względu, w niniejszym badaniu podjęto tematykę dotyczącą wyjaśnienia różnic w mechanizmach separacyjnych zachodzących po zastosowaniu ILs jako modyfikatorów faz ruchomych w trakcie analiz chromatograficznych wybranych antracyklin na fazie stacjonarnej C18, fenylowej i pentafluorofenylowej. Szczegółowa analiza zmian w wartościach parametrów chromatograficznych badanych antracyklin mogła wykazać, czy zastosowanie ILs jako modyfikatorów faz ruchomych w opracowywaniu metod analitycznych opartych na fenylowych fazach stacjonarnych bądź ich pochodnych może rozszerzyć ich potencjał aplikacyjny w oznaczaniu leków cytostatycznych.

***Materiały i metody***

W niniejszym badaniu wpływ ILs na fenylowe fazy stacjonarne zbadano poprzez przeprowadzenie analiz chromatograficznych z udziałem trzynastu różnych struktur, których struktury przedstawiono w Tabeli 1 ([$C_2$MIM][$BF_4$], [$C_6$MIM][$BF_4$], [$C_4$MMIM][$BF_4$], [C4MPyr][BF4], [$C_2$MIM][$PF_6$], [$C_4$MIM][$PF_6$], [$C_6$MIM][$PF_6$], [$C_8$MIM][$PF_6$], [$C_2$MIM][Cl], [$C_6$MIM][Cl], [AllylMIM][Cl], [$C_2$MIM][$CF_3SO_4$], [$C_4$MIM][$CH_3SO_4$]). W badaniach pominięto ILs z anionem [$N(SO_2CF_3)_2$] z uwagi na problemy z przywróceniem warunków początkowych analizy chromatograficznej, o czym wspomniano przy opisaniu wyników zawartych w publikacji 2. Wyselekcjonowane struktury ILs pozwalały dokonać niezależnej oceny wpływu rodzaju anionu oraz kationu na przebieg rozdzieleń

chromatograficznych wybranych antracyklin techniką RP-LC-FL. Aby w pełni określić wpływ charakteru fizykochemicznego IL i użytej fazy stacjonarnej na przebieg rozdzielenia wybranych leków cytostatycznych, analizy przeprowadzono na krzemionkowych kolumnach chromatograficznych z ugrupowaniem fenylowym (Synergi Polar-RP 80A, 150 × 4,6 mm, 4 μm) i pentafluorofenylowym (Fluorophase PFP, 150 × 4,6 mm, 5 μm), a także oktadecylowym (Synergi Hydro-RP 80A, 150 × 4,6 mm, 4 μm), a w niniejszym eksperymencie jako anality użyto doksorubicynę, epirubicynę i daunorubicynę. Wszystkie eksperymenty prowadzono w warunkach chromatograficznych analogicznych do opisanych w publikacji 2, przy czym jako fazę ruchomą zastosowano wyłącznie mieszaninę acetonitrylu i 0,1% kwasu mrówkowego w wodzie (25:75, *v/v*). Do powyższej fazy ruchomej wszystkie ILs były dodawane w stężeniu 2,5 mM. W oparciu o uzyskane wartości parametrów chromatograficznych (*k*, $N_A$, $T_f$ i A) oceniono zależności pomiędzy strukturą IL a wynikiem separacji wspomnianych analitów. Przeprowadzono także hierarchiczną analizę skupień (ang. *Hierarchical Cluster Analysis*, HCA) oraz analizę skupień metodą *k-średnich* (ang. *K-Means Clustering Analysis*, *K-means* CA) dla graficznego zobrazowania wpływu ILs na parametry chromatograficzne trzech wyżej wymienionych antybiotyków antracyklinowych. Analizy chemometryczne wykonano za pomocą programu Statistica 13.3.

***Wyniki***

W pierwszej części niniejszych badań przeprowadzono analizy z użyciem faz ruchomych bez dodatku IL i wykazano, że fenylowe fazy stacjonarne znacznie silniej oddziałują z analizowanymi związkami niż faza alkilowa. Przykładowo, współczynnik retencji idarubicyny na fazie oktadecylokrzemionkowej wynosił 11.89, podczas gdy na fazie fenylowej i pentafluorofenylowej był na poziomie odpowiednio 18.37 i 29.44. W przypadku pozostałych parametrach chromatograficznych także odnotowano zauważalne różnice w wartościach tych parametrów, tzn. fazy fenylowe zapewniały wyższą symetrię piku, uzyskana powierzchnia pików była porównywalna lub wyższa w stosunku do tej osiągniętej na alkilowej fazie stacjonarnej, zaś wartość $N_A$ była najniższa, gdy używano fazę pentafluorofenylową, a najwyższa dla fazy fenylowej. Jednakże należy podkreślić, że wartości parametrów chromatograficznych oznaczanych antracyklin znacząco zmieniły się, gdy do fazy ruchomej dodano ILs w stężeniu 2,5 mM. Ta modyfikacja powodowała zarówno na fenylowej, jak i pentafluorofenylowej fazie stacjonarnej istotne zmiany w retencji analitów, a ich kierunek i wielkość były uzależnione od rodzaju kationu i anionu IL jak też od charakteru fizykochemicznego fazy stacjonarnej. Na fazie pentafluorofenylowej dodatek wszystkich

testowanych ILs powodował zmniejszenie wartości współczynnika retencji badanych analitów, na fazie fenylowej ten efekt uzyskano po zastosowaniu ośmiu struktur ([$C_6$MIM][$BF_4$], [$C_4$MMIM][$BF_4$], [$C_4$MPyr][$BF_4$], [$C_8$MIM][$PF_6$], [$C_2$MIM][Cl], [$C_6$MIM][Cl], [AllylMIM][Cl] oraz [$C_4$MIM][$CH_3SO_4$]), zaś na fazie oktadecylokrzemionkowej znaczące zmiany osiągnięto tylko po zastosowaniu dwóch ILs ([$C_6$MIM][$BF_4$] i [$C_6$MIM][Cl]). Powyższe różnice prawdopodobnie wynikały z faktu, iż w przypadku alkilowej fazy stacjonarnej zmiany w retencji były następstwem oddziaływań hydrofobowych typu Van der Waalsa pomiędzy łańcuchami alkilowymi na powierzchni fazy stacjonarnej i łańcuchami alkilowymi kationów ILs. W przypadku fazy fenylowej i pentafluorofenylowej za ten efekt odpowiadały znacznie silniejsze oddziaływania typu π-π pomiędzy pierścieniem aromatycznych ligandów fazy stacjonarnej a pierścieniem aromatycznym kationów ILs, które były dodatkowo wspierane przez oddziaływania hydrofobowe pomiędzy łańcuchami alkilowymi łączącymi pierścień aromatyczny z grupami krzemionkowymi fazy stacjonarnej a łańcuchami alkilowymi kationów IL. Ponadto, zmiany w retencji analitów po zastosowania ILs były bardziej widoczne na pentafluorofenylowej fazie stacjonarnej w porównaniu do fazy fenylowej. Wynikało to prawdopodobnie z obecności atomów fluoru przyłączonych do pierścienia aromatycznego, które zwiększały aromatyczność pierścienia fenylowego, a tym samym także siłę oddziaływań z kationami IL. Dodatkowo, pentafluorofenylowe ligandy zapewniały możliwość wystąpienia odziaływań typu dipol-dipol oraz/i związanych z transferem ładunku. To dowodzi, że zmiany w retencji analitów posiadających w swojej strukturze aromatyczne ugrupowanie są zależne od rodzaju fazy stacjonarnej i struktury IL, a także wskazują, iż z uwagi na silniejsze interakcje zachodzące w procesie separacyjnym istnieje większa możliwość kontroli retencji analitów za pomocą ILs. Uzyskane w niniejszym badaniu wyniki pozwoliły na klasyfikację anionów i kationów ILs pod względem kierunku zmian retencji analitów na testowanych fazach stacjonarnych. W przypadku kationów ILs, im dłuższy był łańcuch alkilowy podstawnika tych wartość współczynnika retencji analitu była niższa, z kolei w przypadku anionów ten parametr obniżał się w szeregu: [$PF_6$] > [$CF_3SO_4$]> [$BF_4$] > [$CH_3SO_4$] > [Cl]. Ostatecznie, po zastosowaniu [$C_6$MIM][Cl], która zapewniała największą redukcję współczynnika retencji, możliwe było skrócenie czasu analizy o około 8 min na fazie alkilowej, ponad 17 min na fazie fenylowej i ponad 29 min na fazie pentafluorofenylowej. Należy jednak podkreślić, że dodanie IL do fazy ruchomej w większości przypadków nie powodowało znaczących zmian we wielkości i kształcie pików. Do interpretacji uzyskanych wyników wykorzystano także analizę chemometryczną opartą na HCA i metodzie *k-średnich*, które zostały wykonane na znormalizowanych zbiorach danych

opisujących wartości parametrów chromatograficznych ($k$, $N_A$, $T_f$, A) badanych analitów (doksorubicyny, epirubicyny i idarubicyny) wyznaczone w trakcie przebiegu analiz techniką RP-LC-FL bez i z dodatkiem IL do fazy ruchomej. Przeprowadzenie analiz chemometrycznych pozwoliło pogrupować anality (obiekty) i parametry chromatograficzne (zmienne) w klastery/skupiska, które posiadały zbliżone cechy oraz wyróżnić te obiekty/zmienne, które odbiegające od tych zgromadzonych w poszczególnych klasterach/skupiskach. Wykazano m.in., że zróżnicowanie analitów pod względem ich właściwości fizykochemicznych było porównywalne na trzech testowanych fazach stacjonarnych (doksorubicyna i epirubicyna były zgromadzone w jednym klasterze/skupisku, a daunorubicyna i idarubicyna w drugim, co było także skorelowane z ich strukturami chemicznymi), jednakże większe dysproporcje w zachowaniu się daunorubicyny i idarubicyny obserwowano po zastosowaniu kolumny pentafluorofenylowej. Wyniki analiz chemometrycznych wskazały także, że interakcje zachodzące na różnych fazach stacjonarnych z dodatkiem lub bez dodatku IL do fazy ruchomej, prowadzą do ujawienia się odmiennych zależności między poszczególnymi parametrami chromatograficznymi obliczonymi dla badanych antracyklin w zależności od rodzaju użytej fazy stacjonarnej. Zatem, analiza chemometryczna potwierdziła wcześniejsze spostrzeżenia, iż wpływ ILs na wartość parametrów chromatograficznych antybiotyków antracyklinowych zależy od charakteru fizykochemicznego fazy stacjonarnej i struktury chemicznej ILs użytych jako modyfikatory fazy ruchomej.

***Wnioski***

Przeprowadzone badania udowodniły, że zastosowanie ILs jako modyfikatorów fazy ruchomej wywierało silniejszy wpływ na mechanizm separacji analitów w trakcie przebiegu rozdzieleń chromatograficznych z użyciem fenylowych faz stacjonarnych w stosunku do fazy alkilowej, przy czym najsilniejszy efekt odnotowano dla fazy pentafluorofenylowej. Potwierdzono, że modyfikacja składu fazy ruchomej ILs zależnie od struktury chemicznej ILs pozwala znacznie kontrolować retencję analitów, ale jednocześnie nie wpływa negatywnie na wielkość i kształt pików analitów, a wręcz może podnieść efektywność rozdzieleń chromatograficznych. Pozwala to przypuszczać, że zastosowanie ILs jako modyfikatorów faz ruchomych może poszerzyć zakres stosowania fenylowych faz stacjonarnych i ich pochodnych jako interesujących alternatyw względem faz alkilowych w trakcie optymalizacji metod analitycznych do oznaczania substancji biologicznie czynnych i ich aplikacji w badaniach farmaceutycznych, farmakokinetycznych i klinicznych.

**Publikacja 4**

Treder, N., Roszkowska, A., Olędzka, I., Bączek, T., Plenis, A. Effects of $Fe_3O_4$ magnetic nanoparticle functionalization with ionic liquids and a double-chained surfactant on the pretreatment of plasma samples during drug extraction, *Anal. Chem*. 94: (2022) 16587–16595.

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***Wprowadzenie i cel pracy***

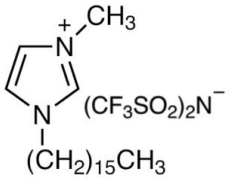
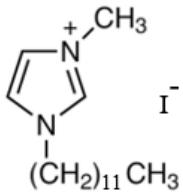
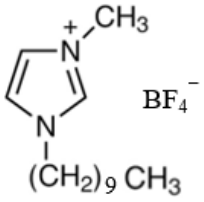
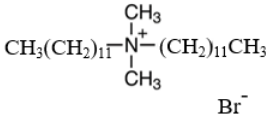
Celem pracy było zbadanie potencjału ILs we funkcjonalizacji magnetycznych nanocząstek (NPs) wykorzystywanych jako magnetyczne sorbenty w ekstrakcji leków cytostatycznych z próbek biologicznych. Podjęcie tej tematyki było związane z poszukiwaniem nowych, prostych i szybkich rozwiązań w przygotowaniu próbek do analizy. Zastosowanie magnetycznych NPs o dużej powierzchni właściwej i magnetycznych właściwościach miało uprościć oraz podwyższyć efektywność tego etapu w trakcie opracowania metody analitycznej. Ponadto, miało także wskazać, czy anionowo-kationowe struktury ILs, które do tej pory były głównie stosowane w ekstrakcji substancji leczniczych z użyciem DLLME, co potwierdziły dane zgromadzone w ramach opracowania publikacja 1, mogą zapewniać odpowiednią wydajność w procedurach opartych na magnetycznej ekstrakcji do fazy stałej (MSPE). Stosując szereg modyfikacji w opracowaniu magnetycznych sorbentów powlekanych ILs dążono także do wykazania zależności pomiędzy strukturą ILs, warunkami syntezy i powlekania NPs, a zdolnością do ekstrakcji analitów oraz do wyjaśnienia, w jaki sposób ILs wpływają na końcowe wyniki rozdzieleń chromatograficznych. W niniejszym badaniu, jako modelowy analit wybrano epirubicynę, którą ekstrahowano z osocza krwi ludzkiej za pomocą NPs uzyskanych zgodnie z nowo opracowanymi protokołami syntezy i funkcjonalizacji. Protokoły te uwzględniały zastosowanie ‘czystych’ NPs bez dalszej ich funkcjonalizacji bądź ich użycie po etapie funkcjonalizacji za pomocą krzemionki i/lub wybranym IL/surfaktantem. Uzyskane dane miały także dostarczyć kompleksowych danych na temat zastosowania ILs we funkcjonalizacji NPs i potencjalnych możliwości aplikacyjnych w analizach farmaceutycznych, zarówno wybranego leku cytostatycznego, jak i innych substancji leczniczych i/bądź ich metabolitów w próbkach biologicznych.

***Materiały i Metody***

Rdzenie magnetycznych NPs $Fe_3O_4$ przygotowano metodą współstrącania z użyciem ultradźwięków, a następnie powleczono krzemionką i jedną z dziewięciu ILs: sześciu struktur

testowanych w publikacji 1 i 2 ([$C_6$MIM][$BF_4$], [$C_6$MIM][$PF_6$], [$C_8$MIM][$PF_6$], [$C_2$MIM][$N(SO_2CF_3)_2$], [$C_2$MPyrr][$N(SO_2CF_3)_2$], [$C_4$MAmm][$N(SO_2CF_3)_2$],Tabela 1) oraz trzech nowych struktur: (1-heksadecylo-3-metyloimidazoliowy bis(trifluorometylosulfonylo)imidek [$C_{16}$MIM][$N(SO_2CF_3)_2$], 1-dodecylo-3-metyloimidazoliowy jodek [$C_{12}$MIM][I] oraz 1-decylo-3-metyloimidazoliowy tetrafluoroboran [$C_{10}$MIM][$BF_4$] (Tabela 2). Do badania zakwalifikowano także surfaktant (didodecylodimetyloamoniowy bromek [$C_{12}C_{12}$MMAmm][Br]) zawierający podwójny łańcuch alkilowym C12. Włączenie tego związku wynikało z założeń projektu, którego celem było także porównanie dwóch materiałów powlekających, z których jeden posiada dwa łańcuchy alkilowe o tej samej długości przyłączone do IV-rzędowego atomu azotu (wspomniany powyżej surfaktant), a drugi to IL z kationem imidazoliowym zawierającym dwa podstawniki alkilowe, z których jeden jest łańcuchem alkilowym o tej samej długości, jak w strukturze surfaktantu (C12) (1-dodecylo-3-metylo-imidazoliowy, [$C_{12}$MIM][I] Tabela 2). Wpływ procedur przygotowania magnetycznych sorbentów na wydajność ekstrakcji wybranego analitu (epirubicyny o stężeniu 500 ng/ml z osocza krwi ludzkiej (0,5 ml)) oceniono na podstawie danych eksperymentalnych uzyskanych po zastosowaniu NPs otrzymanych za pomocą dwóch różnych procedur syntezy opartych na metodzie współstrącania. W procedurze pierwszej zastosowano ultradźwięki i NaOH jako odczynnik strącający, zaś w drugiej procedurze mieszadło magnetycznego i $NH_4OH$. Ponadto oceniono efektywność dwóch różnych procedur funkcjonalizacji NPs krzemionką, w których do jednej procedury użyto 0,1 M HCl w celu aktywacji powierzchni magnetycznych rdzeni przed powlekaniem krzemionką. Przeprowadzono także analizę porównawczą wyników ekstrakcji epirubicyny z próbek osocza uzyskanych po zastosowaniu NPs otrzymanych po użyciu komercyjnie dostępnych rdzeni NPs, które także funkcjonalizowano krzemionką i ILs bądź [$C_{12}C_{12}$MMAmm][Br]. W każdym przypadku, zdolność sorpcyjną przygotowanych sorbentów oceniono na podstawie pola powierzchni piku epirubicyny na chromatogramach uzyskanych techniką RP-LC-FL w warunkach elucji gradientowej. Wprowadzenie elucji gradientowej wynikało z konieczności uniknięcia ewentualnych problemów z przywróceniem warunków początkowych analiz w przypadku testowania silnie adsorbujących ILs na powierzchni fazy stacjonarnej tzn. ILs posiadające długie łańcuchy alkilowe.

*Tabela 2. Struktury cieczy jonowych i surfaktantu włączone do badań w ramach realizacji pracy doktorskiej*

| **CIECZ JONOWA** | | **SKRÓT** | **STRUKTURA** |
|---|---|---|---|
| **Kation** | **Anion** | | |
| 1-heksadecylo-3-metylo-imidazoliowy | bis(trifluorometyl-sulfonylo)imidek | $[C_{16}MIM][N(SO_2CF_3)_2]$ |  |
| 1-dodecylo-3-metylo-imidazoliowy | jodek | $[C_{12}MIM][I]$ |  |
| 1-decylo-3-metylo-imidazoliowy | tetrafluoroboran | $[C_{10}MIM][BF_4]$ |  |
| **SURFAKTANT** | | **SKRÓT** | **STRUKTURA** |
| **KATION** | **ANION** | | |
| Didodecylodimetylo-ammoniowy | bromek | $[C_{12}C_{12}MMAmm][Br]$ |  |

Pozostałe warunki analizy były analogiczne do opisanych w publikacjach 2 i 3, przy czym eksperymenty prowadzono z użyciem kolumny analitycznej Synergi Hydro RP (150 × 4 mm, 5 μm). Następnie, NPs zapewniające największą wydajność ekstrakcji epirubicyny z osocza scharakteryzowano za pomocą dyfrakcji rentgenowskiej (ang. *X-Ray Diffraction*, XRD) oraz transmisyjnej mikroskopii elektronowej (ang. *Transmission Electron Microscope*, TEM) połączonej z mapowaniem spektroskopią rozpraszania energii (ang. *Energy-dispersive X-ray spectroscopy*, EDX), co pozwoliło ustalić skład pierwiastkowy próbki. Ponadto wykonano widma techniką spektroskopii w podczerwieni z transformacją Fouriera (ang. *Fourier Transform Infrared Spectroscopy*, FT-IR) w zakresie 500 – 4000 $cm^{-1}$ oraz przeprowadzono analizę termograwimetryczną (ang. *Thermogravimetric Analysis*, TGA) w zakresie

25 – 300°C, z szybkością 10°C/min, w atmosferze azotu w celu określenia stabilności termicznej przygotowanych sorbentów.

***Wyniki***

Uzyskane wyniki potwierdziły, że kluczową kwestią w zdolności NPs powleczonych ILs do adsorpcji analitów jest długość łańcucha alkilowego stanowiącego podstawnik w kationie ILs. W niniejszym badaniu wraz ze wzrostem długości łańcucha alkilowego podstawnika kationu IL wzrastała wydajność ekstrakcji analitu, przy czym tylko ILs z łańcuchami alkilowymi o długości ≥ C10 ($[C_{16}MIM][N(SO_2CF_3)_2]$, $[C_{12}MIM][I]$, $[C_{10}MIM][BF_4]$) zapewniały ekstrakcję analitu na poziomie wyższym niż odnotowaną bez funkcjonalizacji NPs. Jednakże, gdy stosowano $[C_{16}MIM][N(SO_2CF_3)_2]$, pomimo zastosowania elucji gradientowej pojawiały się zakłócenia sygnału analitycznego prawdopodobnie spowodowane efektem zbyt silnej adsorpcji kationów IL na powierzchni fazy stacjonarnej. Istotnych informacji dostarczyły także wyniki eksperymentów dla NPs powleczonych surfaktantem $[C_{12}C_{12}MMAmm][Br]$, który zapewniał wyższą wydajność ekstrakcji epirubicyny niż IL z jednym łańcuchem alkilowym C12 ($[C_{12}MIM][I]$). Te spostrzeżenia potwierdziły, iż przy zastosowaniu ILs jako materiału powlekającego należy zwrócić uwagę nie tylko na długość podstawnika alkilowego, ale także wybierać ILs, które posiadają w strukturze kationu większą liczbą podstawników o dłuższych łańcuchach alkilowych. Wyniki przeprowadzonych badań także udowodniły, że poza strukturą ILs, na wynik analizy wpływają warunki syntezy i funkcjonalizacji NPs, co było zgodne z wcześniejszymi doniesieniami dotyczącymi wpływu warunków przygotowania NPs na ich właściwości morfologiczne, a zatem także na zdolności sorpcyjne [89]. W przypadku syntezy, najwyższe wartości pola powierzchni piku epirubicyny osiągnięto, gdy zastosowano NPs przygotowane według procedury opartej na ultradźwiękach, podczas gdy najlepszym sposobem funkcjonalizacji okazało się początkowe powlekanie NPs krzemionką, a następnie surfaktantem. NPs uzyskane dwoma sposobami syntezy, dwoma procedurami funkcjonalizacji, powleczone krzemionką i/lub $[C_{12}C_{12}MMAmm][Br]$ zostały także scharakteryzowane za pomocą technik XRD, FT-IR, TG oraz TEM. Pozwoliło to określić kształt i rozmiar NPs, potwierdzić obecność materiału powlekającego na powierzchni magnetycznych rdzeni, jak również określić ich stabilność w warunkach eksperymentalnych. Ostatecznie, analizy XRD wskazały, że optymalna procedura syntezy (z użyciem ultradźwięków i NaOH) pozwoliła na przygotowanie krystalicznych rdzeni $Fe_3O_4$ o wysokiej czystości, powleczonych krzemionką o strukturze amorficznej oraz $[C_{12}C_{12}MMAmm][Br]$ o strukturze krystalicznej, o czym

świadczyły dodatkowe piki na dyfraktogramie pokrywające się z wynikami uzyskanymi dla wzorca [$C_{12}C_{12}$MMAmm][Br]. Obecność grup funkcyjnych rdzeni $Fe_3O_4$ i warstwy powlekającej potwierdziły także widma FT-IR. Wyniki TEM udowodniły, że NPs miały kształt nieregularnych sześcianów o rozmiarach < 50 nm, zaś analiza EDX była spójna z wynikami uzyskanymi w XRD i FT-IR dotyczącymi składu rdzeni $Fe_3O_4$ i ich powłoki (udowodniła obecność atomów tlenu, żelaza, bromu i węgla w badanej próbce). Ponadto, wyniki analizy TG wskazały, że termiczna dekompozycja sorbentów zachodzi powyżej 140°C, zatem przygotowane sorbenty są stabilne w warunkach ekstrakcji.

Na podstawie przeprowadzonych eksperymentów wykazano także, że wykorzystanie funkcjonalizowanych NPs za pomocą [$C_{12}C_{12}$MMAmm][Br] jako materiału sorbcyjnego pozwoliło na ponad 80% odzysk epirubicyny z próbek osocza niezależnie od stężenia analitu w próbie (1 i 500 ng/ml), zaś wysoki stopień selektywności opracowanego sorbentu sprawiał, że matryca biologiczna nie stanowiła przeszkody w izolacji epirubicyny za pomocą NPs. Dodatkowo, porównując wcześniejsze doniesienia na temat procedury ekstrakcji epirubicyny [86,90], zastosowanie funkcjonalizowanych NPs pozwoliło zredukować ilość odczynników użytych w procedurze, a szczególnie ograniczyć zużycie stosowanych rozpuszczalników organicznych.

***Wnioski***

Przeprowadzone badania wykazały potencjał ILs we funkcjonalizacji NPs i możliwość ich wykorzystania w MSPE do ekstrakcji epirubicyny z próbek osocza. Określone zostały kryteria doboru materiału powlekającego oraz wpływ warunków procesu wytwarzania i funkcjonalizacji NPs, jak i warunków prowadzenia procedury ekstrakcji epirubicyny z osocza ludzkiego jako modelowego analitu wywodzącego się z grupy antybiotyków antracyklinowych. Opracowane w trakcie badań procedury syntezy i funkcjonalizacji NPs mogą być użyte do przygotowania NPs stosowanych także do ekstrakcji innych związków, w tym substancji leczniczych z próbek biologicznych. Dodatkowo, badania udowodniły, że zastosowanie powleczonych NPs umożliwia miniaturyzację procedury i ograniczenie zużycia rozpuszczalników organicznych. Te spostrzeżenia dowodzą, że wykorzystanie funkcjonalizowanych NPs w ekstrakcji substancji leczniczych z materiału biologicznego jest odpowiednim kierunek dostosowanym do aktualnych trendów analitycznych zmierzających do opracowania metod przyjaznych środowisku naturalnemu.

## PODSUMOWANIE

Nadrzędnym celem niniejszej dysertacji było zbadanie wpływu ILs na efektywność metod analitycznych stosowanych do oznaczania leków cytostatycznych. Na wstępie przeprowadzono szczegółowy przegląd literatury naukowej, aby rzetelnie ocenić dotychczasowy postęp w zakresie aplikacji ILs w analityce chemicznej. Następnie określono zakres badań i przeprowadzono szereg eksperymentów z udziałem ILs. Efektem badań było uzyskanie wyników doświadczalnych, które stały się tematem przewodnim opublikowanego cyklu publikacji, w skład którego wchodzi: jedna praca przeglądowa dotyczącą aktualnego stan wiedzy na temat ILs w obszarze analitycznym (publikacja 1), dwie prace badawcze związane z zastosowaniem ILs na etapie prowadzenia rozdzielenia chromatograficznego wybranych analitów (publikacja 2 i 3) oraz jedna praca badawcza, w której ILs użyto celem zwiększenia efektywności ekstrakcji leku cytostatycznego w trakcie przygotowania prób do analizy chromatograficznej (publikacja 4).

Podsumowując, pierwsza praca z cyklu pozwoliła na poznanie aktualnych osiągnięć w zakresie stosowania ILs do oznaczaniu substancji leczniczych w próbkach biologicznych i środowiskowych. Spośród zgromadzonych danych literaturowych, najwięcej doniesień dotyczyło zastosowań ILs na etapie przygotowania prób do analizy, ale jednocześnie wskazało na pewien schematyczny sposób postępowania analitycznego. Dotyczyło to zarówno procedury ekstrakcji, którą najczęściej była DLLME, ale także struktur ILs, które często ograniczały się do wąskiej grupy związków. W ramach opracowania pracy przeglądowej szczegółowo przeanalizowano także opublikowane doniesienia dotyczące użycia ILs jako modyfikatorów faz ruchomych w LC. Ten drugi co do popularności sposób ich zastosowania w opracowaniu metod analitycznych nie został także w pełni poznany. Przede wszystkim badano wąską grupę ILs, zaś rozdzielenia chromatograficzne prowadzono głównie z użyciem oktadecylokrzemionkowych faz stacjonarnych. Dodatkowo, opublikowane prace nie zawierały szczegółowych opisów mechanizmów separacyjnych zachodzących w obecności ILs, co znacznie ułatwiłoby projektowanie dalszych metod analitycznych z ich udziałem. Powyższe spostrzeżenia i wnioski stały się podstawą do zaprojektowania planu badań, które ujęto w toku realizacji rozprawy doktorskiej.

W drugiej publikacji z cyklu, będącej jednocześnie pierwszą pracą badawczą, skupiono się na tematyce kontroli retencji analitów na oktadecylokrzemionkowej fazie stacjonarnej, gdy ILs zastosowano jako dodatki do faz ruchomych. Ten rodzaj fazy stacjonarnej był najczęściej opisywany z udziałem ILs, jednakże brakowało danych eksperymentalnych dotyczących

wyników badań uzyskanych dla szerszej grupy ILs, które analizowano w zróżnicowanych warunkach chromatograficznych. Zaplanowano zatem badania z udziałem 17 ILs, które użyto jako modyfikatory faz ruchomych zawierających jako składnik polarny 0,1 % kwas mrówkowy w wodzie bądź bufor fosforanowy o różnym stężeniu (10 i 40 mM) i pH (3, 5, 7). Analizy chromatograficzne prowadzono w warunkach elucji izokratycznej. Powyższe eksperymenty z udziałem szerokiej gamy ILs prowadziły do sformułowania następujących wniosków:

1) Najistotniejszy wpływ na zmiany w retencji analitów wynika ze struktury chemicznej ILs. Decydującą rolę w tej strukturze odgrywał charakter fizykochemiczny anionu, gdyż dopiero w sytuacji, gdy anion nie brał udziału w interakcjach na powierzchni fazy stacjonarnej (tzn. posiadał właściwości kosmotropowe), uwidaczniał się wpływ kationu ILs i jego udział w kontroli retencji analitów. Odnotowano także, że skrócenie czasów retencji analitów było ściśle skorelowane ze wzrostem długości łańcucha alkilowego kationu, co wynikało ze wzrostu siły oddziaływań hydrofobowych pomiędzy nim a łańcuchami octadecylokrzemionkowej fazy stacjonarnej. W konsekwencji, prowadziło to do wypierania analitów bądź utrudnionego dostępu do złoża kolumny analitycznej, co skutkowało zmniejszeniem retencji analitów,
2) Efekt działania ILs mógł być wzmacniany przez zwiększenie jej stężenia, jednak obserwowano występowanie górnej granicy tego stężenia, powyżej którego nie obserwowano zmian w retencji analitów, co prawdopodobnie było spowodowane zjawiskiem wysyceniem miejsc aktywnych na powierzchni fazy stacjonarnej zdolnych do specyficznych interakcji z IL.
3) Wpływ ILs na retencję analitów był także uzależniony od składu fazy ruchomej. Zmiany w retencji analitów były bardziej widoczne, gdy ILs dodawano do 0,1 % wodnego roztworu kwasu mrówkowego lub buforu fosforanowego o stężeniu 10 mM, podczas gdy najmniejszy wpływ ILs był widoczny po dodaniu do buforu fosforanowego o stężeniu 40 mM. Powyższe różnice były prawdopodobnie skutkiem dominującej obecności anionów fosforanowych, które obniżały adsorpcję chaotropowych anionów ILs na powierzchni fazy stacjonarnej lub oddziaływały z kationami antracyklin tworząc z nimi pary jonowe o wyższej zdolności sorpcyjnej do powierzchni fazy stacjonarnej co przeciwstawnie wpływało na retencję analitów i w efekcie obniżało wpływ IL na ten parametr chromatograficzny. W przypadku IL z kosmotropowym anionem zmniejszenie oddziaływania IL na retencję analitów było prawdopodobnie także związane z wystąpieniem silniejszych interakcji anionów fosforanowych z ligandami fazy stacjonarnej, co prowadziło do zwiększonej retencji analitów. Tym samym, efekt ten był

przeciwstawny do wywołanego obecnością kationu IL, gdyż jego interakcje z ligandami fazy stacjonarnej prowadziłyby do wzrostu ładunku dodatniego na powierzchni fazy stacjonarnej i powodowały odpychanie antracyklin, a tym samym obniżenie retencji analitów.

4) ILs wpływały nie tylko na retencję analitów ($t_R$), ale także na pozostałe parametry chromatograficzne uzyskane dla badanych analitów tj.: wielkość sygnału analitu (A), współczynnik asymetrii piku ($T_f$) oraz liczba półek teoretycznych ($N_A$). Wybór odpowiedniej struktury IL pozwalał na zwiększenie intensywności piku oraz poprawę kształtu uzyskanych pików analitów, jak również sprawności układu chromatograficznego.

W trzeciej pracy cyklu kontynuowano badania nad zastosowaniem ILs jako modyfikatorów faz ruchomych, jednakże w tej pracy skupiono się na określeniu mechanizmów zachodzących na powierzchni fenylowych faz stacjonarnych. Ten rodzaj kolumn chromatograficznych w aspekcie oddziaływań ILs był wcześniej znacznie rzadziej rozpatrywany, a ponadto nie dotyczył zastosowań ILs w analizach farmaceutycznych. Dzięki analizom przeprowadzonym na fazie fenylowej, pentafluorofenylowej oraz alkilowej w obecności trzynastu ILs dodanych do fazy ruchomej możliwe było dostarczenie nowych danych eksperymentalnych związanych z aplikacją ILs na etapie separacji chromatograficznej analitów techniką RP-LC. Poniżej przedstawiono kluczowe wnioski z tych badań:

1) Odziaływania typu π-π na fazach stacjonarnych z ugrupowaniem fenylowym sprawiają, że efekt ILs dla tego rodzaju kolumn był silniejszy niż dla kolumn alkilowych. Powyższe oddziaływania prawdopodobnie występowały pomiędzy pierścieniem aromatycznych na powierzchni fazy stacjonarnej a pierścieniem aromatycznym kationów ILs i były dodatkowo wspierane przez oddziaływania hydrofobowe van der Waalsa pomiędzy łańcuchami alkilowymi fazy stacjonarnej a kationami IL. W przypadku faz alkilowych wpływ kationu IL wynikał w głównej mierze ze znacznie słabszych oddziaływań typu van der Waalsa. Tym samym, wpływ kationu IL na retencję analitów był widoczny tylko wówczas, gdy anion IL nie dominował w określonych warunkach chromatograficznych i nie decydował o ostatecznej retencji tych związków,
2) Obecność atomów fluoru w fazie pentafluorofenylowej powodowała dodatkowe interakcje z kationami ILs i w konsekwencji większe zmiany w retencji analitów niż w przypadku faz fenylowych. Ten efekt można wyjaśnić poprzez wzrost aromatyczności pierścienia fenylowego na skutek obecności atomów fluoru przy pierścieniu. W konsekwencji dochodziło do zwiększenia siły oddziaływań typu π-π z kationami IL, a ponadto mogły wystąpić dodatkowe odziaływania typu dipol-dipol oraz transfer ładunku.

3) Zastosowanie IL o tej samej strukturze (1-heksylo-3-metyloimidazoliowy chlorek [$C_6$MIM][Cl]) pozwoliło skrócić praktyczny czas analiz na fazie alkilowej o 8 min, fenylowej o ponad 17 min, zaś pentafluorofenylowej o ponad 29 min,
4) Wpływ ILs na fenylowe fazy stacjonarne powodował także zmiany w wartościach pozostałych parametrów chromatograficznych (A, $N_A$, $T_f$). Kierunki zmian wartości tych parametrów potwierdziły również odmienne interakcje ILs z testowanymi fazami stacjonarnymi. Przykładowo, większość ILs zwiększała wartość $N_A$, gdy analizy prowadzono z użyciem fazy fenylowej, ale w przypadku fazy pentafluorofenylowej zastosowanie ILs powodowało spadek sprawności układu chromatograficznego,
5) Wyniki analizy chemometryczną opartej na HCA i metodzie *k-średnich* potwierdziły, że zmiany parametrów chromatograficznych antybiotyków antracyklinowych po zastosowaniu ILs jako modyfikatorów fazy ruchomej były zależne zarówno od charakteru fizykochemicznego IL jak i rodzaju użytej fazy stacjonarnej.

W czwartej pracy cyklu zajęto się wykorzystaniem potencjału ILs na etapie przygotowania próbek opartym na ekstrakcji typu MSPE, w których ILs zostały użyte jako materiały powlekające magnetyczne sorbenty NPs. Kluczową kwestią dla osiągnięcia wysokiej wydajności ekstrakcji wybranego analitu (epirubicyny) z próbek osocza była odpowiednia funkcjonalizacja NPs determinująca ich pojemność sorpcyjną. Wyniki tych badań pozwoliły wskazać, że:

1) ILs mogą być stosowane do powlekania NPs, jednakże, aby uzyskać wysoką wydajność ekstrakcji, ich struktury powinny być oparte na kationach z długimi łańcuchami alkilowymi,
2) Chcąc projektować procedury z udziałem ILs należy brać pod uwagę nie tylko długość łańcucha alkilowego kationu IL, ale także ilość podstawników obecnych w jego strukturze. Wnioski te wysnuto na podstawie danych uzyskanych dla surfaktantu z dwoma łańcuchami alkilowymi C12 (didodecylodimetyloamoniowy bromek [$C_{12}C_{12}$MMAmm][Br]), który zapewnił wyższą wydajność ekstrakcji analitu niż 1-dodecylo-3-metyloimidazoliowy jodek ([$C_{12}$MIM[I]) z jednymi łańcuchem alkilowym C12.
3) Poza strukturą ILs na uwagę zasługują także procedury syntezy i funkcjonalizacji magnetycznych sorbentów, które również wpływają na wydajność ekstrakcji. Udowodniono, że sposób mieszania roztworu reakcyjnego (łaźnia ultradźwiękowa lub mieszadło magnetyczne), rodzaj zasady strącającej NPs (NaOH lub $NH_4OH$) lub aktywacja powierzchni magnetycznych rdzeni za pomocą 0,1 M HCl przed funkcjonalizacją wpływają na ostateczny profil fizykochemicznych właściwości przygotowanych sorbentów.

Zatem, wyniki badań opisanych w publikacji 4 udowodniły, że po zastosowaniu ILs do powlekania NPs, a następnie użyciu tych sorbentów do ekstrakcji analitu/ów, ich końcowa efektywność jest zależna od wielu zmiennych. Jednakże, możliwe jest opracowanie zminiaturyzowanych procedur zapewniających wysoką wydajność ekstrakcji, które będą zgodne z trendem projektowania metod analitycznych przyjaznych środowisku naturalnemu.

Podsumowując, uzyskane wyniki potwierdziły duży potencjał ILs w opracowaniu metod chromatograficznych dotyczących antybiotyków antracyklinowych, które są jedną z najszerzej stosowanych grup leków cytostatycznych. Umożliwiły dostarczenie nowych danych na temat wpływu ILs na efektywność metod chromatograficznych, co pozwala poszerzyć i usystematyzować wiedzę w obszarze ich aplikacji w naukach farmaceutycznych. Ponadto, poprzez uniwersalność dostarczonych informacji w zakresie stosowania ILs jako dodatków do fazy ruchomej oraz materiałów powlekających magnetyczne NPs w MSPE, przedstawione dane mogą być pomocne w analizie również innych związków chemicznych.

## STRESZCZENIE

Ciecze jonowe (ang. *Ionic Liquids*, ILs) to unikalne cząsteczki złożone z dużych organicznych lub nieorganicznych kationów luźno związanych z małymi nieorganicznymi anionami. Ta z pozoru prosta struktura pozwala na uzyskanie szeregu unikatowych właściwości fizykochemicznych niespotykanych jak dotąd w żadnej innej grupie związków chemicznych. W większości przypadków, ze względu na niską prężność par oraz wysoką stabilność termiczną ILs spełniają także kryteria „*Zielonej Chemii*". Zatem, biorąc pod uwagę niniejsze korzyści, zastosowanie ILs wydaje się interesującym podejściem w poszukiwaniu sposobów na przezwyciężenie różnorodnych ograniczeń w opracowaniu efektywnych metod analitycznych.

Tematem niniejszej dysertacji była ocena wpływu ILs na efektowność metod chromatograficznych stosowanych do oznaczania leków cytostatycznych. Wyniki badań prowadzonych w tym kierunku przedstawiono w formie cyklu czterech publikacjach stanowiących podstawę rozprawy doktorskiej, w tym jednej pracy przeglądowej, w której przedstawiono dotychczasowe osiągnięcia w zakresie zastosowania ILs do opracowania metod analitycznych oraz trzech prac badawczych dotyczących zarówno wpływu ILs na etapie analizy chromatograficznej jak i na etapie przygotowania próbek do analizy.

Pierwszym etapem badań zmierzającym do głębszego poznania wpływu ILs na efektywność metod analitycznych było zastosowanie tych związków jako dodatków do faz ruchomych podczas separacji antybiotyków antracyklinowych (doksorubicyny, epirubicyny, daunorubicyny i idarubicyny) na fazie oktadecylokrzemionkowej. W badaniu wykorzystano

siedemnaście ILs o zróżnicowanej budowie chemicznej, na różnych poziomach stężeń, które dodawano do faz ruchomych o różnym składzie i pH. W oparciu o analizę uzyskanych wartości parametrów chromatograficznych badanych analitów ($t_R$, A, $N_A$ i $T_f$) oceniono wpływ ILs na proces ich separacji. Przedstawione zostały korzyści jak i ograniczenia w stosowaniu tych jonowych modyfikatorów, a także wyjaśniono, w jaki sposób można za ich pomocą kontrolować przebieg i efektywność rozdzielenia chromatograficznego wybranych leków cytostatycznych.

W kolejnym etapie badań przeprowadzono eksperymenty zmierzające do wyjaśnienia wpływu ILs na fazy stacjonarne zawierające ugrupowania fenylowe. Wyniki przeprowadzonych eksperymentów wykazały, że dzięki dodatkowym oddziaływaniom typu π-π pomiędzy kationami ILs a ligandami fenylowych faz stacjonarnych możliwość modyfikacji przebiegu rozdzieleń chromatograficznych wybranych antracyklin była większa niż na fazach alkilowych. Ponadto, w przypadku fazy pentafluorofenylowej efekt ten był jeszcze wyraźniejszy niż obserwowany przy użyciu fazy fenylowej. Stosując odpowiednie struktury ILs można było znacznie redukować czasu analizy bez negatywnego wpływu na selektywność metody oraz istotnej zmiany wartości pozostałych parametrów chromatograficznych (A, N i $T_f$). Ponadto, uzyskane wyniki, wskazały, że zastosowanie odpowiednio dobranej IL jako modyfikatora fazy ruchomej pozwala przezwyciężyć ograniczenia związane z silną retencją analitów na fenylowych fazach stacjonarnych. Tym samym, podczas optymalizacji metod analitycznych te kolumny chromatograficzne mogą być rozważane jako alternatywne rozwiązanie w momencie nieprawidłowej separacji analitów na fazach alkilowych.

Drugi obszar badań związany z oceną wpływu ILs na wyniki analiz leków cytostatycznych dotyczył ich wykorzystania na etapie przygotowania próbek do analizy chromatograficznej. Przeprowadzone badania opierały się na zastosowaniu ILs jako materiałów powlekających magnetyczne NPs, które następnie użyto jako sorbenty w MSPE. Uzyskane wyniki przedstawiały zarówno wpływ procedury syntezy i funkcjonalizacji, jak też struktury ILs, na wydajność ekstrakcji epirubicyny z próbek osocza krwi ludzkiej. Na podstawie przeprowadzonych eksperymentów wykazano, że ILs zbudowane z kationów o odpowiednio długich łańcuchach alkilowych (≥ C10) są odpowiednim materiałem do funkcjonalizacji NPs. Ponadto, opracowując metodę z udziałem funkcjonalizowanych NPs należy uwzględnić procedury ich syntezy i funkcjonalizacji, gdyż warunki przygotowania magnetycznych sorbentów w istotny sposób wpływają na właściwości fizykochemiczne tych sorbentów, a przez to również na wyniki wydajności ekstrakcji. Dla najbardziej efektywnych sorbentów wykonana została analiza XRD, FT-IR, TG i TEM, dzięki którym uzyskano bardziej

szczegółowe informacje na temat kształtu, rozmiaru, stabilności i obecności warstwy powlekającej na powierzchni NPs. Z kolei potwierdzona doświadczalnie użyteczność opracowanego materiału sorpcyjnego wskazała, że nowo opracowane NPs pozwalają na uzyskanie odpowiedniej wydajności ekstrakcji, miniaturyzacji procedury i opracowanie metod przyjaznych środowisku naturalnemu.

Podsumowując, wyniki badań przedstawione w czterech publikacjach stanowiących podstawę niniejszej dysertacji pozwalają rozszerzyć dotychczasową wiedzę na temat ILs oraz ich potencjalnych nowych kierunków zastosowań w opracowywaniu metod analitycznych. Przeprowadzone badania potwierdziły korzystny wpływ ILs na efektywność metod chromatograficznych stosowanych do oznaczania leków cytostatycznych, a także udowodniły, że ich aplikacja jest zgodna z aktualnymi trendami projektowania szybkich, prostych i przyjaznych środowisku procedur analitycznych.

**SUMMARY**

Ionic liquids (ILs) are unique molecules composed of large organic or inorganic cations loosely bound to small inorganic anions. This seemingly simple structure, however, causes that ILs possess a number of unique physicochemical properties not found in any other group of chemical compounds. In most cases, due to low vapor pressure and high thermal stability, ILs are considered as environmentally friendly compounds, according to the criteria of "*Green Chemistry*". Thus, taking into consideration these advantages, the use of ILs seems to be an interesting approach in the search for new ways to overcome various limitations in the development of effective analytical methods.

The subject of this dissertation was the assessment of the influence of ILs on the effectiveness of chromatographic methods used during the determination of cytostatic drugs. The results of research conducted in this direction were presented in the form of a series of four publications constituting the basis of the doctoral dissertation, which includes one review paper presenting current achievements in the use of ILs to develop analytical methods, and three original works concerning the impact of ILs at the stage of chromatographic analysis and also at the stage of sample preparation prior to the analysis of selected cytostatic drugs.

The first stage of presented research was aimed at deeper understanding the impact of ILs on the effectiveness of analytical methods. In this study, various ILs were used as additives to the mobile phases during the separation of anthracycline antibiotics (doxorubicin, epirubicin, daunorubicin and idarubicin) on the octadecylsilica stationary phase. Overall, seventeen ILs with different chemical structures and at different concentration levels were added to mobile

phases of different composition and pH. Based on the obtained chromatographic parameters ($t_R$, A, $N_A$ and $T_f$) for tested analytes, the influence of ILs on the separation process was evaluated. The advantages and limitations concerning the use of these ionic modifiers were presented, and possibilities to use particular ILs in order to control the course and quality of chromatographic separation of selected cytostatic drugs have been explained in this study.

In the next stage of the research, the experiments were carried out to understand the effect of interactions between ILs and stationary phase on the separation mechanism during chromatographic analysis of selected anticancer drugs. The obtained results revealed that due to the additional π-π interactions between the IL cations and the phenyl ligands of the stationary phases, the possibility of modifying the course of chromatographic separations of anthracyclines was greater than for alkyl phases. In the case of the pentafluorophenyl phase, this effect was even stronger than that observed for the phenyl phase. The use of appropriate structure of ILs resulted in significant reduction of the analysis time without affecting the selectivity of the method and changing the values of other chromatographic parameters (A, $N_A$ and $T_f$). The obtained results also showed that properly selected IL as a modifier of the mobile phase allows to overcome the limitations related to the strong retention of analytes on the phenyl stationary phases. It was concluded that such modified chromatographic column can be considered as an alternative when incorrect separation of analytes on the alkyl phases is observed during method optimization.

The second area of research was related to the assessment of the impact of ILs on the extraction efficiency of a cytostatic drug (epirubicin) by the application of those ionic modifiers during sample preparation step prior to chromatographic analysis. ILs were used as coating material for magnetic nanoparticles (NPs), which were next applied as sorbents in magnetic solid-phase extraction (MSPE). The obtained results showed that the synthesis of NPs, the functionalization procedure as well as the structure of ILs affect the extraction efficiency of epirubicin from human plasma samples. Based on the conducted experiments it was shown that ILs built of cations with sufficiently long alkyl chains (≥ C10) are suitable material for the functionalization of NPs. In addition, when developing a method involving functionalized NPs, the procedures for their synthesis and functionalization should also be taken into consideration as the conditions for the preparation of magnetic sorbents can significantly affect the physicochemical properties of the obtained sorbents, and also in consequence the results of extraction efficiency. For the most efficient sorbents, XRD, FT-IR, TG and TEM analyzes were performed, and more detailed information concerning the shape, size, stability and presence of a coating layer on the surface of the NPs was obtained. In turn, the experimentally confirmed

usefulness of the developed sorption material indicated that this newly developed NPs allow adequate extraction efficiency to be reached, while applying miniaturized, environmentally friendly sample preparation procedure

To sum up, the results of research presented in a series of four publications constitute the basis of this dissertation, extend the existing knowledge about the ILs and also designate new directions of their potential application in the development of novel analytical methods. The conducted research confirmed the beneficial effect of ILs on the effectiveness of chromatographic methods used for the determination of cytostatic drugs, and also proved that their application is consistent with the current trends in the design of fast, simple and environmentally friendly analytical procedures.

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**PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ**

*molecules*

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# The Influence of Ionic Liquids on the Effectiveness of Analytical Methods Used in the Monitoring of Human and Veterinary Pharmaceuticals in Biological and Environmental Samples—Trends and Perspectives

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**Abstract:** Recent years have seen the increased utilization of ionic liquids (ILs) in the development and optimization of analytical methods. Their unique and eco-friendly properties and the ability to modify their structure allows them to be useful both at the sample preparation stage and at the separation stage of the analytes. The use of ILs for the analysis of pharmaceuticals seems particularly interesting because of their systematic delivery to the environment. Nowadays, they are commonly detected in many countries at very low concentration levels. However, due to their specific physiological activity, pharmaceuticals are responsible for bioaccumulation and toxic effects in aquatic and terrestrial ecosystems as well as possibly upsetting the body's equilibrium, leading to the dangerous phenomenon of drug resistance. This review will provide a comprehensive summary of the use of ILs in various sample preparation procedures and separation methods for the determination of pharmaceuticals in environmental and biological matrices based on liquid-based chromatography (LC, SFC, TLC), gas chromatography (GC) and electromigration techniques (e.g., capillary electrophoresis (CE)). Moreover, the advantages and disadvantages of ILs, which can appear during extraction and separation, will be presented and attention will be given to the criteria to be followed during the selection of ILs for specific applications.

**Keywords:** ionic liquids; green chemistry; environmental and biological samples; sample preparation; determination of pharmaceuticals; chromatographic methods; electromigration techniques

## 1. Introduction

Analytical chemistry focused on the development of methods for the qualitative and quantitative determination of compounds with different chemical structures is a huge, dynamically developing field of science. The number of available methods and techniques is impressive. However, in addition to successes, there are many limitations regarding the use of such approaches. Problems may appear already at the sample preparation stage. Inadequate selectivity, and the use of large volumes of harmful organic solvents with a high vapor pressure in liquid-liquid extraction (LLE) or solid-phase extraction (SPE) are some of the many reasons for the search for alternatives [1]. The introduction of microextraction combined with the reduction of organic solvents used, and the inclusion of additional

physical and chemical factors (sonication, temperature) have brought enormous progress, but also have several difficulties. Microextraction into both solid and liquid phases is a time-consuming process, and the final results require the indication of many other conditions [2]. For example, in solid-phase microextraction (SPME), commercially available fibers are not always suitable for the target compounds, while for single-drop microextraction (SDME), the stability of the drop in the sample may be a problem [3,4]. These limitations, as well as the need for even greater process control by affecting the retention time, and improving the extraction efficiency and resolution of analytes, are responsible for the attempt to include new structures in the extraction process, which can help to achieve these goals [5]. Modifications, such as the introduction of additional processes in liquid-based sample preparation procedures or changes on the surface of sorbents in SPE-based extraction and microextraction procedures are a good direction in analytics, but often insufficient to achieve the expected effects.

Equally as crucial as sample preparation is the process of the separation and detection of the compounds of interest. Among the many available techniques, chromatography or electrophoresis are most often used for the determination of pharmaceuticals in different matrices. Chromatographic techniques exist in a variety of types: the oldest thin-layer chromatography (TLC), the commonly used high performance liquid chromatography (HPLC) and gas chromatography (GC) as well as the less popular supercritical fluid chromatography (SFC) techniques. These methods can be coupled to various types of detectors, including ultraviolet (UV), fluorescence (FL) or mass spectrometry (MS). There are many important parameters during the development and optimization of methods but the most important include the choice of the stationary phase (the place of separation of the analytes) and the mobile phase composition. If the analytes show excessive column adsorption, tailing of the chromatographic peaks occurs and their width is incorrect [6]. In turn, when choosing a mobile phase, problems can occur with obtaining separate peaks for specific compounds, a too long analysis time and low efficiency [7]. However, other chromatographic conditions, such as the column temperature and the flow rate of the mobile phase as well as the parameters of detection should be carefully selected. This is a particular challenge for pharmaceutical determinations because their diverse structures and rich (despite extraction) matrices, and the necessity to detect many analytes at the same time, are just some of the reasons for difficulties in their separation. In addition, it should be highlighted that the mobile phases in LC often contain large volumes of organic solvents which are highly toxic. An interesting alternative seems to be electromigration techniques such as capillary electrophoresis (CE), micellar electrokinetic chromatography (MEKC) or non-aqueous capillary electrophoresis (NACE). These analytical approaches have been considered to be powerful separation methods due to low sample and reagent consumption, high efficiency, and simplicity. On the other hand, CE-based methods have relatively low sensitivity which makes their application difficult in real clinical and environmental studies. Thus, the above examples show that each stage in the development of an analytical method (both sample preparation and further analysis) can cause problems in performing experiments or in achieving reliable results.

Ionic liquids (ILs) are a relatively new class of compounds that became an object of special attention in the 21st century. Their simple cationic-anionic structure provides unusual and unparalleled properties. Therefore, it should not be surprising that their potential is exploited in many unrelated areas of science, for example, as a catalyst in chemical reactions [8], in drug delivery systems [9], in electroplating processes [10], in treating harmful compounds in wastewater [11], as matrices for analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [12] and many others. Scientists have also become interested in "designer solvents" in response to the constant demand for developing new and better methods, and improving the results obtained. The literature data show that their application is focused on sample preparation by extraction or microextraction as well as chromatography (adding ILs to the mobile phase or to prepare the stationary phase) and electrophoretic techniques (Figure 1).

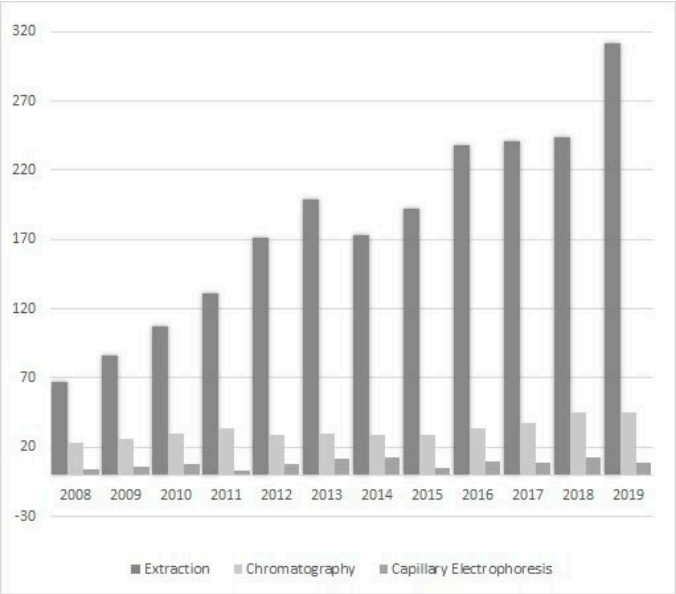



**Figure 1.** Number of publications on the use of ILs in sample preparation (extraction and microextraction) and chromatographic and electrophoretic techniques in 2008–2019 (the authors own elaboration according to ScienceDirect data).

In pharmaceutical sciences ILs can be used for a variety of purposes: as active pharmaceutical substances (API-IL) [13], to determine the solvent residues and impurities in drug quality testing [14] or as a source of information about the presence of pharmaceuticals in biological and environmental samples [15,16]. An important argument supporting their use was also the introduction by Anastas in 1999 of the 12 principles of green chemistry [17]. Attention was drawn to the excessive use of organic solvents and the need to eliminate or reduce environmentally harmful factors. The search for alternatives resulted in the inclusion of ILs in experiments. Negligible vapor pressure, non-flammability, thermal stability, and the possibility of reuse are just some of the properties that have allowed ILs to be described as more environmentally-friendly [18]. It should be highlighted that as newer compounds their literature data are incomplete. However, this does not preclude their use at various stages of analytical testing, from sample preparation to detection and the improvement of results, even for difficult to determine analytes, including the quantification of pharmaceuticals in biological and environmental samples. These substances, with different pharmacokinetic activity, can be delivered directly and indirectly (animal-derived foods) to the human body in very low concentrations. Moreover, pharmaceutical concentrations in urine or bile are different from those in blood or saliva [19]. For this reason, it is necessary to develop a method that will be adequate for the specific biological sample. In the treatment of patients, combination therapy is often used, which results in the presence in the matrix sample of many drugs with different physical and chemical properties making it difficult to choose the best extraction and separation conditions. It should also be remembered that these are not always stable compounds, and to obtain information on their concentrations, it may also be necessary to determine the degradation products and/or metabolites in the presence of many endogenous matrix compounds [20]. Similar considerations can be made in the field of drug determination in environmental samples. According to the data reported in the literature, the sources of pharmaceuticals in wastewater, river waters, lake waters and others are improper drug disposal, hospital wastewater or animal feces. If they occur in an unchanged form, they may cause the risk of typical side effects after they enter the body. One group of drugs often identified in environmental samples are antibiotics, which may be responsible for the development of antibiotic-resistant bacteria [21]. As in biological samples, pharmaceuticals are present in the environment in very low concentrations. Sample purification, the isolation of analytes or the possibility of enriching the sample are crucial and influence the final efficiency of a method.

As already mentioned, pharmaceuticals are compounds with high biological activity, so it is also important to develop simple, reproducible, quick methods, without the need to introduce additional steps to improve the safety of analysts [22]. The inclusion of ILs in their analyses not only improves safety due to the reduction of the use of organic solvent, but also, as confirmed by research, helps to overcome the mentioned difficulties in the analysis of drugs and to improve the validation parameters and efficiency. Therefore, the monitoring of these substances in both the environment and animal and human samples using IL-based environmentally-friendly analytical methods, which also offer reliability, and the qualitative and quantitative sensitivity and selectivity of the compounds of interest is one of the main tasks of modern analytics and chemistry.

The growing number of research papers on ILs has also increased interest in this topic in review articles. Their wide spectrum of possibilities is also clearly visible in the huge variety of subjects of such works. Some of them focused on IL in the context of "green chemistry", pointing to their great potential, but also disadvantages (the need to remove them from the environment, multi-stage synthesis) [23,24]. The reviews very often summarized their applications in sample preparation, especially solid phase microextraction. Most commonly, polymeric ionic liquids (PILs) were evaluated in such applications [25–28]. Some articles considered all the possibilities for using ILs, both at the extraction and detection stages [29–32]. However, the publication selection criteria in the review papers most often concerned analytical methods and techniques or the type of ILs and did not focus on the specific type of analytes or matrices. In addition, it should be noted that the dynamic development of analytical methods using IL requires continuous monitoring of current scientific reports and providing the latest information in current reviews papers. Thus, the purpose of this review was to summarize achievements in the use of ILs for the determination of drugs in biological and environmental samples. In order to properly understand the popularity of ILs in the modern laboratory, the section "Ionic Liquids" presents their history, with the inclusion of their most important features and properties. The basic criteria for choosing articles for the review was the use of ILs during the sample preparation procedure or in the chromatographic/electrophoretic separation of synthetic drugs quantified in biological and environmental samples. The review did not include endogenous compounds, substances responsible for addiction (e.g., nicotine and others) and herbal medicines, except for IL-applications in GC, TLC and SFC. This extension was made in order to fully present the capabilities of ILs and show current trends in the determination of different active biological substances.

## 2. Ionic Liquids

Regarding the history of ILs, and events that are responsible for their presence in many fields of science, it is first of all necessary to define the criteria used in the presentation of this subject. Considering the period of their greatest popularity, that is, the last two decades, we can accept the work of scientists who in their publications focused primarily on modifications of compounds in order to obtain the desired properties and identify their applications. However, to acquire information about the discovery of compounds that were ILs, although no one was aware of this and such a definition was not used, we should return to the mid 19th century. At that time, a by-product known as "red oil" was obtained in the Friedel-Crafts reaction. As shown later, this was the first recorded IL [33]. In the following years, Gabriel and Warner also made an important contribution to the development of ILs. In 1888, for the first time, they synthesized ethanol-ammonium nitrate [34]. Although all previous events were very important, the synthesis of ethylammonium nitrate by Walden in 1914 has most often appeared in publications in the context of the discovery of ILs [35]. Of course, it should be mentioned that in the case of ILs, as in all great discoveries, there are opinions that although Walden synthesized the compounds, he could not use them in practice and his success is over-emphasized [36]. Nevertheless, it was undoubtedly an important stage in the development of ILs. During the following years, there were further syntheses of the compounds and attempts to use them, among others by Yoke and his colleagues [37] and Koch and co-workers [38]. However, in more modern times, with the current compounds that are used in research, it is necessary to focus on analytical methods and

extraction techniques. Considering the application of ILs, Pool's research should be mentioned, in which, using current knowledge, ILs were used in GC as stationary phases [39]. The results of this study prompted the beginning of their further development in this field and became the inspiration for subsequent publications. The 1980s were also important due to the synthesis of ILs based on the imidazolium cation, which are currently widely used in laboratories [40]. This event was important because the existing compounds of ILs had significant limitations in their application, while the imidazolium group provided new opportunities for researchers. Following the trend, subsequent years of research into the use of ILs increased knowledge about them, and consequently led to the introduction of some standards in this area. At the turn of the 20$^{th}$ century, ILs began to function under the name Task-specific Ionic Liquids (TSILs) [41] and companies marketing the first commercially available ILs appeared [42]. Increased access and the positive opinion of the scientific community prompted attempts to apply them to novel projects. In 1998, for the first time, ILs were used as extractants for LLE [43], and in 2005, they were used to coat SPME fibers [44]. Recent years have seen a period of their participation in advanced research, but this will be discussed in detail in subsequent sections. However, it should be highlighted that the most important factor responsible for the rich and long history of ILs is their specific structures, illustrated in Figure 2, which provide the enormous possibilities of these compounds. The cation-anion combinations, described in most definitions, create many possibilities for structure modification, and can thus change the properties of the designed compounds. The cations may have one or more nitrogen, sulfur, phosphorus or oxygen atom in the structure, described as ammonium, sulfonium, phosphonium or oxonium cations, respectively, but in most cases, they are large organic aromatic moieties: pyridinium, piperidinium and the most widely used imidazolium cations. In turn, anions are much smaller and can be both organic and inorganic. In research, tetrafluoroborate ([$BF_4$]), hexafluorophosphate ([$PF_6$]) and halogen anions, and many other compositions appear.

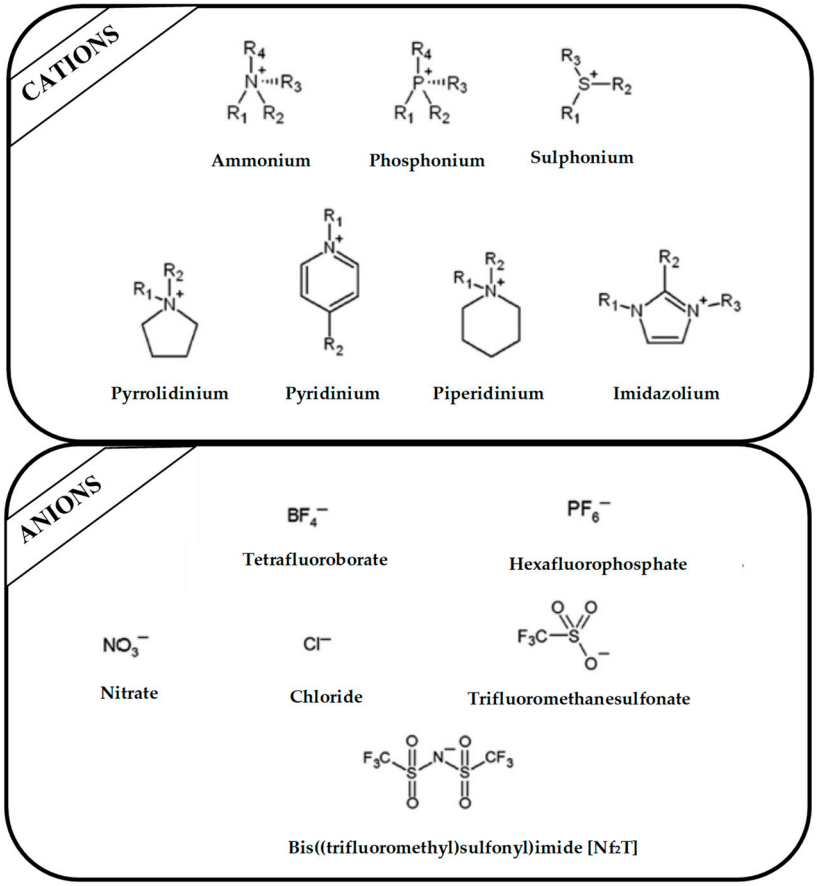



**Figure 2.** Examples of popular anions and cations of ILs used in analytical methods.

Besides the selection of the cation and anion, an important aspect that affects further results is the substituents on the cation, and especially the alkyl chain, the length, branching and position of which have a huge influence on applications of ILs [45]. To fully understand the unique properties of an IL, it is also necessary to pay attention to Coulombic interactions occurring in the molecules, dipole-dipole interactions, Van der Waals forces and hydrogen bonds [46]. It is estimated that the number of available combinations may allow up to 1018 different ILs to be obtained [47]. The differences in the size of the cation and anion, the asymmetry in the structure as well as the mentioned interactions mean that they have no regular, crystalline structure and the delocalization of the cation and anion composition is very possible. Thanks to this, their melting temperature does not exceed 100 °C, and in many cases it is close to room temperature (RTIL) [48]. This feature distinguishes ILs from typical inorganic salts, which, due to the much stronger Coulombic and hydrogen interactions, have a melting point of even above 400 °C. Equally as interesting as their melting point is the viscosity of ILs, which is at a higher level than that of organic solvent. Knowledge of these parameters is necessary when an IL is used in separation and detection techniques. The electrostatic interactions in alkyl chain cations have an enormous impact on viscosity. Coulomb forces, H-bonding and π-π dipole lead to increases in the flow resistance, and additionally, the presence of van der Waals interactions between the cation and anion, depending on the size of the molecule, also causes interactions in the same direction. This property can be modified by changing the temperature or adding an organic solvent [49–52]. Viscosity also influences another property, namely electrochemical conductivity. Thanks to their ionic structure, ILs can carry a charge, but this possibility is not the same for all compounds. When the flow resistance increases, conductivity becomes more difficult. However, increasing the temperature and mixing with organic solvents improves the results. Furthermore, the size of molecules can hinder access to the charge, so it is necessary to select the appropriate cations, which are large ions [53]. ILs are widely used in sample preparation techniques because they can be created as both hydrophilic and hydrophobic compounds that mix with water, and/or organic solvents [54]. It has been proven that the change in the position of the methyl group in the cation determines the change in the acid-base character, and therefore the C2 position is strongly acidic, which affects the interaction with other compounds [55]. The thermal stability of ILs is also important. As studies have shown, the majority of popular ILs are stable even above 300 °C, which is of great importance during GC analysis, where a high temperature is required. As with previous properties, the size and type of ions, pKa, chain length and electrostatic interactions determine the stability of individual ILs. Halogen anions, probably due to their nucleophilic character, have less stability than other inorganic anions, while the most stable is bis(trifluoromethanesulfonyl)imide ([Nf2T]). In turn, among the cations, the stability of pyrrolidinium and piperidinium is lower than that of imidazolium, regardless of the anion used [56–58]. An interesting property is also the insignificant vapor pressure which occurs at elevated temperatures. Zaitaus et al. confirmed the influence of the structure of ILs on vapor pressure. In their study, the absolute vapor pressures for a series of $[C_nMIM][BF_4]$ ionic liquids with (n = 2, 4, 6, 8, and 10) were measured. The results of experiments confirmed that an increase in the number of carbon atoms in the alkyl chain in the imidazolium cation caused a decrease in absolute vapor pressures. However, this effect was different for the homologies of $[C_nMIM][BF_4]$ and $[C_nMIM][Nf_2T]$. Moreover, it was observed that the volatility for $[C_nMIM][BF_4]$ was significantly lower in comparison to $[C_nMIM][Nf_2T]$. In addition, ILs added to organic solvents also reduced their evaporation [59–61].

It should be noted that there are a huge variety of IL combinations, so it is difficult to establish a clear classification. The most popular approach concerns the structure of these compounds (Table 1). A more detailed description concerns three generations of ILs in view of the anion or cation used. The first includes molecules with specific physical properties, which are described in the previous paragraph. The second generation includes ILs for which it is possible to tune their chemical and physical properties and then to use them for a specific purpose, while the last group are compounds with biological activity [62]. From the point of view of analytical applications, it seems reasonable to focus attention on a large and diverse IL group referred to as Task-specific Ionic Liquids. The results of

subsequent tests confirmed that apart from typical ILs, it is necessary to design more specific molecules to achieve a specific goal. This led to the use of ILs in polymerization processes. ILs as monomers can form combinations with other molecules, thus improving the results.

**Table 1.** List of ILs used in modern laboratory for drug analysis.

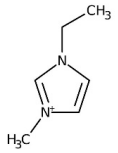
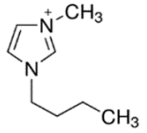
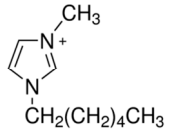
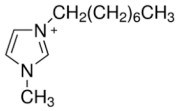
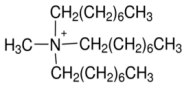
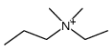
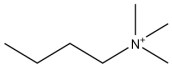
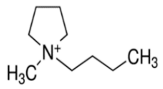
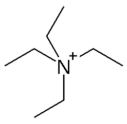
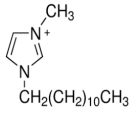
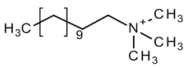
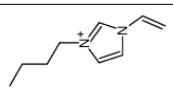
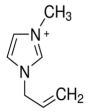
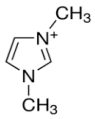
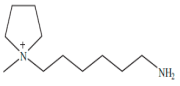
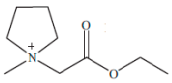
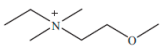
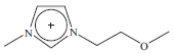
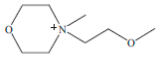
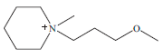
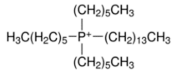
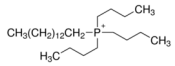
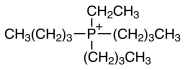
| Cation Structure | Full Name | | Abbreviations |
|---|---|---|---|
| | Cations | Anions | |
|  | 1-ethyl-3-methylimidazolium | hexafluorophosphate, tetrafluoroborate, chloride, bromide, bis(trifluoromethylsulfonyl)imide, methyl sulfate | $[C_2MIM][PF_6]$, $[C_2MIM][BF_4]$, $[C_2MIM][Cl]$, $[C_2MIM][Br]$, $[C_2MIM][Nf_2T]$, $[C_2MIM][CH_3(SO_4)]$ |
|  | 1-butyl-3-methylimidazolium | hexafluorophosphate, tetrafluoroborate, chloride, bromide, bis(trifluoromethylsulfonyl)imide, nitrate, methyl sulfate, octyl sulfate, trifluoromethanesulfonate, dimethyl phosphate, hydroxide | $[C_4MIM][PF_6]$, $[C_4MIM][BF_4]$, $[C_4MIM][Cl]$, $[C_4MIM][Br]$, $[C_4MIM][Nf_2T]$, $[C_4MIM][NO_3]$, $[C_4MIM][CH_3(SO_4)]$, $[C_4MIM][C_8H_{17}(SO_4)]$, $[C_4MIM][CF_3SO_4]$, $[C_4MIM][DMP]$, $[C_4MIM][OH]$ |
|  | 1-hexyl-3-methylimidazolium | hexafluorophosphate, tetrafluoroborate, chloride, bromide, bis(trifluoromethylsulfonyl)imide, tris(pentafluoroethyl)trifluoro-phosphate | $[C_6MIM][PF_6]$, $[C_6MIM][BF_4]$, $[C_6MIM][Cl]$, $[C_6MIM][Br]$, $[C_6MIM][Nf_2T]$, $[C_6MIM][TFP]$ |
|  | 1-octyl-3-methylimidazolium | hexafluorophosphate, tetrafluoroborate, chloride, bromide, bis(trifluoromethylsulfonyl)imide | $[C_8MIM][PF_6]$, $[C_8MIM][BF_4]$, $[C_8MIM][Cl]$, $[C_8MIM][Br]$, $[C_8MIM][Nf_2T]$ |
|  | methyltrioctylammonium | tetrachloroferrate, tetrachloromanganate(II) | $[C_8MAmm][FeCl_4]$, $[C_8MAmm][MnCl_4^{2-}]$ |
|  | ethyl-dimethyl-propylammonium | bis(trifluoromethylsulfonyl)imide | $[NEMMP][Nf_2T]$ |
|  | 1-butyl-3-methylammonium | bis(trifluoromethylsulfonyl)imide | $[C_4M_3Amm][Nf_2T]$ |
|  | 1-butyl-1-methylpyrrolidinium | bis(trifluoromethylsulfonyl)imide, tetracyanoborate, tris(pentafluoroethyl)trifluoro-phosphate | $[C_4MPyrr][Nf_2T]$, $[C_4MPyrr][B(CN)_4]$ $[C_4MPyrr][TFP]$ |
|  | tetraethylammonium | tetrafluoroborate | $[(C_2H_5)_4N][BF_4]$ |
|  | 1-dodecyl-3-methylimidazolium | chloride | $[C_{12}MIM][Cl]$ |
|  | 1-dodecyl-3-methylammonium | chloride | $[C_{12}MAmm][Cl]$ |
|  | 1-vinyl-3-butylimidazolium | chloride | $[ViC_4MIM][Cl]$ |

**Table 1.** *Cont.*

| Cation Structure | Full Name | | Abbreviations |
|---|---|---|---|
| | Cations | Anions | |
|  | 1-allyl-3-ethylimidazolium | bromide | [$AC_2$MIM][Br] |
|  | 1,3-dimethylimidazolium | methyl sulfate | [MMIM]][$CH_3(SO_4)$] |
|  | 1-(6-amino-hexyl)-1-methylpyrrolidinium | tris(pentafluoroethyl)trifluoro-phosphate | [$C_6NH_2$MPyrr][TFP] |
|  | 1-ethoxycarbonyl-methyl-1-methyl-pyrrolidinium | tris(pentafluoroethyl)trifluoro-phosphate | [ECMMPyrr][TFP] |
|  | methoxyethyl-dimethylethyl-ammonium | tris(pentafluoroethyl)trifluoro-phosphate | [MOEDEAmm][TFP] |
|  | 1-methoxyethyl-3-methylimidazolium | tris(pentafluoroethyl)trifluoro-phosphate | [MOEMIM]][TFP] |
|  | 1-methoxyethyl-1-methylmorpholinium | tris(pentafluoroethyl)trifluorophosphate | [MOEMMO][TFP] |
|  | 1-methoxypropyl-1-methylpiperidinium | tris(pentafluoroethyl)trifluorophosphate | [MOPMPP][TFP] |
|  | trihexyltetradecylphosphonium | tetrachloromanganate(II), dicyanamide, bis(trifluoromethanesulfonyl) imide | $[P_{6,6,6,14}{}^+]_2[MnCl_4{}^{2-}]$, $[P_{6,6,6,14}{}^+][N(CN)_2]$, $[P_{6,6,6,14}{}^+][Nf_2T]$ |
|  | tributyl(tetradecyl)phosphonium | *p*-dodecylbenzenesulfonate | $[P_{4,4,4,14}{}^+]$[DDBS]) |
|  | tributyl(ethyl)phosphonium | diethylphosphate | $[P_{2,4,4,4}{}^+][(2O)_2PO_2]$ |

The currently applied analytical methods use the structure and properties of ILs to create polymeric connections with cyclodextrins (CDs) [63] or magnetic imprinted nanoparticles (MILs) [64]. In addition, polymeric ionic liquids (PILs) can also be synthesized by a co-polymerization process [65]. Their participation in the molecular imprinting technique used to develop sorbents of monolithic columns has also been noted. Another, large subclass of ILs are chiral ionic liquids (CILs). Recent scientific reports show that amino acids can be used for the synthesis of CILs. Their carboxyl or amine functional groups determine the chiral nature and function in the structure (cation or anion). The use of amino acids results from the trend of reducing toxicity and the use of natural compounds [66]. In addition, these "designer molecules" are also used as chiral selectors in aqueous two-phase systems (ATPS) [67].

## 3. Sample Preparation

As it was earlier mentioned, the sample preparation procedure is still one of the most important stages in the development of analytical methods. The variety of biological and environmental samples makes them very complicated with regard to gathering all information about the sample preparation stage. Both types of samples are complex analytical matrices, and the stage of their preparation for analysis is multifactorial. It usually requires the performance of various operations and activities both in situ and in the laboratory. Due to the very low concentrations in real samples, the extraction method should have the highest possible recovery. In addition, the sample handling method largely

depends on the chosen final determination technique. Knowing the chemical properties of the drug (or drugs) sought in the analyzed matrix, makes it possible to properly select the organic solvents in order to carry out a successful extraction from the sample, followed by purification, sometimes by back extraction. Considering all these aspects, it is necessary to search for new directions in sample pretreatment procedures. One of these is the use of ILs at the preparation stage of biological and environmental samples to isolate the drugs potentially present in them, both with the use of liquid-liquid based extraction and solid-phase based extraction procedures [68]. ILs are used as liquid phases, extractors, intermediate solvents, mediators and desorption solvents [68–111]. Exemplary applications of individual types of drug extraction from biological and environmental samples with IL-modifications are presented in Table 2. These summarized data clearly indicate that despite the determination of low pharmaceutical concentrations in both types of samples, IL-based extraction procedures go in a different direction. If the matrix is biological fluid, the most common problem is the distribution of peaks, selectivity, shape and of course performance. In turn, environmental samples most often focus on the need to improve extraction efficiency [69,70]. Matrix influence, peak shape and distribution are not the main reasons for using ILs in extraction. A difference also occurs in the volume of the analyzed sample, being much larger for environmental samples [69]. This factor is especially important during the formation of two phases with the participation of ILs, in which the proper volume ratios (aqueous phase, organic phase, IL and others) are needed for the proper phase separation and the subsequent separation [67,112–114]. In the publications presented below, it can also be seen that for environmental samples, there was a much greater variety in the choice of extraction method, especially in the area of dispersive liquid–liquid microextraction (DLLME). In the case of biological samples, they were also extracted by DLLME, but the modifications were much smaller in number.

### *3.1. Liquid-Phase Based Extraction and Microextraction Procedures*

#### 3.1.1. Liquid-Liquid Extraction

LLE is the oldest method of extracting analytes. Unfortunately, despite the simplicity of performance, the method has many disadvantages. It is a very time- and work-intensive process, and the results depend on many additional factors, e.g., the physicochemical character of analytes, the type of extraction solvents, the extraction time and the temperature, which in turn cause reproducibility and repeatability problems. Furthermore, according to the current trend of designing more environmentally-friendly analytical methods, the use of toxic organic solvents should be reduced. As is well known, LLE does not meet this condition. In all probability, this was the reason why in regard to drug quantification in biological and environmental samples, traditional LLE extraction supported by IL modification was rarely considered. To the best of our knowledge, no reports have been published for biological applications, while only one paper can be found in the field of environmental investigations (Table 2).

**Table 2.** Summary of the IL applications in liquid-phase microextraction drugs from biological and environmental samples.

| Drug(s) | Matrices | Tested Ionic Liquids | Extraction Solvent | Analytical Technique | LOD [ng/mL] | Efficiency [%] | Ref. |
|---|---|---|---|---|---|---|---|
| **LLE** | | | | | | | |
| **Environmental samples** | | | | | | | |
| Ranitidine, nizatidine | River water, wastewater | **[$C_4$MIM][$Nf_2$T]** **[$C_4$MIM][$PF_6$]** | MeOH | HPLC-UV | 90<br>430 | 100.4<br>101.2 | [68] |
| **IL-DLLME** | | | | | | | |
| **Biological samples** | | | | | | | |
| NSAIDs | Human urine | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] | MeOH | HPLC-UV | 8.3–32 | 36.8–42.3 | [70] |
| Anti-hypertensive drugs | Rat serum | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_4$MIM][$BF_4$] [$C_6$MIM][Cl] [$C_2$MIM][$CH_3(SO_4)$] | Acetone | HPLC-PDA | 15–20 | 92.8–98.5 | [80] |
| Balofloxacin | Rat serum | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_4$MIM][$BF_4$] [$C_6$MIM][Cl] [$C_4$MIM][Br] [$C_2$MIM][$CH_3(SO_4)$] | ACN | HPLC-DAD | 10 | 99.5 | [81] |
| Rifaximin | Rat serum | **[$C_4$MIM][$PF_6$]** [$C_4$MIM][$BF_4$] [$C_6$MIM][Cl] [$C_4$MIM][Br] [$C_2$MIM][$CH_3(SO_4)$] | MeOH | HPLC-DAD | 10 | 99.5 | [82] |
| Ofloxacin | Human urine and plasma, tablets | **[$C_6$MIM][$PF_6$]** | Ethanol | SFIS | 29 | 89.5–93 | [86] |
| Tetracycline | Eggs | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] [IMIM][$PF_6$] | ACN | HPLC-DAD | 2–12 | 58.6–95.3 | [87] |
| Fluoroquinolone | Chicken, pork and fish meat | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] | ACN | HPLC-DAD | 0.5–1.1 | 60.4–96.3 | [88] |
| Nifurtimox, benznidazole | Human plasma | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] | MeOH | HPLC-UV | 15.7<br>3.66 | 98<br>79.8 | [72] |
| Nifurtimox, benznidazole | Human breast milk | **[$C_8$MIM][$PF_6$]** | MeOH | HPLC-UV | 90<br>60 | 89.7<br>77.5 | [73] |
| Sildenafil, Vardenafil, Aildenafil | Human plasma | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] | MeOH | HPLC-UV | 0.92–2.69 | 100.4–103.9 | [74] |
| Ephedrine, Ketamine | Human urine | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] | ACN | HPCE | 210<br>390 | 79–90 | [89] |
| Daclatasvir | Rat serum | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_4$MIM][$BF_4$] [$C_4$MIM][Br] [$C_6$MIM][Cl] [$C_2$MIM][$CH_3(SO_4)$] | ACN | HPLC-DAD | 15 | 99.4 | [90] |

**Table 2.** *Cont.*

| Drug(s) | Matrices | Tested Ionic Liquids | Extraction Solvent | Analytical Technique | LOD [ng/mL] | Efficiency [%] | Ref. |
|---|---|---|---|---|---|---|---|
| **Environmental samples** | | | | | | | |
| NSAIDs, acetazolamide, caffeine, sulfonamides, carbamazepine, gemfibrozil | Tap water, creek water | **[$C_6NH_2$MPyrr][TFP]** [$C_4$MIM][Cl] [$C_4$MIM][$Nf_2$T] [ECMMPyrr][TFP] [MOEDEAmm][TFP] [MOEMIM][TFP] [MOEMMO][TFP] [MOEMPyrr][TFP] [MOPMPP][TFP] | MeOH/Acetone | HPLC-UV | 0.1–55.1 | 91–110 | [92] |
| Triclosan, triclocarbon | Tap water, wastewater | **[$C_6$MIM][$PF_6$]** [$C_4$MIM][$BF_4$] | MeOH | LC-MS/MS | 0.040–0.58 | 70.0–103.5 | [93] |
| **DLLME modifications** | | | | | | | |
| **Biological samples** | | | | | | | |
| *US-IL-DLLME* | | | | | | | |
| Salmeterol | Dried blood spot | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] | MeOH | HPLC-FL | 0.30 | 90 | [71] |
| Citalopram, nortriptyline | Human plasma | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] | | HPLC-PDA | 10<br>6 | 90–92 | [79] |
| Venlafaxine, amitriptyline | Human plasma | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] | | HPLC-DAD | 0.5<br>0.8 | 91.4–92.6 | [83] |
| Ulipristal | Mice serum, tablets | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] | | HPLC-UV | 6.8<br>9.3 | 95 | [75] |
| Benzodiazepines and benzodiazepine-like | Human blood, post-mortem human blood | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] | | LC-MS/MS | 0.03–4.74 | 24.7–126.2 | [15,76,77] |
| Antidepressants | Human blood | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] [$C_4$MPyrr][$Nf_2$T] [$C_4M_3$Amm][$Nf_2$T] | | LC-MS/MS | 1–2 | 53.11–132.98 | [78] |
| *DLLME (rapid shooting)* | | | | | | | |
| Danazol | Mice serum, capsules | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] | | UV | 55<br>54 | 90.5–103.4 | [84] |
| *TCIL-DLPME* | | | | | | | |
| Piroxicam | Human urine, plasma, and tablets | **[$C_6$MIM][$PF_6$]** | | SFIS | 46 | 95.2–104 | [85] |
| **Environmental samples** | | | | | | | |
| *US-IL-DLLME* | | | | | | | |
| Lovastatin, simvastatin | Tap water, lake water, river water | **[$C_6$MIM][$PF_6$]** | MeOH | HPLC-UV | 0.17<br>0.29 | 90.0–102.2,<br>80.5–112.0 | [95] |
| β-Blockers NSAIDs | Wastewaters | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] | ACN | LC-MS | 0.0002–0.060 | 88–111 | [97] |
| Fluoroquinolones | Groundwater | **[$C_8$MIM][$PF_6$]** [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] | MeOH | HPLC-FL | 0.0008–0.013 | 105–107 | [98] |
| NSAIDs | Tap water, drinking water | **[$C_8$MIM][$PF_6$]**, [$C_4$MIM][$PF_6$] | MeOH | UHPSFC-PDA | 0.62–7.69 | 81.4–107.5 | [99] |

**Table 2.** *Cont.*

| Drug(s) | Matrices | Tested Ionic Liquids | Extraction Solvent | Analytical Technique | LOD [ng/mL] | Efficiency [%] | Ref. |
|---|---|---|---|---|---|---|---|
| | | *MADLLME* | | | | | |
| Derivatization of sulfonamides | River water | **[$C_6$MIM][$PF_6$]**, [$C_4$MIM][$PF_6$], [$C_8$MIM][$PF_6$] | MeOH | HPLC-FD | 0.011–0.018 | 95.0–110.8 | [100] |
| | | *MIL-DLLME* | | | | | |
| Acetaminophen sulfamethoxypyridazine, phenacetin, ketoprofen | Lake water, river water | **[$P_{6,6,6,14}^{+}$]$_2$[$MnCl_4^{2-}$]** [Aliquat$^+$]$_2$[$MnCl_4^{2-}$] [$C_8$MAmm][$MnCl_4^{2-}$] | ACN/MeOH | HPLC-UV | 0.25–1.0 | 42.9–114.7 | [94] |
| | | *IL-DLLME-µ-SPE* | | | | | |
| Antidepressant drugs | Canal water | **[$C_6$MIM][TFP]** [$C_6$MIM][$Nf_2$T] | Methanol | HPLC-UV | 0.3–1.0 | 94.3–114.7 | [101] |
| | | *IL-DLLME-SDS* | | | | | |
| Tetracyclines | River water, fishpond water, leaching water | **[$C_4$MIM][$PF_6$]** [$C_6$MIM][$PF_6$] [$C_8$MIM][$PF_6$] | Methanol | UHPLC-TUV | 0.031–0.079 | 55.1–96.3 | [96] |
| | | *IL/IL-DLLME* | | | | | |
| Triclocarbon, triclosan | Tap, river, snow, Lake water | Hydrophobic: [$C_4$MIM][$PF_6$] [$C_6$MIM][$PF_6$] **[$C_8$MIM][$PF_6$]** Hydrophilic: [$C_2$MIM][$BF_4$] **[$C_4$MIM][$BF_4$]** [$C_4$MIM][$NO_3$] | - | LC-MS/MS | 0.23<br>0.35 | 88–111 | [102] |
| NSAIDs | Tap water, River water | **[$C_4$MIM][$BF_4$]** **[$C_4$MIM][$PF_6$]** [NEMMP][$Nf_2$T] [MOEDEA][TFP] | Methanol | HPLC-DADHPLC-FL | 17–95 | 89–103 | [103] |
| | | **Other liquid phase extraction** | | | | | |
| | | **Biological samples** | | | | | |
| | | *IL-SE-UE-ME* | | | | | |
| Doxepin, perphenazine | Human urine | **[$C_6$MIM][$PF_6$]** [$C_6$MIM][$Nf_2$T] [$C_4$MIM][$PF_6$] | | HPLC-MWD | 100<br>1000 | 89–98 | [104] |
| | | *IL/IL LPME* | | | | | |
| Sulfonamides | Human, chicken, rabbit, cow, pig blood | **[$C_4$MIM][$BF_4$]** **[$C_6$MIM][$PF_6$]** | | HPLC-UV | 3.77–5.21 | 90–113 | [105] |
| | | *dLPME* | | | | | |
| NSAIDS | Human urine | **[$C_4$MIM][$PF_6$]** | ACN | HPLC-UV | 38–70 | 72.8–90.3 | [91] |
| Phenothiazines | Human urine | **[$C_4$MIM][$PF_6$]** | ACN | HPLC-UV | 21–60 | 72–98 | [106] |
| | | **Environmental samples** | | | | | |
| | | *SADBME* | | | | | |
| Diclofenac, ibuprofen | Wastewater treatment plant, river and lake water | **[$C_8$MAmm][$FeCl_4$]** | ACN | HPLC-DAD | | | [64] |
| | | *ILSVA-SME* | | | | | |
| Glucocorticoids | Mineral water, lake water, tap water | **[$C_4$MIM][$PF_6$]** | ACN | HPLC-DAD | 4.11–7.50 | ≥97.24 | [107] |

**Table 2.** *Cont.*

| Drug(s) | Matrices | Tested Ionic Liquids | Extraction Solvent | Analytical Technique | LOD [ng/mL] | Efficiency [%] | Ref. |
|---|---|---|---|---|---|---|---|
| | | *MA-LLME-SIL* | | | | | |
| Sulfonamides | Tap water, lake water, river water, pool water | **[$C_2$MIM][$PF_6$]** | ACN | HPLC-UV | 0.33–0.85 | 75.1–115.8 | [108] |
| Sulfonamides | Wastewater paddy water, river water | **[$C_8$MIM][$PF_6$]** | | HPLC-UV | 0.1–0.4 | | [109] |
| | | *(MBA)-LPME* | | | | | |
| Glucocorticoids | Wastewater | **[$C_4$MIM][$CH_3(SO_4)$]** [$C_4$MIM]$BF_4$] [$C_4$MIM][Cl] [$C_4$MIM][$PF_6$] [$C_4$MPyrr][TFP] [$C_6$MIM][TFP] | MeOH | UHPLC-MS/MS | 0.0128–0.0470 | 49.40–83.1 | [110] |
| | | *IL-AF-µ-EME* | | | | | |
| Antidepressants | Tap water, river water | **[$C_6$MIM][$PF_6$]** [$C_8$MIM][$PF_6$] | MeOH/ACN | HPLC-UV | 0.4 | 88.2–111.4, 90.9–107 | [111] |
| | | *IL-IDME* | | | | | |
| Amitriptyline | Hospital wastewater | **[$C_6$MIM][$PF_6$]** | MeOH/ACN | HPLC-UV | 4.0 | 85.12 | [16] |
| | | **ATPS** | | | | | |
| | | **Biological samples** | | | | | |
| Sulfonamides | Milk (from supermarket) | **[$C_4$MIM][$BF_4$]** [$C_2$MIM][$BF_4$] [$C_6$MIM][$BF_4$] [$C_8$MIM][$BF_4$] | ACN | HPLC-UV | 2.04–2.84 | 72.3–108.9 | [112] |
| Sulfonamides | Pig, rabbit, cow, chicken and human blood | **[$C_6$MIM][Cl]** [$C_4$MIM][Cl] [$C_8$MIM][Cl] | | HPLC-UV | 2.45–4.13 | 85.5–110.9 | [67] |
| | | **Environmental samples** | | | | | |
| | | *IL-ATPF* | | | | | |
| Chlorampheni-col | Lake water, feed water | **[$C_4$MIM][Cl]** [$C_8$MIM][Cl] [$C_4$MIM][$BF_4$] | | HPLC-UV | 0.1 | 97.1–101.9 | [113] |
| | | *MILATPs* | | | | | |
| Chloramphenicol | River water | **[TMG][TEMPO-$OSO_3$]** | | HPLC-UV | 0.14 | 94.6–99.7 | [114] |

[$C_2$MIM]: 1-Ethyl-3-methylimidazolium; [$C_4$MIM]: 1-butyl-3-methylimidazolium; [$C_6$MIM]: 1-hexyl-3-methylimidazolium; [$C_8$MIM]: 1-octyl-3-methylimidazolium; [MMIM]: 1,3-dimethylimidazolium; [$C_4$MPyrr]: 1-butyl-1-methylpyrrolidinium; [$C_4M_3$Amm]: 1-butyl-3-methylammonium; [IMIM]: 1-isopentene-3-methylimidazolium; [$Nf_2T$]: bis(trifluoromethylsulfonyl)imid; [$BF_4$]: tetrafluoroborate; [$PF_6$]: hexafluorophosphate; [Cl]: chloride; [$CH_3(SO_4)$]: methylsulfate; HPCE: high performance capillary electrophoresis; HPLC-FL: high-performance liquid chromatography and fluorescence detection; HPLC-UV: high-performance liquid chromatography and ultraviolet detection; HPLC-DAD: high-performance liquid chromatography and diode array detection; LC-MS/MS: liquid chromatography—tandem mass spectrometry; UV: ultraviolet spectroscopy; HPLC-MWD: high-performance liquid chromatography and multiple wavelength detector; HPLC-PDA: high-performance liquid chromatography and photo-diode array detector; SFIS: stopped-flow injection spectrofluorimetry; TCIL-DLPME: temperature-controlled ionic liquid dispersive liquid phase microextraction; [$C_6NH_2$MPyrr]: 1-(6-aminohexyl)-1-methylpyrrolidinium; [TFP]: tris(pentafluoroethyl)trifluorophosphate; [ECMMPyrr]: 1-ethoxycarbonylmethyl-1-methylpyrrolidinium; [MOEDEAmm]: methoxyethyl-dimethyl-ethylammonium; [MOEMIM]: 1-methoxyethyl-3-methylimidazolium; [MOEMMO]: 1-methoxyethyl-1-methylmorpholinium; [MOPMPP]: 1-methoxypropyl-1-methylpiperidinum; [$Aliquat^+$]$_2$[$MnCl_4^{2-}$]: aliquat tetrachloromanganate(II); [$C_8$MAmm]: methyltrioctylammonium; [$P_{6,6,6,14}^+$]: trihexyltetradecylphosphonium; TUV: tunable ultraviolet detection; UHPLC: ultra-high pressure liquid chromatography; [MOEDEAmm]: ethyl-dimethyl-(2-methoxyethyl)ammonium; [NEMMP]: ethyl-dimethyl-propylammonium; MADLLME: microwave-assisted dispersive liquid–liquid microextraction; [TMG]: 1,1,3,3-tetramethylguanidine; [TEMPO]: 2,2,6,6-tetramethyl piperidine 1-oxyl free radical; IL-AF-µ-EME: Ionic liquid-impregnated agarose film two-phase micro-electrodriven membrane extraction; IL-ATPF: Ionic liquid–salt aqueous two-phase flotation; [DI-SPME]: direct-immersion solid-phase microextraction.

Environmental Samples

Kiszkiel et al. [68] were the only researchers who tested the ionic liquids [$C_4$MIM][$PF_6$] and [$C_4$MIM][$Nf_2T$]) for LLE, showing their ability to selectively isolate nizatidine and ranitidine from wastewater and river waters (Table 2). Based on preliminary studies, an IL with a different anion was selected for each analyte. In the case of nizatidine extraction, [$C_4$MIM][$PF_6$] was used, while for ranitidine—[$C_4$MIM][$Nf_2T$]. Their application allowed methanol consumption to be reduced (1.0 mL for nizatidine and 1.5 mL for ranitidine). During optimization, the appropriate volume of IL was selected and the impact of additional factors such as the effect and mixing time or pH was assessed. The ultimately optimized and validated method allowed over 100% recovery, a wide range of linearity and low LOD values to be obtained for both analytes. Thus, in this paper, the parameter values confirmed that LLE using ILs allows satisfactory results to be achieved.

### 3.1.2. Dispersive Liquid-Liquid Microextraction and Modifications

ILs have more often been applied in new solutions based on liquid-phase microextraction (LPME), and particularly in the increasingly used dispersive liquid-liquid microextraction method (DLLME), introduced for the first time by Rezaee [69]. The most important elements of the most popular microextraction methods are two solvents: extractant and disperser. After their quick injection into the sample, the disperser solvent causes the dispersion of the extraction solvent in the form of fine droplets. The large surface contact with the analyte helps in its adsorption. Then the two formed immiscible layers can be easily separated from each other. The method has many advantages, above all, lower consumption of organic solvents, a faster process and greater sample enrichment. Thus, subsequently, positive properties introduced further modifications leading to even better results. As it was mentioned above, one of them was the use of ILs.

Biological Samples

Cruz Vera and his colleagues [70] were among the first to use ILs in the DLLME of drugs from biological samples. In one-step in-syringe extraction of non-steroidal anti-inflammatory drugs (NSAIDs) from human urine, they used ILs as the extraction solvent and methanol as the disperser solvent. During optimization, they took into account not only the extraction efficiency, but also the enrichment factor and repeatability. Subsequent publications using IL-DLLME are modifications of the matrices and pharmaceuticals (Table 2). However, several repetitive elements of the study can be observed. First, the same group of molecules with the [$PF_6$] anion and the imidazolium cation were most often used to select the ionic liquid with the best results. Differences were related to the length of the cation alkyl chain (1-butyl-3-methylimidazolium ([$C_4$MIM]), 1-hexyl-3-methylimidazolium ([$C_6$MIM]) and 1-octyl-3-methylimidazolium ([$C_8$MIM])) [15,70–72,75–79,84,87–89]. Most often, ILs with a butyl or octyl substituent were qualified for further testing. Probably, the reason for choosing the C4 alkyl chain was the reduced viscosity and the resulting greater transfer of analytes to the IL (compared to C6 and C8) [15,70,71,76,78,80–82,87–90]. On the other hand, as the alkyl chain length increases, solubility in aqueous solutions decreases, and the analyte availability increases. This seems to be the reason for good results for [$C_8$MIM][$PF_6$] IL [72–75,79,83,84] (Figure 3).

However, it should be highlighted that despite the knowledge of the structure-properties, using only one criterion when choosing an IL is impossible. Thus, there is also the opinion that the structures of analytes should influence their choice. Moreover, the volume of ILs is an important factor. In many studies, it has been confirmed that as the volume increases, the efficiency and enrichment factors increase. However, the trend changes at some point and when the volume is too large, the results decrease. Probably, a large volume of ILs reduces the concentration of the analytes. On the other hand, if the volume is too small, the extraction and collection of the IL-analyte phase from the system is also problematic [71]. Therefore, this parameter should also be estimated in each study.

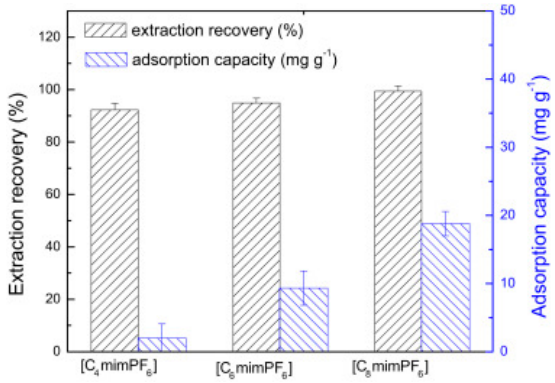



**Figure 3.** Effect of the kind of extraction solvents on ER of UPA and adsorption capacity. Extraction conditions: sample volume, 10.0 mL; sample amount, 10.0 μg; pH, 8.0; ultrasonic temperature, 313 K; ultrasonic time, 10 min; cooling temperature, 278 K; cooling time, 15 min; centrifugation time, 5 min. The error bars were standard deviation. Figure adopted from the reference [75] with copyright permission.

Besides those mentioned above, scientists also tried to include in the study ILs consisting of chloride ([Cl]), bromide ([Br]), [$Nf_2T$] and methyl sulfate ([$CH_3(SO_4)$]) anions [78,80–82]. Only in one publication was there an attempt to replace the imidazolium cation with 1-butyl-1-methyl-pyrrolidinium ([$C_4$MPyrr]) and 1-butyl-3-methylammonium ([$C_4M_3$Amm]) [78]. An important factor presented in the literature is the combination of ILs with organic solvents. Most often they are the dispenser solvent, but they can also be the solvent in back extraction [15,70,74–79,83–86,89]. It is known that ILs are highly viscous compounds. This property can hinder the chromatographic separation and detection of compounds, so the use of acetonitrile, methanol or ethanol is necessary. In some extractions, organic solvents are completely eliminated using instead sonication, controlled temperature, or intensive mixing. Their application helps to disperse ILs and gives as good results as organic solvents [15,74,76–79,83–86]. Gong et al. [75], during the determination of ulipristal acetate, completely eliminated the organic solvent as a dispersing agent. They used ultrasound energy without an organic solvent to disperse, and obtained an extraction recovery over of 95%. The addition of inorganic salts is also used in many works. The salting out process may affect the final results due to ionic strength and associated reactions with $H_2O$ molecules [71,89,91]. The choice of pH is also important, the goal being to have analytes in neutral form, because in ionic form there is less availability for ILs, and the final extraction efficiency decreases [85]. As mentioned before, in addition to several constant elements, there are also several variables, such as analytes and matrices. Studies usually extract drugs commonly used to treat humans and animals, including antibiotics [81,82,86–88], antidepressants [78,79,83], benzodiazepines [15,76,77] and NSAIDs [70,85]. The matrices are most often human urine, plasma and serum (Table 2). An extraction method for a unique kind of matrix was developed by De Boeck and co-workers [15,76–78]. As the authors of several articles related to the use of ILs in DLLME, they started from choosing the best extraction and detection conditions for the determination of benzodiazepines, benzodiazepine-like hypnotics and antidepressants in whole human blood by LC-MS/MS. Then they transferred the optimized conditions for the analysis of postmortem blood samples. Both the matrix type and LC-MS/MS were first used in an IL-based analytical method for determining pharmaceuticals. Drugs used in veterinary medicine were determined in milk, eggs and the meat of pigs, cows, chickens and fish [87,88].

Environmental Samples

Similar to biological samples, two methods of the DLLME procedure can be observed: traditional, using only an IL (extractant) and organic solvent (dispersant) [92,93] or modified, using additional steps, such as ultrasound and others [94–103]. The traditional method was used by Yao et al. [92], who, by performing analyses with different ILs, drew attention to the impact of the character of the analyte on the final results. An IL with the [$Nf_2T$] anion and basic properties allows higher efficiency

extraction for acidic compounds, whereas for compounds containing tertiary amines, the [TFP] anion was better. To further explain this phenomenon, the effect of surfactants on the results was also investigated. The use of the popular sodium dodecyl sulfate (SDS) without a primary amine did not improve the extraction efficiency, but after using a surfactant having such a moiety the result improved significantly. DLLME without modification also allowed the determination of triclosan and triclocarban by Zhoe et al. [93]. During optimization, $[C_6MIM][PF_6]$ was chosen for the analysis because of the higher solubility in water and worse efficiency of the $[C_4MIM]$ cation. The researchers also noted that the addition of an inorganic salt (most often NaCl), which changes the ionic strength, is responsible for two opposite effects. On the one hand, the addition of NaCl causes an increase in the solubility of an IL in water, thus increasing the volume sedimentation phase and consequently, the efficiency decreases, but on the other, there is an increase in analyte enrichment. Thus, the choice of this additive is not obvious.

DLLME modifications in the extraction of environmental samples are much more common. One of them is the use of ultrasound. Parrilla Vázquez and co-workers [97,98] focused on the optimization of this stage. They highlighted that the sonification time (too long may cause degradation) and sample cooling after the process have an impact on improving the results. Mao et al. [95] used high energy ultra-sound instead of normal ultrasound. In all US-IL-DLLME methods, the ILs for further analysis were selected from among the group with imidazolium cations and anions $[PF_6]$ in their structure. The best results were always obtained for ILs with the highest hydrophobicity, therefore the longest alkyl chain. Another modification was the inclusion of SDS in addition to the IL. The surfactant aimed to improve performance by reducing the adhesion of the IL to the walls of the tube. In addition, the novelty was heating the sample to 30 °C after the addition of the IL to completely dissolve the IL and then cooling to form two phases [96]. Yu et al. [94] used MIL to extract various compounds, including pharmaceuticals. They chose the best IL according to several criteria, such as magnetic susceptibility, HPLC compatibility, hydrophobicity needed for phase separation, minimal IL absorbance and minimal anion hydrolysis in the aqueous phase. These conditions were met by $[P_{6,6,6,14}{}^{+}]_2[MnCl_4]$. In order to achieve high efficiency, microwave energy was also used. However, its use could both improve and worsen the results, depending on the volume. Too high a temperature increases the contact of the IL with the aqueous phase and reduces the volume of the sedimentation phase, in consequence reducing the efficiency. The paper also discussed the influence of the dispersant on the final results. The choice of its volume is crucial as too large a volume causes an increase in the solubility of the IL in water, while too small hinders the formation of two phases [100]. Aimed at achieving environmentally-friendly procedures with the best results, methods using two ILs have also been proposed. Toledo-Neira et al. [103] used both $[C_4MIM][BF_4]$ and $[C_4MIM][PF_6]$ to change the polarity of the sample, and as an extractant, respectively. However, this work also uses an organic solvent as a dispersant. In another article with two ILs, one hydrophilic IL was used to disperse the other hydrophobic IL. Finally, only 50 µL of MeOH was used in the method to dissolve the sample prior to HPLC injection, thus the organic solvents were almost completely eliminated [102]. The last method of modification in the context of environmental samples was to combine DLLME with SPE. However the IL, as previously, was only applied as the extractant in DLLME. Among the tested ILs, the best result was obtained with $[C_6MIM][TFP]$ regarding the highest hydrophobicity, which was a constant trend in similar papers [101].

#### 3.1.3. Other Liquid-Phase Extraction

##### Biological Samples

Good research results have encouraged researchers to further modify their extractions with ILs (Table 2). In 2015, doxepin and perphenazine were extracted according to a new procedure: ionic liquid-based surfactant emulsified microextraction accelerated by ultrasound radiation (IL-SE-UE-ME) [104]. Together with ultrasound applied to the surfactant, this led to the creation

of an emulsion with the participation of ILs. The following year, Liu and co-workers [105] determined sulfonamides and used two ILs for extraction. They were the first to add [$C_4$MIM][$PF_6$] together with an inorganic salt, and after forming the precipitate, they added [$C_6$MIM][$PF_6$]. As a result, the analytes could be combined with the ionic liquid. Another equally effective extraction method is ionic liquid-based dynamic liquid-phase microextraction (dLPME). The method, for the extraction of phenothiazine and NSAID derivatives using the high density and viscosity of ILs, was developed by Cruz Vera [91,106]. The sample passes through the ionic liquid placed in a Pasteur pipette and the analytes are separated from the matrix. High viscosity is both an advantage and a limitation here as to perform the extraction it is necessary to reduce its value, therefore the addition of an organic solvent is also used.

Environmental Samples

In some publications, liquid-phase extraction procedures are very similar to DLLME procedures. One such procedure was proposed by Chatzimitakos et al. [64]. They used the potential of MIL [$C_8$MAmm][FeCl] to determine many analytes (including ibuprofen and diclofenac). The authors defined their novel method as stirring-assisted drop breakup microextraction (SADBME). Thus, the IL dispersion element was defined as drop breakup. Although the authors focused on the method itself, they showed that the application of MIL allows for the simplicity of extraction. Due to magnetic property, the separation of the IL-phase was possible by applying an external magnetic field. A similarity to DLLME can also be seen in synergistic centrifugal assisted ionic liquid assisted microextraction (ILSVA-SME). Faster formation of microemulsion (dispersion) with the IL and the surfactant used is achieved by vortex-assisted extraction. The method, as in other cases, allows for better efficiency of results [107]. Song et al. [108] also proposed a similar method of extraction to the above-described DLLME. They used a solid IL to extract sulfonamides and then they dissolved them by microwave energy namely microwave-assisted liquid-liquid microextraction (MA-LLME-SIL), and then cooled them again and dissolved them in acetonitrile. However, as the authors highlighted, this is a different method to DLLME, because a solid IL was used and an organic solvent was not necessary. The dispersion step is present here by shaking the molten IL sample. Thus, they do not define it as DLLME. In search of the best results, only one type of liquid was used, and its appropriate volume, duration of use and microwave power were chosen. Inorganic salt was also added but, as opposed to other works, it was not NaCl, but $Na_2SO_4$.

Another approach to minimize the amount of organic solvents was the modification and adaptation of LPME methods to determine analytes. The first work described the use of ILs in three-phase hollow fiber supported liquid-phase microextraction (HF-LPME). The procedure was based on the transfer of analytes from the donor phase to the acceptor phase through a membrane with an IL placed in the pores. Due to the good solubility of sulfonamides in water, their transfer based on passive diffusion can be difficult. For this purpose, the combination of an IL with tri-n-octylphosphine oxide (TOPO) was used to create a semi-liquid membrane and facilitate the transfer of the analyte to the acceptor phase. During optimization, the IL was compared with n-undecane and dihexyl ether (DHE). The IL, as the most polar compound, allowed the highest efficiency [109]. The second work describing the modification of LPME was related to the addition of an IL to the acceptor phase (IL/n-octanol) in membrane bag-assisted-liquid-phase microextraction ((MBA)-LPME). The extraction set was prepared by the author (a detailed description can be found in the original publication) [110]. The effect of using an IL was an increase in efficiency. Among the tested ILs, the results improved only after using [$C_6$MIM][TFP], which was explained by high hydrophobicity. Therefore, it should be noted that ILs, which are most relevant in DLLME, were not suitable for LPME-modification.

Extraction based on membranes was also proposed by Hanapi et al. [111] using an agarose membrane impregnated with an ionic liquid for electroconvulsive membrane extraction (IL-AF-μ-EME). An IL was used in both the membrane and the acceptor phase. During optimization, [$C_6$MIM][$PF_6$] and [$C_8$MIM][$PF_6$] were used. The cation with a hexyl substituent allowed for better performance. According to the authors, this is due to lower hydrophobicity, and therefore better solubility and

conductivity. In addition to the type and the volume of the IL in the acceptor phase, other conditions (pH, ionic strength, mixing speed) were also optimized in experiments. The method was a fast process allowing for satisfactory validation parameters.

In one publication, ionic liquid-based immersed droplet microextraction (IL-IDME) was also used. Analytes, after transfer to IL droplets and in combination with a MeOH/ACN mixture, were analyzed by HPLC. Only one type of IL was used in the study, determining its optimal volume for analysis. During the optimization of other parameters, as in other papers, attention was paid to the effect of pH. Due to the determination of basic compounds, the samples were adjusted to an alkaline pH because analytes show greater affinity for ILs when in a non-ionized form [16].

#### 3.1.4. Aqueous Two-Phase System

##### Biological Samples

In addition to DLLME extraction, ILs are also used in aqueous two-phase systems (ATPs) [67,112]. These consist of two immiscible water phases enriched with two different substances which affect their physical and chemical properties. These could be polymers, inorganic salts or surfactants. Unfortunately, there are also disadvantages in this process, such as interaction with analytes. Therefore, in searching for new solutions, it was decided to use the potential of ILs here. As in DLLME, ILs retain analytes in one of the phases and help in their separation. The group of tested ILs also remains constant (an imidazolium cation with a different alkyl chain length and a hexafluorophosphate anion); however, the selection criteria change. What is most important is the ability to separate the two phases. Shao et al. [112] chose [$C_4$MIM][$PF_6$] for further experiments. They also tested an IL with a 1-ethyl-3-methylimidazolium ([$C_2$MIM]) cation but then two phases were not formed. In longer alkyl chains, viscosity increased and analyte transfer decreased. In another publication with ATPs extraction, [$C_6$MIM][$PF_6$] was selected, the butyl substituent was too low in polarity and did not form separate phases with the SDS used, while an IL with C8 did not form a stable system. In addition, Yu et al. [67] checked how pH, IL volume, extraction time and the addition of inorganic salt affect ATPs. The results showed that all of the above factors are responsible for the total extraction effect. An increase in the volume of the IL caused an increase in the number of oil drops in the phase, the $K_2HPO_4$ used improved stability, while a change in pH and extraction time determined the final result of the efficiency. As we can also see, in this type of extraction the choice of IL is ambiguous and requires experimental testing. In addition, it is also important to choose a second substance that can determine the availability of analytes for the IL and the presence of two separate phases.

##### Environmental Samples

ATPs, which was described in the extraction of biological fluids, can also be used for environmental samples. Another form of ATPs, referred to as ionic liquid/salt aqueous two-phase flotation (IL-ATPF), was used to isolate chloramphenicol by Han et al. [113] (the solvent sublation apparatus was shown in the original work). The mechanism is based on the transfer of analytes into the IL droplets present in the upper surface phase of the system. As in other ATPs methods, the addition of inorganic salt was necessary and the best results were obtained through $K_2HPO_4$. The most appropriate IL was selected from three types: ([$C_4$MIM][Cl], [$C_8$MIM][Cl], [$C_4$MIM][$BF_4$]). [$C_4$MIM][Cl] was used in further analyses, because of the lowest viscosity and surface tension, which is crucial when analytes must be absorbed by the IL droplets. The particular novelty was also the use of MIL in this type of extraction (1,1,3,3-tetramethylguanidine and 2,2,6,6-tetramethylpiperidine 1-oxyl free radical [TMG][TEMPO-OSO3]). The common effect of MIL is to obtain a rapid extraction by easily collecting the IL-analyte complex with the help of an external magnesium field. The formation of MILATP requires the addition of inorganic salt (as already mentioned in paragraph 3.1.1). In this experiment, after the optimization and interpretation of results, the best addition was $K_3PO_4$. The choice of

temperature was also important, as too high could cause an increase in the solubility of the IL in water, so finally room temperature was chosen [114].

### *3.2. Sorbent-Based Extraction Procedures*

#### 3.2.1. Solid-Phase Extraction

Solid-phase extraction (SPE) is a well-known sample pretreatment technique which ensures the simultaneous enrichment and purification of analytes [115]. In this technique, the compounds of interest and matrix interferences can be differentially desorbed from the SPE sorbent when water, an organic solvent or a mixture of organic solvent with water or salt solution are used as washing/eluting agents. It allows the analytes to be effectively extracted from the sample and the matrix interferences removed. In this extraction procedure, smaller amounts of organic solvent are required, and the risk of the formation of emulsions is decreased compared to LLE-based procedures. In effect, SPE is considered as a more environmentally-friendly method which is able to offer high analyte recoveries. Additionally, the SPE process is rapid and can be easily automated as an off-line SPE or on-line SPE system where direct coupling to chromatographic or electrophoretic separation systems is applied. In on-line SPE, a higher throughput and a more effective reduction of sample contamination or degradation can be obtained, while human exposure to potentially hazardous samples is decreased. On the other hand, the preconcentration and purification of the analytes in SPE may sometimes be ineffective because of the limited selectivity of conventional solid sorbents (e.g., modified silica-based sorbents). For this reason, new SPE materials are systematically developed and introduced to improve selectivity, including molecularly imprinted polymers (MIPs) as well as IL-based sorbents. In most investigations, ILs are immobilized by the covalent attachment of the imidazole group to the silica surface or polymeric support. These IL-based sorbents are considered to be interesting alternatives in SPE for different groups of pharmaceuticals from biological and environmental samples.

##### Biological Samples

Pang et al. [116] fabricated a polymer monolith column with 1-vinyl-3-hexylimidazolium bromide ([ViC$_6$MIM][Br]) IL which was used for the on-line SPE isolation of betamethasone, norgestrel, halcinonide, beclomethasone dipropionate and testosterone propionate from human plasma. The developed SPE-HPLC-UV method offered the effective extraction of the analytes (93–105%), which allowed the target compounds to be quantified with LODs of 1–2 ng/mL. In a study by Liu et al. [117] a poly(ionic liquid-glycidylmethacrylate-coethyleneglycol dimethacrylate) (IL-GMA-co-EDMA) monolithic column with 1-vinyl-3-butylimidazolium chloride ([ViC$_4$MIM][Cl]) was synthesized and applied as an SPE sorbent in the on-line SPE-HPLC-UV method for the determination of nifedipine, nitrendipine and felodipine in human plasma samples. The best extraction of the analytes and purification of the matrix sample was obtained when a methanol/water mixture was used as the eluting agent. It allowed the three antihypertensive drugs in human plasma samples to be determined with LODs of 2–3 ng/mL. Ferreira et al. [118] used 1-vinyl imidazole and 1,4-butane-sultane to create a silica-anchored IL-based material which was applied as a sorbent in an SPE system coupled online with HPLC-MS/MS for the quantification of the antibiotic ceftiofur in bovine milk samples. The extraction efficiency ranged from 70 to 130%, and the LOD was 0.1 μg/L. A sol–gel synthesis of three hybrid materials containing [C$_4$MIM][PF$_6$], [C$_6$MIM][PF$_6$] and [C$_8$MIM][PF$_6$], attached by covalent bonds, was published by da Silva and Mauro Lanças [119]. These IL-based hybrid materials were applied as the sorbents in off-line SPE for the isolation of five sulfonamides and trimethoprim from bovine milk samples. The results indicated that the extraction efficiency of the analytes systematically decreased when the alkyl chain of the IL increased from C4 to C8. This was probably caused by the reduction of the electron density and the steric hindrance from the methyl group on the three-substituent site of imidazole rings, which weakened the π–π interaction between the electron-rich benzene ring of the target compounds and the imidazole rings of the used ILs. The best efficiency was offered by an IL

(C4)-based sorbent which was applied for the isolation and preconcentration of sulfonamide in bovine milk by the on-line SPE-HPLC-MS method. The LODs for the method developed were in the range of 1.5–2.25 μg/mL, with extraction recoveries from 74 to 93%. Yan et al. [120] developed modified dummy molecularly imprinted microspheres (DMIMs) based on [$AC_2$MIM][Br] as the co-functional monomer and phenylephrine as the dummy template. These DMIMs were used as the SPE sorbent for the isolation of clenbuterol and clorprenaline from urine samples. The obtained results confirmed that they were able to more effectively extract the analytes and remove the matrix interferences than with other tested commercial sorbents such as HLB, PCX, C18 and SCX. For the DMIMs, the extraction efficiency ranged from 93.3 to 106%. The developed DMIMs-SPE-HPLC method allowed the analytes to be quantified with LODs of 0.19 and 0.070 μg/L for clorprenaline and clenbuterol, respectively. Ma and Row [1] synthesized a molecularly imprinted monolithic column using levofloxacin and ciprofloxacin as templates, 1-vinyl-3-ethylimidazolium bromide ([$ViC_2$MIM][Br] as the functional monomer, and graphene oxide (GO) as the core material. When the efficiency of the IL-based imprinted monolithic column was tested as the SPE sorbent for the extraction of levofloxacin and ciprofloxacin from human urine, the best results were achieved using water as the washing agent, and a mixture of ethanol/acetic acid (7:3 *v/v*) for the elution of the analytes. The main advantages of the developed SPE protocol were the effective purification of the matrix sample, and the good extraction recovery of the analytes (89.5% and 92.5% for levofloxacin and ciprofloxacin, respectively). However, relatively low sensitivity of the developed SPE-HPLC-UV method was also observed (LODs from 0.06 to 0.27 μg/mL). Wu et al. [121] used an SPE procedure based on hemimicelles and admicelles (mixed hemimicelles) supported by an IL for the simultaneous extraction of five cephalosporins from biological samples. In this technique, the sorbent possesses adsorbed ionic surfactants on the surface of mineral oxides (e.g., SDS or IL) which enables two mechanisms to occur for the retention of the analytes—hydrophobic and electrostatic interactions. In effect, the extraction efficiency can be improved. The authors tested seven different surfactants, such as SDS, cetyltrimethylammonium bromide (CTAB), [$ViC_6$MIM][Br], [$C_4$MIM][Br], [$C_{12}$MIM][$PF_6$], [$C_{16}$MIM][Br] and [$C_{12}$MIM][Br]. The best recoveries were obtained for the long-chain IL [$C_{16}$MIM][Br], which confirms data presented in (Figure 4).

The imidazolium-based IL with a longer alkyl side chain was probably able to strengthen the directionality of hydrogen bonds and van der Waals forces. In consequence, the interactions between the mixed hemimicelles and the hydrophobic regions of target compounds were more intensive and the efficiency increased. Taghvimi et al. [122] prepared mixed hemimicelle magnetic dispersive solid-phase extraction (MHMDSPE) based on carbon-coated magnetic nanoparticles and supported by the IL (IL-C/MNPs) for the extraction of tramadol from urine samples. In this study, MHMDSPE conditions were optimized, including both the selection of the adsorbent type and the solvent used as a desorbing agent. The results indicated that the IL-C/MNPs with [$C_6$MIM][$PF_6$] was more effective than that based on $Fe_3O_4$ NPs.

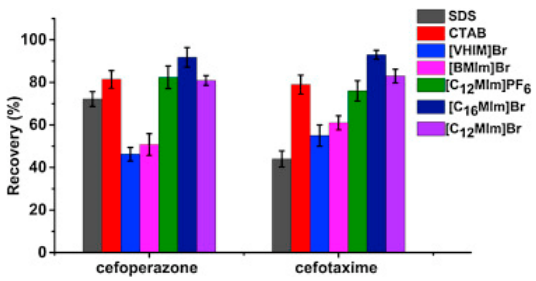



**Figure 4.** Comparison of the types of surfactants on the extraction efficiency of cefoperazone and cefotaxime. Figure adopted from the reference [121] with copyright permission.

This was probably related to the presence of carboxyl and hydroxyl groups on the surface of IL-C/MNPs, which improved the dispersion of the magnetic nano-adsorbent in the urine medium. In effect, stronger interactions between the analyte and the magnetic nano-adsorbent occurred, which

improved the extraction efficiency. The best desorbing solvent was acetone, which allowed a recovery of 94% to be obtained. Yan et al. [123] prepared IL-modified magnetic polymer microspheres (ILMPM) based on $Fe_3O_4$ NPs and $[C_4MIM][PF_6]$ used as a magnetic adsorbent of MDSPE for the determination of sulfamonomethoxine sodium and sulfachloropyrazine sodium in urine samples. The developed ILMPM-SPE sorbent provided a higher purification ability and extraction recovery of the tested analytes compared with magnetic polymers based on using 4-vinyl pyridine, methacrylic acid and acrylamide as monomers. A report was also published describing matrix solid-phase dispersion coupled with homogeneous ionic liquid microextraction (MSPE-HILME) applied for the extraction of sulfamerazine, sulfathiazole, sulfamethazine, sulfadoxine, sulfachloropyridazine, sulfaphenazole and sulfisoxazole from animal tissues [124]. In the study, three kinds of hydrophilic ILs, including $[C_4MIM][BF_4]$, $[C_6MIM][BF_4]$, and $[C_8MIM][BF_4]$ were tested in MSPD and HILME simultaneously. The results confirmed that higher extraction recoveries of the analytes were obtained with the $C_4$ IL than those observed with $C_6$ and $C_8$ ILs. This was related to the significant loss of C6 and C8 ILs in MSPD, which resulted in a small volume of the IL phase and low extraction yields of the target analytes. Compared to $C_6$ and $C_8$ ILs, the $C_4$ IL possesses higher water miscibility and lower viscosity, which facilitates the transfer of target analytes from the sample matrix to the extraction solvent. In this study, this effect was predominant in respect to the extraction capacity of the IL, which often increases with the increase in the alkyl chain length of the IL [125]. Finally, water was selected as the elution solvent in MSPD because of the more effective extraction of sulfonamides, which are water-soluble polar compounds. In this procedure, the C4 IL was mixed with the dispersant and the sample before introduction to the MSPD column, and the IL phase was collected after HILM. When the MSPD-HILME method was coupled to HPLC-UV, the recoveries of the sulfonamides ranged from 85.4 to 118.0%. The LODs for the analytes were 4.3–13.4 g/kg. The application of magnetic core-shell nanoparticles (mag-NPs) of $SiO_2$@$Fe_3O_4$ type, covalently modified with the IL (dimethyl octadecyl [3-(trimethoxysilyl propyl)]ammonium chloride) as the MSPE material for the extraction of tolmetin, indometacin and naproxen from blood samples was also described in the literature [126]. The synthesized mag-NPs were applied as the adsorbent in MSPE according to the protocol presented in Figure 5.

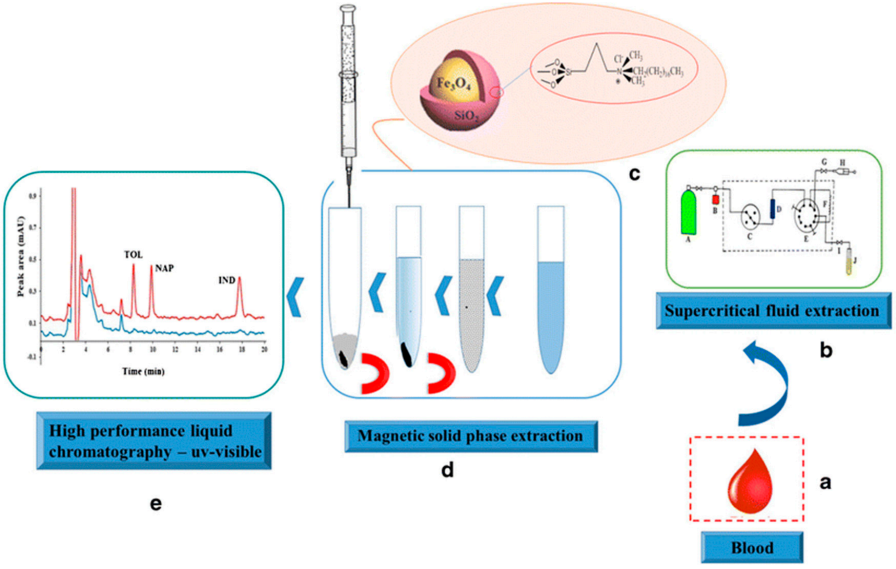



**Figure 5.** Schematic illustration of extraction procedure for tolmetin (TOL), indomethacin (IND) and naproxen (NAP) from blood samples. Figure adopted from the reference [126] with copyright permission.

The results of the study showed that the IL addition provided a more effective extraction of the NSAIDs probably due to an increase in both hydrophobic and π-π dipole or electrostatic interactions between the adsorbent surface and the analytes. On the other hand, the adsorption of the cationic

molecules onto the sorbent was limited because of the repulsion interaction with the adsorbent surface. In consequence, a better purification of the sample was also achieved. The optimized MSPE was coupled to HPLC-UV and used alone or after supercritical fluid extraction (SFE) before HPLC separation. These protocols resulted in LODs between 0.1 and 0.3 µg/L for MSPE-HPLC and 0.2 to 0.3 mg/kg for SFE-MSPE-HPLC, respectively.

Environmental Samples

Fontanals et al. [127] synthesized and applied crosslinked polymer-supported imidazolium trifluoroacetate salt [$MI^+$][CF3$COO^-$] as the SPE sorbent for the extraction of salicylic acid, carbamazepine, nalidixic acid, flumequine, gemfibrozil and four NSAIDs from aqueous samples. In the study, the developed IL-sorbent was tested under weak anion exchange (WAX), strong anion exchange (SAX) and strong cation exchange (SCX) as well as reversed-phase (RP) SPE conditions. The best purification and extraction results of acidic pharmaceuticals from different water samples (ultrapure, tap, river water and effluent wastewater) were obtained when the IL-based SAX material was applied. In the next study, two new imidazolium supported IL phases possessing different anions such as [$CF_3(SO_3)$] and [$BF_4$], were synthesized and applied as SPE-SAX sorbents for the isolation of acidic pharmaceuticals from water samples [128]. The obtained data indicated that [$MI^+$][$CF_3(SO_3)$] and the previously developed [$MI^+$][$CF_3COO^-$]-SAX sorbent gave comparable results, whereas [$MI^+$][$BF_4$] was not able to effectively extract and purify the acidic pharmaceuticals from environmental samples. On the other hand, the application of [$MI^+$][$CF_3(SO_3)$] allowed only comparable efficiency to be obtained and calculated after using the commercially available Oasis MAX column, whereas [$MI^+$][$CF_3COO^-$] was slightly more effective. Hydrophilic ciprofloxacin molecularly imprinted polymer material containing 1-allyl-3-vinylimidazole chloride ([AViMIM][Cl]) IL and 2-hydroxyethyl methacrylate as a bifunctional monomer was synthesized by Zhu and co-workers [129]. This MIP material was able to create strong hydrogen bonds, and electrostatic and π-π dipole interactions with ciprofloxacin in an aqueous solution. It offered excellent molecular recognition for common quinolone antibiotics (ciprofloxacin, levofloxacin and pefloxacin mesylate) in aqueous matrices as well as the selective isolation and separation of trace amounts of ciprofloxacin in real water, soil and pork samples, with recoveries of 87.3–102.5%.

3.2.2. Solid-Phase Microextraction

Solid-phase microextraction (SPME), developed by Pawliszyn and his co-workers in the 1990s [130], is a fast, solvent less-extraction technique for the sampling, cleaning-up and pre-concentration of analytes, which also offers the introduction of the sample to chromatography in a single solvent-free step. The SPME sorbents can be applied in both the headspace mode and the immersion mode. The simplicity of the SPME technique and other advantages, such as high selectivity and effective purification, the relatively low cost of equipment and the possibility of automation, mean that SPME is a powerful tool for the extraction of a wide range of compounds from different matrices. Moreover, new sorbents for SPME based on ILs are also synthesized. Several publications have described the results of their application for improving the extraction efficiency of different groups of pharmaceuticals from biological and environmental samples.

Biological Samples

A paper can be found in the literature describing the use of $SiO_2$@$Fe_3O_4$ functionalized with [$C_4$MIM][$PF_6$] IL for the microextraction of four β-blockers (propranolol, metoprolol, atenolol and alprenolol) from human plasma [131]. In the study, two types of hydrophobic ILs, ([$C_4$MIM][$PF_6$] and [$C_8$MIM][$PF_6$]), were tested. The results show that [$C_4$MIM][$PF_6$] offered an extraction efficiency of 75 to 91%, while for [$C_8$MIM][$PF_6$] these values were significantly lower (about 40%). This can be explained by the higher hydrophobicity of the long-chain IL, which leads to poor dispersion in the aqueous sample. Moreover, [$C_8$MIM][$PF_6$] cannot be completely recovered by MNP, which can additionally decrease the extraction efficiency [132]. In the developed sample preparation procedure,

an effervescent powder composed of sodium dihydrogen phosphate and sodium bicarbonate was also applied for the enhancement of the interaction between the magnetic sorbent and the analytes. When this protocol was coupled with LC-MS/MS, the developed method for the analysis of β-blockers in human plasma was able to monitor the compounds of interest with LODs from 0.03 to 0.62 ng/mL.

Environmental Samples

Serrano et al. [133] published the synthesis of GO functionalized with covalently attached 1-butyl-3-aminopropyl imidazolium chloride IL to GO sheets, and its application as an adsorbent for the dispersive micro SPE of six β-blockers and four anabolic steroids from aqueous samples prior to HPLC separation. It was observed that hydrophobic attraction between the compounds and the GO-IL was the predominant adsorption mechanism of steroids, while for β-blockers, their interactions with the adsorbent were more complicated. For them, both hydrophobic and electrostatic interactions can occur as well as the existence of interactions of electron-donor-acceptor type, which are dependent on the pH used in the extraction process. These mechanisms were more intense on the GO-IL sorbent, which was confirmed by the recovery results for the analytes (87–98%), which were found to be significantly higher than those observed with GO alone and graphene. Yu et al. [65] prepared six neat crosslinked polymeric ionic liquid (PIL) sorbent coatings for the SPME of selected phenolics, insecticides and pharmaceuticals, including phenacetin, ketoprofen, fenoprofen calcium, diclofenac sodium and ibuprofen, from environmental water samples (tap water and lake water). These PIL sorbents were prepared using various IL monomers such as 1-vinylbenzyl-3-hexadecyl-imidazolium chloride ([ViBC$_{16}$IM][Cl]), 1-vinylbenzyl-3-hexadecylimidazolium bis[(trifluoro-methyl)sulfonyl]imide ([ViBC$_{16}$IM][Nf$_2$T]), 1-vinyl-3-(2-hydroxyethyl)imidazolium bromide ([ViC$_2$OHIM][Br]), 1-vinyl-3-(10-hydroxydecyl)imidazolium chloride ([ViC$_{10}$OHIM][Cl]), 1-vinyl-3-(10-hydroxydecyl)imidazolium bis[(trifluoromethyl)sulfonyl]imide ([ViC$_{10}$OHIM][Nf$_2$T]), 1-vinyl-3-(9-carboxynonyl) imidazolium bromide ([ViC$_9$COOHIM][Br]), and crosslinkers like 1,12-di(3-vinyl-benzylimidazolium) dodecane dichloride [(ViBIM)$_2$C$_{12}$]2[Cl]), and 1,12-di(3-vinylbenzyl imidazolium)dodecane dibis[(trifluoromethyl) sulfonyl]imide ([(ViBIM)$_2$C$_{12}$]2[Nf$_2$T]). Next, they were tested in different experimental SPME conditions. The results indicated that all the developed PIL sorbent coatings were stable when the extraction was carried out under an acidic pH using various organic desorption solvents (e.g., methanol, acetonitrile, acetone). However, the best extraction results were obtained using the PIL-based sorbent coating polymerized from the IL monomer [VC$_{10}$OHIM][Cl] and the IL crosslinker [(VBIM)$_2$C$_{12}$]2[Cl]. The extraction efficiencies of pharmaceutical drugs and phenolics were higher when the film thickness of the PIL-based sorbent coating increased from 23 μm to 89 μm, whereas these values were largely unaffected for insecticides. This analysis allowed LODs to be obtained ranging from 0.2 to 2 g/L for the target compounds. A report presenting the synthesis of four different crosslinked PIL-based sorbent coatings by UV polymerization onto nitinol wires was also published in the literature [134]. These PIL coatings possessed either vinylbenzyl or vinyl alkyl imidazolium-based (ViBCnIM- or ViCnIM-) IL monomers with different types of anions, and various dicationic IL crosslinkers. They were used in a direct-immersion solid-phase microextraction (DI-SPME) method for the extraction of a group of polar analytes and non-polar analytes (10 different compounds), including gemfibrozil and carbamazepine. Two studied fibers, such as the polymers PIL–1a from the IL monomer [ViBC$_{16}$IM–Nf$_2$T] and IL crosslinker [(ViBIM)$_2$C$_{12}$–2Nf$_2$T], and PIL–2 based on the IL monomer [ViC$_{16}$IM–Nf$_2$T] and IL crosslinker [(ViIM)$_2$C$_{12}$–2Nf$_2$T] were used for the extraction of the analytes from real tap and river water samples. The results confirmed that these PIL-based fibers offered reproducible and effective extraction of most of the tested analytes from real samples. The extraction can be carried out many times (up to 100 extraction-desorption steps), and at low pH values.

### *3.3. Stir Bar Sorptive Extraction*

In recent years, a sample preparation procedure based on stir bar sorptive extraction (SBSE) has been developed for the extraction of compounds occurring in matrices at trace levels. It should

be noted that the extraction mechanism and the benefits of SBSE are identical to SPME. However, the enrichment factor obtained in SBSE can be significantly higher compared to SPME (~100 times). In SBSE, a glass tube with a magnetic core, coated with a layer of special polydimethylsiloxane (PDMS) tubing is applied to stir aqueous samples. After a certain time, the molecules captured on the bars can be desorbed either thermally for GC or into a solvent for LC. One drawback of SBSE is the low availability of different types of coatings. It should be noted that PDMS, mainly in SBSE, possesses a high affinity to extract non-polar compounds, while polar ones are poorly isolated. To overcome this limitation, new polymeric coatings are introduced, including poly (methyl methacrylate/ ethyleneglycol dimethacrylate) (PA-EG), and IL-based sorbents in order to improve the extraction efficiency of more polar compounds. Another problem of SBSE is the presence of the memory effect (carryover) during the desorption step using an organic solvent. According to the literature data, Talebpour et al. [135], in a comparative study, reported the application of a PA-EG polymeric phase and PDMS-coated stir bar supported by an IL for the extraction of carvedilol in human serum samples. In this investigation, $[C_8MIM][BF_4]$ IL was tested as a modifier in the desorption solvent (methanol) for checking whether better extraction efficiency and the elimination of carryover can be obtained. The results confirmed that carvedilol has a better affinity for the PA-EG phase than for PDMS. Moreover, the addition of $[C_8MIM][BF_4]$ at a concentration of 0.1 M to methanol significantly increased the recovery of carvedilol. Additionally, no carryover effect was observed, whereas it was detected when methanol was used without the IL (about 11% of the initial desorption step). Unfortunately, to the best of our knowledge, no report describing the use of IL-based sorbents for the SBSE extraction of pharmaceuticals from environmental samples has been published.

### *3.4. PASsive Sampling with Ionic Liquids*

Extractions described so far can be classified as extractions with active sampling, because additional mechanisms, such as pressure and so on, are used for the flow of samples through the sorbent. However, the isolation of analytes is also possible in another way. Extraction using passive samplers can be used for the long-term monitoring of pharmaceuticals [136]. A significant difference in this method compared to procedures traditionally used in laboratories is the ability to estimate the time-weighted average concentration (TWAC) of analytes in ecosystems. Currently, the most popular Polar Organic Chemical Integrative Sampler (POCIS) techniques have been enriched with the new PASsive Sampling with Ionic Liquids (PASSIL) technique, developed by a team of scientists from the University of Gdansk. To carry this out, a dosimeter consisting of two disks and a membrane covered with the acceptor phase is necessary. Various ILs or their combinations with other sorbents are used as the acceptor, here.

Caban et al. [137] compared the results of the isolation of analytes (diclofenac, carbamazepine and two sulfonamide antibiotics) using dosimeters in which the membrane was covered only with an IL or a combination of IL and colloidal silica obtained from C18 SPE extraction columns. In the experiment, they tested four ILs using not only the popular imidazolium cation but also the phosphonium cation ($[C_6MIM][Tf_2N]$, $[P_{6,6,6,14}{}^+][N(CN)_2]$, $[P_{4,4,4,14}{}^+]$[DDBS], and $[P_{2,4,4,4}{}^+][(2O)_2PO_2]$). The most important and desirable property of such sorbents was water insolubility. The content of the IL transferred into the donor phase was determined by testing the pH, conductivity and recovery of the phase. In order to select the best extraction conditions, the extraction efficiencies were calculated for all experiments. The results confirmed that when the IL alone was applied (independent of the type of IL), it did not improve the efficiency, and sometimes lower extraction parameters were calculated than those using traditional C18 sorbents (carbamazepine). In contrast, by using the combination of the IL and C18, the efficiency increased, the acceptor phase stability was improved and less IL consumption was possible. The developed method was used to extract analytes from saline water. The use of the matrix, which caused changes in the properties of the IL and analytes due to pH modifications, proved that the final result is a consequence of many components, not only choosing the right sorbent at the stage of method optimization. The same effects were observed in the study using a similar procedure to assess the effect of pH and salinity on the extraction efficiency of β-blockers, NSAIDs and sulfonamides using

the PASSIL technique. It was interesting that the results for samples taken from the donor phase by a dosimeter with the same IL were different depending on the analyte. The extraction of β-blockers was impossible when an IL was used as the sorbent, even after changing the pH. In contrast, for NSAIDs and sulfonamides, the extraction efficiency improved after the appropriate pH modification (Figure 6).

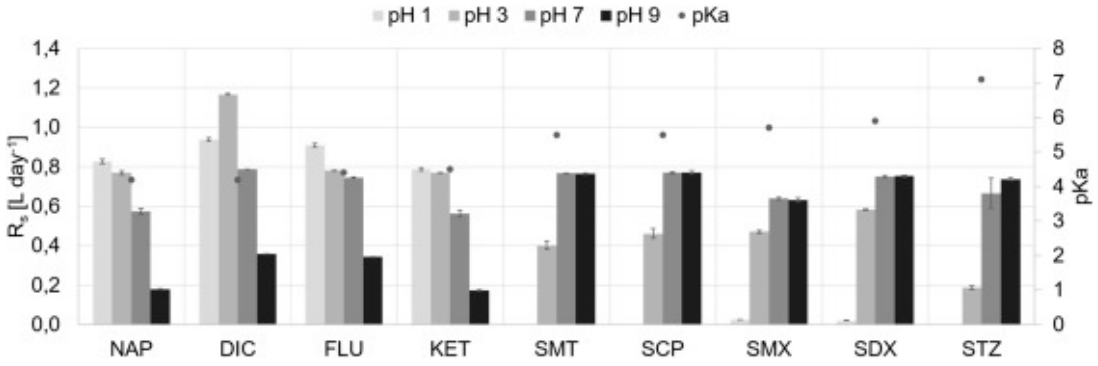



**Figure 6.** Dependency between sampling rate (Rs) values and the salinity of the donor solution for selected sulfonamides and NSAIDs (the pKa values of target compounds are specified by the black dots). Figure adopted from the reference [138] with copyright permission.

The authors suggest that this situation results from the presence of β-blockers in a neutral or cationic form in the solution which cannot be adsorbed on the membrane surface to large [$P_{6,6,6,14}^{+}$]IL cations. In turn, the increase in salinity caused a decrease in the efficiency of analyte extraction due to their competition with the ions of salts present in saline water [138]. Męczykowska et al. [139] also assessed the effect of humic acids, temperature and mixing on the final extraction results of various pharmaceuticals using the PASSIL technique. The results indicated that each of these parameters can decide on the final results. Moreover, the importance was emphasized of polarity or hydrophobic properties as factors affecting these parameters.

## 4. Chromatographic Techniques

### *4.1. High Performance Liquid Chromatography*

#### 4.1.1. IL Additives to the Mobile Phase

Liquid chromatography is the most commonly used technique for determining pharmaceuticals. Most of them are basic and their separation takes place in a reversed-phase using a silica-based column [140–142]. Unfortunately, this involves several serious problems during the analysis. The literature data indicate the main reason to be the presence of free silanol groups, which are negatively charged and can interact with positively charged basic analytes in an ion exchange reaction. Based on experimental research, it can be observed that this is often associated with problems with the resolution and shape of chromatographic peaks or a high retention factor. To prevent or minimize these deleterious effects, a mobile phase is used with additives for blocking free silanols [140]. The most popular additives are various types of amines, such as triethylamine (TEA), dimethyl-octylamine (DMOA) or buffers. The first researchers who noticed that ILs may also have suppressing properties against silanol groups were Kaliszan et al. [143]. In 2005 they published a paper in which they used an additive IL to the mobile phase in drug detection by thin layer chromatography (TLC) and reversed-phase liquid chromatography (RPLC) techniques. Since then, new publications have appeared systematically on similar topics (Table 3). However, considering the topic of ILs in drug determination, it should be highlighted that these works mainly focus on explaining the function of ILs in the suppression process and the drugs are less important as analytes. In addition, only a few works use biological [140,141,144–146] or environmental [142] samples as the matrices; in one, tablets were analyzed [147], but most often they were aqueous solutions [6,143,147–154].

**Table 3.** Summary of the HPLC methods for the determination of pharmaceuticals in biological and environmental samples supported by the addition of ILs to the mobile phase.

| Drug(s) | Matrice(s) | Tested Ionic Liquids | Apparatures | Stationary Phase | Ref. |
|---|---|---|---|---|---|
| Psychotropic drugs | Human serum | **[$C_4$MIM][$BF_4$]** | HPLC-DAD λ = 240 nm | Synergi Polar RP 80A (150 × 4.6 mm, 5 µm) | [140] |
| Fluoroquinolone antibiotics | Bovine, ovine and caprine milk | **[$C_2$MIM][$BF_4$],** [$C_4$MIM][$BF_4$] | HPLC-FL $\lambda_{ex}$ = 280 nm $\lambda_{em}$ = 450 nm | RP Nova-Pak C18 (150 × 3.9 mm, 4 µm) | [141] |
| Fluoroquinolone antibiotics | Mineral and tap water | [$C_2$MIM][$BF_4$], **[$C_4$MIM][$BF_4$]**, [$C_6$MIM][$BF_4$], [$C_8$MIM][$BF_4$], [$(C_2H_5)_4N$][$BF_4$] | HPLC-FL $\lambda_{ex}$ = 280 nm $\lambda_{em}$ = 450 nm | RP Nova-Pak C18 (150 × 3.9 mm, 4 µm) | [142] |
| Antiretroviral drugs | Rats plasma | **[$C_4$MIM][$BF_4$]**, [$C_4$MIM][Br], [$C_4$MIM][$C_8H_{17}(SO_4)$], **[$C_2$MIM][$CH_3(SO_4)$]**, [$C_6$MIM][Cl] | HPLC-DAD | monolithic RP-18e column (250 × 4.6 mm, porous material) | [144] |
| Antidepressants | Urine samples | [$C_4$MIM][$BF_4$], [$C_4$MIM][$PF_6$], **[$C_4$MIM][$CF_3SO_4$]**, | HPLC-UV λ = 254 nm | RP Eclipse X-DB-C8 (150 × 4.6 mm) | [145] |
| Ofloxacin, sparfloxacin, moxifloxacin, levofloxacin, *p*-amino-salicylic acid, ketoprofen, ibuprofen | Human plasma | [$C_4$MIM][Cl], **[$C_6$MIM][Cl]**, [$C_8$MIM][Cl], [$C_{12}$MIM][Cl], [$C_6$MIM][$BF_4$] | HPLC-DAD λ = 235–375 nm | Luna C18(150 × 4.6 mm, 5 µm) | [146] |
| Tricyclic antidepressants | Tablets | **[$C_6$MIM][Cl]**, [$C_6$MIM][$BF_4$] | HPLC-UV λ = 254 nm | Zorbax Eclipse XDB C18 and C8 (150 × 4.6 mm, 5 µm) | [147] |
| β-Blockers | | [$C_2$MIM][Cl], [$C_4$MIM][Cl], [$C_6$MIM][Cl] | HPLC-UV λ = 254 nm | Zorbax Eclipse XDB (150 × 4.6 mm, 5 µm) | [6] |
| β-Blockers | | **[$C_6$MIM][Cl]** | HPLC-UV λ = 254 nm, λ = 300 nm (timolol) | Zorbax Eclipse XDB C18 (150 × 4.6 mm, 5 µm) | [7] |
| Quinine, fluphenazine, thioridazine, chlorpromazine, trifluopromazine, phenazoline, tiamenidine, naphazoline propiomazine | | [$C_2$MIM][$BF_4$], [MMIM][$CH_3(SO_4)$] | HPLC-DAD λ = 254 nm | LiChrospher RP-18 (250 × 4.6 mm, 5 µm) | [143] |
| β-Blockers | | [$C_4$MIM][$BF_4$], **[$C_6$MIM][$BF_4$]** | HPLC-UV λ = 254 nm | Zorbax SB C18 X-Terra MS C18 Kromasil Lichrospher, Nucleosil, Spherisorb | [148] |
| β-Blockers | | [$C_2$MIM][Cl], [$C_4$MIM][Cl], **[$C_6$MIM][Cl]**, [$C_2$MIM][$BF_4$], [$C_4$MIM][$BF_4$], **[$C_6$MIM][$BF_4$]**, [$C_2$MIM][$PF_6$] | HPLC-DADλ = 254 nm, λ = 300 nm (timolol) | Kromasil C18 (150 × 4.6 mm, 5 µm) | [149] |
| β-Blockers | | [$C_2$MIM][$PF_6$], [$C_4$MIM][$PF_6$], [$C_4$MIM][$BF_4$], **[$C_6$MIM][$BF_4$]** | HPLC-UV λ = 254 nm, λ = 300 nm (timolol) | Kromasil C18 (150 × 4.6 mm, 5 µm) | [150] |
| Tricyclic antidepressants | | **[$C_4$MIM][$PF_6$], [$C_4$MIM][Cl],** | HPLC-DAD λ = 254 nm | Gemini-NX C18 (150 x 4.6 mm, 5 µm) | [151] |
| β-Lactam antibiotics | | **[$C_4$MIM][$BF_4$]**, [$C_6$MIM][$BF_4$], [$C_8$MIM][$BF_4$] | HPLC-UV λ = 254 nm | RS Tech C18 (250 × 4.6 mm, 5 µm) | [152] |
| β-Blockers | | **[$C_4$MIM][$BF_4$]**, [$C_8$MIM][$BF_4$], [$C_4$MIM][$PF_6$] | HPLC-UV λ = 254 nm | RP Kromasil C18 (150 × 4.6 mm, 5µm) | [153] |
| Neuroleptic Drugs | | [$C_2$MIM][$PF_6$], **[$C_4$MIM][$PF_6$]**, [$C_4$MIM][Cl] | HPLC-DAD | Zorbax Extend-C18 (150 × 4.6 mm, 5 µm) | [154] |
| Naphazoline, phenazoline, chlorpromazine, fluphenazine; propiomazine, thioridazine | | [$C_6$MIM][$BF_4$], [$C_8$MIM][$BF_4$], [MMIM][$CH_3(SO_4)$], | HPLC-DAD λ = 254 nm | LiChrospher RP-18 (250 × 4.6 mm, 5 µm) | [155] |

[$(C_2H_5)_4N$]: tetraethylammonium; [$C_8H_{17}(SO_4)$]: octylsulfate; [$C_{12}$MIM]: 1-dodecyl-3-methylimidazolium. (The other abbreviations explained under Table 2).

Focusing on the addition of an IL to the mobile phase in LC, it should be noted that the interpretation of the results requires consideration of the influence of both the IL anion and cation. Although the use of the term IL suggests that one large molecule is responsible for the effect, it should be remembered that in the mobile phase the IL dissociates into both the cation and anion, so their combined effect determines the final results. It should also be highlighted that despite the involvement of other physical and chemical factors in the separation process, the largest changes in the chromatogram can be seen when using different kinds of ILs [142]. Their basic mechanism during pharmaceutical analysis is the reaction of IL cations with free silanol groups, the repulsion of IL cations with cations of basic analytes, as well as the reaction of IL anions with cations of analytes [149]. Depending on their type, the mechanism may be a little different than described. The choice of cation, as in the case of extraction (see the Section 3.2) focuses on the selection of an appropriate imidazolium cation with a different alkyl chain length. In one work, the analysis of the imidazolium cation with two methyl substituents was also carried out [143]. The effect of the cation was studied by Herrera et al. [142]. They performed analyses for ILs with the same anion [$BF_4$] and different cations (Table 3). The results showed that an IL with a longer alkyl chain causes a decrease in the retention factor and an increase in efficiency. The effect of changing the retention time is similar in all analyses of basic analytes. The explanation for this effect may be an increase in hydrophobicity along with an increase in the length of the alkyl chain [144]. In turn, in a publication concerning the analysis of β-lactam antibiotics, an increase in the length of the alkyl chain caused an increase in retention. Han et al. [152] highlighted that a different effect may be the result of weak acidic properties and large analyte structures (the decrease in retention in other publications concerned basic analytes). The ester moiety of the antibiotic competed more strongly with the IL used for adsorption, and therefore despite the use of the long alkyl chain of the cation, retention increased. Ubeda-Torres et al. [149] also suggested that the size of the cation is more important than its nature. To study the effects of the IL anion, in other experiments, the same cation but a different anion was used during optimizing the IL selection. The number of anions tested is much greater than cations. The most commonly used are [$PF_6$], [Cl] and [$BF_4$] anions, but the less popular [$CH_3(SO_4)$], octylsulfate ([$C_8H_{17}(SO_4)$]) and [$Nf_2T$] have also been tested (Table 3). The analysis provided several important facts. First, the [$PF_6$] anion showed very strong adsorption on the column and had a stronger effect on the parameters than the present cation. This is probably the result of its strongly chaotropic character [148]. The [$BF_4$] anion is also a chaotropic ion, but with less adsorption than [$PF_6$]. For this reason [$BF_4$] more often qualified for further parts of the experiment. The next popular anion [Cl] belongs to strongly hydrated ions, and does not react with the analyte and the stationary phase; in its presence, the cation is mainly responsible for the mechanism [151]. Although the literature data provide information on the effects of the use of individual anions and cations, their choice is not obvious, not only because of the often antagonistic effect of ions. The use of an ionic liquid, which significantly reduces the retention time, is often associated with a poorer peak shape or resolution. In addition, too short a retention time for biological samples is not recommended because of the interference of analytes and background signals. In turn, improving the shape of the peak is possible at the expense of a higher retention factor. Figure 7 shows the change in retention after the use of two different ILs. Despite the shortening of the retention time by [$C_6$MIM][$BF_4$], an IL with [Cl] was chosen for the study due to better resolution. Therefore, the choice of IL is a kind of compromise, and the choice depends on many factors, including the type and number of analytes, and the type of matrices [147]. The results also show the influence of other factors on the final results. One of the most important modifications is the change in IL concentration. It was observed that a higher concentration leads to an improvement in the shape of the peak and reduces the retention time. However, this effect is more complex. First of all, the crucial factor here is whether the anion or cation has a stronger impact. For example, if the [$PF_6$] anion is used, which has strong adsorption on the stationary phase, the retention time increases, but if the low affinity [Cl] anion and the long alkyl chain cation are used, the retention time decreases. However, it was noted that both with increasing and decreasing retention the effect occurs already at a very low IL concentration, and occurs until the column is completely

filled with the IL. When column saturation occurs, a further increase in the IL concentration in the mobile phase has less of an effect on the results. The retention time is constant or the effect is the opposite to the current one. The mechanism of action of the aforementioned [$PF_6$] in such a situation is explained by the reaction in the stationary phase until the column is saturated, and the reaction of this ion in the mobile phase after its saturation, and consequently, to a decrease in retention time [148,150]. The effect of pH on ILs was also analyzed, and it was found that at a lower pH the retention time is high because a larger number of [$H^+$] ions react with the IL anions and the elution power decreases. This can be both a disadvantage and an advantage, because on the one hand, the separation improves, but on the other hand, the analysis time is too long [152]. In another study, the purpose of which was to assess the effect of buffers on separation parameters in the presence and absence of ILs, it was observed that the IL is mainly responsible for the retention time, while the buffers more strongly affect the final effect without the addition of the IL. However, it should be mentioned that the IL [$C_6$MIM] with a strongly adsorbing cation was used in the experiment [7]. Another publication also suggests that retention is affected by the ratio of unprotonated to protonated silanols [6]. As mentioned above, ILs are an alternative to other mobile phase additives. For this reason, the results of studies with the addition of these compounds and with the addition of ILs are compared. ILs are better than other additives in all tests, but it must be highlighted that TEA the most popular compound, also gives good separation results [151]. In addition, the competitive advantage of ILs over other additives is the lack of effect on pH and, as shown in the literature, the involvement of both cations and anions in suppressing the silanol interaction and improving the results. In addition, the use of ILs is also possible in hydrophilic interaction liquid chromatography (HILIC). The increase in the stationary phase surface polarity obtained after the addition of an IL is responsible for improving the retention and efficiency parameters [146]. The verification of the positive effect of ILs was also presented in studies focusing more on the kind of columns used.

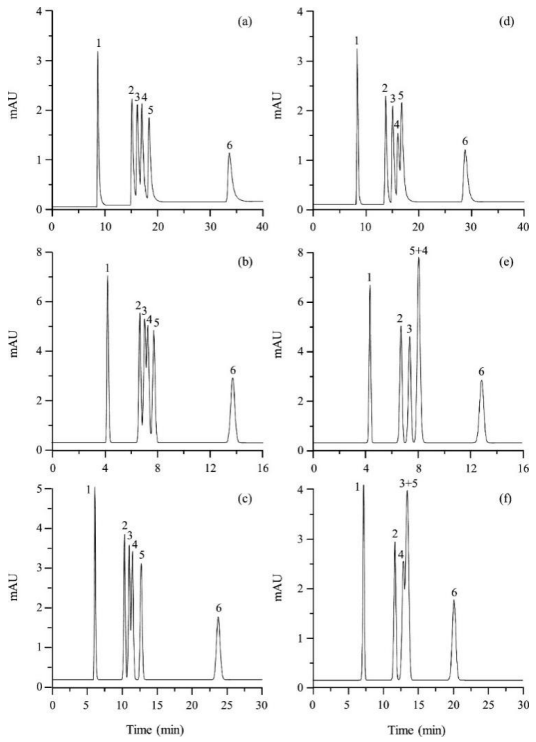



**Figure 7.** Simulated chromatograms for mixtures of the six TCAs using the C18 (**a**–**c**) and C8 (**d**–**f**) columns. Mobile phase composition: (**a**,**d**) 30% acetonitrile, (**b**,**e**) 30% acetonitrile/10 mM HMIM·Cl, and (**c** and **f**) 30% acetonitrile/10 mM HMIM·$BF_4$. Peak identity: (1) doxepin, (2) imipramine, (3) nortryptiline, (4) maprotiline, (5) amitryptiline, and (6) clomipramine. Figure adopted from reference [147] with permission of the copyright holder.

The analyses were performed on monolithic columns [144], popular $C_8$ and $C_{18}$ columns (Figure 7) [147] and six commercially available stationary phases [148]. In each analysis, the addition of ILs improved the results. It was also noted that the results depend on the production process of columns, which decide about the number of free silanol groups. Thus, the best results during the application of ILs in LC are obtained for columns for which the result was worse when using a traditional mobile phase without the addition of an IL [148]. As already mentioned, the application of ILs in LC focuses on the reaction mechanism, and drug determination is not essential here. Apart from a small number of analyses for real samples, the quality of the developed methods is not confirmed by determining the validation parameters. Therefore, the aspects of linearity, repeatability or reproducibility are ignored. To our knowledge, only one work has performed validation [147]. Moreover, only a few anions and cations have been tested in the analyses. There is no information on the effects of less common ILs. In addition, not all works compare the results obtained for ILs with other popular additives. The application of ILs also has several limitations. Although they extend the life of the column by protecting the surface of the stationary phase, conditioning is necessary for several hours to remove adsorbed IL ions and return the column to the starting position [143]. Due to the involvement of both anions and cations in the separation mechanism, other unknown interactions with their participation may occur. In addition, the choice of detector is an important issue during the application of ILs to the mobile phase. The following detectors can be used: FL, UV or *diode array detector* (DAD), but it should be remembered that ILs have a natural ability for ultraviolet absorption, which may affect the final results or prevent the selection of the optimal wavelength for analytes [148,155]. Moreover, the use of mass spectrometry is very problematic, here. However, despite the inconveniences described above, the popularity of ILs is constantly increasing and they are being used in subsequent experiments

#### 4.1.2. Ionic Liquid Stationary Phases

The application of ILs as an addition to mobile phases is not the only way to use them in liquid chromatography. In 2004, the first stationary phase appeared with ILs immobilized on the silica surface [156]. However, despite progress in this area, the use of IL stationary phases is much less popular than IL additives to mobile phases. Several-stage binding reactions are the first step of column preparation, producing as a final product modified IL-silica adsorbents, which finally coat the stationary phase (detailed reaction descriptions can be found in the original papers) [157–159]. Based on previous experience, several similarities can be observed to the previous section. First, the use of an IL stationary phase is the result of the incorrect peak shape, separation, efficiency and retention time obtained on traditional columns. As already mentioned, the same reasons concerned the use of ILs in the mobile phase. Secondly, research shows that both the cation and anion can be involved in the separation process. The imidazolium cation (single or multiple) is most commonly used to modify the stationary phase surface [160]. Furthermore, analytes were also separated on a column prepared using polymeric or chiral ILs [161,162]. There are many ways in the literature for obtaining IL-modified stationary phases based on various chemical reactions and substrates. However, their application is still not common. As mentioned, the number of publications is much smaller than the number of publications describing the suppression of free silanol groups by ILs present in the mobile phase.

This review focuses primarily on the determination of pharmaceuticals in biological and environmental samples, so it should be strongly highlighted here that the number of publications related to the determination of such analytes on IL columns by LC is negligible. Two such articles were published by Rahim et al. [163,164]. They prepared a stationary phase based on β-cyclodextrin and 3-benzylimidazolium tosylate as ILs for the enantioseparation of β-blockers and NSAIDs. The results confirmed an enhanced enantioseparation and better enantioresolution on the novelty stationary phase. Another publication in accordance with the criteria adopted in the review was published in 2019 by Xian et al. [165]. The stationary phase was prepared with photo-initiated thiol-ene click chemistry using the imidazolium cation and anion [$Nf_2T$]. Then, on the prepared column, the sulfonamides were separated by mixed-mode HPLC (MHPLC). The results confirmed good performance and separation

selectivity, and additional research on commercial columns proved that the IL is responsible for a shorter separation time (Figure 8). Although the determination of drugs using IL column modifiers is very rare, their application in the determination of vitamins [166], flavonoids [167], amino acids [168] and many other compounds shows that perhaps in subsequent years these methods will be extended also for such analytes.

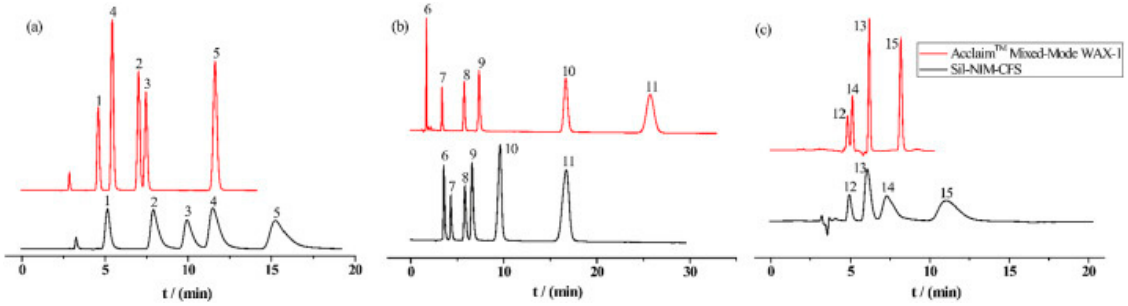



**Figure 8.** Separation of a mixture of nucleosides and nucleic bases, sulfonamides and inorganic anions on Sil-NIM-CFS and Acclaim™ Mixed-Mode WAX-1 columns. (1). uracil; (2). uridine; (3). cytosine; (4). adenine; (5). cytidine; (6). sulfanilamide; (7). sulfamethoxypyridazine; (8). sulfadiazine; (9). sulfathiazole; (10) sulfamethoxazole.; (11). sulfacetamide; (12). potassium bromate; (13). potassium bromide; (14). potassium iodate; (15). sodium iodide; (**a**) Mobile phase for Sil-NIM-CFS: ACN/10 mM ammonium formate (92:8, *v/v*); Mobile phase for Acclaim™ Mixed-Mode WAX-1 columns: ACN/10 mM ammonium formate (80:20, *v/v*); Ph = 5.6, flow rate: 0.6 mL/min, detection wavelength: 254 nm. (**b**) Mobile phase for Sil-NIM-CFS: ACN/$H_2O$ (50:50, *v/v*), flow rate: 0.8 mL/min; Mobile phase for Acclaim™ Mixed-Mode WAX-1 columns: ACN/$H_2O$ (60:40, *v/v*), flow rate: 1.0 mL/min; detection wavelength: 254 nm. (**c**) Mobile phase for Sil-NIM-CFS: ACN/5 mM $Na_2SO_4$ (5:95, *v/v*), pH = 4.28; Mobile phase for Acclaim™ Mixed-Mode WAX-1 columns: ACN/50 mM $Na_3PO_4$ (50:50, *v/v*), Ph = 6.0; flow rate: 0.6 mL/min; detection wavelength: 210 nm; Injection volume: 40 µL, column temperature: 25 °C. Figure adopted with permission from [165].

## *4.2. Other Chromatographic Techniques*

### 4.2.1. Gas Chromatography

In gas chromatography (GC), ILs have found use as stationary phases. This is due to the fact that they have unique properties, such as a wide liquid phase range, low volatility (negligible vapour pressure), high viscosity, good thermal stability and variable polarities, which make them suitable for that purpose [169]. Research into using molten salts in GC started in the 1950s and now IL-based columns are used in the analysis of complex samples [29,170]. A characteristic property of ILs is that they display unusual dual nature retention behavior, separating both non-polar and polar compounds. On the one hand, ILs exhibit a similar behavior to polar stationary phases such as polyethylene glycol or cyanopropyl-substituted polysiloxanes due to their ability to display a high dipolar interaction and hydrogen bonding. On the other hand, they are able to retain non-polar solutes (i.e., alkanes and alkenes), similarly to the low polarity stationary phases such as phenyl substituted dimethyl polysiloxanes [171]. Another important feature of ILs is that varying the cation or anion might significantly affect their physical and chemical properties [169]. For example, imidazolium IL columns using the [$Nf_2T$] anion were the most efficient [29]. ILs formed by less coordinating or nucleophilic anions, such as [$Nf_2T$], tend to be more stable compared to those containing halide salts; in fact, the latter are characterized by a nucleophilic nature, and hence it is possible for them to undergo SN1 or SN2 reactions with the alkyl substituents of the cation. Phosphonium-based ILs, synthesized with a large alkyl chain substituent, have shown outstanding thermal stability; in particular, a dicationic phosphonium IL, namely the dicationic 1,12-di(tripropyl-phosphonium)dodecane bis(trifluoromethylsulfonyl)imide, was synthesized possessing a thermal stability of 425 °C [172]. In addition, it should be noted that dicationic

and tricationic ILs exhibit significantly higher thermal stability compared to monocationic-based ILs [173].

At present, IL-coated capillary GC columns are commercially available under the trade name SLB-IL (Supelco–Sigma-Aldrich, Darmstadt, Germany) (Table 4). These columns are characterized by a different polarity and can work at temperatures passing 200 °C and approaching 300 °C [174]. Studies on the synthesis and properties of new IL-stationary phases are still continuing. Yu et al. [175] analyzed the application of a new triptycene-based amphiphilic material (TP-2IL) as the stationary phase for GC separations. In research, they showed that this column exhibited good performance for analytes from an apolar to a polar nature. Particularly, it has an outstanding capability for resolving critical pairs of anilines and phenols with good peak shapes, and shows distinct advantages over the typical conventional stationary phases. IL columns find many uses in the analysis of flavors and fragrances [176,177], fatty acids [178–182] and petrochemicals [183,184]. González Peredes et al. [185] evaluated different IL columns for the separation of chlorobenzenes and developed an analytical methodology based on the use of the IL stationary phase SLB-IL82 in GC with a microelectron capture detector for the determination of chlorobenzenes in soil samples.

**Table 4.** Selected commercial IL capillary GC columns with matrix active groups.

| GC Capillary Column | Matrix Active Group |
|---|---|
| SLB-IL59 | 1,12-Di(tripropylphosphonium)dodecane bis(trifluoromethylsulfonyl)imide |
| SLB-IL60 | 1,12-Di(tripropylphosphonium)dodecane bis(trifluoromethylsulfonyl)imide |
| SLB-IL61 | 1,12-Di(tripropylphosphonium)dodecane bis(trifluoromethylsulfonyl)imide trifluoromethylsulfonate |
| SLB-IL76 | Tri(tripropylphosphoniumhexanamido)triethylamine bis(trifluoromethylsulfonyl)imide |
| SLB-IL82 | 1,12-Di(2,3-dimethylimidazolium)dodecane bis(trifluoromethylsulfonyl)imide |
| SLB-IL110 | 1,9-Di(3-vinylimidazolium)nonane bis(trifluoromethylsulfonyl)imide |
| SLB-IL111 | 1,5-Di(2,3-dimethylimidazolium)pentane bis(trifluoromethylsulfonyl)imide |
| SLB-ILD3606 | 1,5-Di(2,3-dimethylimidazolium)pentane bis(trifluoromethylsulfonyl)imide |

Do et al. [186] showed that the profiling of all 136 PCDD/Fs is greatly facilitated by using IL columns or combinations including such columns. Boczkaj et al. [187] tested three capillary columns (HP-5Ms, DB-624, SLB-IL 111) in the analysis of oxygenated volatile organic compounds (O-VOCs) in postoxidative effluents from the production of petroleum asphalt. Among the capillary columns investigated, a very polar column, SLB-IL 111, with an ionic liquid as the stationary phase was found to be superior for the separation of O-VOCs, as it has a high selectivity towards n-alkanes and oxygenated volatile organic compounds.

So far, IL stationary phases have not been widely applied in the field of bioanalysis [188]. Destaillats et al. [189] applied an IL-coated SLB-IL 111 column to identify the occurrence of petroselinic acid in human skin, hair and nails. They confirmed that this column can be used to obtain a baseline resolution between petroselinic acid and cis-8 18:1 acid methyl esters.

The use of IL-based stationary phases has extended to multidimensional gas chromatography (GCxGC), ensuring future applications. For example, Zapadlo et al. [190] investigated the use of GCxGC–TOFMS with highly polar IL-based columns for the analysis of polychlorobiphenyls (PCBs). They used a non-polar/ionic liquid column series consisting of poly (50%-n-octyl-50%-methyl) siloxane

(SPB-Octyl) and the ionic liquid SLB-IL59 in the first and second dimension, respectively. As a result, a total of 196 out of 209 PCBs congeners were resolved and all dioxin-like congeners were separated with no interferences from any PCB congener.

ILs have found another significant application as a solvent in headspace gas chromatography (HS-GC), ILs are ideal solvents for HS-GC, a more sensitive method of analysis compared to direct injection. HS-GC avoids direct liquid or solid probing and greatly decreases matrix interference [29]. ILs are excellent solvents and are now used for the analysis of residual solvents in a variety of pharmaceutical products. The detection and quantitation of residual solvents/impurities in drug substances or drug products is an important measure for pharmaceutical quality assurance/quality control, because the residual solvents/impurities that were not totally removed by practical manufacturing techniques always have a potential risk to human health from toxicity. Fink et al. [191] developed a rapid, accurate, IL-based HS-GC method for the determination of water in active pharmaceutical ingredients. The HS-GC method used an IL-based capillary GC column to increase the sensitivity and ruggedness of this method. ILs are also utilized as a headspace solvent. Studies have shown that the sensitivity of the HSGC method is 100 times greater than that of volumetric Karl Fischer titration (KFT) (which is the commonly used technique to determine water content), allowing very small sample sizes (e.g., 4 mg) to be accurately and reproducibly analyzed. In comparison, a typical sample size of 500–1000 mg is used in KFT. Liu and Jiang [192] applied [$C_4$MIM][$BF_4$] as the matrix medium in the analysis of six solvents utilized in the synthesis of Adefovir Dipivoxil: acetonitrile, dichloromethane, N-methyl-2-pyrrolidone ([NMPyrr]), toluene, dimethylformamide (DMF), n-butyl ether. The developed method proved accurate and linear (with $R \geq 0.9993$). All the RSDs were lower than 10%. Moreover, the comparison of [$C_4$MIM][$BF_4$] with DMSO as a matrix medium of headspace GC was also carried out in this study. In this research, it was indicated that DMSO, with its boiling point at 189 °C, has a higher vapor pressure, and the chromatographic peak of the DMSO matrix, with a much higher intensity, always occupies a wider baseline or interferes in the detection of analytes, especially at a higher equilibrium temperature. The impurities and the decomposed products of DMSO at a high equilibration temperature also became interfering substances for the detection of residual solvents.

A great analytical challenge for the pharmaceutical industry is the trace-level analysis of genotoxic impurities (GTIs) in drug substances. Ho et al. [193] used ILs (six compounds: ([$C_4$MPyrr][B(CN)4]), [$C_4$MIM][$BF_4$], [$C_4$MMIM][$BF_4$], [$C_4$MIM][$Nf_2$T], [$C_4$MMIM][$Nf_2$T] and ([$P_{6,6,6,14}{}^+$][$Nf_2$T])) as a new class of diluents for the analysis of two classes of genotoxic impurities (GTIs), namely, alkyl/aryl halides and nitro-aromatics, in small molecule drug substances by headspace gas chromatography coupled with electron capture detection (ECD) without the need for analyte derivatization. The low volatility and high thermal stability of ILs enables these compounds to be used at high headspace oven temperatures with the minimum chromatographic background. Studies have shown that increasing the headspace oven temperatures resulted in varying responses for alkyl/aryl halides and significant enhancements in the responses for all nitroaromatic GTIs. Furthermore, ILs with a conventional high-boiling organic diluent—DMSO, were compared. The chromatographic backgrounds from ILs are significantly lower than the backgrounds from DMSO. The LODs of all analytes obtained using the IL diluents were superior (5 to 500 ppb) to those obtained from pure DMSO. Research on organic solvent residues in drugs was also conducted by Ni et al. [194]. The main focus of this study was to investigate the relationship between analytes (organic solvents) and the matrix medium (ILs) by HS-GC in order to provide guidance in choosing a suitable matrix medium and next to determine the organic residual solvents in ketoconanzole to choose a suitable IL during the process of HS-GC. In research, [$C_4$MIM][$PF_6$] was chosen as the best headspace solvent, because an excellent separation of ethanol, dichloromethane, ethyl acetate, butyl alcohol, pyridine, DMF and DMSO was achieved. The evaluation of ILs for the analysis of residual solvents in pharmaceutical matrices was the subject of research by Laus et al. [195]. The authors chose ([$C_4$MIM][DMP]) as the most suitable ionic liquid as solvent for the HS-GC analysis of solvents with very low vapor pressure, such as dimethylsulfoxide,

*N*-methylpyrrolidone, sulfolane, tetralin, and ethylene glycol which can be found in pharmaceutical products. The limit of quantification (LOQ) of this method was from 59 (tetralin) to 113 μg/g (ethylene glycol), the accuracy was in the range of 96.6–103.7, and it showed high linearity in the tested range of 0.9890–0.9984. The developed method was applied for the detection of traces of sulfolane in a real sample of tablets containing the drug cefpodoxime proxetil. A trace of sulfolane was detected (estimated at 2 μg/g with respect to the tablet mass), which is safely below the regulatory limit of 160 μg/g. To the best of our knowledge, there are only a few reports on the use of ILs as stationary phases for GC separations in the analysis of environmental samples. One of them is the investigation performed by Reyes-Contreras et al. [171], who examined the suitability of ILs as stationary phases for GC–MS, and their application for the determination of nitrosamines and caffeine metabolites in wastewater samples. Studies have shown that the SLB-IL111 column enabled the baseline separation and quantification of 7 nitrosamines in a shorter analysis time compared with the commonly used cyanopropylphenyl polysiloxane. Furthermore, the SLB-IL59 column provided the elution of all the caffeine metabolites analyzed with the highest peak symmetry and an appropriate analysis time. Contaminants in wastewaters were also the subject of research by Domínguez et al. [169]. In this work, the application of IL stationary phases for the determination of benzothiazoles and benzotriazoles was examined. Among the IL columns evaluated, SLB-IL59 provided the total elution of all the target analytes with the highest peak symmetry and the lowest analysis time. Moreover, the lower stationary phase bleeding enabled positive identification and quantification.

In view of the properties of ILs, such as high thermal stability, high viscosity, and tunable selectivity through the modification of their chemical structure, their use, in particular as stationary phases in GC, will increase. New IL stationary phase chemistries that provide unique selectivity towards target analytes are needed to improve the separation performance and versatility of multidimensional GC [170].

#### 4.2.2. Thin-Layer Chromatography

ILs are used in Thin-layer Chromatography (TLC) as stationary phase modifiers, especially in the separation of basic drug compounds. The separation of drug compounds is carried out normally with the use of silica-based stationary phases; however, it is often impossible because of the effect of free silanols on their chromatographic retention [196]. In order to remove this undesirable phenomenon, methods are used such as protonation, the addition of traditional amino quenchers, and changes in the mobile phase composition in order to increase ionic strength. The solution to this problem may be the application of ILs as silanol suppressing agents. The first study on improving separation in TLC by using ILs as mobile phase modifiers was presented by Kaliszan et al. [197]. The efficacy of using ILs as stationary phase modifiers in TLC has also been confirmed in research by Marszałł et al. [198]. The aim of this study was the application of imidazolium-based ILs to reduce the deleterious effects of free silanols on the LC separation of naphazoline nitrate. The authors used $[C_2MIM][BF_4]$ and $[C_6MIM][BF_4]$ as modifiers of the mobile phase. The results showed that ILs with short alkyl-chain lengths are efficient suppressors of free silanols, which are considered to be responsible for the troublesome and irreproducible chromatographic determinations of basic compounds. In the next study, Kaliszan et al. [199] also reported that ILs of the imidazolium tetrafluoroborate class when added to mobile phases blocked silanols and provided excellent TLC separations of strongly basic drugs which were otherwise not eluted, even with neat acetonitrile as the mobile phase. The ILs used by Marszałł et al. [198] as mobile phase modifiers were also tested in the studies reported by Mieszkowski et al. [196,200]. In the first study, 1-alkyl-3-methylimidazolium-based ILs (tetrafluoroborate $[C_2MIM][BF_4]$, L-(+)-lactate $[C_2MIM][LAC]$ and ethyl sulfate $[C_2MIM][ETOSO_3]$) were used as the mobile phase [196]. The subject of the research was the development of a new HPTLC method for the determination of perazine in oral tablets, and a comparative study between these three different ILs with the same cation but different counterions as additives to the mobile phase. In effect, among the selected ILs, the optimum distribution parameters, such as shape and quality of spots,

high precision, and accuracy in qualitative and quantitative determination, characterize the system, with [$C_2$MIM][$BF_4$] as the mobile phase modifier. Summarizing this study, it can be concluded that [$C_2$MIM][$BF_4$] is a valuable and efficient suppressor of free silanols, which are responsible for unwanted interactions of chromatographic stationary phases in the determination of the above compounds. In the second study, the authors compared two TLC methods for the determination of haloperidol in oral drops—the pharmacopeia method (European Pharmacopeia 7.0) and an alternative with IL modifiers of the mobile phase. The addition of [$C_2$MIM][$BF_4$] to the mobile phase gave similar separation and quantitative results with no peak tailing compared to the mobile phase suggested by the European Pharmacopeia 7.0 [200]. Besides the silanol-suppressing potency of [$C_2$MIM][$BF_4$], a lack of interaction and interference with UV densitometric detection was observed. Research on the use of ILs in TLC was also conducted by Lu et al. [201], who used ILs as mobile and stationary phases of TLC to analyze berberine hydrochloride, tetrahydropalmatine and related Chinese patent medicine. In this study, the shape and value of target spots together with the developing duration were compared regarding four mobile phases which were a combination of the ILs ([$C_4$MIM][OH]), [$C_4$MIM][$BF_4$], [$C_4$MIM][Br], [$C_4$MIM][$PF_6$]) and methanol. Moreover, these IL mobile phases were compared with two traditional developing reagents, *n*-hexane-chloroform-methanol and *n*-butanol-acetic acid-water. As a result, it was found that [$C_4$MIM][OH]-methanol has a simpler composition and is more suitable for the simultaneous analysis of two target constituents in a plate. Besides any extra pH additives, the shape of spots was ideal and no tailing occured. [$C_4$MIM][OH] was also used as the stationary phase, which was synthesized based on silica gel. The quantitative method for this kind of IL stationary phase showed a good correlation coefficient ($R^2$ = 0.9971–0.9976), good repeatability (%RSDs of berberine hydrochloride and tetrahydropalmatine were 0.88% and 0.79%, respectively) and method accuracy in terms of 95.91–104.85% (berberine hydrochloride) and 96.02–102.18% (tetrahydropalmatine). Research into the application of ILs as mobile phases in TLC was published by Tuzimski and Petruczynik [202]. The aim of the study was the separation of ten components of a mixture of isoquinoline alkaloids: allocryptopine, berberine, boldine, chelidonine, papaverine, emetine, columbamine, magnoflorine, palmatine and coptisine, using a 2D-TLC (two-dimensional TLC) method. The first dimension used an aqueous mobile phase (RP) (80% methanol–water–0.05 M/L−diethylamine), and in the second dimension a normal phase (NP) (75% methanol, 24.75% ethyl methyl ketone–0.25% IL [$C_4$MIM][$BF_4$]). The addition of ILs to conventional mobile phases caused a decrease in zone broadening and improved the chromatographic resolution. As shown in the results of the experiments, very symmetrical spots and peaks and high system efficiency were obtained. In conclusion, the authors proposed that mobile phase systems containing ionic liquids can be applied to the separation of isoquinoline alkaloids in other natural samples. The use of ILs as stationary phase modifiers can be an effective and more "green" alternative to classical mobile phases such as amines.

#### 4.2.3. Supercritical Fluid Chromatography

Among the numerous applications of ILs, they can also be used in solvent systems composed of ILs and supercritical fluids with an emphasis on supercritical carbon dioxide ($scCO_2$). The specificity of IL–supercritical fluid biphasic systems follows from the availability of several mechanisms for tuning the solvent properties of such systems—apart from the wide selection of IL cations and IL anions to tailor the IL properties, the operating temperature and pressure are also available as variables to adjust the density and the solvent power of the supercritical fluid phase [203]. In an ILs-$scCO_2$ system the product recovery process is based on the principle that scCO2 is soluble in ILs, but ILs are not soluble in $scCO_2$. Since most organic compounds are soluble in $scCO_2$, with the high solubility of $scCO_2$ in ILs, these products are transferred from the IL to the supercritical phase [204]. Ji et al. [99] applied the IL [$C_8$MIM][$PF_6$] and methanol as the extraction and dispersion solvents in a method for the determination of four NSAIDs—nabumetone, ibuprofen, naproxen and diclofenac—in tap water and drinks. The method was based on ultrasound-assisted ionic liquid dispersive liquid–liquid microextraction (US-ILDLLME) followed by ultra-high performance supercritical fluid chromatography

(UHPSFC) coupled to a photo-diode array detector (PDA). The developed method showed rapid separation (2.1 min), good recoveries (81.37–107.47%) and enrichment factors (126–132). The LODs for the analytes were from 0.62 (naproxen) to 7.69 (ibuprofen) ng/mL. This developed procedure was applied to real water samples, tap water, soda, lemon juice and green tea drink. In soda drink, ibuprofen was detected with detection levels of 16.43 ng/mL.

Because SFC can be performed with both polar and nonpolar stationary phases, columns that are marketed for HPLC can be used in SFC [205]. The application of immobilized ionic liquids (IILs) as a class of stationary phases for packed column SFC was studied by Smuts et al. [206]. The authors studied the cation and anion effect. The research was conducted on different IILs: tripropylphosphonium, tributylphosphonium, methylimidazolium, benzylimidazolium, triphenyl-phosphonium and 4,4′-bipyridyl while keeping the counteranion constant, and an immobilized tributylphosphonium with five different anions: acetate, trifluoroacetate (TFA), [Cl], perchlorate and [$Nf_2T$]. The best stationary phase in terms of low retention and good separation efficiency was the IIL tributylphosphonium with the TFA counter anion. Furthermore, the acetate anion exhibited the worst retention time and repeatability, and took the longest to reach baseline stability. [$Nf_2T$]$^-$ displayed poor efficiency in separations for tributylphosphonium-based stationary phases. Chou et al. [207] used covalently bonded 1-octyl-3-propylimidazolium chloride on a silica gel column for the simultaneous separation of acidic, basic and neutral compounds (fenoprofen, ibuprofen, acetaminophen, metoprolol, naphthalene and testosterone) using carbon dioxide subcritical/supercritical fluid chromatography. The data indicated that the IL-modified column, in terms of resolution, was clearly superior to commercial C18 columns. Also, the simultaneous separation of acidic, basic and neutral compounds via SFC was successful with a co-solvent content of 20% MeOH, a pressure of 110 bar, and a column temperature of 35 °C (Figure 9).

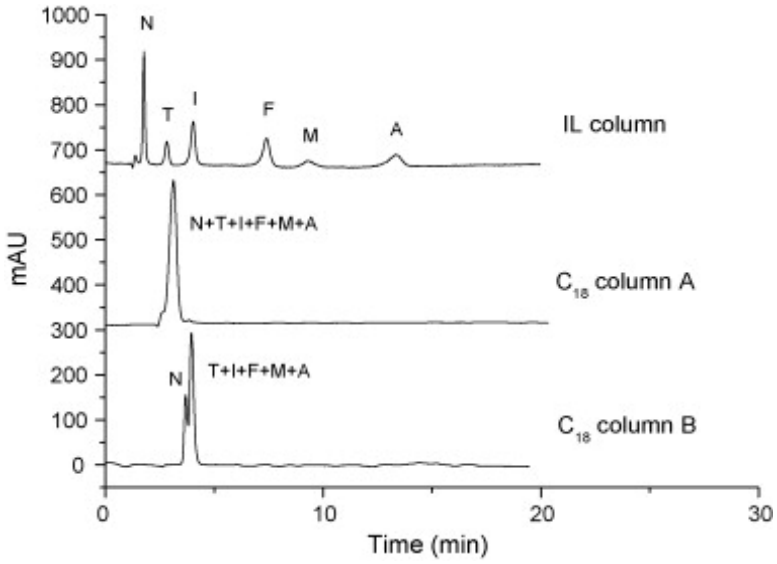



**Figure 9.** Separation of acidic, basic, and neutral compounds via SFC using the IL-modified column and a commercial $C_{18}$ column. Figure adopted with copyright permission from [207].

In conclusion, it should be stated that ILs seem to be good replacements for volatile organic solvents, and the development of new applications utilizing ILs will increase. However, the high cost of ILs and lack of complete data on e.g., toxicity should be noted.

## 5. Electromigration Techniques

### *5.1. Capillary Electrophoresis*

CE, which belongs to electromigration separation techniques, possesses many advantages, such as low sample and reagent consumption, high efficiency, simplicity, short analysis time, automation and inexpensive cost of capillaries in comparison to HPLC columns. CE separations are also extremely effective and allow substances with similar structures to be separated. These advantages mean that this technique has become an interesting alternative analytical tool to other chromatographic methods. Generally, CE analysis is carried out on fused-silica capillaries with silanol groups on the inner surface which are normally negatively charged. This results in the formation of an electroosmotic flow (EOF) that moves compounds toward the cathode when a voltage is applied across a tube filled with an electrolyte solution. Contrary to this effect, electrophoretic mobility exists, which moves a molecule to its opposite electrode. Each ion possesses a specific electrophoretic mobility resulting in a charge-to-mass ratio. However, the effect of EOF is generally predominant in respect to electrophoretic mobility, causing all the molecules to be moved at different speeds toward the cathode. A higher speed can be observed for cations, and neutral analytes take slightly longer to migrate, while negatively charged compounds take the longest to move because of their conflicting electrophoretic mobility. The fact that, simultaneously, both EOF and electrophoretic mobility occur, working on anions in opposite directions during electrophoretic separation, allows greater resolution to be obtained. The main parameters which can affect EOF mobility are the dielectric constant, the zeta potential value and the viscosity of the buffer. The values of these parameters can be regulated by the modification of the background electrolyte (BGE) and/or using different buffer additives, as well as when the physicochemical properties of the wall of the capillary are changed. ILs are considered as good EOF modifiers because of their good electrical conductivity and they are slightly more viscous than organic solvents. In effect, low IL concentrations can be enough for a significant improvement in the electrophoretic separation. According to the literature data, ILs have been applied as the BGE, as additives to the BGE and/or as covalent coating reagents of the capillary. However, taking into account the costs of these modifications, ILs were mainly used as electrolytes or additives to electrolytes to modify the capillary wall. It should be highlighted that both cations and anions of ILs may change the migration behavior of analytes, although the activity of IL cations have a major impact on the resolution in CE. The IL cations, by the modification of the ionic strength of the BGE, can change the EOF, which influences the migration times of the analytes and may improve separation efficiency. Other activity is related to the adsorption of IL cations on the capillary inner surface, which can reduce or even reverse the EOF as well as possibly correcting the peak tailing of some basic enantiomers. Both mechanisms mentioned above allow a significantly better resolution of analytes to be obtained [199,208].

For example, Qin et al. [209] used a 1-methylimidazolium-based IL for covalent bonding of the fused-silica capillary surface wall for reversing the EOF during the development of a CE-MS method for the determination of sildenafil (SL) and its metabolite UK-103,320 (UK) in human serum samples. The most effective separation was obtained with a BGE containing 10 mM of acetic acid (pH 4.5) and with a voltage of 25 kV. The sensitivity and resolution were significantly improved because this approach allowed the elimination of the adsorption of the compounds on the IL-coated capillary wall, which occurred on the bare fused-silica capillary wall. In effect, the analytes passed through the IL-coated capillary with a recovery of 98% and 100% for SL and UK, respectively. Moreover, the resolution between SL and UK was enhanced because of the modification of the EOF. The analytes were separated within 14 min with LODs of 14 and 17 ng/mL for SL and UK, respectively. El-Hady et al. [210] proposed a CE-UV method for the simultaneous determination of four anticancer drugs in human plasma and urine based on [$C_4$MIM][Br] as a component of the BGE. During the study, the parameters of CE separation were optimized. The best results were obtained when the analysis was carried out on a BGE containing a 12.5 mmol/L phosphate buffer at pH 7.4 and 0.1 μmol/L of [$C_4$MIM][Br] (IL), and 20 kV applied voltage. This approach allowed sensitivity to be increased 600 times over that observed

in CE performed without the IL. The developed CE-UV method for the quantification of methotrexate, vinblastine, chlorambucil and dacarbazine in human plasma and urine allowed the analytes to be monitored with the LODs in the range of 0.01 to 0.05 μg/mL.

It should also be noted that excellent separation is particularly required for the analysis of racemic mixtures, including various groups of pharmaceuticals the enantiomers of which can possess significant different pharmacokinetic and pharmacodynamic properties and side effect profiles. The qualitative and quantitative analysis of the compounds in biological and environmental samples is necessary for better understanding the mechanism of their activity in live organisms and their influence on the environment. This issue was a predominant topic of many papers published in recent years in world scientific literature. In those studies, both achiral ILs and chiral ILs (CILs) were applied in combination with various types of chiral selectors (CS) like cyclodextrins (CDs) or their derivatives, antibiotics, polysaccharides or surfactants for the chiral separation of different pharmaceuticals. Typical achiral ILs applied in CE enantioseparation were tetraalkylammonium ILs, alkylimidazolium ILs and alkylpyridinium ILs with inorganic anions such as [OH], [Cl], [Br], [$BF_4$] and [$PF_6$]. Among them, tetraalkylammonium-based ILs are considered as more effective because of their relatively more hydrophilic character, which decreases the likelihood of entering the hydrophobic cavity of the CS. Moreover, their relatively lower conductivity and UV transparency in the wavelength ranges applied for enantiomer detection allow them to be used in higher concentration levels. These data are in accordance with the study reported by Huang et al. [211] who tested alkylpyridinium, tetraalkylammonium and alkylimidazolium-based ILs along with β-CDs for the chiral separation of five β-agonists. The results confirmed that tetraalkylammonium-based ILs were more effective because they could be used at much higher levels than the other tested ILs. Poor resolution was achieved when the long-chain IL, [$C_8$MPyrr][$PF_6$], was used as the BGE modifier. Moreover, the presence of ILs was required for the full enantioseparation of salbutamol, cimaterol and formoterol, which were not resolved using the BGE containing only β-CD as the CS.

Jiang et al. [212] used [$C_2$MIM][$BF_4$] for the coating of a silica capillary during the enantioseparation of ibuprofen, fenoprofen, naproxen and ketoprofen. It enabled the EOF to be modified, which provided the effective resolution of the enantiomers. The tested IL not only affected the EOF but also acted as a discriminator. Moreover, the interaction between hydrogen at the C–2 carbon of the IL and the acid drugs played an important role in the separation. The same type of IL was selected for the enantiorecognition of nine tricyclic antidepressants in the study reported by Tsai et al. [213]. The optimal simultaneous separation of all the tested pairs of enantiomers was achieved with 50 mM of [$C_2$MIM][$BF_4$] as the sole BGE at pH 3. Zhao et al. [214] used three ILs and hydroxypropyl-β-cyclodextrin (HP-β-CD) as the components of the BGE for the enantioseparation of itraconazole, ketoconazole, econazole and miconazole. Compared with [$C_2$MIM][L-lactate] or [$C_2$MPyrr][$BF_4$], [$C_{12}$MAmm][Cl] was the most effective. When this reagent was used along with HP-β-CD it allowed the resolutions of 3.8, 3.5, 2.8 and 2.5 for miconazole, econazole, ketoconazole and itraconazole, respectively, to be obtained.

In the paper published by Liu et al. [215], the effective chiral separation of racemic methyl-ephedrine hydrochloride, thebaine, codeine phosphate and acetylcodeine by capillary electrophoresis with electrochemical detection (CE-ECL) was observed when 0.6% [$C_4$MIM][$BF_4$] as the component of the BGE was applied (Figure 10).

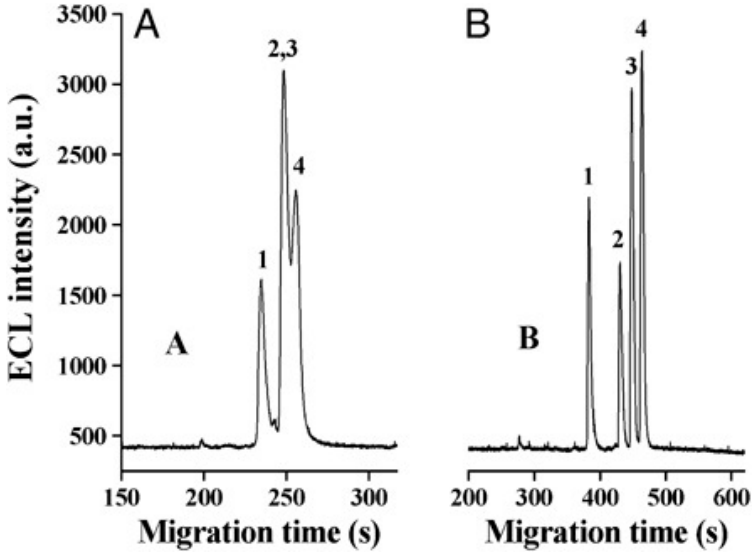



**Figure 10.** Electropherograms of four standard samples: (**A**) without IL in electrophoretic buffer; (**B**) with the use of 0.6% $BIMPF_4$ in the electrophoretic buffer. Peak: 1, 10 μmol/L methylephedrine hydrochloride; 2, 40 μmol/L of thebaine; 3, 25 μmol/L codeine phosphate; 4, 15 μmol/L acetylcodeine. Conditions: electrophoretic buffer, 14 mmol/L phosphate–borax at pH 7.4; electrokinetic injection, 10 s × 10 kV; separation voltage, 15 kV; detection potential, 1.2 V; ECL solution, 5 mmol/L $Ru(bpy)_3^{2+}$ with 50 mmol/L PBS at pH 8.2. Figure adopted from [215] with permission.

The developed method offered the quantification of four drug alkaloids in human urine samples with LODs from $1.4 \times 10^{-7}$ to $6.3 \times 10^{-8}$ mol/L. Jin et al. [216] reported the effective enantioseparation of propranolol, oxprenolol and pindolol by CE when a BGE containing the achiral IL—glycidyltrimethylammonium chloride ([GTMAmm][Cl]) as the modifier along with a dual CDs system based on 2,6-di-O-methyl-β-cyclodextrin (DM-β-CD) and 2,3,6-tri-O-methyl-β-cyclodextrin (TM-β-CD) was applied. The authors also used an on-line sample enrichment technique based on field-enhanced sample injection (FESI) for the improvement of sensitivity. The application of both approaches allowed the LODs of the enantiomers to be decreased from 0.10 to 0.65 nM. Finally, the developed CE method was successfully used for the analysis of spiked urine samples, with good recoveries.

Unfortunately, in many cases, the application of achiral ILs with a single chiral selector was not enough for the effective enantioseparation of the compounds of interest. An interesting alternative approach reported in the literature was using CILs which can possess either a chiral cation or achiral anion, or both. The application of these CILs in combination with traditional chiral selectors allows an extra "enantiorecognition" capability to be obtained while the capability of system modification is retained. In effect, a "synergistic system" occurs during electrophoretic separation, which can significantly improve the resolution of the analytes. The first paper reporting the use of this approach for the enantioseparation of pharmaceuticals was published by François et al. [217]. The authors developed and used two chiral choline-based ILs—ethylcholine bis(trifluoromethylsulfonyl)imide ([EtChol][$Nf_2T$]) and phenylcholine bis(trifluoromethylsulfonyl)imide ([PhChol][$Nf_2T$]) alone or in combination with DM-β-CD or TM-β-CD for the analysis of the anti-inflammatory drugs, 2-arylpropionic acids, as model compounds. The developed CILs were applied as BGE additives, chiral ligands and CSs. Moreover, the enantioseparation efficiency in respect to the type and concentrations of tested CILs and CDs, as well as the methanol addition to the BGE, were evaluated. The results indicated that the effective separation of the analytes was achieved only upon adding one of the CILs containing DM-β-CD or TM-β-CD and methanol to the BGE. Thus, the synergistic effect between the tested chiral choline-based ILs and CDs in the dual separation system was confirmed. In another study, two chiral synergistic systems based on tetramethylammonium-L-arginine (TMA-L-Arg)/glycogen and tetramethylammonium-L-aspartic acid

(TMA-L-Asp)/glycogen were compared with the system containing achiral tetramethylammonium hydroxide (TMA-OH)/glycogen for the chiral separation of nefopam, citalopram and duloxetine [218]. Each tested IL/glycogen synergistic system gave better resolutions of the tested enantiomers compared to those observed for the separation using glycogen alone. However, the addition of TMA-L-Arg to the BGE composition was more effective than TMA-L-Asp, while the TMA-OH/glycogen separation system gave poorer resolution. Zhang et al. [219] tested tetramethylammonium-L-arginine (TMA-L-Arg), tetramethyl-ammonium-L-hydroxyproline (TMA-L-Hyp) and tetramethylammonium-L-isoleucine (TMA-L-Ile) as BGE additives in combination with HP-β-CD for the enantioseparation of amlodipine, nefopam, duloxetine and propranolol. The highest signals of the tested analytes and the best resolution was achieved using a 40 mM Tris/$H_3PO_4$ buffer solution (pH 2.6) containing 20 mM of HP-β-CD and 30 mM of TMA-L-Arg (Figure 11). Zuo et al. [220] reported the enantioseparation of twelve pharmaceuticals using 1-ethyl-3-methylimidazolium-L-lactate ([$C_2$MIM][L-lactate]) and 1-butyl-3-methylimidazolium-L-lactate ([$C_4$MIM][L-lactate]) in combination with β-CD in a BGE. The resolution was better in a dual system based on one of the tested CILs and β-CD compared to the β-CD alone, although the addition of [$C_2$MIM][L-lactate] was more effective. Finally, the BGE composed of 20 mM of [$C_2$MIM][L-lactate] and 10 mM of β-CD at pH 2.5 was selected as optimal for the separation of most analytes.

Only the analysis of homatropine methylbromide was carried out on 30 mM of Tris-$H_3PO_4$ at pH 2.0 (more effective separation), while the enantiomers of venlafaxine and sibutramine were not baseline resolved. Kolobova et al. [221] confirmed that 1-butyl-3-methylimidazolium L-prolinate [$C_4$MIM][L-Pro] as a CS in combination with 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD) allowed a significant improvement in the chiral separation of carvedilol and propranolol.

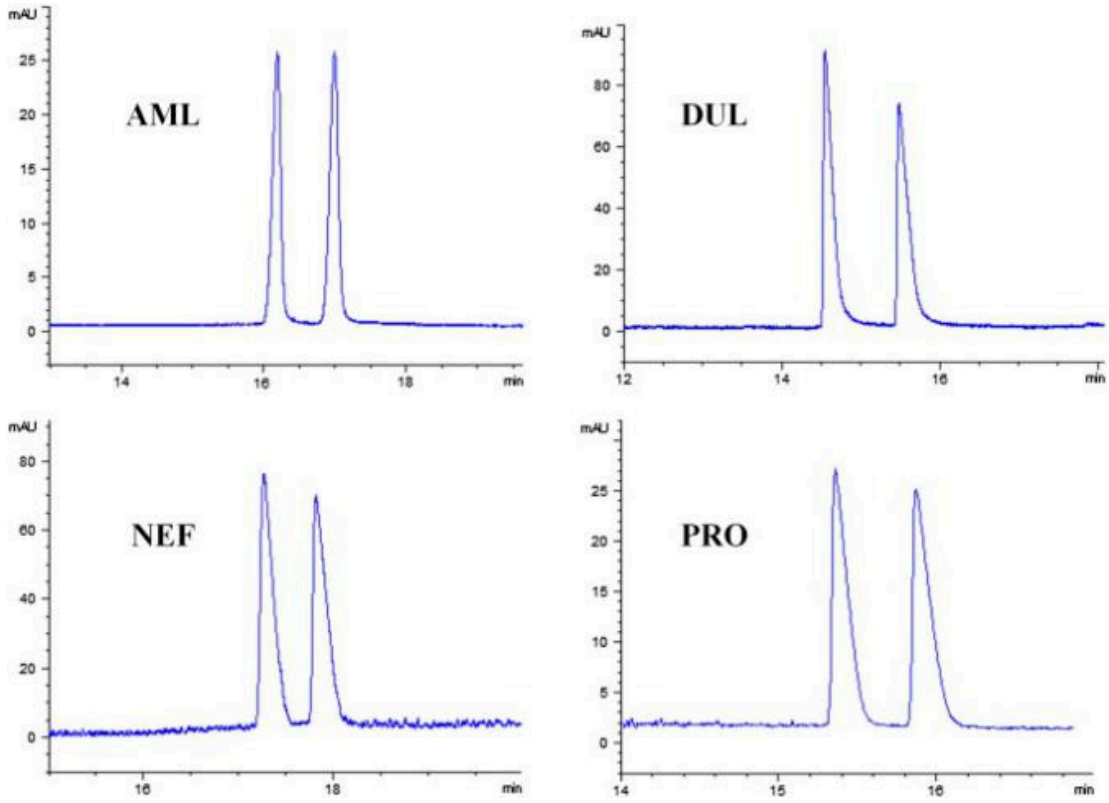



**Figure 11.** Chiral separation of all drug enantiomers in the optimized HP-β-CD/TMA-L-Arg synergic system. Conditions: focused-silica capillary, 50 cm (41.5 cm effective length) × 50 μm i.d; applied voltage, 20 kV; capillary temperature, 15 °C; BGE, 40 mM Tris/$H_3PO_4$ buffer solution (Ph 2.6) containing 20 mM HP-β-CD/TMA-L-Arg. Figure adopted from [219] with copyright permission.

Zhang et al. [222] designed a lactobionic acid LA-based IL, namely tetramethylammonium-lactobionate (TMA-LA), which was applied for the chiral separation of atenolol, metoprolol, propranolol, nefopam and duloxetine. In the study, three combinations, namely a single LA system, β-TMA chloride (TMA-Cl) system and TMA-LA IL system, were tested. The best results were achieved when the IL TMA-LA as the CS was applied. Finally, the BGE containing 40 mM of borax buffer, pH 7.6, 40% *v/v* methanol, 200 mM of TMA-LA and 20 kV applied voltage was selected as the most effective. Zhang et al. [223] tested L-alanine tert-butyl ester bis (trifluoromethane) sulfonamide (L-Ala$C_4Nf_2T$) and L-valine *tert*-butyl ester bis (trifluoromethane) sulfonamide (L-Val$C_4Nf_2T$) as additives to the BGE in combination with M-β-CD, HP-β-CD and glucose-β-CDs (Glu-β-CD) for the enantioseparation of naproxen, pranoprofen and warfarin. Compared to CDs alone, significantly better chiral recognitions of all analytes were obtained, although the resolutions of these dual systems were different. Moreover, the addition of organic modifiers to the BGE additionally improved selectivity. This was probably related to decreasing the EOF, which allowed interactions to be increased between AAILs, M-β-CD and the racemates. The best separations of the analytes were observed when 15 mM of CILs was introduced into the 30 mM sodium citrate/citric acid buffer solution at pH 5.0 containing 20 mM of M-β-CD and 20% ethanol as the organic modifier with a 20 kV applied voltage. The potential synergistic effects of L-Ala$C_4Nf_2T$ and L-Val$C_4Nf_2T$ were also checked in combination with vancomycin during the enantioseparation of naproxen, carprofen, ibuprofen, ketoprofen and pranoprofen [224]. Both dual synergic separation systems were also able more effectively to separate the enantiomers compared to the vancomycin-alone case. Xu et al. [225] applied tetramethylammonium-L-hydroxyproline (TMA-L-Hyp) with clindamycin phosphate (CP) for the separation of a racemic mixture of propranolol, nefopam, citalopram and chlorphenamine. The authors optimized the electrophoretic conditions in terms of the BGE composition, pH, voltage, temperature and UV parameters. The best results were obtained when the CE separation was carried out on an uncoated fused-silica capillary (50 cm total and 41.5 cm effective length × 50 μm i.d.) with a 40 mM borax buffer (pH 7.6) containing 80 mM of CP and 30 mM of TMA-L-Hyp and methanol (20% *v/v*). A voltage of 20 kV and a temperature of 20 °C were used. Nefopam, citalopram, chlorphenamine and propranolol were monitored at 289, 230, 265 and 237 nm, respectively. AAILs based on a tetramethylammonium cation were also tested with maltodextrin for the enantio-separation of pharmaceuticals belonging to different classes. For example, Yang et al. [226] used tetramethylammonium-D-pantothenate (TMA-D-PAN) and tetramethylammonium-D-quinate (TMA-D-QUI) as additives to the maltodextrin-based synergistic systems in a CE method developed for the analysis of racemic mixtures of nefopam, ketoconazole, econazole and voriconazole. For both of the CIL/maltodextrin systems, significantly improved $R_s$ were observed for all the tested enantiomers, although TMA-D-PAN offered better separation results. This synergistic effect was probably related to a decrease in the density of the negative charge as an effect of the adsorption of the CIL cations on the surface of the capillary. This caused increasing complexation between the racemates and the CIL, which improved the resolution for all analytes.

Chen et al. [227] used tetramethylammonium-L-arginine (TMA-L-Arg) and tetramethyl-ammonium-L-aspartic acid (TMA-L-Asp) in combination with maltodextrin for the enantioseparation of nefopam, citalopram, cetirizine, duloxetine and ketoconazole. The most effective chiral separation was observed when a BGE composed of 60 mM of TMA-L-Arg, 7.0% maltodextrin in 50 mM of Tris-$H_3PO_4$ (pH 3.0) and with a voltage of 18.0 kV was applied. Zhang et al. compared the separation systems based on 1-butyl-3-methylimidazolium (T-4)-bis[(2S)-2-(hydroxy-κO)-3-methylbutanoato-κO]borate ([$C_4$MIM][BLHvB]) and 1-butyl-3-methylimidazolium (T-4)-bis[(αS)-α-(hydroxy-κO)-4-methylbenzeneacetato-κO]borate ([$C_4$MIM][BSMB]) along with HP-β-CD [228] as well as dextrin [229] as the CS in CE enantioseparations. In both studies, the addition of the CIL enabled the synergistic effect to occur between them and the used CS, which allowed better resolutions to be obtained and higher peak efficiencies compared to those calculated for the HP-β-CD or the dextrin alone. On the other hand, [$C_4$MIM][BLHvB] was more effective than

[$C_4$MIM][BSMB]. This was probably related to the structure of the [$C_4$MIM][BSMB] anion whose aromatic ring substituent could disturb chiral recognition.

An interesting approach was presented by Zhang et al. [230] who employed IL-dispersed NPs as buffer modifiers for the chiral separation of laudanosine, propranolol, amlodipine, citalopram and nefopam in CE. In the study, [$C_4$MIM]$BF_4$], ([$C_4$MIM][$PF_6$]), 1-dodecyl-3-methylimidazolium chloride ([$C_{12}$MIM][Cl]) and 1-aminoethyl-3-methylimidazolium bromide ([$C_2NH_2$MIM][Br]) ILs were dispersed in multi-walled carbon nanotubes (ILs-MWNTs) and applied as the BGE modifier in combination with chondroitin sulfate E (CSE), as the CS. The obtained results indicated that significantly better separation, selectivity and peak shapes were achieved in the ILs-MWNTs modified system compared to that observed in CSE alone. The parameters affecting the electrophoretic separation were also investigated and optimized. The best results were obtained when CE was carried out on a 20 mM Tris/$H_3PO_4$ buffer solution containing 2.5% CSE and 2.4 μg/mL of ILs-MWNTs at pH 2.8–3.4 and with 15 kV applied voltage.

It should be highlighted that most of the studies described above indicated that the application of CILs alone as BGE modifiers was not able to effectively to separate the enantiomers. However, in the literature there are also a few reports describing the synthesis of novel CIL structures the activity of which was enough to achieve full resolution of drug enantiomers. For example, Yu et al. [231] synthesized a β-CDs-based CIL, 6-O-2-hydroxypropyltrimethylammonium-β-cyclodextrin tetra-fluoroborate ([HPTMA-β-CD][$BF_4$]), and used it as a CS for the enantioseparation of eight pairs of drug enantiomers. The novel CIL offered higher solubility of the analytes in the BGE and gave better stabilization of reversed EOF in CE compared to the parent β-CDs, which allowed a higher intensity of the signals and a more effective resolution to be obtained. The results confirmed that the enantiomers of chlorpheniramine, brompheniramine, promethazine, liarozole, tropicamide, warfarin, pheniramine and bifonazole were more effectively separated with [HPTMA-β-CD][$BF_4$] as the CS than with β-CDs. Recently, a report describing the synthesis of mono-6-deoxy-6-(3-methylimidazolium)-β-cyclodextrin tosylate (β-CDMIMOTs) CIL was also published by Zhou et al. [63]. The authors applied this new CIL as a coating material to modify the EOF in the CE method for the enantioseparation of oxytetracycline, tetracycline, chlortetracycline and doxycycline in environmental samples. The researchers achieved good separation of the analytes due to the multiple functions of β-CD-IL, which enabled the tetracyclines to be entrapped to form an inclusion complex (Figure 12).

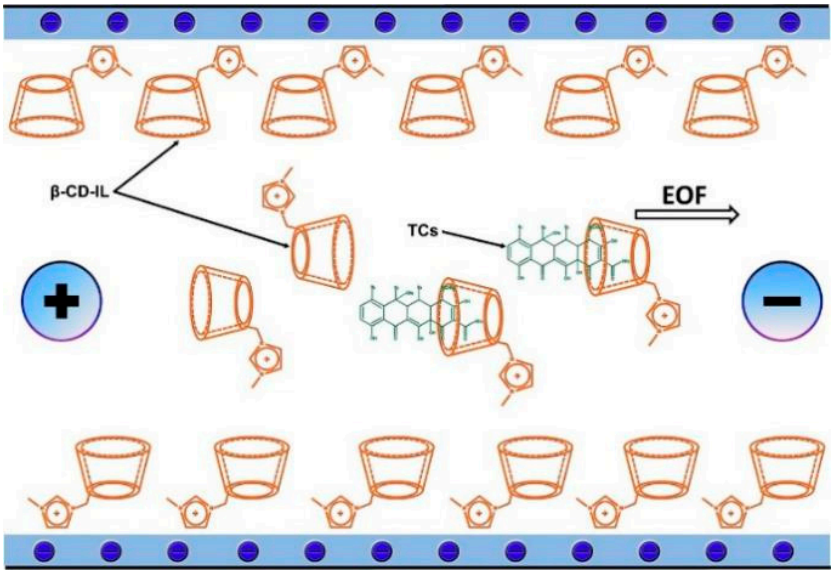



**Figure 12.** Mechanism of separation of four TCs using β-CD–IL as dynamic coating material. Figure adopted with permission from [63].

Compared to β-CD alone, β-CD-IL offered better solubility in an aqueous buffer. A stable suppressed EOF in the capillary was also generated as the effect of the occurrence of hydrogen bonding and the electrostatic interaction with the capillary inner wall. The authors selected the best CE conditions for tetracycline separation, which were achieved when a BGE composed of 10 mmol/L, a pH 7.2 phosphate buffer and 20 mmol/L of β-CD-IL and electrochemical detection at 1 V was used. The developed CE method allowed the compounds of interest to be monitored in environmental water samples with LODs from 0.33 to 0.67 μmol/L.

### *5.2. Micellar Electrokinetic Chromatography*

Considered as a mode of CE, micellar electrokinetic chromatography (MEKC) allows both neutral and charged analytes to be separated. In MEKC, the surfactant monomers are added to the run separation buffer above the critical micelle concentration (CMC), which allows aggregates called micelles to form as a pseudostationary phase. The separation process is based on differences between the analytes partitioning in a micellar stationary phase, and is related to the electrophoretic mobility of the compounds. Therefore, the neutral and hydrophobic analytes incorporated into the micelles gain an apparent electrophoretic mobility and will move at the same velocity as the micelle under electrophoretic conditions. This allows the neutral and charged compounds with the same charge-to-mass ratio to be separated because the migration time in MEKC is dependent on the electrophoretic velocity of the micelle, the distribution ratio and the EOF velocity. The use of additional BGE modifiers can increase efficiency and selectivity. ILs as BGE additions have become interesting alternatives because the long-chain part of the AAILs can act as a surfactant to form a micelle in the BGE when the level of ILs exceeds the CMC. Moreover, the electrostatic interaction between the acidic analyte and the cationic micelle (AAILs) offered a more effective enantiorecognition of the analytes. Higher concentrations of ILs may also be used compared to organic solvent surfactants because of higher conductivity, hydrophobicity and solvation, which decreases the risk of destroying the micellar system in MEKC. In the literature, there are a few papers reporting the use of ILs in MECK. For example, Wang et al. published two consecutive papers [232,233] demonstrating the combination of TM-β-CD with N-undecenoxycarbonyl-L-leucinol bromide (L-UCLB) CIL as a dual chiral selector for the enantiodiscrimination of fenoprofen, indoprofen, ketoprofen, suprofen and ibuprofen. In the study, different levels of CILs and TM-β-CD were tested. The results indicated that TM-β-CD alone could not resolve the enantioseparation of the racemates, whereas the addition of L-UCLB at a concentration of 1.5 to 2.0 mM to the BGE with TM-β-CD provided an excellent resolution. This was related to the competitive inhibition of the interaction between the CIL and the capillary wall in the presence of TM-β-CD. Cui et al. [234] used L-ethyl-3-methylimidazolium-L-lactate, [$C_2$MIM][L-lactate] and 1-ethyl-3-methylimidazolium-L-(β)-lactate [$C_2$MIM][DL-lactate] alone or in combination with HP-β-CD for the chiral resolution of ten analytes belonging to different classes of pharmaceuticals. The results confirmed that the best enantiorecognition was obtained when a BGE composed of 40 mM of HP-β-CD, 50 mM of $NaH_2PO_4$-$H_3PO_4$, pH 2.75, and 30 mM of [$C_2$MIM][L-lactate] was used during the enantiomeric separation. Moreover, this effect was mainly correlated with the cationic activity of the IL, which played an important role in the increased resolution, whereas the anionic part of the CIL possessed a low influence on the chirality and nature of the enantioseparation. Su et al. [235] tested the addition of [$C_4$MIM][Cl], [$C_4$MIM][$PF_6$], [$C_4$MIM][$Nf_2$T] and SDS as modifiers in the BGE during the optimization of MEKC conditions for the separation of seven benzodiazepines. The results confirmed that the BGE containing 170 mM of [$C_4$MIM][$Nf_2$T] and 10 mM of SDS offered the most effective selectivity and resolution of the compounds of interest. This was related to different degrees of association of the tested analytes, which gave a more satisfactory separation compared to the results observed using the IL or SDS alone. The anionic moiety of [$C_4$MIM][$Nf_2$T] probably played a dominant function during the separation process as a heteroassociation site for the benzodiazepines, while the SDS improved the resolution. The developed MEKC method allowed the analytes to be detected in human urine samples with LODs in the range of 2.74 to 4.42 μg/mL.

### *5.3. Non-Aqueous Capillary Electrophoresis*

In recent years, non-aqueous capillary electrophoresis (NACE) has become an interesting separation technique because it allows the detection of water-insoluble analytes which cannot be measured in traditional aqueous CE. Additionally, the analysis time in NACE can be shortened because of the lower viscosity of the buffer solution and the higher EOF as well as the reduction of the electrophoretic current. Moreover, the application of organic solvents allows the analytes to be detected online by MS. As it was earlier mentioned, ILs possess some advantages over conventional organic solvent modifiers, such as good conductivity. Hence, using ILs in NACE can give a better separation effect. These possibilities were confirmed by Ma et al. [236] who applied an ephedrine-based CIL as the CS for the enantiomeric resolution of omeprazole and rabeprazole by NACE. A reversed EOF (anodic flow), probably caused by the adsorption of the cations onto the capillary wall, was observed when (+)-N,N-dimethylephedrinium-bis(trifluoromethanesulfon)imidate ($[DMP]^+[Nf_2T]^-$) was added to the BGE. The best resolution was achieved with the BGE containing an acetonitrile-methanol mixture (60:40, *v/v*) and 60 mM of $[DMP]^+[Nf_2T]^-$. The authors found that the enantioseparation was related to ion-pair interactions dependent on equilibrium constants between the negatively charged enantiomers and DMP cations. Moreover, hydrogen-bonding between the hydroxyl group of $DMP^+$ and the sulfoxide group of the analytes as well as $\pi$–$\pi$ and dipole–dipole interactions were responsible for the separation mechanism.

Summarizing, the application of ILs in electromigration techniques offers new opportunities to solve many analytical problems in the separation field. One of them is the chiral recognition of racemic mixtures of pharmaceuticals having different chemical structures and biological activity. The results of numerous studies based on drug standards confirmed the great potential of ILs in CE applications. On the other hand, there are relatively few reports describing the separations of drugs in real biological and environmental samples. This seems to be caused by the relatively low sensitivity of CE-based methods compared to LC and GC techniques, which may be not enough for many pharmaceutical, clinical and environmental applications. On the other hand, lower LOD values can be obtained in electromigration techniques supported by ILs, which allows a partial resolution for this analytical problem. Moreover, intensive progress is continuing systematically in developing new approaches for improving sensitivity in electromigration techniques based on techniques such as field-enhanced sample injection (FESI), field-amplified sample injection (FASI), field-amplified sample stacking (FASS) or a combination of simultaneous electrokinetic and hydrodynamic injection (SEHI) and field-enhanced sample injection in conjunction with a sweeping technique known as sequential stacking featuring sweeping (SSFS) [237,238]. Probably, when scientists apply both ILs and new technical resolutions in CE, it will allow the required sensitivity to be obtained for clinical and environmental studies. These studies are very important because both CE-based techniques and ILs are environmentally-friendly, so connecting them in one analytical tool could be an important factor supporting the protection of nature.

## 6. Current Trends and Future Perspectives

Pharmaceuticals possess high biological activity and they can take part in various types of interactions, which means that these substances have a huge influence on the functioning of both live organisms as well as whole ecosystems. Therefore, as it was mentioned in Section 1, it is very important to develop sensitive, selective, accurate and precise methods for reliable drug determination in biological and environmental samples. An interesting approach is the application of ILs during method development. According to the data presented in this review, there are several interesting trends in the application of ILs for the determination of pharmaceuticals. First of all, ILs are most often applied at the stage of sample preparation (Table 2). The vast majority of studies concerned the extraction (or actually microextraction) of biological and environmental samples. Moreover, the most common type of analyte extraction from both these matrices was DLLME. Researchers pay a lot of attention to improving these methods by introducing modifications using physical and chemical

factors. As a result, they promote the development of environmentally-friendly solutions in the field of analytical chemistry and the improvement of validation parameters. Unfortunately, it should be noted that despite the development of various IL-based methods, the majority of procedures are still supported by organic solvents. In DLLME, their basic function is the dispersion of ionic liquids. In turn, because of the high viscosity of ILs, sample detection is only possible after dissolving the sample in MeOH, ACN and others. Thus, the application of ILs leads to improved validation parameters, but the developed methods are not completely eco-friendly. The results prove that despite moving in the right direction, this area requires further development. Improving the results is possible not only by proper sample preparation, but also by the application of ILs in chromatographic and electrophoretic techniques. The addition of ILs to mobile phases is the main way of using them for the determination of pharmaceutical drugs by chromatographic techniques. As the results show, the suppression of the interaction of silanol by use of ILs is a huge advance in the problematic analysis of basic drugs (Table 3). The use of ILs in the BGE in electrophoretic techniques, which in many respects are compatible with green chemistry, although their sensitivity still remains a challenging task for the analyst, seems to be promising. It may be surprising that despite the existence of commercially available and described methods for the self-preparation of IL-based chromatographic columns and capillaries for electrophoresis, such methods of their use is very rare for pharmaceuticals. If the huge potential of ILs is to be discovered, it should also be noted that in addition to the above detection methods, researchers are trying to use them with other chromatographic techniques. Although such applications are not yet widespread in the analysis of pharmaceuticals, their dynamic development may cause such experiments to be performed in the future. In addition to trends in the design of analytical methods, the qualification of ILs with similar structures to a specific stage of analysis is the constant rule. In many works, optimization concerns the selection of a specific IL from a large diverse group of IL molecules. However, according to the data presented in different reports, the final optimization effect leads to the selection of the same IL. For example, an IL with hydrophobic properties was sought for liquid-phase extraction and the best results were often achieved for the imidazolium cation and anion [$PF_6$]. In turn, as an addition to the mobile phase, the selection of the IL [$PF_6$] was not suitable due to too strong adsorption on the column and was replaced by [$BF_4$]. It must be highlighted that these are trends for most, but not all papers (detailed in Tables 2 and 3). However, the fact is that despite access to a vast amount of ILs, only a few have been tested in experiments, and the final selection focuses on a small number. As mentioned, the samples are analyzed by various chromatographic and electrophoretic techniques, while a UV detector is almost always used for analyte detection, rarely FL and almost never MS/MS.

The above examples confirm that there are no ideal solutions in the design of analytical methods for the determination of pharmaceuticals in biological and environmental samples. However, in the case of ILs, their advantages over disadvantages and also the incomplete data on them prove the need for continuous interest and development in this area.

## 7. Conclusions

ILs as molecules with unique properties have been the subject of increased interest in recent years. Undoubtedly, the key issue is "green chemistry", which has set the direction of current research. Due to their huge potential, it is natural to use ILs in the search for solutions to many problems in modern laboratories, including their participation in analyzing pharmaceuticals in real samples. The use of analytical methods at various stages confirms the universality and enormous potential of this "solvent design". The application of various chromatographic and electrophoretic techniques and extraction methods together with the possibility of the use of ILs for a wide range of analytes prove that their contribution to the development of analytical methods is not overestimated. At the same time, the limitations that appear during their use show that success in experiments is not easy and this field of research requires further development.

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**Conflicts of Interest:** The authors declare that there are no conflict of interest.

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# Control of retention mechanisms on an octadecyl-bonded silica column using ionic liquid-based mobile phase in analysis of cytostatic drugs by liquid chromatography

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ABSTRACT

This study assesses the potential of using ionic liquids (ILs) as mobile phase additives to control the retention mechanism of four cytostatic drugs: doxorubicin hydrochloride (DOX), epirubicin hydrochloride (EPI), daunorubicin hydrochloride (DAU) and idarubicin hydrochloride (IDA). Chromatographic separations were performed on a C18 analytical column (Discovery C18 150 × 4.6 mm, 5 µm) using six IL anions and four methyl-substituted IL cations with different alkyl chain lengths (alone or with the additional methyl group on the aromatic ring), or with an allyl group added as a cationic substituent. Thus, a total of 17 different ILs were assessed. The aqueous formic acid solution and phosphate buffer were used to compare how mobile phase composition affected the behavior of the analyzed cytostatic agents in the presence of ILs. In addition, the impacts of IL concentration, phosphate buffer concentration, and phosphate buffer pH on the final results were also considered. The ability to change analyte retention without negatively impacting peak shape or analytical efficiency was also controlled via the tailing factor and number of theoretical plates. Based on the results, the tested ILs were classified as either effective or ineffective mobile phase additives for separation of anthracyclines and identification by LC-FL technique.



## 1. Introduction

Liquid chromatography (LC) is one of the most commonly used analytical techniques for determining a wide range of natural and synthetic compounds. LC is based on the interaction between the stationary phase, solute, and solvents, which determines the retention of analytes and, ultimately, the obtained chromatographic separation results in respect to selectivity, resolution and column efficiency. For example, the addition of common organic solvents such as methanol or acetonitrile to the aqueous solution in binary or ternary mobile phase systems allows for an increase in elution strength [1,2]. The retention time can be modified by changing the composition of the aqueous component of the mobile phase. As an example, Jones et al.'s analysis of various component counterions in mobile phases [3], including $ClO_4^-$, $H_2PO_4^-$, $BF_4^-$, $CF_3CO_2^-$, and $PF_6^-$, revealed that the interaction between analytes and counterions is dependent on the type of anion used, as their final results were significantly influenced by the anions' chaotropic properties. An important factor in understanding the underlying mechanisms of retention is the limitations that result from the presence of free silanol groups on the surface of stationary phases, which can lead to non-Gaussian shape and tailing of peaks, longer analysis times, and lower detection efficiencies [4].

Recently, a group of compounds – namely, ionic liquids (ILs) – has been applied as a mobile phase additive due to their ability to modify analyte retention and suppress silanol interactions [5]. In addition, ILs possess a number of physicochemical properties that make them environmentally friendly, including being non-flammable, thermally stable, and recyclable [6,7]. The structure of ILs is composed entirely of ions with an exactly equal number of positive and negative charges. These compounds consist of a large dissymmetrical organic cation (e.g. ammonium, sulfonium, phosphonium or oxonium cations, but in most cases, pyridinium, piperidinium and imidazolium cations having different alkyl chains) and a small organic (e.g. methylsulfate [$CH_3SO_4$], trifluoromethylsulphate [$CF_3SO_4$] and bis(trifluoromethylsulfonyl)imide [$N(SO_2CF_3)_2$]) or inorganic (e.g. chloride (Cl), tetrafluoroborate [$BF_4$], hexafluorophosphate [$PF_6$]) anion. Both IL ions are involved in the separation process [8]. Furthermore, the high viscosity and fluorescence capacity of ILs does not affect the pressure during chromatographic analysis or preclude their use in fluorescence detection [9,10]. In addition, despite a lack of complementarity of ILs

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with MS detection and problematic disposal of fluorine-based compounds, the potential of ILs in other instrumental analysis has been comprehensively investigated. Initial studies exploring the potential use of IL-based mobile phases as retention modifiers were performed by Kaliszan et al. [11], who examined ILs composed of imidazolium cations and [$BF_4$] or [$CH_3SO_4$] anions. Their results showed that these compounds influenced the retention factor differently during drug analysis using reverse-phase liquid chromatography (RP-LC) and thin-layer chromatography (TLC). Moreover, other studies have examined how different IL concentrations in the mobile phase influence the retention of particular compounds, how IL structure impacts the final analyte separation, and the degree to which the effectiveness of ILs depends on the pH or nature of the buffer that is used [12–15]. In previous reports concerning the development of chromatographic methods with the use of ILs as an additive to the mobile phase, only a limited number of IL-based anions, namely [$BF_4$], [$PF_6$], or [Cl] were tested [16]. Studies examining the addition of IL to the mobile phase have also confirmed changes in retention behavior for various types of stationary phases, with decreases being observed for both pentafluorophenyl (PFP) and octadecyl alkyl chain (C18) stationary phases [17].

ILs have been applied to control retention on the surface of chromatographic columns in research focusing on a wide range of analytes, including toxic substances, trace elements, bioactive compounds, and pharmaceuticals [18–20]. Predominant groups of pharmaceuticals have hydrophobic properties; hence, RP-LC utilizing an alkyl-bonded phase (C18 or related) is typically applied for the analysis of these compounds. On the other hand, a lot of them are basic compounds, which are positively charged at the time of separation and can interact with silanol groups on the surface of the stationary phase [21,22]. As already mentioned, this may decrease the column efficiency making the chromatographic analysis more complicated. In consequence, specific chromatographic conditions are required for effective separation of these basic drugs. Therefore, to correctly understand IL-based separation mechanisms, different analytical conditions should be carefully considered. For pharmaceuticals, methods utilizing ILs are most commonly applied for the separation of $\beta$-blockers or antibiotics [12,16]; in contrast, little is known about their application for the separation and analysis of anticancer drugs. Monitoring the level of anticancer drugs in bodily fluids is highly important, as these drugs have a narrow therapeutic index. Thus, appropriate dosage regimens and information on drug pharmacokinetics are crucial in pharmacotherapy, as they can improve treatment efficacy and help to avoid dangerous side effects [23,24].

In the present work, ILs were added to the mobile phase during LC-FL in order to assess their ability to enhance the C18 stationary phase's efficiency in analyzing four selected cytostatic drugs (i.e., anthracycline antibiotics): epirubicin hydrochloride (EPI), doxorubicin hydrochloride (DOX), daunorubicin hydrochloride (DAU), and idarubicin hydrochloride (IDA). Specifically, the influence of anions, cations, and substituents at the cation on the chromatographic determination of selected anticancer drugs was estimated for 17 different ILs. This comprehensive analysis included: six different IL anions ([$BF_4$], [Cl], [$PF_6$], [$N(SO_2CF_3)_2$], [$CF_3SO_4$], and [$CH_3SO_4$]); four different IL cations (imidazolium, pyrrolidynium, pyridinium, and ammonium) with various substituents, such as alkyl (alkyl = ethyl, butyl, hexyl, octyl) and allyl chains; and two methyl groups (methylimidazolium, dimethylimidazolium). Changes in the retention mechanisms were tested at different IL concentrations in the mobile phase. Furthermore, this study also examined the effect of different mobile phase pH values and phosphate buffer concentrations. While the determination of pharmaceuticals is often performed using phosphate buffers or acidified aqueous solutions [25], previous studies have also shown that buffer components and pH values can also affect IL behavior [14]. Finally, changes in the retention of the selected cytostatic drugs were evaluated by calculating the retention time ($t_R$), tailing factor ($T_f$), and a number of theoretical plates ($N_A$). It should be emphasized that the chromatographic separation of this group of drugs using an eluent containing ILs has not been previously reported in the literature.

## 2. Experimental

### 2.1. Chemicals and reagents

Epirubicin hydrochloride (EPI) (>98% purity), idarubicin hydrochloride (IDA) (>98% purity), and doxorubicin hydrochloride (DOX) (>98% purity) were purchased from Cayman Chemical Company (USA). Daunorubicin hydrochloride (DAU) (>98% purity) was obtained from Tocris Bioscience (Bristol, United Kingdom), while the HPLC-grade acetonitrile (ACN) and methanol (MeOH) were provided by J.T. Baker (Phillipsburg, NJ, USA). Analytical-reagent-grade disodium phosphate ($Na_2HPO_4$), sodium dihydrophosphate ($NaH_2PO_4$), and *ortho*-phosphoric acid ($H_3PO_4$) (85%) were purchased from POCH (Gliwice, Poland), while formic (HCOOH) acid was acquired from Sigma-Aldrich (St. Louis, MO, USA). The water used in the experiments was deionized using a Milli-Q system (Molsheim, France). The ILs used in this study were provided by Sigma-Aldrich (St. Louis, MO, USA) (Table 1).

### 2.2. Apparatus and chromatographic conditions

All experiments were carried out on an ACME 9000 system (Younglin Instrument Corporation, Anyang, The Republic of Korea) consisting of a pump (SP 930D), autosampler, (CTS30) thermostat, and fluorescence detector RF-20A XS (Schimadzu, Japan). Data analysis was performed using AutoChro-3000 software. The analytical column used in this research was a Discovery HS C18 (150 Å~ 150 × 4.6 mm, 5 µm, surface area: 300 $m^2$/g, carbon load: 20%) purchased from Supelco (Bellefonte, USA). The column temperature was set at 30 °C.

The tested aqueous phase components consisted of a 0.1% aqueous solution of formic acid or 10 mM and 40 mM phosphate buffer (adjusted to pH 3, 5, or 7) with the IL (see Table 1). To prepare tested mobile phases, each was mixed with acetonitrile in the proportion of 75:25 (*v/v*). All ILs containing [$BF_4$], [Cl], [$CH_3SO_3$], and [$CF_3SO_3$] anions were added to a 0.1% aqueous solution of formic acid and phosphate buffer at concentrations of 2.5, 5.0, and 10 mM. In addition, [AllylMIM][Cl] was used as the mobile phase component at a concentration of 20 mM, and ILs with [$PF_6$] and [$N(SO_2CF_3)_2$] anions were also added to the final solution at a concentration of 1.25 mM. Mobile phase without the addition of an IL was used as the reference mobile phase, and the pH phosphate buffer was made acidic by adding concentrated *ortho*-phosphoric acid (85%). The flow rate of the mobile phase was 1.3 mL/min, with an injection volume of 15 µL. Analytes were measured via FL detection, with an excitation wavelength of 487 nm and an emission wavelength of 555 nm. Each experiment for the tested ILs was conducted in triplicate.

### 2.3. Preparation of stock and standard solutions

Stock standard solutions of the anthracycline drugs were prepared in MeOH to a concentration of 100 µg/mL. Working standard solutions containing each of the four compounds were prepared by diluting the stock standard solutions with MeOH to the desired volumes. The concentration of each analyte in the mixture was 2.5 $\mu$g/mL. The working standard solutions at 2.5 and 1 ng/mL for each analyte were prepared by an appropriate dilution of the stock

**Table 1**
ILs used as mobile phase additives.

| IL | Cation | Anion | Molecular weight [g/mol] |
|---|---|---|---|
| $[C_2MIM][BF_4]$ | 1-ethyl-3-methylimidazolium | tetrafluoroborate | 197.97 |
| $[C_4MIM][BF_4]$ | 1–butyl–3-methylimidazolium | | 226.02 |
| $[C_6MIM][BF_4]$ | 1-hexyl-3-methylimidazolium | | 254.08 |
| | | | 240.05 |
| $[C_4MMIM][BF_4]$ | 1–butyl–2,3-methylimidazolium | | |
| | | | 237.05 |
| $[C_4MPyr][BF_4]$ | 1–butyl–4-methylpyridinium | | |
| $[C_2MIM][PF_6]$ | 1-ethyl-3-methylimidazolium | hexafluorophosphate | 256.13 |
| $[C_4MIM][PF_6]$ | 1–butyl–3-methylimidazolium | | 284.18 |
| $[C_6MIM][PF_6]$ | 1-hexyl-3-methylimidazolium | | 312.24 |
| $[C_8MIM][PF_6]$ | 1-octyl-3-methylimidazolium | | 340.29 |
| $[C_2MIM][Cl]$ | 1-ethyl-3-methylimidazolium | chloride | 146.62 |
| $[C_6MIM][Cl]$ | 1-hexyl-3-methylimidazolium | | 202.72 |
| [AllylMIM][Cl] | 1-allyl-3-methylimidazolium | | 158.63 |
| $[C_2MIM][N(SO_2CF_3)_2]$ | 1-ethyl-1-methylimidazolium | bis(trifluoromethylsulfonyl)imide | 391.31 |
| $[C_2MPyrr][N(SO_2CF_3)_2]$ | 1-ethyl-3-methylpyrrolidinium | | 394.35 |
| $[C_4MAmm][N(SO_2CF_3)_2]$ | 1–butyl–3-methylammonium | | 396.37 |
| $[C_2MIM][CF_3SO_4]$ | 1-ethyl-3-methylimidazolium | trifluoromethanesulfonate | 260.23 |
| $[C_4MIM][CH_3SO_4]$ | 1–butyl–3-methylimidazolium | methylsulfate | 250.32 |

standard mixture of 2.5 µg/mL with MeOH. All stock and standard solutions were stored at −20 °C until analysis.

## 3. Results and discussion

### 3.1. Effect of ILs structure on retention mechanism

The retention behavior of analytes in the presence of an IL-based mobile phase should be considered as the separate effect of cations and anions on chromatographic separation due to their dissociation in aqueous solutions. Although the simultaneous influence of both ions ($[C_2MIM]$, $[C_4MIM]$, and $[C_6MIM]$ cations and $[PF_6]$ and $[BF_4]$ anions) has been observed in previous works [16,26-27], it should be emphasized that these studies placed more focus on the cationic substituents and limited the IL selection process to testing those with different alkyl chain lengths, which are also the most common substituents in imidazolium ILs [26].

#### 3.1.1. IL cations and their substituents

The present study separately examines the influence of cations and their substituents on the chromatographic behavior of four anthracycline antibiotics. To this end, analyses were performed for four different IL cations: imidazolium $[C_4MIM][BF_4]$, pyridinium $[C_4MPyr][BF_4]$, pyrrolidinium $[C_4MPyrr][N(SO_2CF_3)_2]$, and ammonium $[C_4MAmm][N(SO_2CF_3)_2]$ (Table 1). The obtained results confirmed that analyte retention time ($t_R$) was lower after using IL at the concentration of 2.5 mM with the $[C_4MPyr]$ (from 4.85 min for DOX to 19.70 min for IDA) than obtained for IL with the $[C_4MIM]$ cation (from 5.23 min for DOX to 20.38 min for IDA) (Fig. 1A, Table 2). Previous studies on the behavior of ILs in separation chromatography have attributed the phenomena involving the alkyl chain of cations to electrostatic attraction between the IL alkyl chain and free silanol groups, and to repulsion between the positively charged column surface and the cationic solute [16]. The results of the current study relating to ILs with the same anion and alkyl chain length (i.e., $[C_4MIM][BF_4]$ and $[C_4MPyr][BF_4]$) revealed that an aromatic pyridinium ring with a 4′ methyl group could also affect the result (Table 2) s. For $[C_4MIM]$ cation, the methyl group was located in 3′ position of the imidazolium ring. Thus, in both tested ILs the methyl group was located in various positions in respect to butyl group located in 1′position of aromatic ring.

Findings of studies focusing on ILs with different alkyl chain lengths also suggest that the size of the cation may be more important than its chemical structure [16]. This is consistent with the results of the present study in reference to the sized cations that had different aromatic rings (imidazolium and pyridinium) and the same substituents. Therefore, the retention differences for cytostatic drugs were obtained for $[C_4MIM][BF_4]$ and $[C_4MPyr][BF_4]$ (5.23 vs. 4.85 min for DOX, 6.42 vs. 6.02 min for EPI, 12.92 vs. 12.33 min for DAU and 20.38 vs. 19.70 min for IDA, respectively – Fig. 1A). In addition, higher differences were observed in the analyte retention times obtained for the ILs with different anions ($[C_4MIM][BF_4]$ and $[C_4MPyr][BF_4]$ vs $[C_2MPyrr][N(SO_2CF_3)_2]$ and $[C_4MAmm][N(SO_2CF_3)_2]$). In the case of $[C_2MPyrr][N(SO_2CF_3)_2]$ and $[C_4MAmm][N(SO_2CF_3)_2]$, the presence of the $[N(SO_2CF_3)_2]$ anion seemed to decisively influence the high retention factor. After using both of these ILs, the retention time for the first analyte was always over 40 min (Fig. S1, Supplementary data). These differences in retention times of DOX (44.80 min after using $[C_4MAmm][N(SO_2CF_3)_2]$ and 55.10 min, when $[C_2MPyrr][N(SO_2CF_3)_2]$ was applied) were probably related to the presence of the butyl and ethyl chain for $[C_4MAmm][N(SO_2CF_3)_2]$ and $[C_2MPyrr][N(SO_2CF_3)_2]$, respectively.

As noted above, previous studies have emphasized the influence of the alkyl chain length on the mechanisms occurring on the surface of the stationary phase [28]. Fig. 1 shows that the retention time decreases as the alkyl chain length increases (1A vs 1B vs 1C), regardless of the type of anion that is used. For instance, when the separation was performed with the use of IL additive of $[C_6MIM]$, $[C_4MIM]$ and $[C_2MIM]$ cations with $[BF_4]$, the retention times of DAU were 8.63/12.33/14.55 min, respectively. When using the same IL cations, but with $[PF_6]$ those parameters were at the level of 8.67/23.50/28.72 min, respectively. In the case of using $[C_6MIM][Cl]$ and $[C_2MIM][Cl]$ the retention times were 7.12 and 10.20 min, respectively. This finding confirms previous results in this area. However, in order to better understand the role of substituents, which can also modify the interaction occurring during the chromatographic separation, we conducted analyses using mobile phases containing an IL with three substituents on the aromatic ring ($[C_4MMIM][BF_4]$) (Fig. 1A) and an allyl moiety ($[Allyl MIM][Cl]$) (Fig. 1C). A comparison of retention time results for the mobile phase with the same anion and alkyl chain length ($[C_4MIM][BF_4]$ (Fig. 1A)) enabled an independent assessment of the additional substituent's effect on $[C_4MMIM][BF_4]$. Thus, the retention times of the analytes after using $[C_4MMIM][BF_4]$ vs. ($[C_4MIM][BF_4]$ as the additive to the mobile phase were 4.75 and 5.23 min for DOX, 5.86 and 6.42 min for EPI, 11.90 and 12.92 min for DAU, and 16.87 vs. 20.38 min for IDA, respectively. As the results show, the presence of an additional methyl group on the aro-

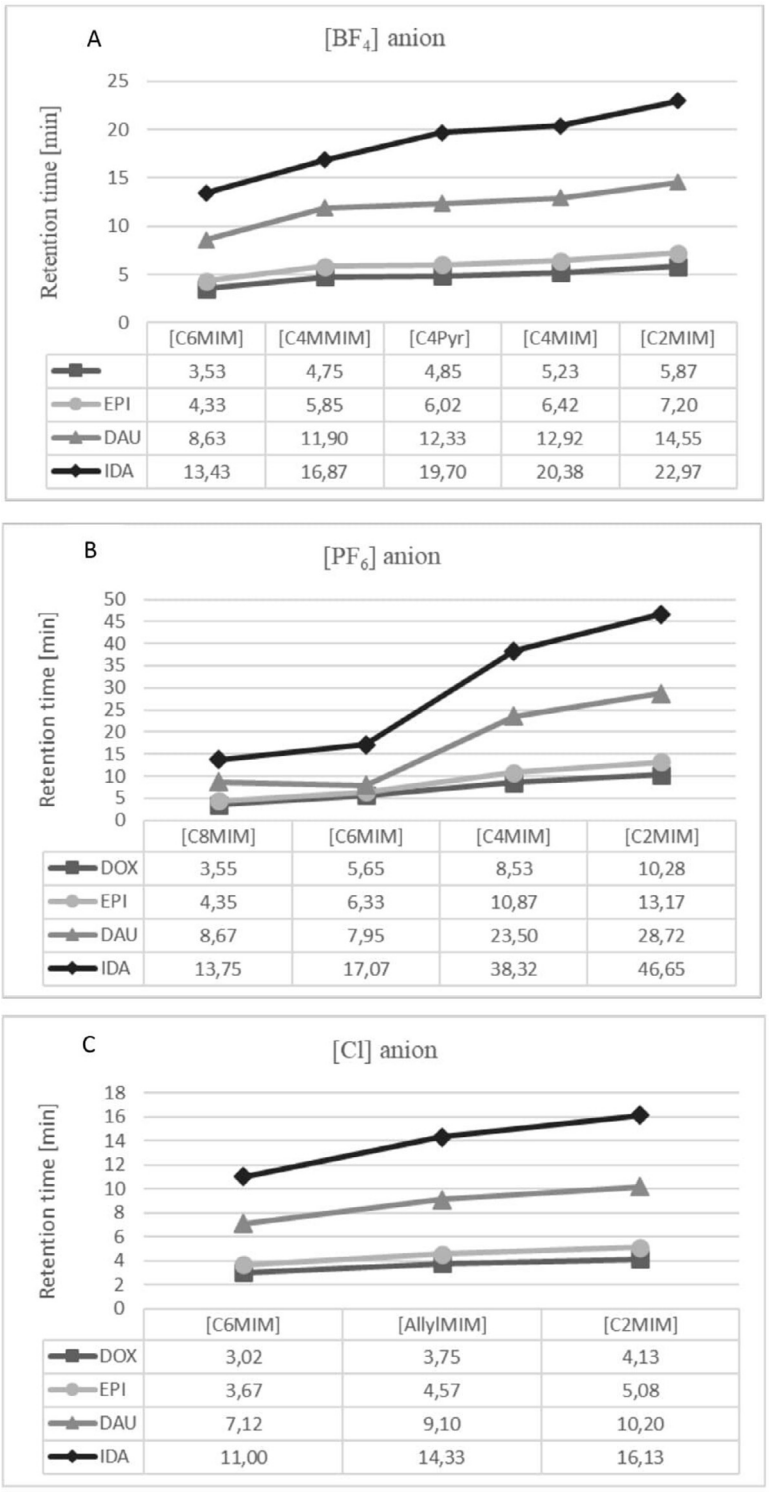


**Fig. 1.** Mean retention times of four anthracycline cytostatics obtained after chromatographic separation using a mobile phase with an IL consisting of: **(A)** a [$BF_4$] anion and [$C_6$MIM], [$C_4$MMIM], [$C_4$MPyr], [$C_4$MIM], or [$C_2$MIM] cations at concentrations of 2.5 mM; **(B)** a [$PF_6$] anion and [$C_8$MIM], [$C_6$MIM], [$C_4$MIM], or [$C_2$MIM] cations at concentrations of 1.25 mM; and **(C)** a [Cl] anion and [$C_6$MIM], [AllylMIM], or [$C_2$MIM] cations at concentration of 2.5 mM ($n$= 3).

matic ring of the ILs cation reduces the retention time of cytostatic agents, thus proving the involvement of both methyl groups in the cationic solute repulsion reaction. Therefore, the selection of the most efficient IL for use in a mobile phase should mainly depend on the substituent directed to both the surface of the chromatographic column and the analyte. Analyte retention for the mobile phase containing [AllylMIM][Cl] additives was stronger than that of the mobile phase containing [$C_6$MIM][Cl] (3.75 vs. 3.02 min; 4.57 vs. 3.67 min; 9.10 vs. 7.12 min and 14.33 vs. 11.00 min for DOX, EPI, DAU and IDA, respectively); however, it was weaker than the retention time for the mobile phase containing [$C_2$MIM][Cl] (4.13 5.08, 10.20 and 16.13 min for DOX, EPI, DAU and IDA, respectively (Fig. 1C). Thus, the presence of an unsaturated bond was not an important factor in this case, and the allyl substituent can be

**Table 2**
Mean values for retention time, peak height, number of theoretical plates, and tailing factor for four anthracyclines on a Discovery C18 column using a mobile phase composed of 0.1% formic acid and ACN (75:25, *v/v*), with different concentrations of ILs added to both mobile phase components ($n$= 3).

| IL | ILs conc. [mM] | DOX | | | | EPI | | | | DAU | | | | IDA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $t_R$ [min] | H | $N_A$ | $T_f$ | $t_R$ [min] | H | $N_A$ | $T_f$ | $t_R$ [min] | H | $N_A$ | $T_f$ | $t_R$ [min] | H | $N_A$ | $T_f$ |
| [$C_2$MIM] | **0** | **3.73** | **504.86** | **5755** | **1.06** | **4.62** | **156.75** | **5725** | **1.11** | **9.42** | **186.97** | **4518** | **1.05** | **15.13** | **172.87** | **8090** | **0.91** |
| [$BF_4$] | 10 | ↑ 6.37 | 264.95 | 9842 | 0.88 | 8.03 | 85.23 | 8997 | 0.86 | 17.25 | 82.77 | 7574 | 0.77 | 27.87 | 68.49 | 7174 | 0.80 |
| | 5 | ↑ 6.23 | 283.77 | 10,952 | 0.90 | 7.75 | 93.28 | 11,847 | 0.86 | 16.08 | 93.52 | 8652 | 0.77 | 25.60 | 74.25 | 6569 | 0.78 |
| | 2.5 | ↑ 5.87 | 317.77 | 12,205 | 0.92 | 7.20 | 99.56 | 11,711 | 0.87 | 14.55 | 102.94 | 9118 | 0.81 | 22.97 | 84.14 | 8202 | 0.81 |
| [$C_4$MIM] | 10 | ↑5.20 | 328.20 | 8920 | 0.94 | 5.50 | 110.39 | 9511 | 0.88 | 13.70 | 112.60 | 7275 | 0.77 | 22.22 | 94.21 | 6912 | 0.79 |
| [$BF_4$] | 5 | ↑5.18 | 304,71 | 7366 | 0.91 | 6.47 | 96.49 | 7678 | 0.90 | 13.62 | 96.77 | 7377 | 0.79 | 21.90 | 78.55 | 6958 | 0.79 |
| | 2.5 | ↑5.23 | 294.02 | 10,258 | 0.93 | 6.42 | 94.88 | 9506 | 0.87 | 12.92 | 95.07 | 8700 | 0.77 | 20.38 | 76.94 | 8194 | 0.81 |
| [$C_4$MPyr] | 10 | ↑ 5.57 | 322.62 | 7759 | 0.89 | 6.97 | 110.87 | 9213 | 0.91 | 14.60 | 118.80 | 7289 | 0.79 | 23.37 | 86.36 | 6711 | 0.79 |
| [$BF_4$] | 5 | ↑ 5.05 | 350.84 | 8468 | 0.90 | 6.30 | 125.13 | 7576 | 0.92 | 13.10 | 138.60 | 7666 | 0.78 | 20.98 | 102.79 | 6952 | 0.78 |
| | 2.5 | ↑ 4.85 | 348.82 | 8448 | 0.88 | 6.02 | 126.30 | 8448 | 0.88 | 12.33 | 137.77 | 7658 | 0.82 | 19.70 | 102.08 | 6749 | 0.80 |
| [$C_4$MMIM] | 10 | ↑ 5.08 | 357.97 | 10,272 | 0.94 | 6.3 | 117.87 | 11,183 | 0.94 | 13.13 | 122.6 | 8883 | 0.78 | 20.93 | 98.261 | 8455 | 0.77 |
| [$BF_4$] | 5 | ↑ 4.98 | 362.25 | 10,198 | 0.90 | 6.13 | 120.98 | 11,348 | 0.93 | 12.45 | 130.03 | 9069 | 0.78 | 19.67 | 102.90 | 8424 | 0.76 |
| | 2.5 | ↑ 4.75 | 365.54 | 9648 | 0.93 | 5.85 | 120.92 | 10,527 | 0.95 | 11.9 | 136.09 | 9463 | 0.79 | 18.77 | 104.94 | 8967 | 0.79 |
| [$C_6$MIM] | 10 | ↓ 3.35 | 476.17 | 8061 | 1.06 | 4.05 | 181.15 | 11,630 | 1.03 | 7.88 | 183.09 | 9175 | 0.83 | 12.73 | 152.12 | 8089 | 0.80 |
| [$BF_4$] | 5 | ↓ 3.47 | 457.25 | 8447 | 0.96 | 4.22 | 152.70 | 8548 | 0.96 | 8.28 | 188.71 | 9155 | 0.79 | 12.95 | 159.56 | 9362 | 0.79 |
| | 2.5 | ↓ 3.53 | 437.71 | 7438 | 1.07 | 4.33 | 150.39 | 8627 | 0.94 | 8.63 | 181.85 | 10,753 | 0.82 | 13.43 | 154.83 | 9358 | 0.78 |
| [$C_2$MIM] | 10 | ↑ 4.58 | 410.73 | 8054 | 0.93 | 5.68 | 147.14 | 7318 | 0.91 | 11.62 | 172.49 | 8172 | 0.81 | 18.45 | 126.91 | 7608 | 0.82 |
| [Cl] | 5 | ↑ 4.33 | 417.33 | 8382 | 0.97 | 5.35 | 147.18 | 8725 | 0.96 | 10.90 | 170.70 | 7698 | 0.83 | 17.32 | 128.89 | 8276 | 0.84 |
| | 2.5 | ↑ 4.13 | 444.35 | 6881 | 0.95 | 5.08 | 166.58 | 9299 | 0.92 | 10.20 | 195.01 | 8433 | 0.87 | 16.13 | 146.73 | 8114 | 0.81 |
| [AllylMIM] | 10 | ↑ 4.22 | 410.33 | 7110 | 1.02 | 5.20 | 153.07 | 9059 | 0.91 | 10.50 | 188.32 | 8821 | 0.83 | 16.63 | 139.47 | 8322 | 0.83 |
| [Cl] | 5 | ↑ 4.08 | 424.76 | 6691 | 1.02 | 5.03 | 161.90 | 8804 | 0.98 | 10.12 | 200.70 | 8611 | 0.85 | 15.93 | 153.58 | 8467 | 0.84 |
| | 2.5 | ↑ 3.75 | 456.62 | 10,329 | 0.97 | 4.57 | 174.46 | 9017 | 1.00 | 9.10 | 211.36 | 8354 | 0.86 | 14.33 | 156.26 | 8465 | 0.88 |
| [$C_6$MIM] | 10 | ↓ 2.95 | 580.78 | 6820 | 1.00 | 3.55 | 214.48 | 8255 | 1.03 | 6.88 | 318.04 | 8316 | 0.87 | 10.67 | 267.47 | 8000 | 0.79 |
| [Cl] | 5 | ↓ 3.02 | 577.30 | 5132 | 1.10 | 3.65 | 208.46 | 8321 | 1.03 | 7.10 | 308.59 | 9553 | 0.81 | 10.97 | 275.00 | 8779 | 0.79 |
| | 2.5 | ↓ 3.02 | 589.07 | 8090 | 1.05 | 3.67 | 219.16 | 8682 | 1.02 | 7.12 | 320.94 | 9927 | 0.86 | 11.00 | 284.66 | 9010 | 0.80 |

$t_R$ – time retention; H – height of the peak; $N_A$ – number of theoretical plates; $T_f$ – tailing factor.

considered as the standard substituent with three C atoms in the alkyl chain. As already mentioned, the shortest retention times for the anthracycline antibiotics were obtained using the IL with the longest alkyl chain; however, the use of ILs with longer alkyl chains has some limitations. For example, hydrophobicity increases as the length of the alkyl chain increases [29]. Consequently, it can be impossible to obtain a homogeneous aqueous phase by increasing the concentration of ILs with longer alkyl chains, despite the expected positive retention effect caused by more repulsion with cationic analytes, which gives shorter retention time of the analytes. This outcome was observed for the [$C_8$MIM][$PF_6$] with the octyl chain (Fig. 1B). Due to IL's high hydrophobicity among [$PF_6$]-based ILs, the highest concentration of it that could be tested in the mobile phase was 1.25 mM. However, it should also be noted that [$C_8$MIM][$PF_6$] yielded the shortest retention time for the tested analytes.

### 3.1.2. IL anions

Changes in the retention of analyzed compounds are due to the involvement of both ions. Furthermore, as previously reported, the interaction between the stationary phase and the solute increases or decreases depending on whether the IL cations or anions adsorb on the surface of the stationary phase. Anions that do not adsorb to column surface remain in the eluent in free form and can still react with the analytes. In our experiments, we tested IL-based mobile phases with six different anions. This group included popular anions ([$PF_6$], [$BF_4$], [Cl]), as well as those that are less frequently used ([$CF_3SO_4$], [$CH_3SO_4$]), and one that was being applied for the first time ([$N(SO_2CF_3)_2$]) (Table 1). The effect of the IL anions present in the mobile phase was briefly explained in Section 3.1.1. As Fig. 1 clearly illustrates, the addition of the popular ions led to a decrease in the retention of the cytostatic drugs such that [$PF_6$]> [$BF_4$]> [Cl]; this finding is consistent with those reported in the literature [27,30–31]. For example, the retention times of IDA in the presence of 2.5 mM [$C_2$MIM][$PF_6$], [$C_2$MIM][$BF_4$], and [$C_2$MIM][Cl] additives in the mobile phase were 46.65, 22.97 and 16.13 min, respectively. This effect is explained by the position of anions in the Hofmeister series [32]. A series showing the classification of ions according to their ability to salt out proteins is also used to determine their behavior in aqueous solutions. Thus, the cosmotropic (strongly hydrated) [Cl] anions were not adsorbed on the column, whereas the strongly chaotropic [$PF_6$] ions and the slightly weaker chaotropic [$BF_4$] anions exhibited strong adsorption on the column, thereby increasing analyte retention. Next, the analyses were extended to the application of less common anions, namely, [$CF_3SO_4$] and [$CH_3SO_4$]. The chromatograms shown in Fig. 2 were obtained for four anthracycline drugs with a mobile phase containing ILs consisting of 1-ethyl-3-methylimidazolium ([$C_2$MIM]) with [$CF_3SO_4$], [$BF_4$], and [Cl] anions, while Fig. 3 shows the results for these analytes with a mobile phase containing ILs consisting of 1–butyl–3-methylimidazolium ([$C_4$MIM]) with [$BF_4$] and [$CH_3SO_4$] anions. This approach made it possible to perform an independent assessment of these anions' effects on the final chromatographic separation for the four tested drugs. The results of this assessment indicated that the use of [$CF_3SO_4$] resulted in stronger retention (8.20/10.43/22.23/34.92 min for DOX, EPI, DAU and IDA, respectively – Fig. 2A) compared to the use of [$BF_4$] (5.80/7.20/14.55/22.91 min for DOX, EPI, DAU and IDA, respectively Fig. 2B) and [Cl] (4.13/5.08/10.20/16.13 min for DOX, EPI, DAU and IDA, respectively – Fig. 2C). Furthermore, the results showed that the use of [$BF_4$] led to a longer analyte retention times compared to [$CH_3SO_4$] (5.23 vs. 4.37 min for DOX, 6.42 vs. 5.47 min for EPI, 12.92 vs. 11.18 min for DAU and 20.38 vs. 17.63 min for IDA, respectively) (Fig. 3A vs. 3B). Analyses were also performed for three ILs containing [$N(SO_2CF_3)_2$]: [$C_2$MIM][$N(SO_2CF_3)_2$], [$C_2$MPyrr][$N(SO_2CF_3)_2$], and [$C_4$MAmm][$N(SO_2CF_3)_2$]. The results of these analyses indicated extremely strong adsorption of [$N(SO_2CF_3)_2$] on the column surface regardless of the cation present in the mobile phase, and, consequently, extremely long retention times (data not shown). In addition, the strong [$N(SO_2CF_3)_2$] adsorption required the col-

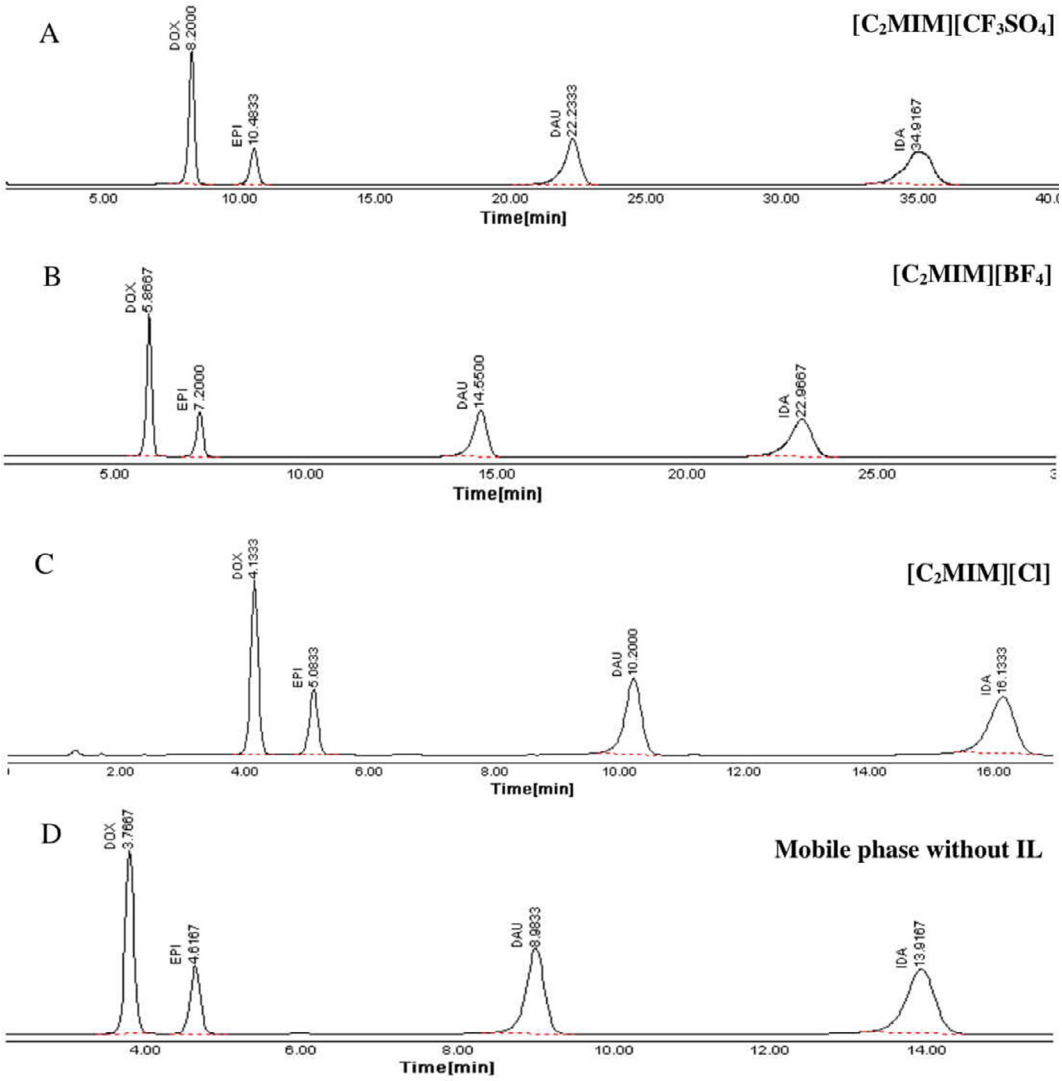


**Fig. 2.** Chromatograms of four anthracycline cytostatics with a mobile phase containing ILs consisting of a [$C_2$MIM] cation and **(A)** [$CF_3SO_4$], **(B)** [$BF_4$], or **(C)** [Cl] anions at a concentration of 2.5 mM. **(D)** Mobile phase without IL.

umn to be conditioned for several hours to restore it to its initial conditions. Finally, the results obtained for all of the tested anions revealed that they influence retention mechanisms, and have a greater impact on analyte retention compared to cations and their substituents. The decisive influence of the alkyl cation substituents on the final analyte separation process could only be observed when [Cl] and [$CH_3SO_4$] anions were present. It should also be emphasized that the [Cl] anion with a hexyl alkyl chain at the imidazolium ring reduces the retention time of cytostatic agents compared to the mobile phase without IL (Fig. S2). Therefore, in order to control retention mechanisms, researchers should focus on the anion when choosing mobile phase additives, because it may have impact on overall IL behavior in specific chromatographic conditions, and indirectly determine the IL cation effect on final separation of the tested analytes.

### *3.2. Effect of IL concentrations on retention mechanism*

Retention mechanisms can also be impacted by the concentration of ILs in the mobile phase. However, as noted above, altering the retention mechanisms in this way is only possible for ILs with moderate-to-low hydrophobicity. Preparing hydrated mobile phases that contain high concentrations of highly hydrophobic ILs was impossible due to problems with their solubility in water. As indicated in the previous section (Section 3.1.), the shortest retention times for the analyzed drugs were obtained when [Cl]-based ILs were tested. Thus, in order to verify the effect of IL concentration on analyte retention, chromatographic separations were performed for IL-based [Cl] anions ([$C_2$MIM][Cl], [AllylMIM][Cl], [$C_6$MIM][Cl]) and moderately chaotropic anions ([$C_2$MIM][$BF_4$], [$C_6$MIM][$BF_4$], [$C_4$MIM][$BF_4$], [$C_4$MMIM][$BF_4$], and [$C_4$MPyr][$BF_4$]). Table 2 shows the results of the analyses performed for the four anthracyclines using mobile phases containing ILs at concentrations of 2.5, 5.0, and 10 mM. The selected chromatograms obtained from the results are shown in Figs. S2-S4 (Supplementary Data). These data indicate that the retention times of the tested anthracyclines increased after IL addition to the mobile phase. Additionally, higher differences for analyte retention parameters were observed for LC separations performed with the mobile phases containing 2.5 and 5 mM of the tested ILs than those containing 5 and 10 mM concentrations of ILs (Table 2). For example, the retention times were 5.87/6.23/6.37 min for DOX, 7.20/7.75/8.03 min for EPI, 14.55/16.08/17.25 min for DAU and 22.97/25.60/27.87 min for IDA when [$C_2$MIM][$BF_4$] at the concentrations of 2.5, 5 and 10 mM was present in the mobile phase. In the experiments based on [AllylMIM][Cl] additive at the concentrations of 2.5, 5

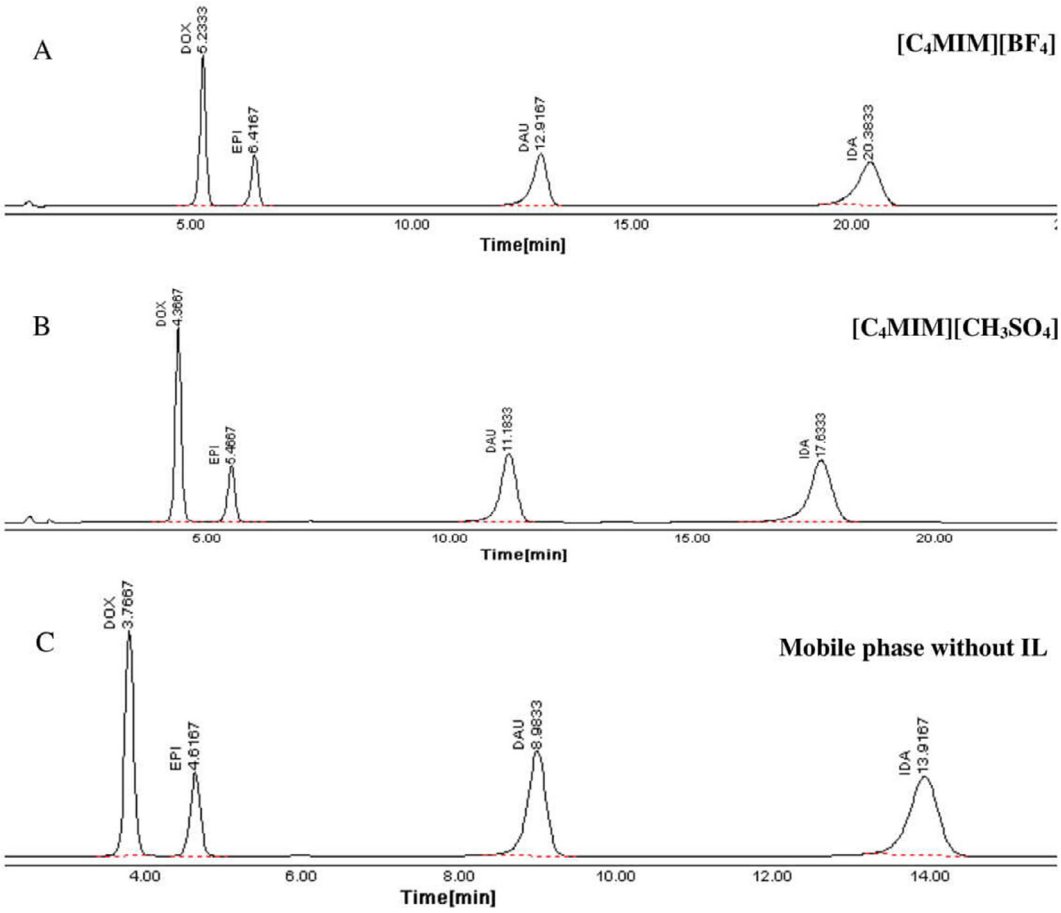


**Fig. 3.** Chromatograms of four anthracycline cytostatics with a mobile phase containing ILs consisting of a [$C_4$MIM] cation and **(A)** [$BF_4$] or **(B)** [$CH_3SO_4$] anions at a concentration of 2.5 mM. **(C)** Mobile phase without IL.

and 10 mM, the analyte retention times were 3.75/4.08/4.22 min for DOX, 4.57/5.03/5.20 min for EPI, 9.10/10.12/10.50 min for DAU and 14.33/15.93/16.63 min for IDA, respectively. The relationships between the concentration of IL and the analyte retention may be attributed to the following factors and mechanisms. When the concentrations of ILs increase slightly, interactions of IL cations with the alkyl groups of the stationary phase gradually strengthen, and an increase in the carbon content of stationary phase occurs, which finally results in the increase of the retention of analytes. The second factor affecting an increase in analyte retention is correlated with ILs possessing chaotropic anion, which may come from ion-pair creation between protonated cationic analyte and the counter-anion. Formation of neutral ion-pairs is possible when the environment possesses a lower dielectric constant. Thus, the presence of acetonitrile in the mobile phase composition decreases permittivity of the mobile phase (dielectric constant of acetonitrile – 37.5 and water – 78) and results in increasing strength of electrostatic interactions at these conditions [33]. In effect, IL having chaotropic anion, including also weak chaotropic anion such as [$BF_4$], can create ion-pair which as uncharged molecule is able for stronger interaction with the stationary phase, and this results in an increase in retention of the analytes. Above-mentioned processes are described by the anti-Langmuir isotherms achieving saturation level [34–39], after which the additive concentration does not influence on the retention. The results reported in Table 2 and the mechanisms of interactions indicated that a further increase in the concentration of IL will probably resulted in minimal changes in analyte retention. Therefore, the additive of IL to the mobile phase at the concentration of 10 mM was established as the limit. It should be also noted that the results of these analyses revealed two main trends. First, they indicated that the retention of the tested cytostatic drugs increased alongside the concentrations of [$C_2$MIM][$BF_4$], [$C_4$MPyr][$BF_4$], [$C_4$MIM][$BF_4$], [$C_4$MMIM][$BF_4$], [$C_2$MIM][Cl], and [$C_2$MIM][Cl] (Table 2). Thus, this effect was comparable for [Cl] and [$BF_4$] anions in the presence of ethyl or butyl alkyl chains. Second, the results indicated that analyte retention decreased as the concentration of [$C_6$MIM][$BF_4$] and [$C_6$MIM][Cl] increased. Both of these ILs have a hexyl substituent on the imidazolium cation, which makes it likely that this fragment of their structure was responsible for the observed effect. As previously reported, IL cations with longer alkyl chain are adsorbed more on the surface of the stationary phase (stronger hydrophobic interaction), thus lower IL concentration in the mobile phase can lead to a decrease in analyte retention as the results of repulsion forces between the adsorbed IL cations on the adsorbent surface and protonated analytes. Creation of bilayer electronic surface between IL cations (through electrostatic interaction) with IL cations connected with residual silanols also decreases analyte retention under the repulsive interactions (higher positive charged the stationary phase). This process is enhanced by cosmotropic IL anions. In consequence, a notable decrease in retention time was observed for all tested cytostatic drugs after the addition of [$C_6$MIM][Cl] to the mobile phase, and only a slight decrease in their retention times was noticed for [$C_6$MIM][$BF_4$] in comparison to the mobile phase without IL. Thus, the retention times of DOX, EPI, DAU and IDA without the presence of IL were 3.73/4.62/9.42/15.13 min, respectively, whereas after addition of [$C_6$MIM][Cl] to the mobile phase at the concentrations of 2.5, 5 and 10 mM, this parameter

was equal to 3.02/3.02/2.95 min (DOX); 3.67/3.65/3.55 min (EPI); 7.12/7.10/6.88 min (DAU) and 11.00/10.97/10.67 min (EPI), respectively. In the case of [$C_6$MIM][$BF_4$] added to the mobile phase at the levels of 2.5, 5 and 10 mM, the retention times of DOX, EPI, DAU and IDA were 3.53/3.47/3.35 min; 4.33/4.22/4.05 min; 8.63/8.28/7.88 min, and 13.43/12.95/12.73 min, respectively. The obtained results confirmed that changes in the retention of the selected anthracycline antibiotics depended on the dominant effect of the cation substituent (decrease in retention) or anion (increase in retention), and that this effect was enhanced at higher concentrations in the mobile phase. These data are consistent with previous reports in this area [5,13,30]. As it was mentioned above, another noteworthy finding was that the greater changes in retention of the analytes were observed when ILs were used at 2.5 and 5 mM, than at 5 and 10 mM. Thus, the results prove that low concentrations of ILs in the mobile phase are sufficient for modifying the retention of cytostatic drugs. Ultimately, our findings indicate that the optimal strategy for improving the retention of the four examined anthracyclines is the addition of [$C_6$MIM][Cl] to the mobile phase at a concentration of 2.5 mM. The separation of the tested cytostatics was performed in chromatographic conditions described in Section 2.2, and the application of 2.5 mM [$C_6$MIM][Cl] to the mobile phase containing 0.1% HCOOH facilitated the detection of anthracyclines at a concentration of 1 ng/mL, while the use of the same mobile phase without [$C_6$MIM][Cl] addition at 2.5 mM allowed the detection of these analytes at a concentration of 2.5 ng/mL.

### 3.3. Effect of IL concentrations on peak shape and tailing factor

Changes in retention require other separation parameters to be controlled, including tailing factor ($T_f$) and the number of theoretical plates ($N_A$) of the peaks. $T_f$ is a coefficient of asymmetrical peaks that provides information about whether a peak is fronting ($T_f < 1$) or tailing ($T_f > 1$) [21,22]. While ideal peak symmetry is obtained at $T_f = 1$, values in the range of 0.9–1.2 are also acceptable in most studies. Table 2 presents the $T_f$ values obtained via chromatographic separations of four anthracyclines without IL additive and after using eight different IL additives to the mobile phase. Thus, the $T_f$ values for the tested analytes without IL were in the range of 0.91–1.11. When the IL-based mobile phases were applied, the $T_f$ values for the tested analytes were within the acceptable range for the first two analytes (from 0.88 to 1.10 for DOX and between 0.86 and 1.03 for EPI, respectively). Slightly lower values were observed for the DAU and IDA peaks (0.77–0.87 and 0.76–0.88, respectively). Thus, for analytes with longer retention times, peak fronting occurs when IL is added to the mobile phase. It indicates that the presence of ILs in the mobile phase can cause a fronting peak, although other factors should be also considered (fronting peak of IDA without IL). The literature data indicate that both tailing and fronting peaks are related to surface heterogeneity of the stationary phase which depends on the manufacturing process of the chromatographic column [34]. When the adsorbent surface is covered with two types of adsorption sites having different adsorption constants, the interactions with different energies take part in these sites because of different equilibrium isotherms and different rates of mass transfer kinetics. In the case of tailing peak effect, silanol groups take part in the interaction occurring during chromatographic separation [21,22]. In the literature there are few reports reported by Gritti and Guiochon who fully described peak disturbances, including fronting peak [35–38]. This effect may appear when the two types of sites correspond most probably to two different environments in or around the alkyl ligands bonded to the adsorbent surface. Because the saturation capacity of the low energy sites is large, these sites correspond most probably to simple interactions with an alkyl group bonded to the surface. The involvement of free silanol groups at the silica surface in the formation of the high-energy sites is unlikely in these interactions. The authors also highlighted that only adsorbate interactions could explain the observed anti-Langmuir behavior of the isotherm with S-shape. Thus, adsorbate interactions are possible due to the formation of neutral ion pair complexes in the mobile phase, between the analyte cations and the anions presented in the mobile phase. The size, the valence, and the charge of the ions dissolved in the solution have an important influence on the isotherm parameters and possibly on the mass transfer kinetics. The fronting issue was also raised by Ruiz-Angel and Berthod [39], who used ILs as analytes. This previous study showed that the retention mechanism was based on a combination of hydrophobic and ionic interactions which produced concave adsorption isotherms with the Kromasil C18 stationary phase. In effect, severely fronting peaks for all tested imidazolium ILs were observed. The authors correlated these results with the chaotropicity of anions and hydrophobicity of cations, and the fact, that such type of isotherm is created when a synergistic effect occurs in the stationary phase related to adsorption of neutral ion-pair complex by the C18 stationary phase. These data are in accordance with observations in our study. Fronting peak was probably related to the ion-pair interactions between protonated cationic analyte and the counter-anion having chaotropic character, including weak [$BF_4$], which are stronger in the presence of acetonitrile as organic modifier in the mobile phase. In effect, the interaction between these unionized species and the stationary phase were also stronger and the sorption process could be described by adsorption isotherm resulted in a fronting peak. The data presented in Table 2 indicate that this mechanism was mostly responsible for this effect as higher differences in $T_f$ parameters in respect to calculated without IL were found for the ILs with [$BF_4$] than [Cl] anion. In addition, analyses of three IL concentration levels (2.5, 5, 10 mM) showed similar peak symmetries, indicating that IL concentration has little to no effect under such conditions. This is in accordance with the anti-Langmuir shape of isotherms describing the ion-pair creation process and adsorbate interactions providing full saturation capacity of the stationary phase, which are described by S-shape isotherms. Thus, when [$C_2$MIM][$BF_4$] at the concentration of 2.5, 5 and 10 mM was added to the mobile phase, $T_f$ values for DOX and EPI were 0.92/0.90/0.88 and 0.87/0.86/0.86, respectively. In the case of [$C_2$MIM][Cl] additive used at the same concentrations this parameter was 0.95/0.97/0.93 for DOX and 0.92/0.96/0.91 for EPI, respectively. Moreover, the greater fronting peak observed after the use of IL with the ethyl chain compared to the hexyl chain with the same anions can be explained by less spherical blocking of ion-pairs to interact with the stationary phase by IL having cation with shorter alkyl chain. Therefore, when the separation was carried out using 2.5, 5 and 10 mM of [$C_6$MIM][$BF_4$] additive to the mobile phase, $T_f$ parameter was 1.07/0.96/1.06 for DOX and 0.94/0.96/1.03 for EPI, respectively. In the case of the addition of [$C_6$MIM][Cl] at the same concentrations, the values for DOX were 1.05/1.10/1.00 and 1.02/1.03/1.03 for EPI, respectively. $N_A$ is the second parameter that is used to control column efficiency during retention time modification, with higher $N_A$ values being indicative of narrower and sharper chromatographic peaks. This parameter was calculated according to the Eq. (1):

$$N = \left(\frac{tr}{W}\right)^2 \quad (1)$$

where: $t_R$ – retention time, and W – peak width at baseline using the tangent line method. In most cases, the presence of IL improved the column efficiency for all tested analytes (Table 2). Thus, $N_A$ values for DOX, EPI, DAU and IDA were 5755/5225/4518/8090, respectively, when the separation was performed without IL. This parameter increased to 9648/10,527/9463 and 8967 for DOX,

EPI, DAU and IDA, respectively, after the addition of 2.5 mM [$C_4$MMIM][$BF_4$], and also from 8090 to 9927 for each respective analyte when 2.5 mM [$C_6$MIM][Cl] was used as the additive to the mobile phase. Among all of the analyzed drugs, the lowest NA values were observed for IDA. Nevertheless, seven out of eight ILs provided increased column efficiency for IDA when added to the mobile phase at the concentration of 2.5 mM compared to the mobile phase without the presence of IL. In summary, both the $T_f$ and NA made it possible to estimate the influence of IL in the mobile phase as a retention modifier, with no or slightly negative effects being observed with respect to $T_f$, whereas in most analysis with IL additive to the mobile phase, the $N_A$ parameter was elevated for each analyte.

### 3.4. Effect of IL-based mobile phase composition on retention mechanism

It is common practice to modify the composition of the mobile phases, especially through the addition of different buffers (mainly phosphate buffer). These buffers comprise the aqueous component of the mobile phase, and are effective at improving the separation results. In our previous work, 40 mM of phosphate buffer provided the best separation for one of the anthracyclines (EPI) [40]. The influence of phosphate buffer on the behavior of ILs during chromatographic separation has been detailed in [14]. In particular, the data suggested that the final retention of the compound of interest during analysis using a mobile phase with IL should be considered a summary effect of both the selected IL and the "environment" in which the IL is deployed. In the current study, a comparative analysis of the effect of adding [$C_4$MIM][$BF_4$] and [AllylMIM[[Cl] to phosphate buffer at various concentrations and pH levels during anthracycline separation was performed. The results of these comparative analyses are presented below.

#### 3.4.1. Effect of phosphate buffer concentration on retention mechanism

Figs. 4 and 5 show the chromatograms obtained for four anthracycline cytostatics when the LC analysis was performed on a mobile phase consisting of the phosphate buffer at concentrations of 10 mM and 40 mM (pH 3) with addition of [$C_4$MIM][$BF_4$] and [AllylMIM][C*l*], respectively. These ILs were selected based on previous findings, and two different exemplary retention behaviors: [$C_4$MIM][$BF_4$] features an adsorbing anion, which visibly increased retention; and [AllylMIM][Cl] features a cosmotropic, non-adsorbing [Cl] anion, which causes only a slight increase in retention. In the absence of IL, analyte retention increased when using 10 mM of phosphate buffer (Fig. 4A, Table S1) in relation to 0.1% aqueous formic acid (Table 2). This effect was further enhanced by increasing the phosphate buffer concentration to 40 mM (Fig. 5A, Table S1). Therefore, the retention times of the analytes calculated after using 0.1% HCOOH, 10 mM buffer phosphate at pH 3 and 40 mM buffer phosphate at pH 3 without IL as the aqueous component of the mobile phase were 3.73/4.45/4.80 min for DOX, 4.62/5.52/5.90 min for EPI, 9.42/11.20/12.10 min for DAU and 15.13/17.83/19.27 min for IDA respectively (Table 2, Table S1). The obtained results are in line with previous theoretical work regarding phosphate anion adsorption on the surface of the stationary phase [28]. Phosphate anions interact with cationic solute and enhance retention; however, when ILs are introduced, the effect of phosphate anions on the separation of analytes can change the interactions that occur on the surface of the stationary phase and with the cationic solute. The addition of 2.5 mM of [$C_4$MIM][$BF_4$] in phosphate buffer at 10 mM (pH 3) increased the retention of all analytes (5.07/6.32/13.18/21.02 min for DOX, EPI, DAU and IDA, respectively) (Fig. 4B, Table S1), while changes in retention due to the addition of an IL were negligible at higher phosphate buffer concentrations (5.00/6.17/12.78/20.38 min for DOX, EPI, DAU and IDA, respectively) (Fig. 5B, Table S1). The observed increase in retention was the result of the presence of the [$BF_4$] anion, which, similar to the phosphate anion, increased retention due to its adsorption onto the surface of the stationary phase and its interaction with the cationic solute. As already mentioned, the presence of acetonitrile in the mobile phase decreases water dielectric constant, which in turn increases the strength of electrostatic interactions between the ionized species increases at these conditions [33]. However, the chaotropic character of [$BF_4$] is stronger than phosphate anion, thus the ion-pair molecule based on this anion interacts more effectively with the stationary phase. It should be highlighted that the retention of analytes increased more effectively after the addition of 2.5 mM [C4MIM][BF4] to 10 mM phosphate buffer in comparison to IL addition to 40 mM phosphate buffer. Phosphate anions are able to suppress IL effects probably by more efficient competition of phosphate anions with [BF4] to interact with cationic solute, and hence the more concentrated phosphate buffer the more dominant impact of phosphate anions on chromatographic separation is observed. In effect, the stationary phase was blocked by phosphate anion based pairs, whereas the interactions with [BF4] based molecules with the stationary phase were reduced. This mechanism explains the reason why the effect of increasing analyte retention by IL additive was suppressed when 40 mM of phosphate buffer was applied. Changes in retention also occurred after the addition of 20 mM of [AllylMIM][Cl] to the phosphate buffer (Fig. 4C). To evaluate the effect of ILs with minimal anion adsorption to the stationary phase on analyte retention, which is additionally suppressed by the phosphate buffer, a higher concentration of IL was tested. The results of this test showed that, compared to the buffer without IL, the addition of [AllylMIM][Cl] to a 10 mM phosphate buffer reduced the retention of cytostatic drugs (4.25/5.22/10.55/17.13 min for DOX, EPI, DAU and IDA, respectively, Fig. 4C). Conversely, the observed changes in retention were negligible when this IL was added to a 40 mM phosphate buffer (4.63 vs. 4.80 min for DOX, 5.72 vs. 5.90 min for EPI, 12.10 vs. 11.70 min for DAU, and 18.68 vs. 19.27 min for IDA, respectively) (Fig. 5C vs Fig. 5A). Thus, the effects of higher concentrations of [AllylMIM][Cl] were muted by the respective effect of 40 mM of phosphate buffer. The decreased analyte retention observed for 10 mM of phosphate buffer and [AllylMIM][Cl] was probably due to the cosmotropic properties of the anion [Cl], which include a lack of adsorption affinity for the stationary phase. In addition, this effect can be supported by the IL cation and repulsion of the cationic solute. The presented results show that the IL's structure enables the reversal or reduction of the phosphate buffer's effects on retention time at lower concentration values. However, at higher phosphate buffer concentrations (e.g., 40 mM) the effect of the phosphate anions outweighs the effect of the IL, regardless of the IL's concentration. In this case, the observed effect was probably related to the interaction of phosphate anions with IL cations adsorbed on the surface of the stationary phase, which suppressed the cationic solute repulsion and in consequence weakened the effect of IL cations on the retention of analytes.

#### 3.4.2. Effect of the mobile phase pH on retention mechanism

The mobile phase pH affects the amount of ionized and non-ionized analytes as well residual silanols on the alkyl silica surface in a column, which means that also influences the retention mechanism. Residual silanols with a pKa between 3 and 7, depending on the type of silica, are weakly to strongly ionized within the working pH of typical RP-LC columns (2.5 ⟨ pH ⟩ 7.5). This gives rise to a negatively charged stationary phase, which can interact as a weak cation-exchanger and increase the retention of cationic solute as well as give broadening and tailing of chromatographic peaks. Thus, in the case of anthracyclines, which are basic drugs

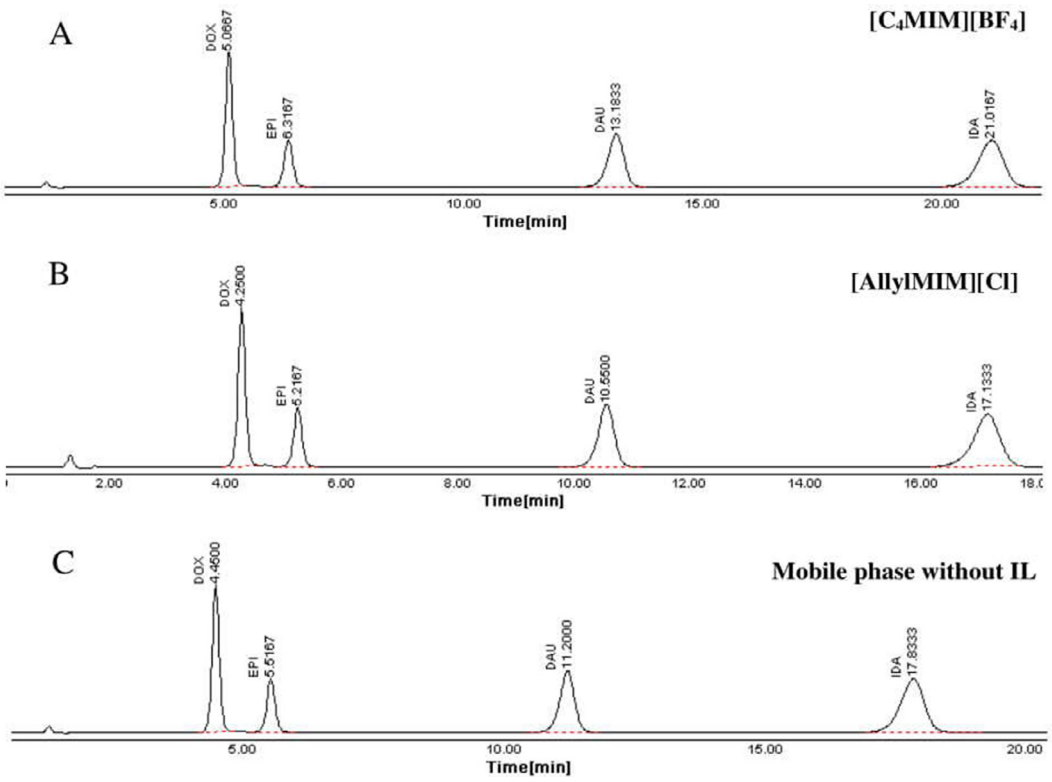


**Fig. 4.** Chromatograms of four anthracycline cytostatics with a mobile phase consisting of 10 mM of phosphate buffer (pH 3) **(A)** without IL, **(B)** with [$C_4$MIM][$BF_4$] at a concentration of 2.5 mM, and **(C)** with [AllylMIM][Cl] at a concentration of 20 mM.

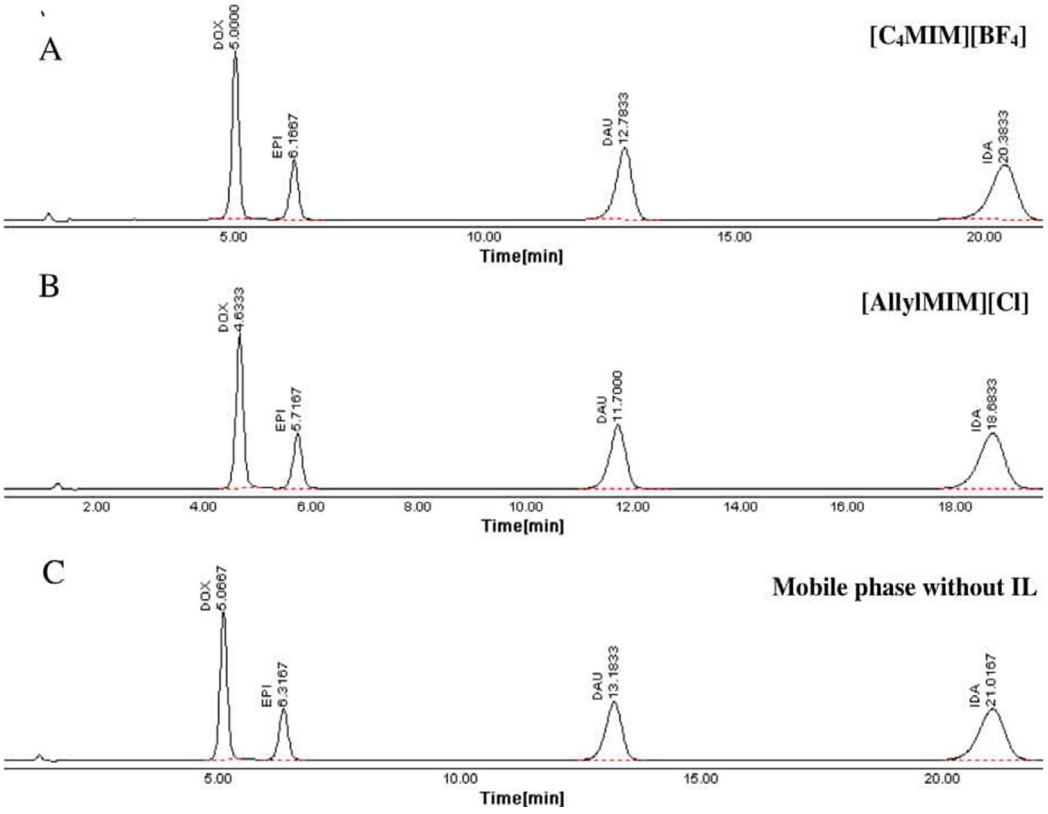


**Fig. 5.** Chromatograms of four anthracycline cytostatics with a mobile phase consisting of 40 mM phosphate buffer (pH 3) **(A)** without IL**, (B)** with [$C_4$MIM][$BF_4$] at a concentration of 2.5 mM, and **(C)** with [AllylMIM][Cl] at a concentration of 20 mM.

with a pKa of > 8, mobile phases with acidic pH can decrease the silanol-cationic solute interaction and consequently result in lower analyte retention rates and improved peak profiles (Fig. S5, Supplementary Data). In addition, the suppression of silanol ionization may also depend on the concentration of additives and, therefore, the amount of ionized species in the mobile phase that can interact with the free silanol groups, which results in more effective decrease in analyte retention time and reduction of tailing of analyte peaks. The representative chromatograms obtained during this part of the study are provided in Figs. S6 and S7. Table S1 shows the changes in time retention observed for 40 mM of phosphate buffer with and without [$C_4$MIM][$BF_4$] at three pH levels (pH 3, 5, and 7), as well as for 10 mM phosphate buffer at a pH of 3. For the mobile phases containing 40 mM of phosphate without and with IL, the retention of the tested anthracyclines at pH 3 was shorter than at pH 7 (e.g., 4.80 and 5.00 vs. 5.13 and 5.27 min for DOX; 12.10 and 12.78 vs. 13.22 and 13.62 min for DAU, respectively), which confirms reduction of ionization of free silanol groups and their subsequent interaction with basic analytes at lower pH. On the other hand, the addition of IL only slightly increased analyte retention at pH of 3, 5, and 7, which suggests that the amount of residual silanols on the surface of the tested Discovery HS C18 column was low. Moreover, the concentration of the phosphate buffer was likely a more dominant factor for all of the tested pH values than was the addition of IL. These data are consistent to those observed in the separation of anthracyclines using 10 and 40 mM phosphate buffers at pH 3.

When effect of the mobile phase pH on retention mechanism is considered, it should be also noticed that pH of the mobile phase depends on the volume of organic fraction in the mobile phase as well as the used IL additive because they generally have low dielectric constants (between 10 and 20 for the tested ILs). In effect, the permittivity and buffer capacity is lower, pH of the mobile phase decreases or increases depending on the used type of buffer, and decides about dissociation of the analytes [33,41]. In consequence, these factors can change IL behavior in specific chromatographic conditions in respect to described in our experiments, although the dominant group of IL used as additive to the mobile phase is not able to influence pH.

*3.4.3. Effect of the mobile phase pH on peak shape and tailing factor*

Changes in the retention of the tested anthracyclines using 10 and 40 mM of phosphate buffer at different pH values both with and without addition of IL were also evaluated using $T_f$ and $N_A$ values. The evaluation of the peak shape based on $T_f$ values clearly showed that the addition of IL to both 40 mM and 10 mM of phosphate buffer was appropriate for each tested pH value as it resulted in improved peak shape, or no difference in $T_f$ value was observed. Thus, the $T_f$ values for the tested analytes without IL additive to the mobile phase ranged from 0.85 to 1.16 for 40 mM phosphate buffer, and were between 0.89 and 1.04 for 10 mM phosphate buffer (Table S1). Additionally, in the experiments based on IL additive to phosphate buffer, little differences in $T_f$ depending on used pH were observed, although these values systematically increased for all analytes with increasing pH value. This trend was in accordance with a theory that more silanol groups are ionized at higher pH and the probability of tailing peak effect is increased [21,22]. The obtained results also confirmed that Discovery HS C18 column has probably low residual silanol groups, although surface heterogeneity of this stationary phase created anti-Langmuir shape of isotherm confirming the presence of adsorbate interactions between neutral ion pairs of cation analytes with IL anion and the surface of the stationary phase. This observation is consistent with data reported in Section 3.4.2. Furthermore, the $T_f$ values were close or within the acceptable range of 0.9–1.2 for both variants - with and without IL. Based on the obtained results, it can be concluded that, similarly as in the presence of ILs in aqueous solution of 0.1% HCOOH, addition of ILs to the phosphate buffer also enables the appropriate peak shape for all analyzed drugs.

The NA values for mobile phase without IL were higher for 40 mM of phosphate buffer with pH of 3 and 5, whereas the obtained values at a pH of 7 were comparable to those calculated for 10 mM of phosphate buffer at pH 3 (Table S1). For example, this parameter for DOX and EPI was calculated at the levels of 9217/7897/6152 and 9729/8431/6580, respectively, when 40 mM phosphate buffer at pH 3, 5 and 7 without IL was applied as an aqueous mobile phase component. In the experiments based on 10 mM phosphate buffer (pH 3) without IL, the NA values were 6160 for DOX and 6781 for EPI, respectively. Next, the NA parameters of the phosphate buffer (10 mM concentration at pH 3 vs 40 mM concentration at pH 3, pH 5, and pH 7) with [$C_4$MIM][$BF_4$] were compared; the results of this comparison revealed a number of complex differences. First, in the presence of IL, higher NA values were obtained for DOX and EPI when 40 mM of phosphate buffer with pH of 3 and 5 were used (8824 and 8307 for DOX as well as 8005 and 8687 for EPI). In contrast, the use of a mobile phase with a pH of 7 resulted in lower values for DOX (5528) and EPI (6614), comparable for DAU (8858) but higher values for IDA (10,681). Moreover, the use of IL in 10 mM of phosphate buffer (pH 3) more effectively improved peak performance for DOX and EPI (7525 and 7697, respectively) while the NA values for DAU and IDA were comparable to found without IL (8618 vs. 8830 for DAU and 7361 vs. 7436 for IDA, respectively). Contrary, the addition of IL to 40 mM of phosphate buffer at all tested pH values resulted in slightly lower NA values for most of the analytes.

As a summary, the analytical results were obtained for 17 ILs, namely pyridinium, piperidinium, ammonium, imidazolium IL cations, additional substituents at the cation, alkyl and allyl chains as well as for rarely tested anions, such as [$CF_3(SO_4)$], [$CH_3(SO_4)$], [$N(SO_2CF_3)_2$]. Moreover, the impact of different IL cations and anions on the separation of the analytes in various chromatographic conditions (i.e., 0.1% HCOOH in water or 10 and 40 mM phosphate buffers at pH 3 and 5) has been presented. The results showed that in order to decrease the retention of the analytes, it is recommended to use IL with non-adsorbing anions on the stationary phase, such as [Cl] anions. Additionally, the retention of analytes on the column can be controlled by the length of the alkyl chain at the IL cation (the retention of the analytes decreases as the length of the alkyl chain increases). To enhance analyte retention, the best option is to use IL with anions, such as [$BF_4$] or [$CF_3(SO_4)$]. In turn, the use of IL with [$N(SO_2(CF_3)_2$] anion is not recommended due to difficulties in restoring the initial chromatographic conditions. In addition, the application of IL with [$PF_6$] anion can be problematic as similarly to [$N(SO_2(CF_3)_2$] anion, it strongly adsorbs on the stationary phase, therefore this type of anion should be linked to the long-alkyl chain cation. Overall, using the [$C_6$MIM][Cl] at the concentration of 2.5 mM as the modifier of the mobile phase containing 0.1% HCOOH in water and acetonitrile (75:25, v/v) allowed to obtain the lowest retention time of the compounds of interest (3.02/3.67/7.12/11.00 vs. 3.73/4.62/9.42/15.13 min for DOX, EPI, DAU and IDA, with and without the IL, respectively), higher heights of the peaks (H) (589.07/219.16/320.94/284.66 vs. 504.86/156.75/186.97/172.87 for DOX, EPI, DAU and IDA with and without IL, respectively), and also higher values of number of theoretical plates ($N_A$) (8090/8682/9927/9010 vs. 5755/5725/4518/8090 for DOX, EPI, DAU and IDA with and without [$C_6$MIM][*Cl*], respectively) (Table 2). Therefore, the addition of [$C_6$MIM][Cl] to the mobile phase resulted in higher and narrower chromatographic peaks of the cytostatic drugs, and also facilitated a separation of compounds from the baseline noise signal. In consequence, the addition of [$C_6$MIM][Cl] at the concentration of 2.5 mM to the mobile phase facilitated the detection of all investigated cytostatic drugs

at the level of 1 ng/mL, while the use of the mobile phase in the same chromatographic conditions, but without the addition of IL allowed to detect the analytes only at a concentration of 2.5 ng/mL. Moreover, reduction of the retention of analytes by the addition of [$C_6$MIM][Cl] to the mobile phase allowed to decrease acetonitrile consumption during chromatographic separation. In addition, this study confirmed that the simultaneous use of ILs and phosphate buffer at higher concentrations in the mobile phase reduces the effect of IL addition due to the suppression caused by phosphate ions. Thus, the use of IL as an additive to the phosphate buffer is justified only at lower concentrations of this buffer, as at higher concentrations, the phosphate anion competes with chaotropic anion of ILs for the interaction on the stationary phase or, alternatively, interaction with alkyl chains of IL in the presence of cosmotropic anion can be observed, which ultimately also results in the suppression of the IL effects.

## 4. Conclusion

This study analyzed the impact of IL-based mobile phases on the retention mechanism of four cytostatic drugs: DOX, EPI, DAU, and IDA. Specifically, this research examined how the structure and concentration of 17 different ILs influenced analyte retention, and how the pH and composition of the mobile phase impact IL behavior during the chromatographic separation of drugs. The results indicated that the retention of analytes can be controlled by selecting the appropriate IL structure. Although anions and cation substituents are both involved in this process, their participation is not equal, as the behavior of anions in relation to the stationary phase and the mobile phase determines the effect of cations. Reducing the retention time of cytostatic agents when using a cation with a hexyl alkyl chain is possible only in the presence of anions not involved in interactions on the column surface. In addition, ILs with anions that possess moderate adsorption properties or that do not adsorb onto the column (i.e., [Cl], [$BF_4$], and [$CH_3SO_4$]) may be used as potential stationary phase modifiers for anthracycline separation; conversely, anions with strong adsorption properties (i.e., [$PF_6$], [$N(SO_2CF_3)_2$], and [$CF_3SO_4$]) are not appropriate for the pharmaceutical analysis of these cytostatic drugs. Furthermore, combining different types of cations (imidazolium, pyridinium, piperidine, and ammonium) with adsorbing anions ([$BF_4$] or [$N(SO_2CF_3)_2$]) did not affect the final results. Moreover, separation involvement was also demonstrated for substituents on cations aside from alkyl chains. Retention time was successfully reduced through the addition of an imidazolium cation methyl group. The anthracycline determination performed at different IL concentrations revealed that the most changes in retention occurred at the lowest IL concentrations. For most of the ILs, higher concentrations either increased the retention time of the analytes (strongly adsorbing anions) or did not affect it (cosmotropic anion and long alkyl chain). The influence of changes to the mobile phase composition (0.1% formic acid solution or phosphate buffer) on analyte retention was also observed, with the most important changes occurring after the addition of IL to the aqueous formic acid (0.1%) solution. In addition, the results also showed that the use of phosphate buffer suppressed IL performance, especially at higher concentrations. The findings also indicated that IL behavior is independent of changes in the phosphate buffer's pH. Retention control with ILs also ensures adequate separation efficiency, as evidenced by the obtained $T_f$ and $N_A$ parameters. Clear differences in $N_A$ values were observed when IL was added to the mobile phases, including improved column efficiency in most cases. Additionally, the shape of the peak was retained in mobile phases with and without IL, which was confirmed by the $T_f$ values. Thus, ILs can be used to control the retention mechanisms of anthracycline separation and improve the overall performance of the LC method without negatively affecting any other parameters. Moreover, currently, many harmful reagents are used in everyday laboratory practice, including large amounts of organic solvents, mainly acetonitrile or methanol. A large group of compounds in the form of ILs is generally considered to be environmentally friendly, although the thinking of ILs as completely "green" has changed in recent years [42]. The use of them at low levels as "green solvents" in the development of analytical methods will allow to change the trend and help reduce the use of harmful solvents.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Natalia Treder:** Conceptualization, Methodology, Investigation, Software, Writing – original draft, Writing – review & editing. **Ilona Olędzka:** Data curtion, Writing – original draft, Writing - review & editing. **Tomasz Bączek:** Investigation, Project administration. **Alina Plenis:** Conceptualization, Methodology, Writing – original draft, Writing - review & editing, Project administration, Supervision.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462257.

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# Practical and theoretical considerations of the effects of ionic liquids on the separation properties of phenyl-based stationary phases in reversed-phase liquid chromatography

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ABSTRACT

The use of ionic liquids (ILs) as mobile phase additives in reversed-phase liquid chromatography (RP-LC) can influence the dynamic coating of the stationary phases and even allow them to change their chromatographic properties. In this study, the interaction between thirteen different ILs added to 0.1% HCOOH (mobile phase) and the functional groups on phenyl and pentafluorophenyl stationary phases was investigated via RP-LC analysis and the fluorescence detection (FL) of four basic aromatic analytes (anthracycline antibiotics). In addition, an octadecyl column was included in the experiments to investigate potential differences in how the aromatic and alkyl stationary phases interacted with the tested ILs. Changes in behaviors of the studied analytes in the absence and presence of tested ILs were evaluated based on retention factor (*k*), peak area (A), number of theoretical plates ($N_A$) and tailing factor ($T_f$). Furthermore, hierarchical cluster analysis (HCA) and *k*-means approach were used to better visualize found relationships between the chromatographic parameters of anthracyclines for each column. The obtained results indicated that π-π interactions occurring on the phenyl-based stationary phases resulted in more pronounced effects from the ILs compared to those occurring on the alkyl stationary phase. Different subclusters of the k, A, $N_A$, and $T_f$ parameters detected by the HCA confirmed that the addition of ILs leads to various changes in the chromatographic parameters of the studied analytes. These observations were additionally confirmed by k-means calculations. Overall, our findings show that selecting the appropriate IL for addition to the mobile phase can significantly reduce analysis time and improve peak shape and column performance when using aromatic stationary phases.

## 1. Introduction

Silica-based stationary phases functionalized by different alkyl chain lengths are the most common type of stationary phases used in reversed-phase liquid chromatography. As such, much prior work has focused on separation mechanisms that are based on hydrophobic interactions between these stationary phase and the analytes and/or retention modifiers present in the mobile phase [1–3]. Unfortunately, the exclusive use of alkyl columns can lead to insufficient chromatographic separation of the analytes [4]. This limitation clearly indicates the need to examine and evaluate other types of stationary phases, particularly those capable of offering a greater number of interactions with analytes and mobile-phase modifiers compared to alkyl-bonded stationary phases. For instance, phenyl-based stationary phases are able to provide different separation, as they contain an electron-rich phenyl ring and facilitate strong π-π interactions with the analytes in the mobile phase [5–8,9]. The greater polarizability of phenyl-based phases also fosters dispersion interactions between the stationary phase and the analytes, which can further support these π-π interactions. Consequently, compared to alkyl columns, phenyl-based columns provide much stronger analyte retention, albeit with longer overall analysis times. Furthermore, the presence of π-π interactions can protect against adverse interactions between analytes and silanol residues on the column, thus enabling improved efficiency and symmetry among the acquired peaks [10]. However, the term, "phenyl phase" refers to a large group of columns that use molecules with an aromatic ring in their structure. Thus, the final

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physicochemical properties of a column can vary due to a variety of factors, including: the morphology of its silica substrate; its bonding density; the length of the chain of the spacer arm between the silicon and phenyl ring; whether an oxygen atom has been incorporated into the spacer arm (e.g., phenoxyethyl); and whether the column has been endcapped with a reagent containing neutral or hydrophilic substituent groups [11]. Pentafluorophenyl-based stationary phase is a type of phenyl phase that can be used as an alternative to alkyl phases, as it possesses both normal-phase and retention-reversed-phase features for polar solutes, though its use is strongly dependent on the composition of the mobile phase. Both phenyl- and pentafluorophenyl-based phases contain a phenyl ring that enables π-π interactions with the analytes, but the strong inductive effect of the fluorine (F) atom on the carbon–fluorine bond increases the aromaticity of this ring and creates dipole-type and charge-transfer interactions, which further enhance analyte retention [6,12].

Various mobile-phase additives are routinely applied in liquid chromatography to improve analyte separation on the stationary phase [13]. This group of mobile phase additives is constantly being enriched with new compounds, including different ionic liquids (ILs), and subsequently tested for their efficacy in LC-based analysis [14]. In contrast to other additives, cations and/or anions of particular ILs can contribute to analyte separation, as they can adsorb onto the stationary phase depending on their position in the lyotropic order. For ILs composed of cations with an imidazolium ring and a kosmotropic anion (e.g., [Cl]), only the cations are preferentially adsorbed onto the hydrophobic stationary phase ligands due to their chaotropic character (they are weakly hydrated, so their position in the lyotropic order is high), while the adsorption of [Cl] is minor (they are strongly hydrated). Consequently, IL cations are mainly involved in the interactions occurring on the surface of stationary phase. On the other hand, adsorption on the stationary phase is consistent with both the chaotropy of the anion and the lyotropicity of the cation when an IL with a chaotropic anion is used, which results in the formation of a double-layered electronic structure on the column surface [14]. Thus, the interaction between a cation or anion in an IL and the stationary phase is directly dependent on its hydrophobicity. However, current knowledge relating to the interaction between ILs in the mobile phase and the surface of the stationary phase is largely limited to alkyl columns [3,14–16]. Indeed, very little is known about the simultaneous use of IL-based mobile phases and their effects and interactions with aromatic stationary phases. The potential of ILs in chromatographic analyses using aromatic stationary phases has been demonstrated for the separation of nucleic acid analogs [11] or plant alkaloids [17], while Stepnowski et al. have demonstrated the use of phenyl-based stationary phases to separate ILs used as analytes, but not as mobile phase additives [18].

Notably, no prior work has examined the use of ILs as mobile phase additives in basic pharmaceuticals analyses employing aromatic stationary phases. Thus, the present study comprises a profound and detailed investigation of the chromatographic properties of phenyl-based columns in the presence of ILs as mobile phase additives. To this end, thirteen different ILs were selected, and the effects of their anions and cations were independently evaluated with respect to the chromatographic separation of four aromatic anthracyclines with different polarities, namely, doxorubicin hydrochloride (DOX), epirubicin hydrochloride (EPI), daunorubicin hydrochloride (DAU), and idarubicin hydrochloride (IDA). The following parameters were calculated for all analytes in each phenyl- and pentafluorophenyl-based stationary phase with and without the evaluated ILs: retention factor ($k$), peak area (A), number of theoretical plates ($N_A$), and tailing factor ($T_f$). Furthermore, the use of an octadecyl stationary phase was also analyzed to obtain a deeper understanding of how ILs interact with aromatic and with alkyl stationary phases. The obtained datasets were then used to perform a hierarchical cluster analysis (HCA) and $k$-means method to better visualize how the presence of particular ILs altered the chromatographic parameters for each analyte. To the best of our knowledge, an analysis of this depth—namely, the use of thirteen different ILs with structures as mobile phase additives during chromatographic separation employing phenyl-based stationary phases—has not previously been reported in the literature.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals used in this work were analytical grade. Standards of epirubicin hydrochloride (EPI), idarubicin hydrochloride (IDA), and doxorubicin hydrochloride (DOX) were purchased from Cayman Chemical Company (USA), and a standard of daunorubicin hydrochloride (DAU) was purchased from Tocris Bioscience (Bristol, United Kingdom). Acetonitrile (ACN) and methanol (MeOH) were provided by J.T. Baker (Phillipsburg, NJ, USA), while formic acid and uracil were acquired from Sigma-Aldrich (St. Louis, MO, USA). The water used in the experiments was purified using a Milli-Q system (Molsheim, France). The ionic liquids (ILs), namely, 1-ethyl-3-methylimidazolium tetrafluoroborate [$C_2$MIM][$BF_4$] (IL1) (>98% purity), 1-hexyl-3-methylimidazolium tetrafluoroborate [$C_6$MIM][$BF_4$] (IL2) (>97% purity), 1-butyl-2,3-dimethylimidazolium tetrafluoroborate [$C_4$MMIM][$BF_4$] (IL3) (>97% purity), 1-butyl-4-methylpirydynium tetrafluoroborate [$C_4$MPyr][$BF_4$] (IL4) (>97% purity), 1-ethyl-3-methylimidazolium hexafluorophosphate [$C_2$MIM][$PF_6$] (IL5) (>99% purity), 1-butyl-3-methylimidazolium hexafluorophosphate [$C_4$MIM][$PF_6$] (IL6) (>97% purity), 1-hexyl-3-methylimidazolium hexafluorophosphate [$C_6$MIM][$PF_6$] (IL7) (>97% purity), 1-octyl-3-methylimidazolium hexafluorophosphate [$C_8$MIM][$PF_6$] (IL8) (>95% purity), 1-ethyl-3-methylimidazolium chloride [$C_2$MIM][Cl] (IL9) (>98% purity), 1-hexyl-3-methylimidazolium chloride [$C_6$MIM][Cl] (IL10) (>97% purity), 1-allyl-3-methylimidazolium chloride [AllylMIM][Cl] (IL11) (>97% purity), 1-ethyl-3-methylimidazolium trifluoromethanesulfonate [$C_2$MIM][$CF_3SO_4$] (IL12) (>98% purity), and 1-butyl-3-methylimidazolium methylsulfate [$C_2$MIM][$CH_3SO_4$] (IL13) (>97% purity) were obtained from Sigma-Aldrich (St. Luis, MO, USA). The chemical structures of the tested ILs are provided in Table S1.

### 2.2. Columns

The phenyl-based stationary phases—Synergi Polar-RP 80A and Fluorophase PFP—used in this study were acquired from Phenomenex (Torrance, USA) and Thermo Electron (Massachusetts, USA), respectively. The alkyl-bonded stationary phase (Synergi Hydro-RP 80A) used in this work was supplied by Phenomenex (Torrance, USA). The detailed parameters of all applied columns are listed in Table 1.

### 2.3. Apparatus and chromatographic conditions

All experiments were performed on an ACME 9000 system (Younglin Instrument Corporation, Anyang, The Republic of Korea) equipped with pump (SP 930D), autosampler, (CTS30) thermostat, and fluorescence detector RF-20A XS (Schimadzu, Japan), while experimental data was collected and integrated using AutoChro-3000 software. The mobile phase consisted of a 0.1% aqueous solution of formic acid with and without the addition of particular ILs and ACN (75:25, *v/v*). The concentration of each IL in the mobile phase was 2.5 mM, the flow rate was 1.3 mL/min, the injection sample volume was 15 μL, and the column temperature was 30 °C. The analytes were measured via FL detection with an excitation wavelength of 487 nm and an emission wavelength of 555 nm. For each tested stationary phase, the mobile phase without added IL was used as the reference phase. To confirm that the initial conditions of LC-FL system were stable prior to the analysis of next IL as mobile phase additive, the analysis of the working mixture solution of anthracyclines at 2.5 mM concentration using mobile phase without additives were performed. Moreover, 20 min equilibration of LC-FL

**Table 1**
Basic characteristics of the columns tested.

| Abbreviation | Trade Name | Bonded Phase | Surface Area ($m^2/g$) | %C | Dimensions, Particle Size | Poro Size (A) |
|---|---|---|---|---|---|---|
| Phenyl | Synergi Polar-RP 80A | Ether-linked phenyl with polar endcapping | 475 | 11 | 150 × 4.6 mm, 4 μm | 80 |
| Pentaflouorophenyl | Fluorophase PFP | Pentafluorophenylpropyl, endcapped | N.A. | 12 | 150 × 4.6 mm, 5 μm | 100 |
| Alkyl | Synergi Hydro- RP 80A | C18 with polar endcapping | 475 | 19 | 150 × 4.6 mm, 4 μm | 80 |

N.A. – data are not available.

system was applied each time before the analysis of the mobile phase with new tested IL. The same procedure was used before each successive IL-based mobile phase analysis.

### 2.4. Preparation of stock and standard solutions

Standard stock solutions for DOX, EPI, DAU, and IDA at a concentration of 100 μg/mL were prepared in MeOH. The working mixture solution of the four anthracyclines was obtained by combining and diluting the stock solutions in MeOH to a concentration of 2.5 μg/mL. All stock and standard solutions were stored at − 20 °C until analysis.

### 2.5. Data analysis

Hierarchical cluster analysis (HCA) with Ward's method of agglomeration and the Chebyshev distance measure as well as *k*-means method were employed for the chemometric assays of the chromatographic parameters (*k*, A, $N_A$, and $T_f$) of the anthracyclines obtained without IL and after adding each of the thirteen ILs to the mobile phase in the phenyl (Table S2), pentafluorophenyl (Table S3), and alkyl (Table S4) columns. Conversely, Statistica 13.3 software (StatSoft, Tulsa, USA) was applied for the multivariate analyses. Prior to the HCA, each variable was standardized and the chemometric calculation was performed using matrix data of 4 objects × 56 variables for each tested column. The tested stationary phases are detailed in Table 1, and the numbering of the ILs reported in Section 2.1 and Table S1 remained unchanged in the chemometric calculations.

## 3. Results and discussion

The current dynamic development of various mobile phase additives highlights the need for a thorough analysis of mechanisms underlying the interactions that occur during chromatographic separations. However, prior studies examining the interactions between stationary phases and the ILs used as mobile phase additives have mainly focused exclusively on alkyl-based stationary phases [14,15,19–21]. The results of these studies have clearly indicated that changes in analyte retention following the addition of ILs to the mobile phase are related either to the hydrophobic interactions between the alkyl chains in the stationary phase and the alkyl chains of the IL cations, or to the alkyl chains of the stationary phase and IL anions. Conversely, knowledge relating to the interactions between ILs added to the mobile phase and phenyl-based stationary phases is highly limited. Nonetheless, a few studies have emphasized that changes in retention are related to more complex interactions, possibly due to the presence of a phenyl ring on the surface of the stationary phase [11,17,18]. Additionally, it should be noted that even if ILs are rarely used in LC-MS methods because their addition can be responsible for IL condensation and pollution in ionization source in mass spectrometry and their toxicity of is still under discussion [22,23], however, their application of as additive to the mobile phase at low concentration could assist in performing analyzes using the ultraviolet (UV), electrochemical (EC) and fluorescence (FL) detectors by the improvement of chromatographic separation and increasing sensitivity of these detectors when potential risk for environmental can be considered to be minimal. This paper examines theoretical considerations that can enable a more in-depth understanding of how ILs affect analyte retention in aromatic stationary phases, as well as the practical aspects of using particular ILs in the analysis of anthracyclines. Anthracyclines represent basic, high molecular weight analytes, which structurally belong to the tetracyclic molecules with an anthraquinone backbone linked to a sugar moiety by a glycosidic bond (Fig. S1). Due to such a structure, they may interact in numerous ways during chromatographic separation; thus, they are optimal analytes for use in analyses of how ILs can impact separations performed using phenyl and pentafluorofenyl stationary phases (Table 1). To compare how the addition of particular ILs impacted both types of stationary phases, the analyses with the alkyl stationary phase were also performed using the same chromatographic conditions. In the first stage, experiments were performed without the addition of ILs in order to assess how the physicochemical properties of tested stationary phases influenced the behavior of the analytes. Once these analyses had been completed, a second round of tests was conducted with the addition of 13 selected ILs with different chemical structures (Table S1).

### 3.1. Mobile phase without IL additives

In order to differentiate between interactions resulting from the presence of ILs in the mobile phase and those occurring in the absence of ILs, initial analyses were performed using a mobile phases consisting of 0.1% HCOOH in water and ACN (75:25, *v/v*), with no other additives. The *k*, A, $N_A$, and $T_f$ values for each analyte were calculated based on the obtained chromatograms. The *k* value was established according to the formula,

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

where $t_R$ is the retention time of the analyte, and $t_0$ is the retention time for the unretained peak (uracil). The $N_A$ value was calculated according to the formula,

$$N = \left(\frac{t_R}{W}\right)^2 \quad (2)$$

where W is the peak width at baseline as determined via the tangent line method. The $T_f$ value was calculated according to the following equation:

$$T_f = \frac{W_{0.05}}{2f} \quad (3)$$

$$T_f > 1.0 = Tailing$$

$$T_f < 1.0 = Fronting$$

where $W_{0.05}$ is the width of the peak at 5% height, and *f* is the distance from the point at peak midpoint to the tailing edge.

The chromatographic parameters of the four anthracyclines calculated for the two phenyl-based stationary phases (i.e., Synergi Polar column and Fluorophase column), without the addition of IL to the mobile phase, are presented in Table S2 and Table S3, respectively. The results obtained for alkyl-bonded stationary phase (i.e., Synergi Hydro) are presented in Table S4. Fig. 1 A-D shows the values of the *k*, A, $N_A$, and $T_f$ parameters for IDA obtained in the above-described experiments.

According to the results, the retention of anthracyclines was much stronger for phenyl-based columns than that for the alkyl column. Specifically, analyte retention was strongest in the pentafluorophenyl-based

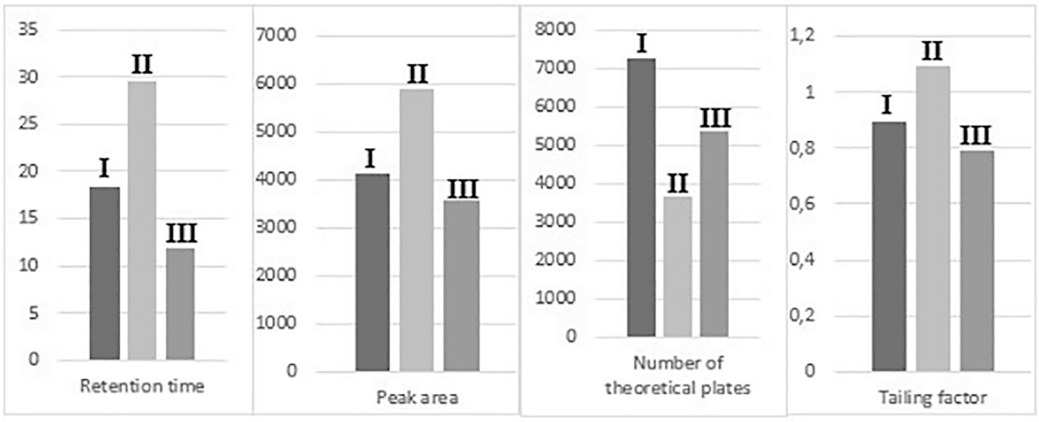


**Fig. 1.** Retention factor (*k*), peak area (A), number of theoretical plates ($N_A$), and tailing factor ($T_f$) for IDA obtained on phenyl (I), pentafluorophenyl (II), and alkyl (III) stationary phases using of mobile phases without the addition of IL (n = 3).

column, followed by the phenyl-based column, and finally the alkyl column (Table S2-S4). One potential explanation for this outcome may be the presence of π-based electrons in the phenyl-based stationary phase and not in the alkyl stationary phase, as these electrons can create additional retention mechanisms. The examined anthracyclines are aromatic compounds (Fig. S1) and may participate in π-π interactions with the aromatic ring of the phenyl-based columns. In addition, the presence of an ether bond in the Synergi Polar column increases the aromaticity of the phenyl group and ensures stronger π-π type interactions with conjugates. Furthermore, the ethoxy group on the surface of the Synergy Polar column facilitates hydrogen bonding with the analytes [12] and may be an important mechanism during anthracycline separation, as these compounds, due to their $pK_a > 8$, may occur in the acid mobile phase (0.1% HCOOH) in the form of positively charged ions ($–NH_3^+$). In such conditions, H-bonding interactions may occur between the oxygen atom in the stationary phase's ethoxy group and the hydrogen atom in the $NH_3^+$ group of the analytes. However, based on the calculated *k* values, such interactions were significant only for IDA (Table S2, Fig. 1A), likely due to the lack of a methoxy group in position 4 in IDA's chemical structure (Fig. S1). Ultimately, the pentafluorophenyl-based stationary phase's (Table S3) stronger analyte retention was likely due to the phenyl ring's increased aromaticity in the presence of fluorine atoms, which in turn created stronger π-π interactions with the aromatic analytes, as well as the occurrence of additional dipole-type interactions and/or the formation of electron donor–acceptor complexes between the anthracyclines and the stationary phase.

Despite having large widths (no data shown), the peak areas of the analytes separated using phenyl-based stationary phases were comparable or higher than the peak areas of those separated on the alkyl stationary phase (Tables S2-S4, Fig. 1B). However, the pentafluorophenyl column's low column efficiency ($N_A < 5000$) resulted in insufficient separation of the analyzed compounds (Table S3, Fig. 1C). The calculated $T_f$ values for the anthracyclines indicated that the chromatographic separations using the aromatic stationary phases yielded better peak symmetry compared to those using the alkyl column (Tables S2-S4, Fig. 1D). With the exception of the peaks for DAU and IDA obtained on phenyl column (0.88 and 0.89, respectively. Table S2), the $T_f$ parameters for most of the experiments using aromatic stationary phases showed no significant deviation from 1.0.

### 3.2. Mobile phase with IL additives

In this part of the study, ILs with different alkyl chain lengths at the imidazolium ring ($C_n$MIM, $n = 2, 4, 6, 8$) were applied individually to investigate how the IL cation influences the phenyl- and pentafluorophenyl-based stationary phases. The results of these tests were then compared to those obtained for the separation of analytes using an octadecyl stationary phase. In addition, the impact of the allyl ([AllylMIM]), butyl, and methyl ([$C_4$MMIM]]) substituents on the imidazolium ring, as well as the butyl substituent on the pyridinium ring ([$C_4$MPyr]), of the ILs was also investigated (Table S1). Fig. 2 presents typical chromatograms obtained from the experiments using [$C_2$MIM][Cl] (IL9) to test the stationary phases. The chromatographic parameters for the studied anthracyclines are summarized in Tables S2 (phenyl column), Table S3 (pentafluorophase stationary phase), and Table S4 (octadecyl column). The addition of ILs to the mobile phase variously affected the chromatographic parameters (*k*, A, $N_A$, and $T_f$) calculated for the anthracyclines.

#### 3.2.1. The influence of IL addition on k parameters

As previously reported, the cation and anion of an IL can both interact with the stationary phase. The cation, through hydrophobic (dispersive/π-π) interactions with the stationary phase, causes the repulsion of basic analytes, thus shortening analyte retention. In contrast, the anion possesses chaotropic properties, which enables it to adsorb onto the surface of the stationary phase and interact via electrostatic interactions with cationic analytes, thus increasing their retention on the column [19]. Similar to previous reports, in this study the use of ILs with the same type of anion led decreased analyte retention as the length of their alkyl chains increased. This finding held independently of type of stationary phase used (Fig. 2, Table S2-S4) [18,20,24]. However, the interaction strength between the ILs and the stationary phases was different for the phenyl-, pentafluorophenyl-, and alkyl-based stationary phases.

For the phenyl stationary phase, three of the four ILs with a [$BF_4$] anion decreased the retention factor of the four anthracyclines, although the most distinct differences in the *k* parameter (from 18.37 to 8.22) were observed when IL2 (based on the [$C_6$MIM] cation) was used (Fig. 2A, Table S2). In the case of the pentafluorophenyl stationary phase, each of tested ILs with a [$BF_4$] anion decreased the *k* value of the analytes, with IL2 once again providing the most effective results (from 29.44 to 12.06) (Table S3). Notably, the results of the tests with the octadecyl stationary phase showed that only two of the ILs with a [$BF_4$] anion caused a significant reduction in the *k* value (Table S4). This finding confirms that the use of an alkyl column results in hydrophobic interactions between the anthracyclines and the surface of the alkyl phase. To change this tendency in analyte retention, ILs based on an imidazolium cation with a longer alkyl chain (C6 – IL2) or pirydynium cation substitutes with a C4 chain and an additional methyl group (IL4) should be applied. On the other hand, the results for the phenyl-based stationary phases showed that the π-π interactions between the IL cations and electron-rich phenyl ring—which were facilitated by the

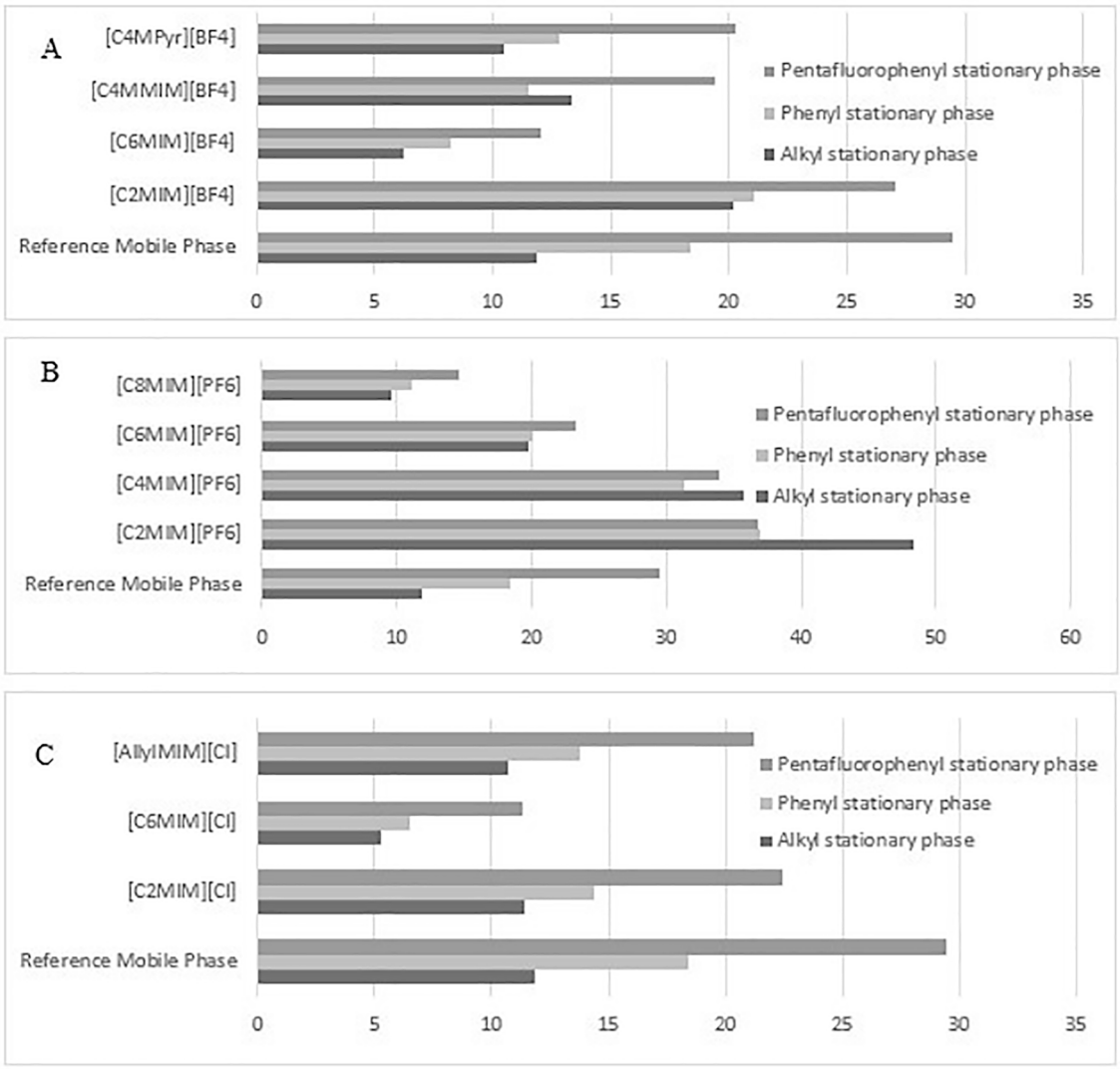


**Fig. 2.** Retention factor of IDA obtained during chromatographic separation with phenyl, pentafluorophenyl, and alkyl stationary phases without added IL (Reference Mobile Phase) and with IL added to the mobile phase (at 2.5 mM concentration). The added ILs consisted of: **(A)** a [$BF_4$] anion and [$C_2$MIM] (IL1), [$C_6$MIM] (IL2), [$C_4$MMIM] (IL3), and [$C_4$MPyr] (IL4); **(B)** a [$PF_6$] anion and [$C_2$MIM] (IL5), [$C_4$MIM] (IL6), [$C_6$MIM] (IL7), and [$C_8$MIM] (IL8); and **(C)** a [Cl] anion and [$C_2$MIM] (IL9), [$C_6$MIM] (IL10), and [AllylMIM (IL11) cations.

hydrophobic interactions of their alkyl chains—were stronger than those observed between the tested analytes and the phenyl groups present on the surface of the aromatic stationary phases. The exception to this trend was the interactions calculated for the use of IL1 with the phenyl column (Table S2). Additional interactions caused by the presence of fluorine atoms in the structure of the pentafluorophenyl stationary phase contributed to a stronger reduction in analyte retention (Table S3).

The use of ILs with a strong chaotropic [$PF_6$] anion resulted in the same correlation between the IL cations and all stationary phases (Fig. 2B), although a reduction in analyte retention was only possible when IL8, which contained the C8 alkyl chain, was used for all columns (Tables S2-S4), or when IL7 was added to the mobile phase during the tests with the pentafluorophenyl column (Table S3). For IDA (i.e., the analyte with the strongest retention), retention decreased from 18.37 to 11.14 when the phenyl column was used, and from 29.44 to 14.65 when the pentafluorophenyl column was used; however, it was only slightly reduced (from 11.87 to 9.53) for the tests using the alkyl column (Fig. 2B).

The effect was similar for ILs with a [Cl] anion and a cation based on imidazolium with either a C2 (IL9) or C6 (IL10) alkyl chain; however, the addition of these ILs resulted in reduced analyte retention for each column due to the presence of [Cl], which is kosmotropic. In particular, the greatest reduction in analyte retention occurred with the C18 column, followed by the phenyl- and pentafluorophenyl-based stationary phases, respectively. In the case of ILs with an [AllylMIM] cation (IL11), the changes in the *k* value for IDA were comparable to those observed for IL9, which suggests that the presence of an unsaturated bond did not alter the strength of the interactions between the IL cation and the ligands in the stationary phase (Fig. 2C). Of all the tested ILs, the addition of [$C_6$MIM][Cl] (IL10) facilitated the shortest analysis times on the aromatic stationary phases (Fig. 3).

Less common IL anions, such as [$CF_3SO_4$] (IL12) and [$CH_3SO_4$] (IL13), were also examined in this study. The findings of these tests revealed that the addition of [$CF_3SO_4$] (IL12) increased the retention of all analytes on the phenyl, pentafluorophenyl, and alkyl columns, while the addition of [$CH_3SO_4$] (IL13) caused a decrease in the retention of all four anthracyclines on all stationary phases. Moreover, an important difference between the aromatic columns and the C18 column is the opposite effect of some of the tested ILs. For instance, the additive [$C_6$MIM][$PF_6$] increased the *k* value in the C18 column while decreasing it in the case of aromatic columns.

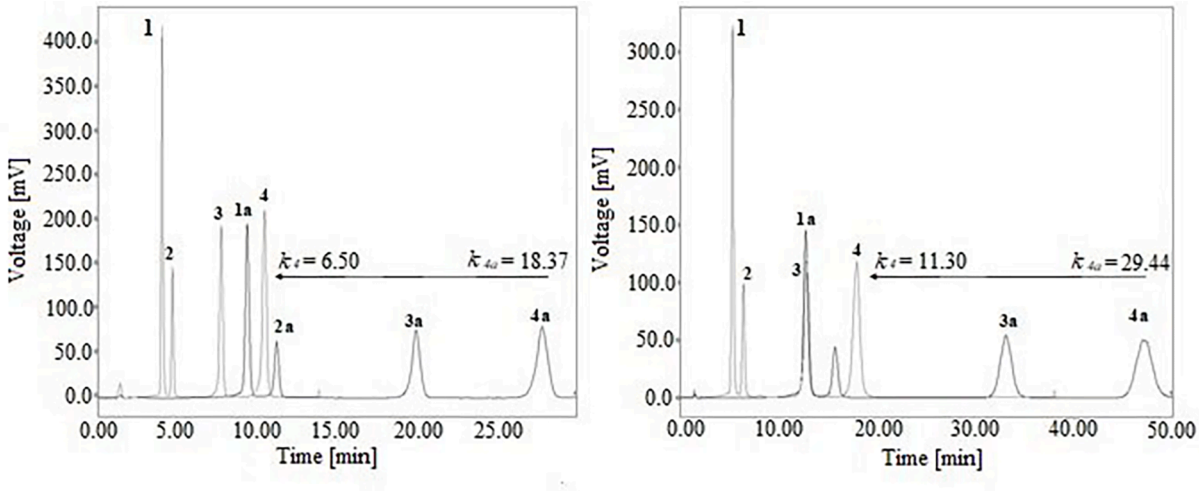


**Fig. 3.** Chromatograms obtained during LC-FL analysis of four anthracycline cytostatics at a concentration of 2.5 μg/mL tested on different stationary phases: (**A**) phenyl column; (**B**) pentafluorophenyl column. Each anthracycline was present at 2.5 mM IL10 (1–4) and without IL addition (1a-4a). 1, 1a – DOX; 2, 2a – EPI; 3, 3a – DAU; 4, 4a – IDA.

On the whole, the results of this study clearly show that analyte retention is influenced by both the anions and cations of ILs added to the mobile phase. These findings are consistent with those of prior studies, which show that the cation causes the repulsion of basic analytes and reduced analyte retention via hydrophobic interactions with the alkyl chains of the stationary phase. Similarly, the findings of this work also align with prior findings showing that anions with chaotropic properties can be adsorbed onto the surface of the stationary phase, thus increasing the retention of cationic analytes on the column through electrostatic interactions [19–21]. With regards to the influence of individual anions combined with the same cation ([$C_2$MIM] or [$C_4$MIM]), the results of this study showed that analyte retention decreased in the order of [$PF_6$] > [$CF_3SO_4$] > [$BF_4$] > [$CH_3SO_4$] > [Cl] for each stationary phase; however, the smallest changes in analyte retention occurred on the alkyl phase. The results also confirmed that the π-π interactions between the IL cations and the electron-rich phenyl ring of the stationary—which is supported by the hydrophobic interaction of their alkyl chains—were stronger than those between the analytes and the phenyl group on the surface of the aromatic stationary phases. This finding also aligns with those of previous research [11], and may be explained by the fact that stationary phases with an aromatic ring enable the creation of π-π interactions with analytes possessing aromatic structures and the ILs, which are also a source of π electrons. Thus, ILs are able to effectively decrease π-π interactions between the stationary phase and the analytes, consequently reducing their retention on the column. Additionally, in the phenyl stationary phase, the aromatic ring is linked to the silica by an alkyl chain. Stepnowski et al. [18] have suggested that hydrophobic interactions may occur between the respective alkyl chains in the stationary phenyl phase and the IL. These interactions may explain why greater reductions in analyte retention on the phenyl stationary phase were observed for IL cations with longer alkyl chains. Moreover, hydrogen bonds may also form between IL cations and the ethoxy group on the surface of the phenyl phase; such bonds create stronger repulsive forces, which further decrease analyte retention. In contrast, the addition of ILs to the mobile phase caused variations in the retention of analytes on the pentafluorophenyl stationary phase. This outcome may be related to the presence of F atoms on the aromatic ring in the structure of the ILs, as these atoms increase the aromaticity of the column's phenyl ring, which in turn produces stronger π-π interactions with the IL. These stronger reactions decrease the aromatic solute's access to the stationary phase, consequently reducing analyte retention. Moreover, additional dipole-type interactions and/or the formation of electron donor–acceptor complexes between the IL and the stationary phase also serve to enhance this effect.

The above-described relationships indicate that the final *k* parameter value for the anthracyclines was determined by both the physiochemical characteristics of the stationary phase and the properties of the employed IL's cations and anions. Furthermore, different ILs produced different interactions during chromatographic separation, which adds complexity to the final data analysis, especially with respect to the values of the other chromatographic parameters.

### 3.2.2. *The influence of IL addition on a parameters*

In the case of the phenyl column, the addition of IL5-IL8 (which contained a [$PF_6$] anion) and IL1 and IL4 (which contained a [$BF_4$] anion) to the mobile phase increased the A parameter of the aromatic solutes compared to no increase in the chromatographic separations without the addition of ILs (Table S2). The best results were obtained for IL6 and IL7 ([$PF_6$] anion and [$C_4$MIM] or [$C_6$MIM] cations, respectively), while the lowest A value was calculated for IL12. In the case of the pentafluorophenyl stationary phase, the addition of ILs to the mobile phase resulted in smaller peak areas for the analytes in almost all applied conditions compared to the reference mobile phase (Table S3). The lowest A values for the anthracyclines were obtained with the use of ILs based on a [$PF_6$] anion (IL5-IL8). In addition, the observed differences in the A parameter were larger for almost all ILs using the pentafluorophenyl stationary phase than those observed with the phenyl- and alkyl-based stationary phases (Tables S2 and S4, respectively). In the case of the C18 column, most ILs only caused small differences in the peak areas of the analytes when added to the mobile phase (Table S4), with IL2 yielding the lowest A values for all analyzed anthracyclines. Danielson et al. [25] reported that enhancement or weakening of fluorescence during the instrumental analysis is dependent on the structure of the analytes. Moreover, the background fluorescence in the UV region of IL may play a key role for ILs able to emit radiation in the absorption ranges of the analytes. According to literature data, all imidazolium ionic liquids are characterized by absorption in the entire UV region and a long absorption tail that extends into the visible region [26]. These absorption characteristics are attributed to the presence of several energetically different associated forms of the imidazolium ions in the IL. Thus, the short wavelength emission is due to the monomeric form of imidazolium ion when the long component is due to its associated forms that are energetically different. Moreover, with decrease in the concentration of the IL, the relative intensity of the long-wavelength band decreases and ultimately vanishes at high dilution (~4 mM) [27]. Additionally, moderately polar and polar conventional solvents,

including acetonitrile, are capable of breaking these structures providing to vanish fluorescence. In the presented study, the studied analytes possess structural similarity, and each compound exhibited natural fluorescence. On the other hand, the tested ILs were added to the mobile phase at low concentration of 2.5 mM, when acetonitrile was applied as organic modifier. It suggests that the fluoroescence activity of imidazolium ILs was limited. However, both IL cation and anion of IL can interact with the components of the mobile phase, the solutes and stationary phase ligands. In consequence, the absorption capability of particular analytes can be modified by the IL addition what results alter the emission properties of tested compounds. On the other hand, the differences in the effects of ILs on the peak efficiency presented in this study, were mainly dependent on the type of stationary phase used. As mentioned above, the results obtained for phenyl stationary phase shown that the enhancement of fluorescence by IL was often achieved. The use of ILs with highly chaotropic [PF6] anion resulted in enhancement of the fluorescence. Additionally, some of ILs tested, namely [C2MIM][BF4] (IL1) and [C4MPyr][BF4] (IL4) provided higher A values. The interactions between cationic solute and the chaotropic anion of those ILs were probably responsible for this effect. Of note, the additional π-π interactions on this stationary phase occurring due to the presence of phenyl groups on the surface of the stationary phase, result in a different affinity of binding of the cationic analytes to the column. In the case of pentafluorophenyl stationary phase, only [C6MIM][Cl] (IL10) and partially [AllylMIM][Cl] (IL11) did not decrease the peak efficiency, while other ILs decreased the A values. The presence of F-atoms increases the electronegativity of the phenyl ring and provides stronger and richer interactions at the surface of the stationary phase. These interactions make stronger aromatic character of the molecule, and in consequence, probably more effective absorption of excitation wavelength leading to higher emission. On the other hand, the addition of IL as additive to the mobile phase, changes the peak area of analytes, and one of possible explanation of this effects may be related to strong interaction of IL cations with the pentafluorophenyl ligand, which consequently weakens analyte retention, and ultimately affect the radiation absorption and emission by the analytes during FL analysis. For the stationary alkyl phase, a significant increase in the A value for all tested analytes in comparison to the reference values was obtained for (IL6) and (IL7), while (IL5) and (IL12) increased it for two of the four tested analytes (DAU, IDA). In turn, for this type of column, (IL2), (IL3), (IL10) and (IL13) decreased the A value for the analyzed compounds. It may point out that the fluorescence activity of the molecules on alkyl stationary phase was also mainly dependent to the interaction between the analyte and chaotropic anion, which increases the retention of the analytes on the chromatographic column.

In summary, if the anion does not bind to the stationary phase ligands and solute, its presence in the column space leads to the weakening/extinction of the analytes' fluorescence. However, more studies are needed to fully understand whether the increase in fluorescence is responsible for its enhancement by the IL cation, or weaker absorption of the analyte in the presence of IL, or perhaps unbound anions should be treated as extinction coefficients. Regardless different possible mechanisms affecting the change in peak area in the presence of IL in the mobile phase, the interpretation of the results should comprise the effects of stationary phase in addition to the effects observed for free IL or IL bound to the analyte.

### 3.2.3. The influence of IL addition on $N_A$ parameters

The performance data for phenyl column shows that the addition of ILs increased the $N_A$ parameter for all analytes (Table S2). Specifically, IL5 was the most effective for the separations of EPI and DAU (increase of the $N_A$ value from 7643 to 10,588 for EPI and from 9009 to 14,598 for DAU), while the use of IL13 yielded the highest $N_A$ parameter value for IDA (increase of the $N_A$ value from 7247 to 12641). In contrast, the pentafluorophenyl column produced the smallest variations in $N_A$ value, with decreases in column efficiency being observed for the addition of nearly all ILs to the mobile phase (Table S3); of the ILs, IL8 provided the worst results for this column (e.g. decrease of the $N_A$ value from 3634 to 2433). Notably, IL13 was the lone exception to this trend, increasing the $N_A$ value from 3768 to 4016 for DOX and from 3815 to 4201 for EPI. In contrast, the addition of ILs to the mobile phase resulted in increased the $N_A$ values of all the studied analytes. For instance, the addition of IL5, IL6, IL7, and IL12 significantly improved the alkyl column's efficiency with respect to DOX and EPI, while IL8 and IL9 were most effective for increasing the column's efficiency for DAU and IDA, respectively (Table S4). Only in a few cases did the addition of ILs result in lower $N_A$ parameters (e.g, for DOX when using IL8) than those obtained for the analyses performed without ILs.

### 3.2.4. The influence of IL addition on $T_f$ parameters

The interactions occurring on the surface of the stationary phases in the presence of ILs are also described by the $T_f$ values for each analyte, as this parameter provides information about possible peak fronting ($T_f < 1$) or tailing ($T_f > 1$). Analyte peak fronting or tailing effects are caused by the diversity of the stationary phase's surface, which causes specific interactions that require energy that can be described by other equilibrium isotherms and different rates of mass transfer kinetics [28,29,30]. The changes in $T_f$ observed after adding ILs to the mobile phase are likely due to the interactions between the ILs, the analytes, and the phenoxyethyl, pentafluorophenyl, octadecyl chains, and end-capping groups on the surface of stationary phases. The experimental results showed that adding ILs to the mobile phase yielded different $T_f$ values for the four anthracyclines compared to the chromatographic separations without ILs (Tables S2-S4). In particular, reduced $T_f$ values were especially observed with the use of anion-dominated ILs (i.e., ILs consisting of a highly chaotropic anion and a short alkyl chain). The use of IL5 ($[C_2MIM][PF_6]$) foregrounded this effect most prominently: for the phenyl-based stationary phase, the use of IL5 reduced the $T_f$ value for EPI from 0.92 to 0.74 (Table S2), while for the pentafluorophenyl column it decreased the $T_f$ value for EPI from 0.99 to 0.85 (Table S3). On the other hand, the $T_f$ value increased when the IL cation facilitated stronger effects on the surface of the stationary phase (e.g. from 0.92 to 1.02 on phenyl and from 0.99 to 1.10 on pentafluorophenyl stationary phase for EPI in the presence of IL10 in mobile phase). This result suggests that more antagonistic interactions between anions and IL cations on the stationary phase surface may lead to greater thermodynamic disturbances in the resultant reaction and, consequently, greater the asymmetry in the peaks. However, these correlations were mainly observed in the analytes with the lowest retention factor (i.e., DOX and EPI). In the case of DAU and IDA, which showed stronger retention on the columns, a decrease in $T_f$ values was often observed; this tendency was in the following order of columns used: pentafluorophenyl > phenyl ≫ alkyl. In consequence, the largest reduction in the $T_f$ values of DAU and IDA were observed for the pentafluorophenyl-based stationary phase (Table S3). Overall, the peak shapes of IDA and DAU were more symmetrical for the pentafluorophenyl-based stationary phase than for the phenyl- (Table S2) and alkyl-based columns (Table S4) under the same chromatographic conditions.

The results also demonstrated that adding ILs to the mobile phase during chromatographic analyses with aromatic stationary phases improved the separation parameters for the studied anthracyclines (vs. alkyl columns), regardless of the IL's structure. The additional interactions between the stationary phase and the aromatic ring of the ILs enabled a significant reduction in analyte retention and analysis time. Notably, the use of IL10 enabled the analysis time for all analytes separated via aromatic stationary phases to be reduced by more than half. In addition, the obtained results also clearly indicated that the use of ILs in conjunction with aromatic stationary phases greatly influences the chromatographic parameters. For instance, selecting an IL with a specific structure can not only increase the peak area (A) of the studied analytes, and but it can also improve column efficiency ($N_A$). However, it is worth emphasizing that ILs a performed better with respect to the

values of the A and $N_A$ parameters in the analyses with the phenyl than pentafluorophenyl stationary phases. Furthermore, the application of ILs also impacted the $T_f$ parameter, especially for unsymmetrical peaks. Under the applied chromatographic conditions with ILs added to the mobile phase, significant improvements in analyte peak shapes were obtained using the phenyl-based stationary phases.

In summary, both the chemical structure of the IL and the physicochemical properties of the stationary phase can affect the interactions that occur during RP-LC separation, thus modifying the chromatographic parameters of the analytes. For a deeper understanding and visualization of these correlations, chemometric analysis via HCA was conducted using the dataset with the chromatographic parameters calculated for the four anthracyclines with the three examined columns.

### 3.3. HCA and k-means assay of chromatographic data

In this study, a chemometric analysis of chromatographic parameters for four analytes obtained under various LC conditions was performed using HCA and classical k-means method. The HCA is a multivariate tool that allows the relationships between variables and/or objects to be visualized in graphical form without losing any significant information. The major goal of the HCA is to spontaneously assign objects/variables to optimal groups, and within each group the most similar objects are found and formed clusters are different from each other as established by HCA [31,32].

In contrast to HCA, where the number of groups is initially unknown, in a classical k-means method being a supervised pattern recognition method, a priori establishment of the number of groups for data interpretation is required. Such approach allows to find the separation of cases by k-means clustering using only a small number of descriptors (variables) selected from a large set of descriptors [33].

#### 3.3.1. HCA results

Dendrograms based on the calculated *k*, A, $N_A$, and $T_f$ parameters for DOX, EPI, DAU, and IDA using the phenyl (Table S2), pentafluorophenyl (Table S3), and octadecyl stationary phases (Table S4) with and without the addition of ILs to the mobile phase produced by HCA are shown in Fig. 4A and B, Fig. 5A and B and Fig. 6A and B, respectively. For each HCA calculation, less restrictive significance criterion of Sneath's index (2/3 of $D_{max}$, where $D_{max}$ is a maximum distance) was selected [34]. Thus, according to the Sneath's test results for the objects, two clusters were distinguished on the dendrograms calculated for Synergi Polar (Fig. 4A) and Synergi Hydro (Fig. 6A) columns, respectively, while only one was found for Fluorophase column (Fig. 5A). According to the HCA results, IDA and DAU were located together (phenyl and alkyl column) or as separated objects (pentafluorophenyl stationary phase) on the left of the dendrogram in each case, while EPI and DOX were consistently clustered on the right of the dendrogram. The HCA results also indicated on higher differences in the chromatographic behavior between IDA and DAU than for EPI and DOX, especially in the experiments performed with the use of Fluorophase column (Fig. 4A). These results are consistent with the chemical structures of these compounds, as EPI and DOX are diastereoisomers with a hydroxyacetyl group in position 9 (Fig. S1), while IDA and DAU possess an acyl group. Additionally, IDA lacks a methoxy group in position 4, which may explain its different chromatographic behavior.

One notable feature of the dendrograms (Fig. 4B-6B) is that according to Sneath's index (2/3 $D_{max}$), the variables are located in two clusters (1 and 2) characterized by different structures; this grouping indicates that different groups of chromatographic parameters enabled comparable differentiation of the analytes on the tested columns. The HCA results for the phenyl column showed 23 variables in cluster 1, with the $T_{f\_without\ IL}$ and $k_{\_without\ IL}$ parameters being positioned in subcluster 1.1, and 33 variables in cluster 2, with $N_{A\_without\ IL}$ and $A_{without\ IL}$ being located in subcluster 2.2 (Fig. 4B). This pattern suggests that comparable results were obtained when using the $T_{f\_without\ IL}$ and $k_{\_without\ IL}$ parameters to describe the analytes, but that that the description obtained using the values for $N_{A\_without\ IL}$ and $A_{\_without\ IL}$ differed. The HCA results also show that subcluster 1.1 includes the *k* parameter after the addition of IL1, IL7, and IL12 and the $T_f$ obtained after the addition of IL7, IL1, IL10, and IL12. The description of the analytes provided by the parameters in subcluster 1.2 differs slightly. This subcluster includes the *k* parameter calculated after the use of IL5 and IL6, as week as the $N_A$ and A values of the analytes in the presence of IL5 and IL1. According to the HCA, the largest group of parameters is located in subcluster 2.2, which includes: the $N_A$, *k*, and A values calculated for the analytes in the presence of IL2, IL3, and IL10; the *k*, A, and $T_f$ values calculated for the analytes with the addition of IL9; and the values for $N_A$, $T_f$, and A acquired following the addition of IL11 to the mobile phase. The location of these parameters in the dendrogram suggest that the abovementioned ILs comprised the most affected chromatographic parameters, thus providing the greatest differentiation of the anthracyclines. On the other hand, while these changes were significant for the *k* and $T_f$ parameters, they were only minor for the $N_A$ and A parameters without the addition of IL to the mobile phase (i.e., $N_{A\_without\ IL}$ and $A_{\_without\ IL}$ were included in the same subcluster).

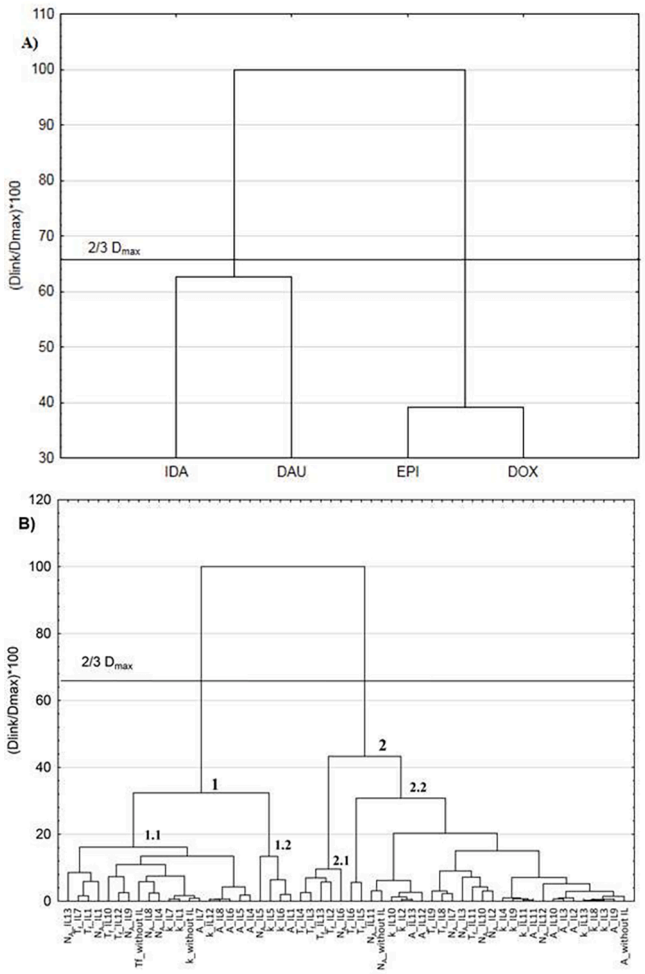


**Fig 4.** Dendrograms obtained by HCA utilizing Ward's method of agglomeration and the Chebyshev distance measure for objects (**A**) and variables (**B**) based on the data set containing the chromatographic parameters of the analytes calculated for phenyl column without ILs and in the presence of 13 ILs added to the mobile phase at a concentration of 2.5 mM.

The dendrogram showing the chromatographic parameters calculated for the anthracyclines separated on pentafluorophenyl stationary phase (Table S3) shows 13 variables in cluster 1 and 43 parameters in cluster 2 with 43 parameters (Fig. 5B). Thus, the results of the

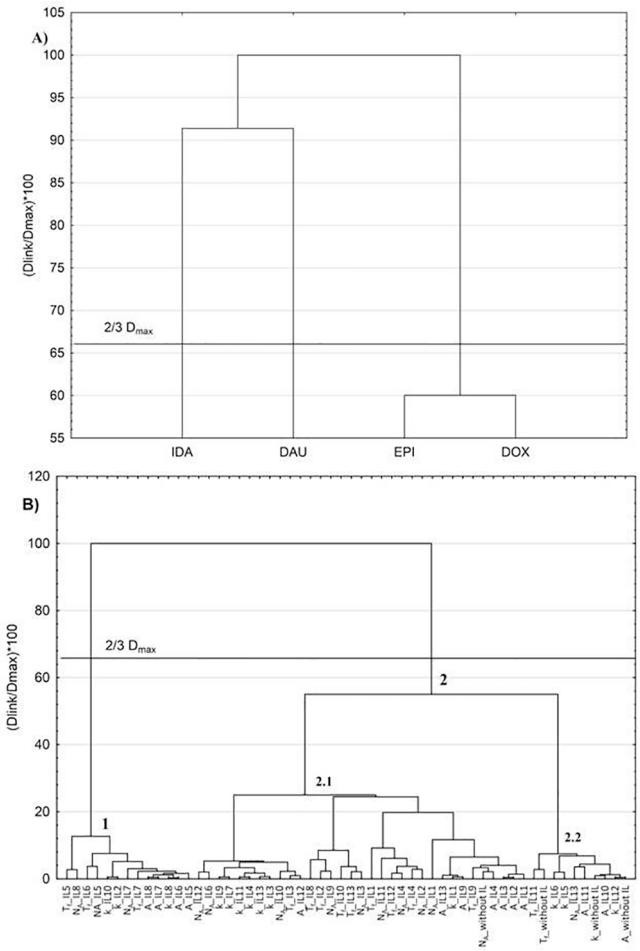


**Fig 5.** Dendrograms obtained by HCA using Ward's method of agglomeration and the Chebyshev distance measure for objects (**A**) and variables (**B**) based on the data set containing the chromatographic parameters of the analytes calculated for the pentafluorophenyl column without IL and in the presence of 13 ILs added to the mobile phase at a concentration of 2.5 mM.

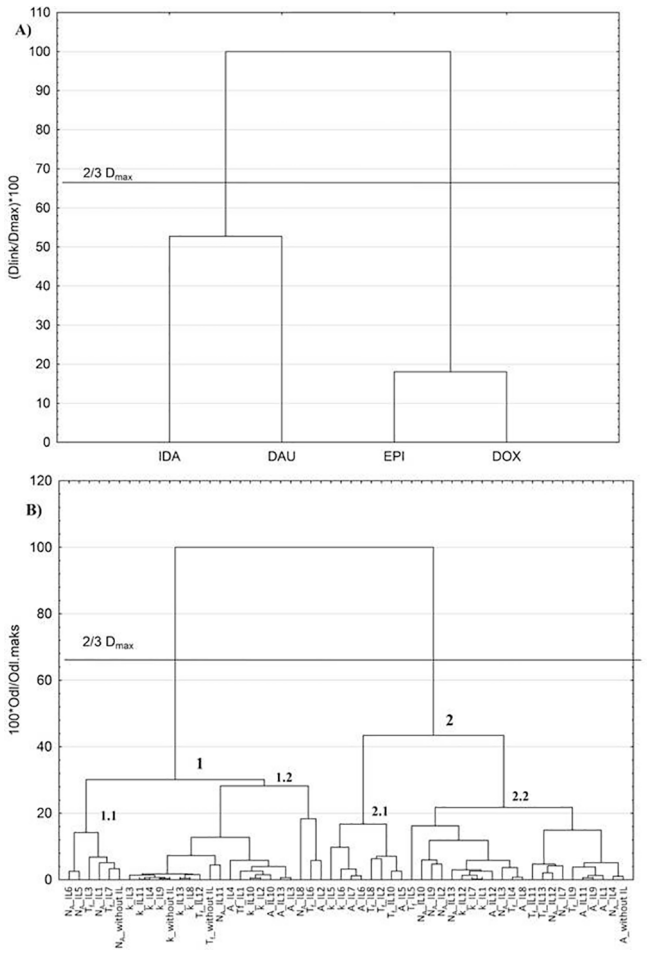


**Fig 6.** Dendrograms obtained by HCA using Ward's method of agglomeration and the Chebyshev distance measure for objects (**A**) and variables (**B**) based on the data set containing the chromatographic parameters of the analytes calculated for the alkyl column without IL and in the presence of 13 ILs added to the mobile phase at a concentration of 2.5 mM.

separations on the pentafluorophenyl stationary phase showed different relationships than the results obtained using the phenyl stationary phase (Fig. 4B). It should be highlighted that most parameters included in cluster 1 were calculated after the addition of IL5 and IL7 (A, $T_f$, $N_A$) or IL8 (A, $N_A$, *k*) to the mobile phase (Fig. 5B), which suggest that the most significant changes in the interactions during separation with the use of ILs were observed in contrast to the values calculated without IL addition. Subcluster 2.1 was the largest, containing 33 parameters (seven A, nine $T_f$, ten $N_A$, and seven *k*), including $N_{A\_without\ IL}$ and all parameters calculated after using IL1, IL3, IL4, and IL9. Moreover, this subcluster also included three from four ones when IL12 ($N_A$, $T_f$ and A) and IL13 ($T_f$, *k* and A) were tested. Additionally, seven other variables were also clustered nearby along with the parameters obtained for the anthracyclines without IL such as $T_f$, *k*, and A (subcluster 2.2). Thus, only a few parameters provided comparable differentiation of the analytes with respect to the values calculated without IL addition.

For the HCA based on the chromatographic parameters obtained using the alkyl column (Table S4), 26 variables were included in cluster 1, with subcluster 1.1 containing $N_{A\_without\ IL}$ and other $N_A$ parameters with the addition of IL1, IL5, IL6, as well as $T_f$ obtained with the addition of IL3 and IL7. Subcluster 1.2 mainly contained *k* parameters, including $k_{\_without\ IL,}$ but it also included A obtained in the presence of IL2-IL4, IL10 and IL13 and $N_A$ after the addition of IL8 and IL11. Moreover, subcluster 1.2 also included the $T_{f\ without\ IL}$ and $T_f$ values for the analytes tested with the addition of IL1, IL6, and IL12. Thus, cluster 1 included other groups of parameters found on the dendrograms calculated using the phenyl and pentafluorophenyl stationary phases (Fig. 4B and Fig. 5B, respectively). The same outcome was observed in cluster 2, where cluster 2.1 included A and *k* calculated for IL5 and IL6 and three $T_f$, and A parameters calculated with the addition of IL7, while subcluster 2.1 mainly included the $N_A$ and A parameters. Thus, the addition of ILs modified the chromatographic parameters for the analytes established on the alkyl column to a greater extent than those calculated using the phenyl-based stationary phases.

### 3.3.2. *K-means Results*

Taking into consideration the HCA results, two clusters were selected a priori for k-means calculations based on the chromatographic parameters established for the tested stationary phases without and with IL addition to the mobile phase. The detailed information regarding the k-means analysis for each column are included in the Supplementary Material (K-means results). In Figures S3-S5 are shown plots of mean values for each object (A) and for each variable (B) for each of the clusters found by k-means mode based on data sets calculated for tested Synergi Polar, Fluorophase and Synergi Hydro column, respectively. These data confirmed that for each tested column, the k-means results for the objects were consistent to the HCA (cluster 1: DOX and EPI and cluster 2: DAU and IDA). The data obtained for pentafluorophenyl column also shown that the similarities between DAU and IDA were lower in respect DOX and EPI, which is in accordance with the HCA

calculations. Moreover, for each object, the difference between the average values for both clusters was statistically significant.

The k-means calculations for the variables based on data set established for Synergi Polar showed that 29 and 27 parameters were included into cluster 1 and 2, respectively. Thus, for this stationary phase, six parameters (10.7%), such as $N_{A\_IL6}$, $N_{A\_IL7}$, $T_{f\_IL2}$, $T_{f\_IL4}$, $T_{f\_IL9}$ and $T_{f\_IL13}$ were found by k-means method to be in different positions in respect to the dendrogram calculated by the HCA (Fig. 4B). According to k-means calculations, the difference between the average values for both clusters were statistically significant for the parameters: $A_{\_IL5}$, $Tf_{\_without\ IL}$, $Tf_{\_IL2}$, $T_{f\_IL13}$, $k_{\_IL1}$ and $k_{\_IL5}$ for the cluster 1 as well as $A_{\_without\ IL}$, $T_{f\_IL5}$, $T_{f\_IL6}$, $k_{\_IL3}$, and $k_{\_IL10}$ for the cluster 2.

When k-means method was used for data set calculated for Fluorophase column, 21 variables were positioned to cluster 1, while 35 variables were located in cluster 2. Thus, eight parameters such as $N_{A\_IL6}$, $N_{A\_IL10}$, $T_{f\_IL2}$, $T_{f\_IL8}$, $k_{\_IL3}$, $k_{\_IL4}$, $k_{\_IL11}$ and $k_{\_IL13}$ (14%) were differently positioned by k-means method comparing to the HCA. Moreover, the difference for variables between the average values for both clusters were statistically significant for three parameters located in cluster 1 ($T_{f\_IL2}$, $k_{\_IL10}$ and $k_{\_IL13}$) and for four variables from cluster 2 ($A_{\_without\ IL}$, $N_{A\_IL3}$, $k_{\_without\ IL}$, $k_{\_IL6}$).

Finally, the k-means results for Synergi Hydro column were calculated, and show that 26 and 30 variables were included into the cluster 1 and 2, respectively. For this stationary phase, four parameters ($N_{A\_IL2}$, $N_{A\_IL5}$, $N_{A\_IL6}$ and $T_{f\_IL5}$) (7.1%) were differently located by k-means than that observed for the HCA analysis. According to k-means method, the difference for variables between the average values for both clusters were statistically significant for four parameters from the cluster 1 ($N_{A\_IL8}$, $T_{f\_IL6}$, $T_{f\_IL7}$ and $k_{\_IL9}$) as well as five parameters located in the cluster 2 ($A_{\_IL5}$, $A_{\_IL9}$, $A_{\_IL11}$, $T_{f\_IL8}$, $T_{f\_IL13}$), respectively.

Overall, the HCA dendrograms for objects calculated using the data sets obtained for four anthracyclines via three stationary phases confirm that adding ILs to the mobile phase provided similar differentiation of the analytes although slightly higher differences between DAU and IDA were found for pentafluorophenyl column (Fig. 4A-6A), for which the HCA results revealed the presence of other groups of chromatographic parameters in clusters 1 and 2 (Fig. 4B-6B). Thus, the HCA facilitated the separation of specific groups of parameters, which in turn enabled comparable differentiation of DOX, EPI, DAU, and IDA on each tested column. These results also confirm that the interactions occurring on the various stationary phases—with or without the use of ILs as mobile phase additives create different relationships between the chromatographic parameters calculated for the studied anthracyclines. These data were comparable to the classification based on k-means method, where all objects and most variables were positioned to the same clusters performed according to the HCA algorithm.

## 4. Conclusions

This study provides insight into the complex interactions between the ligands of phenyl-based stationary phases and ILs in the mobile phase. Specifically, thirteen different ILs were tested in an attempt to develop a more robust understanding of the interactions that occur on aromatic columns during the chromatographic separation of anthracyclines. The results were also compared to those obtained using an alkyl (C18) column, with the findings revealing that the addition of IL caused greater changes in the retention on the aromatic stationary phase than on the alkyl column, and for some IL the effect on the aromatic columns was opposite to that obtained for the C18 column. Therefore, the strong analyte retention observed on the phenyl and pentafluorophenyl columns, mainly due to π-π interactions, was significantly reduced in the presence of the majority of the tested ILs, with the exception of those containing strongly adsorbing anions, such as $[PF_6]$ or $[CF_3SO_4]$, or those with short alkyl chains at the cation. Notably, the use of $[C_6MIM][Cl]$ shortened the analysis time by over 17 min for the phenyl stationary phase, and by over 29 min for the pentafluorophenyl stationary phase, while the decrease in retention observed for alkyl column was only about 8 min. The findings also indicated that selecting an IL with an appropriate structure results in increased analyte peak areas and significantly improves the performance of aromatic columns. Interestingly, if the IL effect was determined by the anion, the fronting of the analyte peaks deepened. In contrast, if the effect was determined by the IL cation, $T_f$ increased. Moreover, the HCA and k-means results showed that the anthracyclines were similarly differentiated, although differences in chromatographic behavior between DAU and IDA were found to be higher when pentafluorophenyl stationary phase was used. Overall, this study considers the theoretical aspects of interactions occurring with and without the addition ILs to the mobile phase during RP-LC analysis using phenyl-based stationary phases, as well as the practical aspects related to the structure of ILs for the analysis of anthracyclines, which was not been previously reported in the literature. Nevertheless, the presented results could be considered as case specific as the reported data were obtained for one type of analytes, while each type of stationary phase was represented by one chromatographic column. Considering that different analyte structures or columns from different manufacturers the potential impact of ILs on chromatographic separation can differs. Therefore, further experiments will be extended on diverse group of analytes, considering their pKa values, molecular weight and structures. Also, as a next step, the experiments utilizing the stationary phases obtained from different manufacturers and with different parameters (i.e. carbon loading, ligand length determining the spatial arrangement of the phenyl ring on the surface of the stationary phase and others) will be performed for better understanding the retention mechanisms on the chromatographic columns.

### *CRediT authorship contribution statement*

**Natalia Treder:** Conceptualization, Methodology, Investigation, Software, Writing – original draft, Writing – review & editing. **Ilona Olędzka:** Data curation, Writing – review & editing. **Anna Roszkowska:** Visualization, Writing – original draft, Writing – review & editing. **Piotr Kowalski:** Writing – review & editing. **Tomasz Bączek:** Investigation, Project administration. **Alina Plenis:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Project administration, Supervision.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2022.107396.

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# Effects of $Fe_3O_4$ Magnetic Nanoparticle Functionalization with Ionic Liquids and a Double-Chained Surfactant on the Pretreatment of Plasma Samples during Drug Extraction

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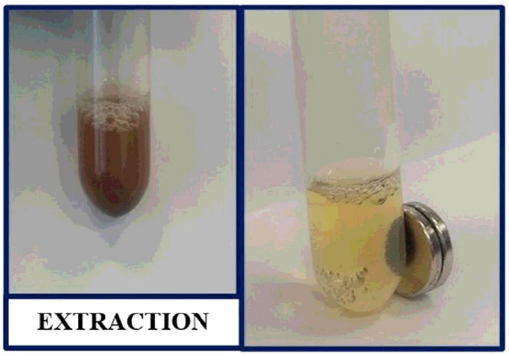


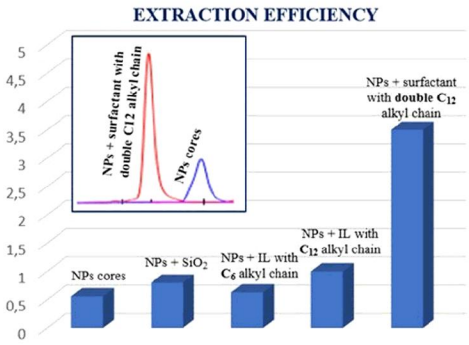


**ABSTRACT:** Ionic liquids (ILs), also known as "designer solvents," comprise a large group of compounds that can improve overall sample preparation performance due to their unique physical and chemical properties. Some of them have a comparable structure to surfactants, which can be also considered as effective extraction solvents. In this study, nine different ILs and a double-chained surfactant were investigated as potential coating materials for iron oxide-based nanoparticles (NPs) used in the pretreatment of human plasma samples. Various methods of synthesizing and functionalizing NPs were employed in fabricating the magnetic sorbents, with the physicochemical properties of the resultant extraction phases (i.e., naked NPs, NPs coated with silica, and NPs coated with silica and selected IL or surfactant) being characterized via X-ray diffraction (XRD), Fourier transform infrared (FT-IR) spectroscopy, thermogravimetric analysis (TG), and transmission electron microscopy (TEM). The effectiveness of the developed NP-based extraction phases was tested by applying them for the extraction of epirubicin hydrochloride (EPI) from plasma samples, followed by analysis via liquid chromatography with fluorescence detection (LC-FL). The results showed that NPs coated with both silica and IL or silica and surfactant provided significantly higher extraction efficiency compared to naked NPs and NPs coated solely with silica. Additionally, the findings also revealed that the adsorption of analytes depends not only on the coating procedure but also on the type of coating material used to functionalize the NPs. Among the tested structures, didodecyldimethylammonium bromide provided the best performance for the functionalization of NP sorbents previously coated with silica.

## INTRODUCTION

In recent years, research related to magnetic nanoparticles (MNPs) has been growing, including work examining their synthesis and characteristics, as well as their potential application in environmental, food, and biomedical studies.[1,2] MNPs comprise a large and diverse group of molecules that includes particles with an iron, cobalt, or nickel core, and oxides such as graphene oxide, iron oxide (II), and iron oxide (III), among others. Nanoparticles (NPs) with an iron core (i.e., maghemite ($Fe_3O_4$) or magnetite ($Fe_2O_3$)) are among the most frequently used, as they are easy to prepare and inexpensive to synthesize.[3,4] Iron-based NPs can be obtained using a variety of different methods, such as coprecipitation, hydrothermal synthesis, microemulsion, and thermal decomposition;[1] however, many researchers have emphasized the importance of selecting the optimal synthesis method and conditions, as both factors play a critical role in determining the physicochemical properties of the resultant NPs. Indeed, the shape, size, and composition of the synthesized NPs are influenced by numerous factors, including the type of iron salt, temperature, pH, time reaction, and the speed with which the reactants are mixed. Furthermore, the efficiency of the synthesis reaction is determined by the selected synthesis

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method, with the coprecipitation method providing the highest efficiency and the microemulsion process offering the lowest.[5] In addition, certain procedures require the use of specific conditions or equipment; for example, the hydrothermal method requires the use of a stainless steel autoclave, while the thermal decomposition method requires the use of high temperatures.[6] Among the available methods for preparing $Fe_3O_4$, coprecipitation is the most frequently used due to its simplicity, low cost, short reaction time, and high efficiency.[7−9] However, problems with oxidation and the tendency of iron-based NPs to aggregate have been reported in prior studies,[10] making the preparation of $Fe_3O_4$ complicated with respect to the employed synthesis conditions.

Once synthesized, NPs undergo further modifications. Structural functionalization is one of the most frequently used methods for adapting NPs to the conditions of the experiments in which they are being used. In this step, a wide range of compounds, including polymers, surfactants, and silica, are applied as coating materials onto the surface of the NP, either alone or in combination (i.e., silica with a polymer).[11−13] One interesting recent approach to structural functionalization has been the use of ionic liquids (ILs). ILs possess several unique properties (e.g., low vapor pressure, high thermal stability, and low melting points) that make them more eco-friendly than many other commonly used reagents,[14] some can be toxic, especially at higher concentrations.[15] Furthermore, ILs possess a wide range of structures, which enable them to be applied multidirectionally. Nonetheless, the different behaviors of ILs under different experimental conditions make it difficult to select the appropriate structure for a given application.[16,17] Structurally, ILs belong to a large and diverse group of ion combination molecules, which include also quaternary ammonium-based ionic liquids (QaILs) and a class of 1-alkyl-3-methylimidazolium ILs with surfactant properties (surface-active ionic liquids, SAILs). Nonetheless, the studies concerning the application of ILs and surfactants in functionalization of NPs are limited. The current knowledge about IL- and surfactant-based functionalization of NPs concerns ILs with a short alkyl chain,[5,18] whereas the most frequently used surfactants are cetyltrimethylammonium bromide (CTAB), which is a cationic surfactant, and sodium dodecyl sulfate (SDS), which is an anionic surfactant.[19−22]

The search for new solutions for use in sample preparation has led to the application of MNPs for the pretreatment of biological samples. The main challenge in this area is to develop environmentally friendly methods that can be implemented quickly and simply for real sample analysis. A sampling device consisting of a properly coated core of magnetic NPs can effectively and quickly extract analytes from a sample matrix through the application of an external magnetic field. The first use of magnetic solid-phase extraction (MSPE) during sample preparation was reported by Safarikowa and Safarik, who demonstrated that reactive copper phthalocyanine dye attached to silanized magnetite can enable the isolation of target analytes.[23] Magnetic sorbents have since been successfully applied to extract analytes from food matrices (i.e., milk, fish, honey, meat)[5] and in environmental analyses of pesticides in soil and waste water samples.[2] Moreover, NPs have also been used to isolate peptides, biomarkers, and pharmaceuticals from biological matrices, which are particularly difficult materials to analyze due to the presence of many ballast substances (i.e., sugars, lipids, salts, proteins) and their low analyte concentrations. In MSPE protocols for biological samples, NPs are mainly functionalized with common materials, such as silica. To date, only a few studies have reported the use of NPs coated with ILs for the analysis of pharmaceuticals in biological samples.[24−26] However, in these studies, only a limited number of ILs were tested during the isolation of pharmaceuticals (i.e., nonsteroidal anti-inflammatory drugs, $\beta$-blockers, and venlafaxine) from biofluids. In the case of $Fe_3O_4$-based NPs functionalized with surfactants, mainly CTAB and SDS were previously applied for the analysis of pharmaceutics (e.g., indomethacin, anagliptin, nystatin, and naproxen) in biological samples.[19−22]

In this paper, we present a comprehensive study of the use of ILs as a coating material for iron-based magnetic NPs. Specifically, the present study aims to assess how the use of ILs affects the physical properties of the $Fe_3O_4$ cores, as well as the results of NP-based extraction. To this end, nine ILs with different structures were examined, including those with different anions and cations, aromatic rings, and lengths and amounts of their alkyl substituents. Additionally, double-chained surfactant didodecyldimethylammonium bromide ($[C_{12}C_{12}MMAmm][Br]$) was also tested for the evaluation of whether a cationic surfactant is more effective than tested imidazolium-based ILs with amphiphilic properties. The magnetic $Fe_3O_4$-based NP cores were synthesized with the help of sonication and functionalized with silica. Additionally, experiments were also performed to identify the most optimal compound for use as a coating for magnetic iron-based NPs. In these experiments, NPs prepared via other synthesis and functionalization procedures were applied to assess how the tested structures affected the NPs' properties and, thus, the final extraction results. To examine the influence of ILs or a surfactant on the extraction efficiency of silica-based NPs, the performance of uncoated NPs and NPs coated with silica only was analyzed. For the purposes of comparison, commercially available NPs were also functionalized with the selected ILs or surfactant and used in MSPE to assess how NPs impact the extraction results. Next, the five NPs, namely, those synthesized with the use of ultrasounds or a magnetic stirrer, bare NPs, NPs coated with silica, and NPs coated with silica and additional coating material (IL or surfactant), were subjected to detailed characterization via X-ray diffraction (XRD), Fourier transform infrared (FT-IR) spectroscopy, thermogravimetric analysis (TG), and transmission electron microscopy (TEM). Finally, the potential of NPs functionalized with the ILs or surfactant for sample pretreatment was assessed by applying them to extract the anthracycline antibiotic, epirubicin hydrochloride (EPI), from the human plasma samples, followed by analysis via liquid chromatography coupled to a fluorescence detector (LC-FL). To the best of our knowledge, this is the first work to demonstrate the potential use of a large group of compounds with ionic structures to functionalize NPs, including nine different ILs and one cationic surfactant with a double alkyl chain, in addition to comparing how using the most effective structure to functionalize commercial NPs and NPs synthesized using various methods impacts the extraction results.

## EXPERIMENTAL SECTION

**Materials.** All chemicals used in this work were analytical grade. Iron (III) chloride hexahydrate ($FeCl_3 \times 6\ H_2O$) and iron (II) sulfate heptahydrate ($FeSO_4 \times 7\ H_2O$) were obtained from Chempur (Piekary Śląskie, Poland), while tetraethyl orthosilicate (TEOS), formic acid, and acetonitrile (ACN)


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were supplied by Merck (Poznań, Poland). Sodium hydroxide (NaOH), 100% ethanol (EtOH), ammonium hydroxide solution ($NH_4OH$, 25%), hydrochloric acid (HCl) at a concentration of 36.6%, and acetone were purchased from POCH (Gliwice, Poland). Eight of the ILs used in this work, namely, 1-hexyl-3-methylimidazolium tetrafluoroborate ($[C_6MIM][BF_4]$), 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM][PF_6]$), 1-octyl-3-methylimidazolium hexafluorophosphate ($[C_8MIM][PF_6]$), 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ($[C_2MIM][N(SO_2CF_3)_2]$), 1-ethyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide ($[C_2MPyr][N(SO_2CF_3)_2]$), butyltrimethylammonium bis(trifluoromethylsulfonyl)imide ($[C_4MAmm][N(SO_2CF_3)_2]$), 1-decyl-3-methylimidazolium tetrafluoroborate ($[C_{10}MIM][BF_4]$), and 1-dodecyl-3-methylimidazolium iodide ($[C_{12}MIM][I]$), and surfactant, didodecyldimethylammonium bromide ($[C_{12}C_{12}MMAmm][Br]$), were supplied by Sigma-Aldrich (St. Louis, MO), while the remaining IL, 1-hexadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ($[C_{16}MIM][N(SO_2CF_3)_2]$), was obtained from IoLiTec Ionic Liquids Technologies GmbH (Heilbronn, Germany). The chemical structures of the tested ILs and surfactant are presented in the Supporting Material (Table S1). Epirubicin hydrochloride (EPI) (>98% purity) was purchased from Cayman Chemical Company, and iron (II, III) oxide—commercially available NPs—was acquired from Sigma-Aldrich (St. Louis, MO). The water used in the experiments was deionized using a Milli-Q system (Molsheim, France). Solutions consisting of 1.5 M NaOH and 0.1 M HCl were prepared by dissolving an appropriate amount of solid NaOH and an appropriate volume of concentrated HCl in pure water, respectively. A set of three standard solutions was then prepared to obtain the EPI working solutions (500, 100, 1 ng/mL) by appropriately diluting the stock solution (10 $\mu$g/mL). All stock and standard solutions were kept frozen at −20 °C until use. The control human plasma (citrated plasma, cat. no. P9523-5ML) was purchased from Merck (Poznań, Poland).

**Synthesis of NPs.** The ultrasonic-based (synthesis I) and magnetic stirrer-based (synthesis II) MNP synthesis methods were performed with coprecipitation following previously reported methods, with some modifications.[27−29] Briefly, in the ultrasonication-based approach (synthesis I), 4.6 g $FeCl_3 \times 6\ H_2O$ and 2.3 g $FeSO_4 \times 7\ H_2O$ were weighed and dissolved in 100 mL of redistilled water, followed by the addition of 0.85 mL of 36.6% HCl. The prepared mixture was then slowly transferred into a flask containing 250 mL of NaOH (1.5 M) and placed in an ultrasonic bath at 80 °C (Polsonic, Warsaw, Poland). The final mixture was sonicated for 1 h and then cooled to room temperature. Finally, the prepared NPs were separated from the solution with the use of neodymium magnets, washed several times with a mixture of EtOH and water (80:20, v/v), and dried in an oven at 60 °C for 2 h (POL-EKO, Wodzisław Śląski, Poland). In the stirring-based method (synthesis II), the same amounts of iron salt were dissolved in 100 mL of redistilled water and transferred to a flask. The flask was then placed on a magnetic stirrer (IKA Ret Control-Visc, Warsaw, Poland) (1000 rpm, 80 °C) and 10 mL of 25% $NH_4OH$ was slowly added dropwise. The resulting NP solution was stirred for an additional 1 h and then cooled to room temperature. The remaining steps in the synthesis II approach were the same as those in the synthesis I method.

**Functionalization of NPs.** Silica-coated $Fe_3O_4$ was prepared using one of two methods (functionalization I and II), which were based on the modified reports.[10,30,31] For the functionalization I approach, 0.5 g of bare $Fe_3O_4$ was initially dispersed in 50 mL of 0.1 M HCl and sonicated for 10 min. The NPs were then separated from the 0.1 M HCl solution and added to 50 mL of an EtOH and water (80:20, v/v) solution along with 1 mL of 25% $NH_4OH$ and 0.250 $\mu$L of TEOS and stirred for 8 h at room temperature. Finally, the coated $Fe_3O_4$ was separated from the solution using a magnet, washed with a mixture of water and EtOH (80:20, v/v), and dried in an oven at 60 °C. In the second coating procedure (functionalization II), 0.5 g of prepared or commercially available NPs was dispersed in 50 mL of EtOH and sonicated for 1 h. Next, 10 mL of 25% $NH_4OH$, 8 mL of $H_2O$, and 0.250 $\mu$L of TEOS were added and the mixture was sonicated for another hour, followed by stirring at room temperature for 8 h. Finally, the functionalized NPs were separated, washed, and dried as in the functionalization I method.

The bare (prepared or commercially available) and silica-coated $Fe_3O_4$ were functionalized with the ILs or surfactant using a modified version of the protocol previously reported by Jashmidi et al.[25] Briefly, each of the tested compounds was dissolved in acetone and then added to the NPs at a ratio of 1:30:1.5 (IL/surfactant/acetone/NPs, m [mg]/v [$\mu$L]/m [mg]). In this study, 10 mg of IL/surfactant, 300 $\mu$L of acetone, and 15 mg of NPs were used for the one extraction. Finally, the prepared mixture was stirred (1200 rpm, 40 °C) until the acetone was completely evaporated and the NPs were dry.

**Characterization Techniques.** XRD measurements were performed using an Empyrean diffractometer (Malvern Panalytical company, Malvern, U.K.) with monochromatized radiation Cu K$\alpha$ ($\lambda$ = 1.5406 Å) and a scintillation detector. Analysis of FT-IR spectra over a range of 500−4000 $cm^{-1}$ was performed using a Thermo Scientific Nicolet 6700 FT-IR spectrometer (Boston, MA) with an ATR accessory. In total, 32 scans were run for each sample at a resolution of 4 $cm^{-1}$. TG analysis was performed on a Mettler Toledo thermogravimetric analyzer with a small furnace (RT: 1100 C) (Warsaw, Poland). The measurements were carried out with a heating rate of 10 °C/min in a nitrogen atmosphere (30 mL/min) and a temperature ranging from 25 to 300 °C. Finally, a Tecnai $G^2$ 20 X-Twin electron microscope (Thermo Fisher Scientific) operating at an acceleration voltage of 200 keV was used to record the TEM images and collect the EDX elemental mappings.

**Extraction Procedures.** About 0.5 mL of plasma was enriched with EPI (10 $\mu$g/mL) to a level of 0.5 $\mu$g/mL. Next, 15 mg of NPs (prepared or commercially available bare $Fe_3O_4$, $Fe_3O_4$_$SiO_2$, $Fe_3O_4$_$SiO_2$_IL/surfactant, $Fe_3O_4$_IL/surfactant) was added to the plasma, vortexed for 30 s, and mixed for 15 min (3000 rpm/min). After mixing, the NPs were separated from the matrix using an external magnetic field, the solution was decanted, and 100 $\mu$L of ACN was added to the NPs over 5 min with mixing on a vortexer. After a 5 min desorption period, the solution was separated from the NPs using an external magnetic field, transferred to the inserts, and analyzed.

**Chromatographic Conditions.** LC measurements were performed using an Agilent Technologies 1220 Infinity LC system equipped with a gradient pump, an autosampler, and a thermostat (Agilent 1200 G4290C, Agilent Technologies, CA)


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along with an FL detector operating at excitation and emission wavelengths set at 487 and 547 nm, respectively. Separation was performed using a Synergi Hydro-RP 80A column (150 mm × 4.5 mm, 4 μm) (Phenomenex, Torrance) and a mobile phase consisting of ACN (phase A) and 0.1% aq solution formic acid (phase B). The gradient program used for separation was as follows: 0−2 min, 5−30% A; 2−8 min, 30−50% A; 8−10 min, 50−95% A; 10−10.1 min, 95−5% A; and 10.1−16 min, 5% A (column equilibration). The injection volume of the analytical solution was 15 μL, the flow rate was set at 1.0 mL/min, and the column temperature was 30 °C. The resultant data was analyzed using ChemStation software.

## RESULTS AND DISCUSSION

**Selection of Optimal Compounds for NP Functionalization.** Previous studies examining the use of ILs in the sample pretreatment (e.g., in SPE or DLLME extraction) have found that the extraction efficiency of the coating is strongly dependent on the type and structure of ILs.[14] To evaluate how the IL structure influences the sorption capacity of magnetic NP sorbents, nine IL structures consisting of various anions, $[BF_4]$, $[PF_6]$, $[N(SO_2CF_3)2]$, [Br], and [I], and cations ($[C_nMIM]$, $[C_nMPyr]$, $[C_nMAmm]$, $n$ = 2, 4, 6, 8, 10, 12, or 16) (Table S1) were tested. In addition to different cations and anions, the selected IL structures were also evaluated with respect to the influence of their alkyl chain length, number of alkyl substituents, and their aromatic rings. We also selected a commercially available surfactant $[C_{12}C_{12}MMAmm][Br]$ with the same length of alkyl chain as one of the tested IL ($[C_{12}MIM][I]$) to compare the effects of cationic surfactant with double alkyl chain on extraction efficiency in respect to the effects observed for an amphiphilic imidazolium-based IL with one dodecyl chain. So far, such comparative study has not been reported in the literature. To this end, magnetic sorbents ($Fe_3O_4$) were produced using the synthesis I approach, and the resulting bare NPs were coated with silica to form an intermediate layer between the core and external layer of NPs (functionalization I). Next, the NPs were functionalized using each of the 10 tested coating materials following the procedure detailed in the section Functionalization of NPs. The potential of the resultant IL- or surfactant-coated NPs was evaluated based on their extraction efficiency during the pretreatment of human plasma spiked with 0.5 μg/mL EPI, which was verified via LC-FL analysis. Relative standard deviation (RSD%) for all measurements carried out in triplicate was estimated below 2.5%, which confirmed the repeatability of the analysis.

To further evaluate the effects of using the ILs or surfactant as a coating material for $Fe_3O_4$_$SiO_2$-based NPs, the extraction efficiency results were also compared to the data obtained for non-IL-coated $Fe_3O_4$_$SiO_2$ NPs (i.e., bare NPs and NPs coated with silica only) and commercially available NPs coated with the optimal coating material. The results of these tests confirmed that the structure of coating material significantly affected extraction efficiency: six of the nine IL-coated NPs had lower sorption capacities compared to the bare $Fe_3O_4$_$SiO_2$ case, while increased extraction efficiency was observed for the remaining three ILs and one surfactant (Figure 1). Interestingly, the use of ILs with the same anion but different cations resulted in an increase or decrease in the sorption capacity of NPs. For instance, $[C_6MIM][BF_4]$ resulted in lower EPI extraction, while $[C_{10}MIM][BF_4]$ increased it. The same results were observed in the case of the IL with an

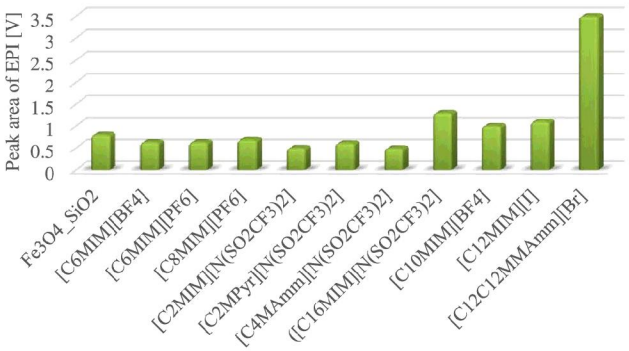


**Figure 1.** ILs and the surfactant used to functionalize $Fe_3O_4$_$SiO_2$-based NPs and their impact on the extraction efficiency of EPI from plasma samples (measurements in triplicates, RSD < 2.5%).

$[N(SO_2CF_3)2]$ anion. When this anion was combined with a $[C_{16}MIM]$ cation, the extraction efficiency for EPI increased; however, when it was combined with a $[C_2MIM]$ cation, the extraction efficiency decreased. These results clearly show that the IL cation, or more precisely, the length of the alkyl chain used as a cationic substituent, is a dominant influence on the adsorption capacity of the functionalized NPs. All of the ILs that reduced the adsorption capacities of NP cores had alkyl chain lengths in the range of C2−C8, while those that resulted in improved extraction efficiency had alkyl chain lengths of C10, C12, or C16. These results are noteworthy, as they clearly indicate that the adsorption capacity of $Fe_3O_4$ coated with a double layer is influenced not only by the alkyl chain length but also by the number of alkyl chains in the structure of the coating material. The surfactant consisting of a cation with two C12 alkyl chains ($[C_{12}C_{12}MMAmm]$) showed much greater analyte adsorption ability compared to tested ILs. For instance, the extraction efficiency after using an surfactant with a didocedyl alkyl chain was three times higher than after using an IL with a dodecyl chain. Additionally, a comparison of the performance of ILs with imidazolium, pyridinium, or ammonium cations showed that the presence of these cations did not significantly affect extraction efficiency. Thus, achieving optimal extraction with modified NPs is primarily dependent on the length and number of alkyl chains in the structure of the coating material. Increasing in the alkyl chain length of the IL to enhance extraction efficiency was previously proposed by Liu et al.,[32] who used ILs with different alkyl chains to functionalize graphene oxide (GO)-based NPs during SPE extraction. Liu et al.'s findings revealed that the IL with the longest alkyl chain provided the best extraction efficiency, as the longer chain created a stronger hydrophobic interaction between the analytes and the magnetic sorbent. In our study, a similar mechanism may explain the improved extraction efficiency observed with the use of double-chain alkyl surfactant. The use of the highly hydrophobic $[C_{12}C_{12}MMAmm][Br]$, which possesses two C12 alkyl chains, provided greater affinity between EPI and the tested NPs. In addition, the double alkyl chains expand the surface area of the NPs, which further improves extraction efficiency. Nevertheless, it should be emphasized that the literature contains few reports of the use of ILs with longer alkyl chains to perform extractions. Indeed, investigations of commercially available ILs have been mostly limited to those with shorter alkyl chains, such as $[C_4MIM][PF_6]$, $[C_6MIM][PF_6]$, $[C_8MIM][PF_6]$, $[C_4MPyr][N(SO_2CF_3)2]$, or $[C_4MIM][Cl]$.[25,33−35] In turn,


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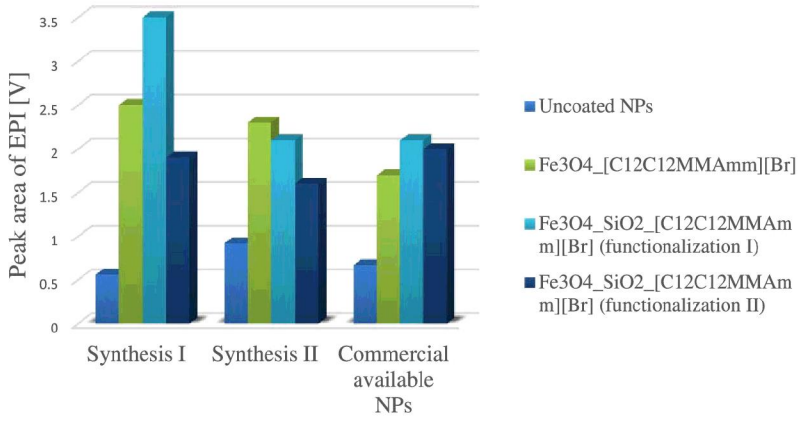


**Figure 2.** Sorption capacity of $[C_{12}C_{12}MMAmm][Br]$-coated NPs based on the synthesis and functionalization procedure.

the use of double-chain surfactant, i.e., $[C_{12}C_{12}MMAmm][Br]$, in the functionalization of magnetic sorbents was not reported in previous studies.[36]

**Optimization of the Synthesis and Functionalization of NPs.** After selecting the most effective compound for use in coating the NPs, the two synthesis and functionalization procedures were examined to assess whether the method of preparing the NPs also affected the extraction of EPI from plasma samples. Regarding the synthesis procedure, the performance of naked NPs prepared using the synthesis I approach (sonication) was compared to that of naked NPs prepared using the synthesis II approach (magnetic stirrer). Since the preparation conditions can affect the physicochemical properties of the NPs, we examined whether the type of base (NaOH or $NH_4OH$) or the order in which the reagents were added (i.e., base to salt solution or salt solution to base) influenced the formation of the NPs and, ultimately, the extraction efficiency. In addition, prior findings have shown that a strong alkaline environment ensures adequate magnetite stability; thus, in the present study, synthesis was performed in an oxidation environment while maintaining a molar ratio of $Fe^{3+}/Fe^{2+}$ = 2:1.[9] This approach allowed for the simplification of the procedure, and it also eliminated the influence of the molar ratio on the differences in the properties of the NPs. Next, the functionalization procedures were examined by comparing NPs functionalized with $SiO_2$ (without the use of 0.1 M HCl; functionalization II), followed by the application of the coating material, and NPs functionalized directly (without an $SiO_2$ layer) with the coating material. Each of the functionalization procedures was performed on NPs prepared using the synthesis I and synthesis II methods. All of the prepared NPs were then coated with the optimal structure (i.e., $[C_{12}C_{12}MMAmm][Br]$) and their respective extraction efficiencies were compared. Additionally, the influence of the synthesis method on the sorption capacity of the NPs was assessed by comparing the extraction efficiency results with those obtained for commercially available NPs with size particles ranging between 50 and 100 nm (as specified by the manufacturer).

The results obtained for the uncoated NPs prepared via the synthesis I (sonication) and synthesis II (magnetic stirrer) approaches were compared with those obtained for uncoated, commercially available NPs. The results of this comparison indicated that the NPs produced via the synthesis I approach provided slightly lower extraction efficiency compared to the commercially available NPs, while the uncoated NPs obtained via the synthesis II method provided slightly higher extraction efficiency (Figure 2). However, it should be emphasized that these differences were not significant, which confirms that the procedure used to synthesize $Fe_3O_4$-based NPs does not adversely affect their properties and application potential. In contrast, the results also confirmed that, regardless of the synthesis method, the extraction efficiency mainly depends on the functionalization approach. The adsorption of EPI from human plasma (Merck, Poland) was significantly higher when NPs were directly coated with both surfactant ($Fe_3O_4$_$[C_{12}C_{12}MMAmm][Br]$) and $SiO_2$ ($Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$). Significant differences in adsorption capacity were observed between the $Fe_3O_4$-based NPs that had been directly functionalized with $[C_{12}C_{12}MMAmm][Br]$ ($Fe_3O_4$_$[C_{12}C_{12}MMAmm][Br]$) and those that had first been functionalized with a base layer of $SiO_2$ ($Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$) before the application of the surfactant with a double alkyl chain. The lowest extraction efficiency was obtained for the NPs prepared using the synthesis II (magnetic stirrer) and functionalization II (without 0.1 M HCl) procedures. NPs prepared with the use of sonication (synthesis I) and the functionalization I method (i.e., applying an $SiO_2$ layer to bind the $[C_{12}C_{12}MMAmm][Br]$ to the NPs' surface) provided better extraction results compared to the NPs prepared using functionalization II or those that had been directly coated with $[C_{12}C_{12}MMAmm]$-$[Br]$. Slightly different results were also obtained after the functionalization of the commercially available NPs, as the extraction efficiency was observed to increase in series: $Fe_3O_4$_$[C_{12}C_{12}MMAmm][Br]$ < $Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}$-$MMAmm][Br]$ (functionalization II) < $Fe_3O_4$_$[C_{12}C_{12}$-$MMAmm][Br]$ (functionalization I). The obtained results suggest that the functionalization step is determined by the morphological properties of the NPs and that both steps in the NP preparation procedure should not be considered separately.

Based on the obtained results, the preparation of NPs via synthesis I, followed by functionalization with $SiO_2$ according to functionalization I and the application of the optimal structure ($Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$) is the best protocol for maximizing extraction efficiency.

**Application of Characterization Techniques for NPs.** To gain the insight into the relationship between the physicochemical properties of magnetic sorbents and the extraction results, the prepared NPs (i.e., bare $Fe_3O_4$ NPs and


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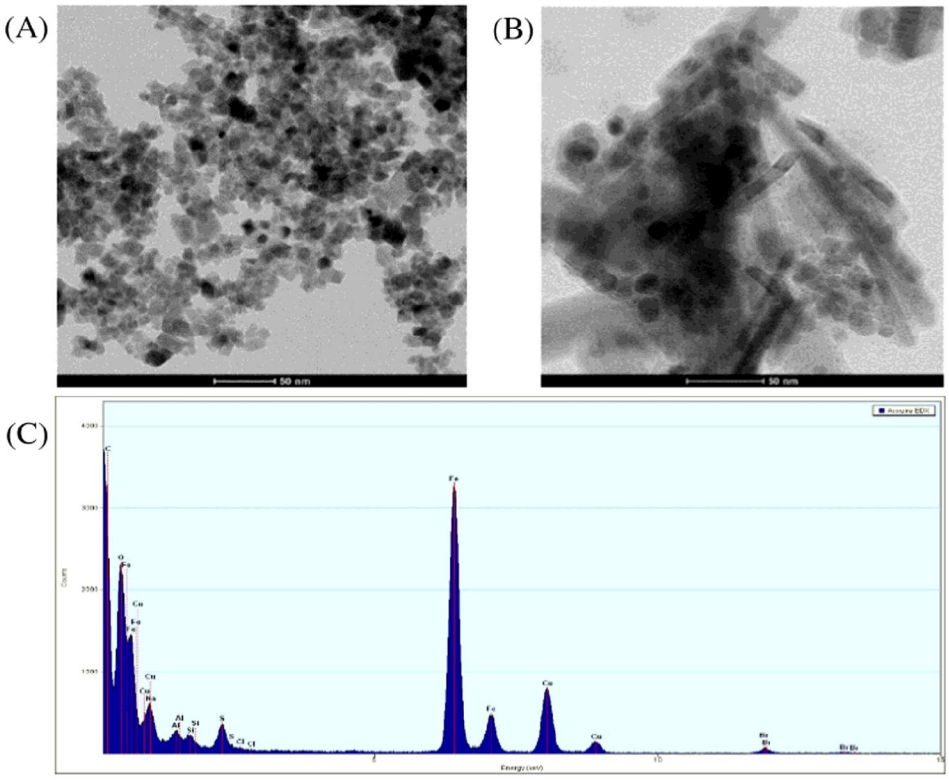


**Figure 3.** TEM image of (A) bare $Fe_3O_4$ and (B) $Fe_3O_4$_$[C_{12}C_{12}MMAmm][Br]$ NPs and (C) elementary EDX spectrum for $Fe_3O_4$_$[C_{12}C_{12}MMAmm][Br]$ NPs prepared with sonification.

$Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$) were characterized using the XRD, FT-IR, TG, and TEM techniques.

**XRD Analysis.** Figure S1 shows the XRD patterns for the bare $Fe_3O_4$ samples prepared according to the synthesis I (Figure S1A) and synthesis II (Figure S1B) methods, as well as those for $Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$ formed via the synthesis I and functionalization I methods (Figure S1C). Diffraction peaks characteristic of the $Fe_3O_4$ cores in the $2\theta$ range at 31, 36, 43, 54, 57, and 63° were obtained after preparation via sonification (S1A) and with the use of a magnetic stirrer (Figure S1B), which confirms that both procedures are able to produce the magnetic $Fe_3O_4$. The XRD peaks were sharp and distinct for the $Fe_3O_4$ samples prepared according to the synthesis I approach (Figure S1A), indicating good crystallinity and homogeneity. In addition, no impurity peaks from other iron oxides were observed, which implies good purity. However, additional diffraction peaks were observed for the NPs produced using the synthesis II method (Figure S1B). A comparison of these peaks with the XRD data card (JCPDS-International Centre for Diffraction Data) indicated the presence of goethite, FeO(OH), in the sample. The diffractograms for the NPs prepared according to the synthesis I and II methods also indicated differences in the intensity of the peaks, which may suggest the influence of the synthesis conditions on the morphology of the NPs. The functionalization of $Fe_3O_4$_$SiO_2$ with $[C_{12}C_{12}MMAmm][Br]$ resulted in additional diffraction peaks (main at $2\theta$ = 22°) (Figure S1C), which may be related to the crystalline structure of the ILs. Furthermore, changes in the intensity of the characteristic diffraction peaks for $Fe_3O_4$ were also observed, suggesting a change in the orientation of the crystalline faces.[37] Moreover, in comparison to naked $Fe_3O_4$-based NPs prepared with the same synthesis procedure, a slight broadening of the baseline and a decrease in the $2\theta$ range of 0−15°, followed by a slight increase, was observed for $[C_{12}C_{12}MMAmm][Br]$-based NPs (Figure S1A vs C), which may indicate the presence of amorphous $SiO_2$ on the surface of the latter $Fe_3O_4$ NPs. However, it should be emphasized that neither $SiO_2$ nor the IL affected the six characteristic peaks of bare $Fe_3O_4$, which proves that the crystal structure of these NPs has not been changed.

**FT-IR.** The presence of the $Fe_3O_4$ core and $[C_{12}C_{12}MMAmm][Br]$ shell functional groups was determined via FT-IR (Figure S2). In this study, a 544 $cm^{-1}$ wavelength band for $Fe_3O_4$ (Figure S2A), a 553 $cm^{-1}$ wavelength band for $Fe_3O_4$_$[C_{12}C_{12}MMAmm][Br]$ (Figure S2C), and a 550 $cm^{-1}$ wavelength band for $Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$ (Figure S2D) were analyzed, with results indicating the presence of Fe−O stretching vibrations. The Fe−O band shifted for the presence of the $Fe_3O_4$ core and $[C_{12}C_{12}MMAmm][Br]$-coated NP samples (from 544 to 553 or 550 $cm^{-1}$), which is suggestive of interactions between the maghemite core and the surfactant shell. In addition, the FT-IR spectra for $Fe_3O_4$_$[C_{12}C_{12}MMAmm][Br]$ (Figure S2C) and $Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$ (Figure S2D) were compared to that of the $[C_{12}C_{12}MMAmm][Br]$ standard (Figure S2B). All main bands characteristic of $[C_{12}C_{12}MMAmm][Br]$ were present in both NP samples (i.e., 2958, 2919, 2851, 1466, 1398, 975, 889, 726 $cm^{-1}$ for $Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$), thus confirming the presence of $[C_{12}C_{12}MMAmm][Br]$ on the surface of the magnetic cores. For $Fe_3O_4$_$SiO_2$_$[C_{12}C_{12}MMAmm][Br]$, characteristic bands of Si−O−Si were also observed (1631 and 1019 $cm^{-1}$), although their intensities were lower than


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those for $Fe_3O_4$_$SiO_2$ (Figure S3) prepared according to the same procedure.

**TEM/EDX Analysis.** The morphology, shape, and size of the NPs were defined based on TEM images. Figure 3A (bare $Fe_3O_4$—synthesis I) and B ($Fe_3O_4$_$SiO_2$_[$C_{12}C_{12}$-MMAmm]—synthesis I, functionalization I) shows that the NPs are shaped like irregular cubes that tend to form clusters. In Figure 3B, the dark fields for bare $Fe_3O_4$ are blurred and covered by a semitransparent layer, which indicates the presence of coating material and proves the presence of [$C_{12}C_{12}$MMAmm][Br] on the surface of a $Fe_3O_4$ core. Although functionalization is one method of preventing the agglomeration of NPs, the obtained TEM images still show the occurrence of the clusters. This finding may be related to the sample preparation procedure applied before TEM analysis (i.e., sonication of NPs in methanol). However, similar behavior has been observed among coated NPs in previous studies, suggesting that the presence of an NP coating does not provide sufficient protection against the agglomeration of NPs.[7] Although the obtained images also confirmed the "nano" size of the tested particles (<50 nm), their exact diameters could not be determined due to their agglomeration. EDX measurements were also performed to identify the elementary decomposition of the sorbents. The EDX spectrum of the nanostructure of the NPs is presented in Figure 3C. The signals for oxygen and iron, as well as those for bromide and carbon ions, confirmed the expected composition of the $Fe_3O_4$ core and its IL shell. Thus, the obtained results were consistent with those of the XRD and FT-IR analyses, indicating appropriate preparation of the nanocomposite.

**TG Analysis.** The thermal stability of the prepared NPs was determined by TG analysis. The TG curves for bare $Fe_3O_4$ and $Fe_3O_4$_[$C_{12}C_{12}$MMAmm][Br], $Fe_3O_4$ NPs prepared with sonication and $Fe_3O_4$_[$C_{12}C_{12}$MMAmm][Br] prepared with the use of sonification, are shown in Figure S4A,B. The measurements revealed that, within the tested temperature range (25−300 °C), the single-stage weight loss for NPs was 6.52%, which can be attributed to the loss of adsorbed water. Thus, naked $Fe_3O_4$ can be considered thermally stable. The thermogram obtained for $Fe_3O_4$ NPs coated with [$C_{12}C_{12}$MMAmm][Br] showed a thermal decomposition weight loss of 32.88% at 300 °C. Greater weight loss was observed for the bare NPs prepared using a magnetic stirrer (synthesis II) (2.63, 3.11, and 7.92% for 118, 203.2, and 300 °C, respectively), with an overall weight loss of 13.68% (Figure S5A). The differences in the results obtained for the NPs prepared with the magnetic stirrer and those prepared via sonication (synthesis I) may not only be attributable to the loss of water but also be due to the presence of goethite and salammoniac, as shown in the XRD results. TG curves were also prepared for the $Fe_3O_4$_$SiO_2$ NPs (synthesis I and functionalization I), with a weight loss of up to 3.82% being observed in the tested range of temperatures (Figure S5B). The obtained results confirmed the strength of the Si−O−Fe bonds and thus the thermal stability of $Fe_3O_4$ coated with $SiO_2$.

Overall, the NP characterization results confirmed the preparation of functionalized $Fe_3O_4$-based NPs coated with [$C_{12}C_{12}$MMAmm][Br], as the XRD, FT-IR, and EDS spectra showed the signals characteristic for maghemite and surfactant. Moreover, the synthesis and functionalization procedures had a significant impact on the parameters that were used to characterize the NPs. For instance, only maghemite was obtained for the sorbent cores prepared using synthesis I (sonication), while an additional iron compound (goethite) was detected in the cores prepared via synthesis II (magnetic stirrer). However, since both procedures were carried out under the same oxygen conditions, the influence of the environmental conditions on the obtained results can be omitted. Indeed, the observed differences were most likely due to the effect of the employed synthesis procedures. The differences in the morphologies of NPs obtained via synthesis I and synthesis II were consistent with the analyte extraction results and may explain the differences observed in the extraction efficiencies of different types of NPs (i.e., higher extraction efficiencies were observed for NPs obtained via synthesis I). In addition, the FT-IR spectrum confirmed the presence of Si−O−Si bands on the surface of the $Fe_3O_4$ cores coated only with $SiO_2$, as well as the stability of $Fe_3O_4$_$SiO_2$ in surfactant functionalization conditions.

Based on the obtained results, the most efficient extraction of EPI was obtained when TEOS and then [$C_{12}C_{12}$MMAmm]-[Br] were used to functionalize the NP cores. These NPs were then compared to other prepared NPs ($Fe_3O_4$_[$C_{12}C_{12}$-MMAmm][Br] or $Fe_3O_4$_$SiO_2$_[$C_{12}C_{12}$MMAmm][Br]—functionalization II) (Figure 1). The presence of silica on the surface of the NPs before [$C_{12}C_{12}$MMAmm][Br] functionalization may suggest that silica is an important effective mediator in [$C_{12}C_{12}$MMAmm][Br]-based NP functionalization. In turn, the TG measurements for $Fe_3O_4$ coated with $SiO_2$ and [$C_{12}C_{12}$MMAmm][Br] showed that the stability of these sorbents is determined by the thermal decomposition of the surfactant. The TG results indicated that thermal decomposition does not begin until 140 °C for the prepared NPs, which should be sufficient for their use in extractions. Meanwhile, the TEM images established the size of the NPs along with high surface specificity; however, the TEM images also showed that NPs have a tendency to aggregate. Nevertheless, it was possible to achieve high extraction efficiency. Such results may suggest that the presence of coating materials and their high affinity for analytes compensate for the influence of NP aggregation on extraction efficiency or perhaps that these aggregates break down during extraction, thus increasing the specific surface area of these sorbents. While these are interesting possibilities, they require further research to determine their plausibility.

**Comparison of [$C_{12}C_{12}$MMAmm][Br]-Based NPs' Application to Other Methods.** To verify the potential of the synthesized $Fe_3O_4$_$SiO_2$_[$C_{12}C_{12}$MMAmm][Br]-based NPs for isolating analytes from biological samples, extractions from plasma samples were performed using EPI as a model substance. Due to the basic physicochemical properties and high molecular weight, it can be consider as a representative for a huge group of pharmaceuticals. Additionally, for the patient treated with EPI, the monitored therapy is recommended; therefore, its use in this study allows to demonstrate the application potential for analytical methodology based on functionalized NPs. In this study, the analysis of EPI samples was performed using the FL detector, which on the one hand is a popular alternative to the ultraviolet (UV) (lower sensitivity) and MS detector (higher maintenance costs) and on the other hand is suitable for EPI detection, which shows natural fluorescence. Finally, the obtained results proved that extraction of this drug from plasma samples with the use of functionalized NPs was possible at different concentration levels (1, 30, and 500 ng/mL) (Figure S6), which could be


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expected in real patient samples. Moreover, the absolute extraction recovery of EPI from plasma samples was also calculated by comparing the results for samples spiked with a standard solution of EPI at 1 ng/mL ($n$ = 3) and 500 ng/mL before and after extraction ($n$ = 6). Based on the obtained results, the absolute recovery of EPI using NPs was estimated at over 80% for each concentration level with RSD% at 7.75 and 3.45% for 1 and 500 ng/mL, respectively. Finally, the results clearly indicated the adequate adsorption of EPI on the prepared sorbents, as well as the fact that complex matrices, such as plasma samples, do not disturb the extraction of analytes via NPs.

To date, NPs modified with ILs have been mainly used in the preparation of environmental samples in studies aiming to isolate industrial dyes, drugs, or metals[33−35] (Table S2). Indeed, the literature contains only two studies wherein magnetic sorbents are used in the preparation of plasma or serum samples,[24,25] while in a third study, Amiri et al.[26] used this approach to analyze dried blood samples. In the aforementioned studies, sample volumes of 10[24] and 2 mL,[25] extraction materials of 40[25] and 60 mg,[26] and higher volume of desorption solvents were required for analyte extraction (Table S2). For example, the applied procedures allowed for determination of venlafaxine (5 ng/mL) with a recovery of 90%[24] and also $\beta$-blockers (1, 5, and 10 ng/mL) in the range of 75−91%.[25] In turn, the use of surfactants as a coating material for NPs for the extraction of pharmaceuticals from biological samples is limited to the application of SDS and CTAB (Table S3). Thus, no previous study has reported the use of double-chained surfactants for that purpose. In addition, similarly to extraction procedures utilizing NPs coated with ILs, most surfactant-based procedures use large volumes of sample, extraction material, and desorption solvent. In the current study, EPI was extracted from 0.5 mL of plasma sample at a concentration of 1 ng/mL using only 15 mg of $Fe_3O_4$_$SiO_2$ magnetic sorbent and 10 mg of [$C_{12}C_{12}$MMAmm][Br] (per sample), with desorption being performed using 100 $\mu$L of ACN. Thus, the use of [$C_{12}C_{12}$MMAmm][Br] to functionalize $Fe_3O_4$-based NPs in the design of miniaturized pretreatment procedures is a novel and promising approach for the analysis of trace levels of analytes in biological samples.

## CONCLUSIONS

The experiments presented in this study have demonstrated that long alkyl chain ILs or a double alkyl chain surfactant is a suitable coating material for iron oxide-based magnetic sorbents. Compared to NPs functionalized with silica only, which is one of the most commonly used coating material, the use of newly synthesized NPs improved the extraction efficiency for EPI from biological samples. The key issue in preparing new NPs is selecting the appropriate structure of coating material. It was observed that, while the sorption capacity increased alongside the alkyl chain length of the IL cation, the IL anion did not significantly affect the results. This effect was even more pronounced when the compound with a higher number of long alkyl chains was used. Ultimately, in this study, [$C_{12}C_{12}$MMAmm][Br] classified as a cationic surfactant provided significantly improved extraction efficiency and was selected for further analysis. The selection of a method for synthesizing and functionalizing the NPs was also critical, with NPs prepared by sonication, followed by functionalization with silica and coating with [$C_{12}C_{12}$MMAmm][Br] being identified as the optimal sorbent. In addition, a comparison of the NP cores prepared in this study and commercially available NPs confirmed the viability of preparing magnetic sorbents in house. It should also be emphasized that the use of NPs functionalized with [$C_{12}C_{12}$MMAmm][Br] allows for the miniaturization of the whole procedure, as only a small volume of matrix, sorbent, and organic solvent was used. Thus, this proposed approach aligns with the current emphasis on designing eco-friendly analytical methods. Moreover, magnetic sorbents can be removed from the matrix quickly and easily with the use of an external magnetic field. In sum, the high extraction efficiency and the "green" aspects of miniaturization of the sample preparation with the use of IL- or surfactant-based NPs confirm the usefulness of these sorbents as an extraction material in the design of future analytical methods.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c01985.

ILs and the surfactant tested in the study; XRD patterns for bare $Fe_3O_4$ NPs produced using sonification, bare $Fe_3O_4$ NPs synthesis with a magnetic stirrer, and $Fe_3O_4$_$SiO_2$_[$C_{12}C_{12}$MMAmm][Br]; FT-IR spectra of bare $Fe_3O_4$ NPs, [$C_{12}C_{12}$MMAmm][Br], $Fe_3O_4$_[$C_{12}C_{12}$MMAmm][Br], and $Fe_3O_4$_$SiO_2$_[$C_{12}C_{12}$MMAmm][Br]; TGA curves for bare $Fe_3O_4$ and $Fe_3O_4$_[$C_{12}C_{12}$MAmm][Br]; chromatograms for blank sample and plasma sample spiked with EPI at 1 and 30 ng/mL after extraction using [$C_{12}C_{12}$MMAmm][Br]-based NPs; previous reports using $Fe_3O_4$-based NPs functionalized with commercially available ILs, and previous reports using $Fe_3O_4$-based NPs functionalized with surfactants (PDF)

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### Notes

The authors declare no competing financial interest.

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