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Kamil Mateusz Łuczykowski

Nowe możliwości diagnostyczne w monitorowaniu funkcji wątroby od pobrania narządu do przeszczepienia

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

Promotor:
Prof. dr hab. Barbara Bojko

Bydgoszcz, 2025 r.

Składam serdeczne podziękowania
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Spis treści

1. Wykaz skrótów

DCD – dawca po śmierci krążeniowej *(ang. donation after circulatory death)*

EASL – Europejskie Stowarzyszenie Badań nad Wątrobą *(ang. European Association for the Study of the Liver)*

ECD – dawca o rozszerzonych kryteriach *(ang. extended criteria donor)*

FDA – Agencja ds. Żywności i Leków *(ang. Food and Drug Administration)*

HBD – dawca z bijącym sercem *(ang. heart beating donor)*

HMP – hipotermiczna perfuzja maszynowa *(ang. hypothermic machine perfusion)*

HOPE – hipotermiczna perfuzja z podaniem tlenu *(ang. hypothermic oxygenated perfusion)*

IRI – uszkodzenie niedokrwienno-reperfuzyjne *(ang. ischemia-reperfusion injury)*

LC-MS – chromatografia cieczowa sprzężona ze spektrometrią mas *(ang. liquid chromatography-mass spectrometry)*

LPC – lizofosfatydylocholina *(ang. lysophosphatidylcholine)*

LPE – lizofosfatydyloetanoloamina *(ang. lysophosphatidylethanolamine)*

MOI – otwarty interfejs mikroprzepływowy *(ang. microfluidic open interface)*

NEVLP – normotermiczna perfuzja wątroby *ex vivo (ang. normothermic ex vivo liver perfusion)*

NMP – normotermiczna perfuzja maszynowa *(ang. normothermic machine perfusion)*

SCD – dawca o standardowych kryteriach *(ang. standard criteria donor)*

SCS – hipotermia prosta *(ang. static cold storage)*

SPME – mikroekstrakcja do fazy stałej *(ang. solid-phase microextraction)*

TFME – mikroekstrakcja cienkowarstwowa *(ang. thin-film microextraction)*

2. Wykaz publikacji stanowiących podstawę postępowania w sprawie o nadanie stopnia naukowego doktora

P. 1. **K. Łuczykowski**, N. Warmuzińska, B. Bojko: ***Current approaches to the analysis of bile and the determination of bile acids in various biological matrices as supportive tools to traditional diagnostic testing for liver dysfunction and biliary diseases***. TrAC-Trends in Analytical Chemistry, 2021, 142, 116307 – praca poglądowa

Punktacja MNiSW: 140, IF: 14,908

P. 2. **K. Łuczykowski**, N. Warmuzińska, B. Bojko: ***Solid phase microextraction – a promising tool for graft quality monitoring in solid organ transplantation***. Separations, 2023, 10, 153 [Dwóch równorzędnych pierwszych autorów] – praca poglądowa

Punktacja MNiSW: 20, IF: 2,500

P. 3. **K. Łuczykowski**, N. Warmuzińska, D. Kollmann, M. Selzner, B. Bojko: ***Biliary Metabolome Profiling for Evaluation of Liver Metabolism and Biliary Tract Function Related to Organ Preservation Method and Degree of Ischemia in a Porcine Model***. Int. J. Mol. Sci., 2023, 24, 2127 – praca oryginalna

Punktacja MNiSW: 140, IF: 4,900

P. 4. **K. Łuczykowski**, N. Warmuzińska, K. Jaroch, D. Kollmann, M; Selzner, B. Bojko: ***Recent solid-phase microextraction-based analytical approaches for the profiling of biliary bile acids in pre-transplant assessments of liver grafts subjected to normothermic ex vivo liver perfusion***. Anal. Chim. Acta, 2024, 1318, 342954 – praca oryginalna

Punktacja MNiSW: 100, IF: 5,700

*Łączna wartość punktów Ministerstwa Nauki i Szkolnictwa Wyższego (*MNiSW*) dla prac wchodzących w cykl: 400.*

Łączna wartość wskaźnika impact factor (IF) dla prac wchodzących w cykl: 28,008.

3. Streszczenie w języku polskim

Transplantacja wątroby stanowi obecnie jedną z najskuteczniejszych metod leczenia schyłkowej niewydolności tego narządu. Ogromny postęp w zakresie technik chirurgicznych, immunosupresji oraz opieki nad pacjentem przekłada się na stale rosnącą liczbę biorców kwalifikowanych do zabiegu. Niestety, liczba dostępnych narządów nie jest w stanie sprostać zwiększającemu się zapotrzebowaniu, co skutkuje wysoką śmiertelnością pacjentów oczekujących na transplantację. W odpowiedzi na istniejącą dysproporcję, centra transplantacyjne zwiększają pulę dostępnych narządów sięgając po narządy pochodzące od dawców o rozszerzonych kryteriach. Niestety, organy takie obarczone są większym ryzykiem powikłań pooperacyjnych, a brak jednoznacznych i wiarygodnych metod utrudnia ocenę ich jakości przed przeszczepieniem. W związku z tym, największym wyzwaniem współczesnej transplantologii jest opracowywanie i udoskonalenia metod konserwacji narządów oraz nowych rozwiązań analitycznych prowadzących do znalezienia parametrów lub związków, które pozwoliłyby na skuteczniejszą ocenę stanu przeszczepianych narządów.

Celem rozprawy była ocena wpływu różnych metod prezerwacji wątroby oraz stopnia niedokrwienia organu na zmiany profilu metabolomicznego żółci z wykorzystaniem wieprzowego modelu zwierzęcego, co pozwoliłoby na wytypowanie potencjalnych biomarkerów zmian zachodzących w konserwowanych narządach w okresie okołotransplantacyjnym. Do przygotowania próbek żółci wykorzystano technikę mikroekstrakcji do fazy stałej (SPME, ang. solid-phase microextraction), a analizy metabolomiczne przeprowadzono za pomocą chromatografii cieczowej sprzężonej ze spektrometrią mas (LC-MS, ang. liquid chromatography-mass spectrometry).

Uzyskane wyniki wykazały, że zarówno stosowana metoda konserwacji, jak i czas niedokrwienia narządu wpływają na metabolom żółci. W przeciwieństwie do normotermicznej perfuzji maszynowej, próbki żółci produkowanej przez wątroby poddane uprzednio hipotermii prostej wykazywały wyższe poziomy poszczególnych kwasów żółciowych, m.in. chenodeksycholowego, tauroursodeoksycholowego czy glikohyocholowego, a także licznych lipidów należących do grupy lizofosfatydylocholin i lizofosfatydyloetanoloamin. Wymienione metabolity były już opisywane w literaturze w kontekście chorób wątroby i pierwotnego stwardniającego zapalenia dróg żółciowych. Ponadto, normotermiczna perfuzja maszynowa ograniczała negatywny wpływ niedokrwienia na funkcje narządu, co potwierdza

korzyści z jej zastosowania w przypadku organów pochodzących od dawców o rozszerzonych kryteriach. Biorąc pod uwagę fakt, że wpływ niedokrwienia objawia się zmianą w poziomach, m.in. kwasów żółciowych będących głównym składnikiem żółci, dalsze eksperymenty obejmowały opracowanie celowanych metod oznaczania metabolitów z tej grupy. Analizy przeprowadzone w pełni zwalidowaną metodą LC-MS wykazały, że niedokrwienie narządu spowodowało istotnie różnice w poziomach kwasu taurocholowego, glikocholowego oraz glikochenodeoksycholowego w próbkach pobranych już w czasie perfuzji. Ponadto, wyniki te korelowały z wynikami oznaczania izomerów kwasów żółciowych otrzymanymi nowo opracowaną metodą wykorzystującą bezpośrednie sprzężenie SPME ze spektrometrem masowym za pośrednictwem otwartego interfejsu mikroprzepływowego (MOI, ang. microfluidic open interface).

Podsumowując, w niniejszej rozprawie doktorskiej udało się zaproponować protokoły analizy próbek o wysokiej przepustowości oparte na SPME i z powodzeniem zastosować je do przygotowania próbek żółci. Badanie zidentyfikowało metabolity warte rozważenia jako potencjalne markery zmian zachodzących w konserwowanych przeszczepach w przyszłych analizach przeprowadzonych na ludzkich graftach wątroby. Zaproponowana metoda SPME-MOI-MS pozwala na uzyskiwanie wyników w czasie niemal rzeczywistym, oferując obiecujące narzędzie do oceny funkcji narządów i wczesnej predykcji ewentualnych powikłań po przeszczepie.

Słowa kluczowe: transplantacja wątroby, perfuzja maszynowa, żółć, mikroekstrakcja do fazy stałej (SPME), metabolomika.

4. Streszczenie w języku angielskim

New diagnostic capabilities for monitoring liver function from organ procurement to transplantation.

Liver transplantation currently represents one of the most effective treatments for end-stage liver failure. Significant advances in surgical techniques, immunosuppression, and patient care have led to a steadily increasing number of recipients qualifying for the procedure. Unfortunately, the number of available organs cannot meet the growing demand, resulting in high mortality rates among patients awaiting transplantation. In response to this disparity, transplant centers are expanding the donor pool by utilizing organs from extended criteria donors. However, such organs are associated with a higher risk of postoperative complications, and the lack of unequivocal and reliable methods hinders the assessment of their quality prior to transplantation. Therefore, the greatest challenge in modern transplantology lies in developing and improving organ preservation techniques and analytical tools to identify parameters or compounds that could enable more effective evaluation of transplanted organs.

The aim of this dissertation was to assess the impact of various liver preservation methods and the degree of organ ischemia on changes in the metabolomic profile of bile using a porcine animal model. This approach aimed to identify potential biomarkers of changes occurring in preserved organs during the peri-transplant period. Bile samples were prepared using solid-phase microextraction (SPME), and metabolomic analyses were performed using liquid chromatography coupled with mass spectrometry (LC-MS).

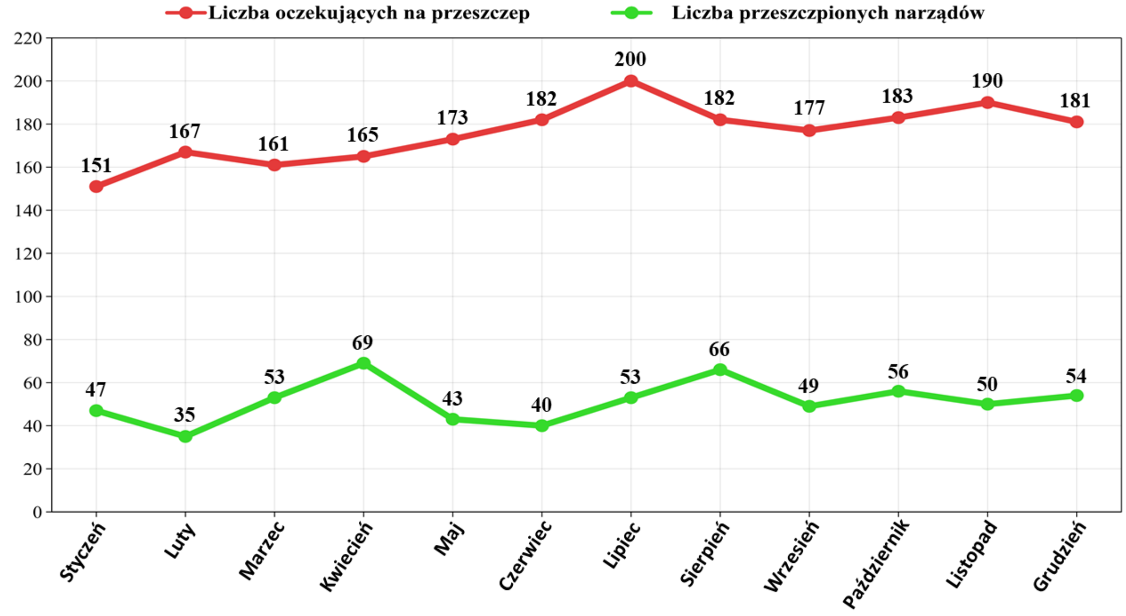
The results showed that both the preservation method and ischemia time influenced the bile metabolome. Unlike normothermic machine perfusion, bile samples from livers previously subjected to static cold storage exhibited higher levels of specific bile acids—such as chenodeoxycholic acid, tauroursodeoxycholic acid, and glycohyocholic acid—as well as various lipids, including lysophosphatidylcholines and lysophosphatidylethanolamines. These metabolites have already been described in the literature in the context of liver diseases and primary sclerosing cholangitis. Furthermore, normothermic machine perfusion mitigated the negative effects of ischemia on organ function, confirming its benefits for organs obtained from extended criteria donors. Given that ischemia impacts the levels of bile acids—key components of bile—further experiments focused on developing targeted methods for quantifying metabolites from this group. Analyses conducted using a fully validated LC-MS method demonstrated that organ ischemia significantly affected taurocholic, glycocholic, and glycochenodeoxycholic acid levels in samples collected during perfusion. Moreover, these findings correlated with results from analyses of bile acid isomers obtained using a newly developed method that combines SPME directly with mass spectrometry via a microfluidic open interface (MOI).

In summary, this doctoral dissertation successfully proposed high-throughput sample analysis protocols based on SPME and applied them to the preparation of bile samples. The study identified metabolites worth considering as potential markers of changes occurring in preserved grafts, which could be further evaluated in future studies involving human liver grafts. The proposed SPME-MOI-MS method enables near real-time results, offering a promising tool for assessing organ function and predicting potential post-transplant complications at an early stage.

Keywords: liver transplantation, machine perfusion, bile, solid-phase microextraction (SPME), metabolomics.

5. Wstęp

Przeszczepianie wątroby jest obecnie wysoce skutecznym sposobem leczenia różnych postaci schyłkowej niewydolności tego narządu. Od czasu pierwszej próby przeszczepu wątroby w 1963 r. obserwujemy stały postęp w tej dziedzinie medycyny. Dzięki rozwojowi technik chirurgicznych, strategii immunosupresyjnych oraz znacznej poprawie w zakresie opieki nad pacjentami liczba kandydatów kwalifikowanych do zabiegu transplantacji w ciągu ostatnich dziesięcioleci stale wzrasta, podczas gdy liczba dawców pozostaje na stałym poziomie [1]. Statystyki Polskiego Centrum Organizacyjno – Koordynacyjnego do Spraw Transplantacji „Poltransplant" za rok 2024 pokazują, że liczba pacjentów oczekujących na przeszczep wątroby znacznie przekracza liczbę dostępnych dawców (Ryc. 1.). Przykrą konsekwencją tej dysproporcji między zapotrzebowaniem na organy a ich dostępnością jest fakt, że każdego roku wielu pacjentów umiera w oczekiwaniu na przeszczep.



Rycina 1. Statystyka liczby oczekujących na przeszczep wątroby oraz liczby przeszczepionych narządów od zmarłych dawców w miesiącach 2024 r. wg Poltransplant.

W związku z powyższym, największym wyzwaniem współczesnej transplantologii jest zwiększenie liczby narządów dostępnych do przeszczepu [2,3]. W krajach Ameryki Północnej i Europy Zachodniej dominuje przeszczepianie organów od dawców zmarłych, zatem aby rozszerzyć pulę dostępnych narządów centra transplantacyjne poza organami od dawców o standardowych kryteriach coraz chętniej sięgają po organy suboptymalne [4]. Niestety, problemem pozostaje brak wiarygodnych metod oceny jakości narządów przed przeszczepem. Obecnie chirurg decyduje o przyjęciu lub odrzuceniu potencjalnej wątroby na podstawie interpretacji ostatnich badań laboratoryjnych dawcy i oceny wizualnej narządu [3,5]. W niektórych przypadkach w celu bezpośredniej oceny tkanki stosowana jest biopsja. Zabieg ten jest rzadki ze względu na jego inwazyjność i ryzyko powikłań [5,6]. W związku z tym, opracowywane są zarówno lepsze sposoby konserwacji narządów, jak i nowe metody oceny jakości graftu przed transplantacją w celu zmniejszenia wskaźnika ich odrzucania [7]. Dla odmiany, w wielu krajach azjatyckich dominującą formą transplantacji jest przeszczep wątroby od żywego dawcy. Wśród technicznych innowacji, które sprawiają, że tego typu donacja staje się bezpieczniejsza i bardziej akceptowalna są chirurgia laparoskopowa i robotyczna [4].

5.1. Rodzaje dawców

Wątroby przeznaczone do zabiegu transplantacji pozyskiwane są zarówno od żywych jak i zmarłych dawców. Jakość organów pobranych od dawców żywych oceniana jest pod kątem ogólnego stanu zdrowia dawcy, stopnia stłuszczenia oraz odpowiedniej anatomii naczyń i dróg żółciowych. Ocena takich narządów jest łatwiejsza w porównaniu do organów pobranych post mortem, ale sam zabieg transplantacji wymaga zapewnienia bezpieczeństwa zarówno biorcy, jaki i dawcy [8]. Wśród organów pozyskiwanych od zmarłych dawców najlepiej rokują narządy pobrane od tzw. dawców wątroby o standardowych kryteriach (SCD, ang. standard criteria donor). Do grupy tej zaliczamy osoby spełniające określone warunki zapewniające, że narząd jest w stosunkowo dobrym stanie i daje większe szanse na powodzenie przeszczepu. W tym przypadku typowe kryteria to najczęściej młody wiek, stwierdzenie śmierci mózgowej bez ciężkich uszkodzeń narządów wewnętrznych, brak przewlekłych chorób wątroby w wywiadzie, oraz krótkie przechowanie poza organizmem pobranego już organu [9]. Niestety, stale rosnąca liczba kandydatów do przeszczepu, a także pojawianie się nowych wskazań, w tym pierwotnych i wtórnych guzów wątroby, szybko wyczerpują pulę organów od dawców SCD. W związku z tym, aby obsłużyć tak dużą grupę oczekujących na przeszczep, lekarze przyjmują wątroby dawców o zwiększonym ryzyku, klasyfikowanych obecnie jako dawcy wątroby o rozszerzonych kryteriach (ECD, ang. extended criteria donor). Według europejskiej organizacji „Eurotransplant” oraz Europejskiego Stowarzyszenia Badań nad Wątrobą (EASL, ang. European Association for the Study of the Liver) kryteriami, których przekroczenie klasyfikuje dawcę do grupy ECD są: wiek > 65 lat, BMI > 30 kg/m^2, ALT > 105 U/l, AST > 90 U/l, stężenie sodu w surowicy > 165 mmol/l, bilirubina w surowicy > 3 mg/dl, pobyt dawcy na oddziale intensywnej terapii z wentylacją > 7 dni, stłuszczenie wątroby > 40%, krążeniowa przyczyna zgonu oraz czas zimnego niedokrwienia narządu > 14 godzin [9,10]. Niestety, wykorzystywanie narządów ECD obarczone jest zwiększonym ryzykiem powikłań po zabiegu na skutek zwiększonej podatności na uszkodzenia niedokrwienno-reperfuzyjne (IRI, ang. ischemia-reperfusion injury), które mogą skutkować pierwotnym brakiem funkcji, wczesną dysfunkcją przeszczepu, zwężeniem dróg żółciowych, czy niedokrwienną cholangiopatią [11]. W związku z tym, aby jak najlepiej wykorzystać pulę dostępnych organów ECD oraz zminimalizować ryzyko powikłań potransplantacyjnych istnieje ciągła potrzeba opracowywania i udoskonalenia metod konserwacji narządów oraz nowych rozwiązań analitycznych prowadzących do znalezienia parametrów lub związków, które pozwoliłyby na skuteczniejszą ocenę jakości organów przed zabiegiem.

5.2. Metody prezerwacji wątroby przed transplantacją

Współczesna transplantologia wątroby stawia przed lekarzami i naukowcami wyzwanie związane z optymalnym przechowywaniem narządu w okresie poprzedzającym przeszczep. Efektywna prezerwacja ma kluczowe znaczenie dla zachowania funkcji wątroby, zmniejszenia ryzyka jej uszkodzenia oraz poprawy wyników przeszczepienia. Wśród obecnie stosowanych metod wyróżnia się trzy główne strategie konserwacji narządu: przechowywanie w zimnym roztworze prezerwacyjnym – hipotermię prostą (SCS, ang. static cold storage), hipotermiczną perfuzję maszynową (HMP, ang. hypothermic machine perfusion) oraz normotermiczną perfuzję maszynową (NMP, ang. normothermic machine perfusion) [7,12].

Hipotermia prosta, ze względu na łatwo dostępne zaplecze logistyczne oraz niski koszt, jest standardową, najczęściej stosowaną metodą konserwacji w transplantacji wątroby. Procedura ta polega na natychmiastowym przepłukaniu naczyń narządu specjalnym roztworem konserwującym i po schłodzeniu przechowywaniu go w nim w temperaturze około 4°C. Mechanizm ochronny tej metody polega na zmniejszeniu zużycia energii komórkowej poprzez obniżenie zapotrzebowania metabolicznego tkanki, co pozwala na zachowanie integralności strukturalnej i funkcjonalnej narządu, zapewniając jego prawidłową funkcję po reperfuzji [12]. Skład roztworu konserwującego ma na celu ograniczenie obrzęku komórek oraz ich lizy, które mogą wystąpić w wyniku chłodzenia. W przypadku organów SCD metoda

SCS jest wystarczająca, ponieważ narządy te są w stanie tolerować umiarkowany poziom IRI, co przekłada się na zadowalające wyniki transplantacji oraz wysoki wskaźnik przeżycia. Niestety, wątroby od dawców ECD gorzej znoszą niedokrwienie, doświadczając silniejszej odpowiedzi zapalnej i poważniejszego uszkodzenia reperfuzyjnego, co prowadzi do gorszych wyników zarówno w krótkim, jak i długim okresie po przeszczepieniu [13]. Ponadto, ze względu na statyczny charakter procedury i warunki odbiegające od fizjologicznych, metoda ta daje ograniczone możliwości oceny jakości wątroby podczas konserwacji i bezpośrednio przed przeszczepem.

Alternatywą dla SCS jest hipotermiczna perfuzja maszynowa, która w odróżnieniu od biernego chłodzenia narządu, opiera się na ciągłym przepływie płynu konserwującego przez naczynia wątroby. Metoda ta używana jest najczęściej w dwóch wariantach, jako zwykła perfuzja bez natlenienia lub hipotermiczna perfuzja z podaniem tlenu (HOPE, ang. hypothermic oxygenated perfusion). Podczas HMP komórki wątroby nadal pozostają w stanie obniżonej aktywności metabolicznej, jednak mechaniczna cyrkulacja płynu wspomaga ochronę struktur komórkowych, zmniejsza stres oksydacyjny oraz poprawia przepływ mikrokrążeniowy. Poprzez usuwanie toksycznych produktów przemiany materii nagromadzonych w okresie niedokrwienia, dostarczanie kluczowych substratów do zachowania funkcji komórkowych oraz opcjonalne natlenianie tkanek, metoda ta pomaga ograniczyć uszkodzenie niedokrwienne oraz poprawić jakości narządów, zwłaszcza tych pochodzących od dawców ECD [12,14,15].

Najnowszą ze współcześnie stosowanych technik prezerwacji organów jest maszynowa perfuzja normotermiczna. Ta innowacyjna metoda ma na celu optymalne utrzymanie funkcji wątroby poprzez stworzenie warunków jak najbardziej zbliżonych do fizjologicznych. NMP polega na utrzymywaniu narządu w temperaturze ciała (ok. 37°C) przy jednoczesnej ciągłej perfuzji wzbogaconym w tlen płynem zawierającym erytrocyty, niezbędne składniki odżywcze oraz leki, co pozwala na podtrzymanie procesów metabolicznych wątroby. Takie warunki konserwacji umożliwiają wydłużenie czasu samej prezerwacji, sprzyjają rewitalizacji organu oraz zmniejszają ryzyko wystąpienia IRI [16]. W przeciwieństwie do SCS i HMP, metoda ta umożliwia monitorowanie kluczowych parametrów funkcjonalnych, takich jak produkcja żółci, metabolizm glukozy oraz poziom markerów uszkodzenia wątrobowego przez cały okres prezerwacji, co pozwala na ocenę jakości graftu przed transplantacją [12]. Niestety, NMP nie jest jeszcze szeroko stosowana w praktyce klinicznej ponieważ wymaga bardziej skomplikowanego sprzętu, wykwalifikowanego personelu oraz odpowiednich warunków laboratoryjnych, co przekłada się na wysokie koszty operacyjne. Ponadto, technologia ta jest stale rozwijana, poprzez badania nad optymalnymi parametrami perfuzji oraz składem płynów perfuzyjnych, a stwarzane przez nią nowe możliwości monitorowania funkcji organu stanowią wyjście do poszukiwania najbardziej wiarygodnych wskaźników oceny żywotności wątroby [17,18].

5.3. Żółć jako materiał do badań dysfunkcji wątroby i dróg żółciowych – P.1.

Żółć jest nieustannie produkowanym przez wątrobę płynem, którego główną funkcją jest wspomaganie trawienia lipidów w jelicie cienkim. Pełni również rolę w procesie wydalania związków endogennych i egzogennych, zarówno w ich pierwotnej postaci, jak i jako produkty metabolizmu wątrobowego. Głównym składnikiem żółci wątrobowej jest woda (ok. 95%), natomiast pozostałą część stanowią kwasy żółciowe, bilirubina, lipidy, niewielkie ilości białek oraz inne metabolity. Pomimo jej istotnej roli w funkcjonowaniu organizmu, wykorzystanie żółci jako matrycy biologicznej w badaniach naukowych jest ograniczone ze względu na trudności w preparatyce tego materiału biologicznego wynikające z jej konsystencji, wysokiej zawartość lipidów oraz dużej zmienność właściwości fizykochemicznych. Niemniej jednak liczne badania podkreślające wysoką wartość naukową analizy żółci zwróciły uwagę zarówno naukowców, jak i klinicystów, prowadząc do wzrostu zainteresowania tym obszarem.

Ze względu na fakt, że skład żółci w dużej mierze determinowany jest stanem fizjologicznym wątroby i dróg żółciowych, płyn ten jest przedmiotem badań mających na celu wyjaśnienie mechanizmów patogenezy chorób tych narządów. Najnowsza literatura opisuje wykorzystanie tego materiału biologicznego w szerokim zakresie badań medycznych i środowiskowych, skupiając się również na metodach przygotowania próbek i technikach instrumentalnych umożliwiających analizę żółci w takich dziedzinach jak mikrobiologia, farmakokinetyka czy nauki omiczne.

Poza całościową analizą żółci, uwaga badaczy skupia się również na celowanej analizie kwasów żółciowych, będących głównym organicznym składnikiem żółci. Ponadto, związki te przechodzą liczne przemiany metaboliczne po czym zostają wydzielane do krążenia wątrobowo-jelitowego i ogólnoustrojowego. Ich ciągła cyrkulacja w organizmie sprawia, że są obecne w różnych matrycach biologicznych, a ich ilościowe oznaczanie może dostarczyć cennych informacji klinicznych, wspomagających diagnostykę chorób wątroby i dróg żółciowych.

Zagadnienia związane z analizą żółci i kwasów żółciowych w ocenie funkcji wątroby i dróg żółciowych zostały opisane w pracy poglądowej pt. „Current approaches to the analysis of bile and the determination of bile acids in various biological matrices as supportive tools to traditional diagnostic testing for liver dysfunction and biliary diseases” (TrAC-Trends in Analytical Chemistry, 2021, 142, 116307) będącej częścią cyklu publikacji prezentowanych w niniejszej rozprawie doktorskiej (publikacja P.1.).

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Current approaches to the analysis of bile and the determination of bile acids in various biological matrices as supportive tools to traditional diagnostic testing for liver dysfunction and biliary diseases

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ABSTRACT

Bile acids produced in the liver undergo numerous metabolic changes before being secreted into enterohepatic and systemic circulation. The continuous circulation of these compounds throughout the body, means that they will be present in various biological matrices; therefore, the quantification of these compounds may yield important clinical information that can help improve the diagnostic process for liver and biliary diseases. However, each of these complex matrices has different properties, which necessitates the use of appropriate sample preparation and analytical methods. On the other hand, bile, which is not a routinely used biological material, has been applied in a wide variety of medical and environmental research via both traditional and new and innovative analytical approaches. This review summarizes the wide range of strategies that have been used for the analysis of bile, as well as for the determination of bile acids in various biological matrices.

1. Introduction

Bile is a fluid that is constantly produced by the liver in order to aid lipid digestion in the small intestine. Bile's secondary function is to help excrete endogenous and exogenous compounds, either as native substances or as products resulting from hepatic metabolism. Hepatic bile is mainly composed of water (~95%), with the remainder consisting of bile acids, bilirubin, lipids, small amounts of proteins, and other metabolites [1]. Unfortunately, it is difficult to prepare and collect bile for use as a biological matrix, largely due to its consistency, its high lipid content, and high variability with respect to its physicochemical properties; consequently, bile is used less often in scientific research compared to other matrices, such as blood and urine. However, reports detailing bile analysis' high scientific value have attracted the attention of scientists and clinicians, and made it the object of a number of recent studies. Furthermore, it is assumed that changes in the concentration of bile acids, which are the main organic component of bile, may correlate with pathological changes in the body.

The first part of this paper reviews the various sample preparation methods and analytical platforms that researchers have developed to enable the use of bile in medical and environmental research (Fig. 1), particularly for the diagnosis of liver and biliary diseases (Table 1). The second part of this paper provides an overview of the qualitative and quantitative methods that have been employed for the analysis of bile acid in various biological matrices (Fig. 2), and how these methods have been implemented to support the diagnosis of liver dysfunction (Table 2).

2. Current approaches to the analysis of bile

2.1. Bacteriology/virology

In recent years, bile has been used as a matrix in numerous studies that have analyzed microbial flora and viral infections. Most of these microbiological studies have employed conventional aerobic and anaerobic culture methods, using blood agar, MacConkey agar, or Schaedler agar as the medium [2,3]. In contrast, Perez-Cameo et al. implemented an automated system in blood culture bottles (BACT/ALERT, bioMerieux) to evaluate how microorganisms isolated in the donor's bile duct culture impacted the incidence of infections during the early post-liver-transplant period [4]. In all

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Abbreviations

3-α-HSD	3-α-hydroxysteroid dehydrogenase
BA	bile acid
BTC	biliary tract cancer
CA	cholic acid
CC	cholangiocarcinoma
CDCA	chenodeoxycholic acid
CDL	choledocholithiasis
DCA	deoxycholic acid
DPV	differential pulse voltammetry
EDC	endocrine disrupting chemical
EHC	extrahepatic cholangiocarcinoma
FXR	farnesoid X receptor
GBA	glycine conjugated BA
GCAS	glycine cholic acid 3-sulfate
GLCAS	glycine lithocholic acid 3-sulfate
HEV	hepatitis E virus
IBD	inflammatory bowel disease
LCA	lithocholic acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NICCD	neonatal intrahepatic cholestasis caused by citrin deficiency
oxPL	oxidized phospholipid
PAH	polycyclic aromatic hydrocarbon
PSC	primary sclerosing cholangitis
PXR	pregnane X receptor
SBA	sulfated bile acid
SPCE	screen printed carbon electrode
TBA	total bile acid
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TGR	takeda G-protein Receptor
TLCAS	taurolithocholic acid 3-sulfate
VDR	vitamin D receptor

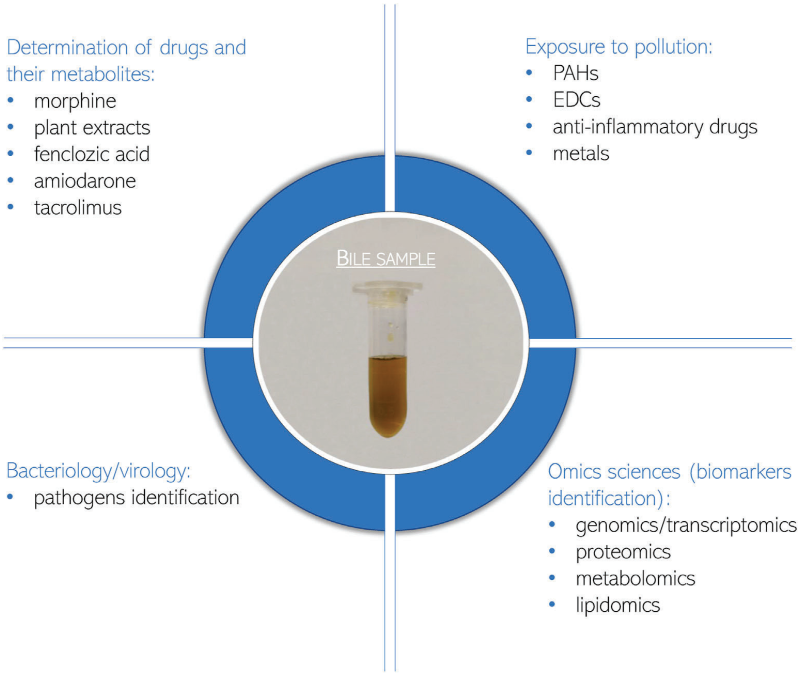

Fig. 1. The use of bile in medical and environmental research.

the cited studies, the system based on colorimetric methods or MALDI-TOF-MS was used to identify pathogens. Molecular genetics was also applied in the identification of microorganisms. For example, Lee et al. used polymerase chain reaction (PCR) to identify *H. pylori* in the bile of patients with cholecystitis [5], while Zheng et al. performed whole-genome shotgun sequencing of *K. pneumoniae* that had been isolated from bile samples taken from cholangiocarcinoma patients [6]. In addition, molecular techniques have also been used to identify herpesviruses and the hepatitis E virus (HEV) in biliary fluid samples [7,8]. However, the detection and identification of bacteria following conventional isolation and culturing methods can be delayed up to 4 days, and molecular techniques based on multiplex PCRs are often expensive and only aimed at specific pathogens. Thus, there is a pressing need for new methods for rapidly detecting bacteria in biological fluids. One example of such an approach can be seen in Rubio et al‘s application of flow cytometry as a screening method in the detection of bacteria in human bile [9].

2.2. Exposure to pollution

The biliary excretion of compounds of environmental concern may be used as an alternative indicator of environmental exposure in animals, as bile often contains substances in the blood and liver that have not been filtered by the kidneys, such as polycyclic aromatic hydrocarbons (PAHs), endocrine disrupting chemicals (EDCs), and metals [10]. Fish bile has often been used to study the impact of environmental pollution on living organisms. For example, Hemachandra et al. evaluated the potential of using bile fluorescence patterns in feral Nile tilapia as biomarkers of exposure to PAH contaminations in tropical waters [11]. Researchers have employed fixed fluorescence (FF) and synchronous fluorescence scanning (SFS) techniques to detect PAH metabolites in fish bile, as these methods only require a simple sample preparation step in which bile is diluted with a methanol solution to decrease self-absorption and the quenching of the fluorescence signal. Additionally, Ruczyńska et al. have proposed the use of high-performance liquid chromatography with fluorescence detection (HPLC-F) for the quantification of 1-OH-pyrene [12]. Although FF and SFS are easy and inexpensive screening methods, more comprehensive analytical methods such as gas chromatography-mass spectrometry (GC-MS), HPLC-F, or liquid chromatography-mass spectrometry (LC-MS) analysis are required to accurately quantify the concentrations of various PAH or EDC metabolites in bile. The use of GC-MS as an analytical platform for the determination of PAHs, EDCs, and their metabolites has been described in Refs. [13–15]. In each of these papers, sample analysis was preceded by enzymatic hydrolysis with glucuronidase and sulfatase to render the unconjugated compounds, as well as a derivatization step. The further addition of solid-phase extraction (SPE) and a cleaning step made it possible to use this protocol to simultaneously determine a large variety of emerging and persistent EDCs in fish bile samples. This approach was proposed by Ros et al., who used a 200 mg Plexa cartridge and a 1 g Florisil cartridge for the SPE and clean-up steps, respectively [15].

In recent years, the SPE-UPLC-MS platform has been widely used to determine EDCs and pharmaceutical residues present in fish bile due to environmental water pollution. For instance, da Silva et al. used a 6 mL Strata-X cartridge containing 200 mg of polymeric reversed-phase sorbent to prepare bile samples obtained from fish in urban and non-urban areas [16], while Brozinski et al. used Oasis HLB and MCX cartridges to purify acidic and basic compounds, respectively, in their study of anti-inflammatory drug concentrations in bile and lake water [17]. Togunde et al. proposed solid-phase microextraction (SPME) as an alternative sample preparation method in their study of the bioconcentrations of several pharmaceuticals in bile samples. Their results showed that the use of SPME (with PDMS and C18 coated fibres) is advantageous for analyses of complex matrices such as biliary fluid, as its amalgamation of extraction and clean-up into a single step results in no or minimal matrix effects [18].

Bile has also been used as a bioindicator for metal exposure in fish. Hauser-Davis et al. assessed whether measuring biliary metal concentrations in fish could be useful strategy for biomonitoring metal contamination. To this end, they assessed the effectiveness of inductively coupled plasma mass spectrometry (ICP-MS) on its own and in combination with size-exclusion high-performance liquid chromatography (SEC-HPLC) for determining metals in this biological fluid [10,19].

2.3. Determination of drugs and their metabolites

The liver plays a major role in metabolizing drugs and other compounds; after being metabolized in the liver, metabolites of these drugs and compounds are excreted into the bile tract. As such, knowledge of biliary drug deposition is essential to understanding the relative importance of the various metabolic pathways and their relationship to total body clearance [20]. This relationship has led to bile becoming a frequently used matrix in the study of the metabolism of xenobiotics and their derivatives.

A combination of liquid chromatography and mass spectrometry has been the main analytical platform used to analyze drugs in bile, with only a few works describing the use of GC-MS for this purpose. For instance, in their evaluation of bile concentrations of morphine in heroin overdose cases, Mercurio et al. used mixed-mode 130 mg/10 mL columns for sample preparation and correlated the resultant levels with blood and liver concentrations [21].

Dilution and centrifugation—with the subsequent use of the resultant supernatant—is the simplest sample preparation method prior to LC-MS analysis. This method has been used in many applications, such as the determination of bile metabolites formed after the administration of *Scutellaria baicalensis* extract [22] or rhubarb decoction [23], and the determination of fenclozic acid and amiodarone metabolites [24,25]. Methanol, ultrapurified distilled water, and acetonitrile are the solvents most often used for dilution. In previous studies, the centrifugation step has been performed either before or after dilution. Alternatively, other studies have employed enzymatic hydrolysis, concentration, or evaporation using a gas stream, followed by reconstitution with organic reagents. Another widely employed bile sample preparation method is liquid-liquid extraction. Liu et al. attempted to optimize the LLE protocol using ethyl acetate, chloroform, and diethyl ether for the purpose of determining bile coumarin concentrations after the oral administration of *Radix Glehniae* extract [26]. Their results showed that ethyl acetate provided the highest extraction efficiency and the lowest co-extracted interferences; therefore, ethyl acetate was selected as the extraction solvent. The LLE method was also implemented in combination with clean-up step using a PSA SPE cartridge [27] or protein precipitation (PP) with a mixture of acetonitrile/zinc sulfate 0.05 M in water [28]. The latter protocol was used to quantify the concentration of tacrolimus in bile samples taken from human liver transplant recipients, with the results showing that whole-blood concentrations of the drug imperfectly reflected its concentrations at the effect site. Thus, the evaluation of biliary concentrations of tacrolimus appears to be a good alternative pharmacological marker of immunosuppressive activity [28].

Nonetheless, pharmacokinetic studies require the concentrations of drugs or metabolites in biological fluids to be continuously monitored. Microdialysis—which is an analytical technique that enables the simultaneous collection and preparation of samples—is one approach that has been successfully applied in pharmacokinetic research [29,30]. Notably, microdialysis rarely requires a sample clean-up step prior to analysis, as the dialysate usually lacks cells, cellular debris, and proteins. Recent works have described *in vivo* microdialysis coupled with LC-MS as a method for the rapid and sensitive analysis of metabolites in rat bile. The rats were placed under urethane anesthesia, a microdialysis probe was implanted into their bile duct and perfused with Ringer's solution (147 mM NaCl, 2.4 mM $CaCl_2$, and 4.0 mM KCl with a pH of 7.3) at a flow rate of 2.0 μL/min in order to allow the diffusion of spinosin into the dialysate [29]. Perfusion solutions contain a high concentration of inorganic salts, which results in considerable ion interference; as such, it is not possible to analyze the dialysate using a mass spectrometer. To avoid ion suppression, the bile samples were

Table 1
The use of bile fluid in medical and environmental research.

Application	Species (method of bile collection)	Sample preparation	Analytical platform	Author (reference)
Microbiological analysis of bile and its impact in patients with SSC-CIP	Human (ERCP)	culturing under aerobic conditions (36°C) on 5% Columbia sheep blood agar, McConkey agar and anaerobic conditions (36°C) on Schaedler agar	Colorimetric method	Voigtlander et al. [2]
Microbiological analysis of bile in patients with benign and malignant biliopancreatic diseases	Human (gallbladder puncture)	culturing on blood agar, MacConkey agar and Mannitol agar	Colorimetric method	Alves et al. [3]
Routine bile culture from liver donors as screening of donor- transmitted infections in liver transplantation	Human (during bile duct resection)	culturing using an automated system in blood culture bottles (BACT/ALERT, bioMérieux) and on agar plates, and liquid enrichment media	MALDI-TOF-MS	Perez-Cameo et al. [4]
Identification of *H. pylori* in bile of patients with cholecystitis	Human (during therapeutic cholecystectomy)	centrifugation (10 min, 14000 rpm), incubation with lysis buffer and proteinase K (55°C, 8 h), DNA extraction with phenol-chloroform, purification using a QIAamp DNA kit	RT-PCR	Lee et al. [5]
Genome sequencing of the tigecycline-resistant *K.pneumoniae* strain isolated from the bile samples of a patient with cholangiocarcinoma	Human (n.a.)	cells lysing with EDTA, lysozyme, and detergent treatment, digestion with proteinase K and RNase, DNA extraction using a DNeasy Blood & Tissue Kit	HiSeq 2000 Sequencing System	Zheng et al. [6]
Identification herpesviruses in bile after liver transplantation and elucidation their role in graft complications after transplantation	Human (ERCP)	extraction of Herpesvirus-DNA using QIAamp DNA blood mini-kit	RT-PCR	Rauber et al. [7]
Evaluation of the presence of HEV in bile samples	Pig (gallbladder puncture)	extraction of viral RNA using a combination of phenol/ chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods	RT-PCR	Gardinali et al. [8]
Evaluation of flow cytometry for the detection of bacteria in bile	Human (n.a.)	culturing on blood agar, chocolate agar, MacConkey agar, Schaedler agar and thioglycollate broth; no sample preparation for flow cytometry is required	MALDI-TOF MS; Sysmex UF500i flow cytometer	Rubio et al. [9]
Determination of metals in bile sample	Fish (gallbladder puncture)	addition of concentrated HNO_3, water bath (80°C, 2 h), addition of H_2O_2, second water bath, dilution	ICP-MS or SEC-HPLC-ICP-MS	Hauser-Davis et al. [10,19]
Analysis of bile fluorescence profiles of fish as biomarkers of exposure to PAH	Fish (gallbladder puncture)	A. dilution with EtOH or MeOH solution; B. addition of β-glucuronidase and arylsulfatase, addition of MeOH and centrifugation (10 min 5000 rpm)	A. FF and SFS B. HPLC-F	Hemachandra et al. [11], Ruczyńska et al. [12]
Analysis of PAHs in bile	Fish (gallbladder dissection)	incubation with β-glucuronidase and sulfatase, extraction with EtOAc, derivatization with BSTFA, evaporation	GC-MS	Vaaland et al. [13], Blanco et al. [14]
Determination of EDCs or anti-inflammatory drugs in bile	Fish (gallbladder puncture)	A. incubation with β-glucuronidase, sulfatase and β-glucosidase, SPE and clean-up step, derivatization/ evaporation B. protein precipitation, incubation with β-glucuronidase and sulfatase, SPE C. incubation with β-glucuronidase and arylsulfatase, SPE	A. GC-MS B. LC-MS C. LC-MS	Ros et al. [15], da Silva et al. [16], Brozinski et al. [17]

Table 1 (*continued*)

Application	Species (method of bile collection)	Sample preparation	Analytical platform	Author (reference)
Determination of pharmaceutical residues in bile	Fish (n.a.)	incubation with β-glucuronidase, sulfatase and β-glucosidase, SPME (C18 and PDMS) or liquid extraction (MeOH)	LC-MS	Togunde et al. [18]
Evaluation of bile concentrations of morphine in cases of heroin overdoses	Human (n.a.)	dilution with H_2O, incubation with β-glucuronidase, SPE, derivatization with BSTFA	GC-MS	Mercurio et al. [21]
Determination of metabolites after administration of drugs or extracts	Rat (bile duct cannula), Human (T-tube)	A. addition of MeOH or ACN, centrifugation, optional hydrolysis with β-glucuronidase B. LLE with ethyl acetate in combination with protein precipitation (with a mixture of acetonitrile/zinc sulfate 0.05 M in H_2O) or SPE C. microdialysis (perfusion with Ringer's solution), SPE	A. LC-MS B. LC-MS C. LC-MS	Du et al. [22], Song et al. [23], Martin et al. [24], Deng et al. [25] Liu et al. [26], Zhou et al. [27], Tron et al. [28] Ma et al. [29], Wen et al. [30]
Transcriptomics in search of biomarkers of bile duct diseases	Human (ERCP, T-tube)	A. RNA extraction using phenol-chloroform-isoamyl alcohol (25:24:1) mixture, ethanol precipitation, RNAiso Plus kit B. centrifugation, RNA extraction using mirVana RNA isolation kit	A. RT-PCR B. RT-PCR	Shigehara et al. [31] Baraniskin et al. [32]
Analysis of DNA methylation of bile fluid in EHC patients	Human (ERCP, T-tube)	genomic DNA extraction with a High Pure Viral RNA kit	MethyLight PCR	Shin et al. [33]
Evaluation of the genomic alterations of diffusely infiltrating type CC using NGS	Human (ERCP)	genomic DNA extraction with QIAGEN© QIAamp® DNA blood maxi kit	NGS	Lee et al. [34]
Comparative proteomic analysis of bile in biliary disease patients	Human (ERCP)	centrifugation, SDS-Page/2-DE, in-gel digestion	LC-MS	Zhang et al. [35], Shen et al. [36], Navaneethan et al. [37]
Evaluation of the protein profile of PSC and CC patients	Human (ERCP)	dilution, addition of n-butanol/iso-propyl ether (40:60), centrifugation, dilution of aqueous phase with 8 M urea and H_2O_2, filtration, lyophilization, resuspension in CE-MS running buffer	CE-MS	Lankisch et al. [40]
Comprehensive analysis of bile fluid from patients with PSC	Human (ERCP)	removing of albumin, IgG and lipophilic substances, 2D-DIGE	LC-MS	Rupp et al. [41]
Metabolic profiling of bile in CC patients	Human (ERCP)	no sample preparation is required	NMR	Sharif et al. [44]
Bile fingerprinting during human liver transplantation	Human (during organ retrieval)	SPE	LC-MS	Legido-Quigley et al. [46]
Metabolic profiling of bile	Rat, dog (bile duct cannula)	Dilution with ACN, ethyl acetate or hexane, centrifugation	SFC-MS	Jones et al. [47]
High throughput metabonomic screening of bile	Rat, dog (bile duct cannula)	no sample preparation is required	ASAP-MS	Twohig et al. [48]
Lipidomic profiling of bile in biliary disease patients	Human (ERCP)	Bligh and Dyer extraction method with butylated hydroxytoluene and diethylenetriaminepentaacetic acid, evaporation and resuspension in MeOH solution	LC-MS	Navaneethan et al. [49]

pre-treated via SPE using Waters Oasis HLB columns prior to instrumental analysis [30].

2.4. Omics sciences used in systems biology

2.4.1. Genomics/transcriptomics

Genomics and transcriptomics approaches have also been used to analyze bile samples from patients with biliary and liver disorders, as they are able to provide some understanding of the mechanisms underlying pathological changes in the bile ducts. The advent of genome-wide analyses using next-generation sequencing (NGS) technologies enabled the successful identification of gene mutations, amplifications, and rearrangements, as well as epigenetic alterations in the biliary tract related to various disorders of different etiologies. Shigehara et al. proved that bile is an attractive biological material for miRNA analysis by performing an RNA extraction using a phenol-chloroform-isoamyl alcohol (25:24:1) mixture followed by ethanol precipitation and RT-PCR. Their approach enabled the identification of 10 miRNAs, with the expression of these miRNAs being significantly higher in the group of malignant biliary tract disease patients than in the benign group. Shigehara et al.‘s results thus demonstrate that miR-9 and miR-145* are excellent diagnostic markers for biliary tract cancer (BTC) [31]. Other studies have determined RNU2-1f levels with the aim of

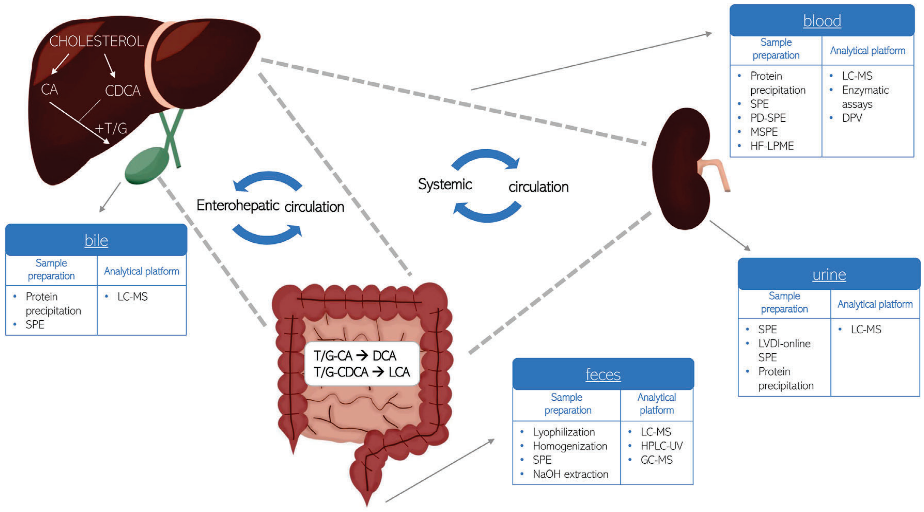

Fig. 2. Methods of bile acid analysis in various biological matrices.

differentiating patients with cholangiocarcinoma (CC) from patients with choledocholithiasis (CDL) or primary sclerosing cholangitis (PSC) by quantitative RT-PCR technique [32]. Furthermore, researchers have also developed a panel of DNA methylation markers that can be used to detect extrahepatic cholangiocarcinoma (EHC) with an overall sensitivity of 75.6% and a specificity of 100%. This five-gene panel (TWIST1, CDH13, GRIN2B, CCND2, RUNX3) may detect EHC cases that are missed by bile cytology may thus increase the sensitivity of bile-based detection of EHC by up to 39% [33]. Finally, NGS-based genomic bile analysis has also been used to assess genomic alterations of diffusely infiltrating-type CC, which could aid early detection and treatment adjustment [34].

2.4.2. Proteomics

Proteomics is the most commonly used method for identifying disease-specific biomarkers. Although the complex composition of bile can limit proteomic analysis, recent refinements in technology have facilitated major advances in this field. Proteomic analyses of bile generally consist of an initial sample preparation step, protein separation via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional gel electrophoresis (2-DE), and analysis using an LC-MS platform (MALDI-TOF or LTQ-Orbitrap) [1,35–37]. Navaneethan et al. employed proteomics to search for protein biomarkers differentiating malignant from benign biliary strictures [37]. Other comparative “bottom-up” proteomic bile profiling methods have enabled the identification of proteins associated with gallstone formation [35], and yielded results suggesting SSP411 protein as a potential biomarker that can be used to diagnose CC [36]. However, one important step in proteomic studies is the preparation of bile samples prior to electrophoretic separation. Cheng et al. showed that the application of an acetone precipitation method followed by the use of a commercially available 2D Clean-Up kit enables the effective concentration of bile proteins, the removal of abundant proteins and debris, and the clear presentation of nonabundant proteins and their isoforms on 2DE gel images [38]. SDS-PAGE can be combined with other fractionation methods for comprehensive proteomic analysis. For example, Barbhuiya et al. were able to describe 2552 biliary proteins in non-cancerous bile samples using a multipronged approach (SDS-PAGE, strong cation exchange, and OFFGEL™) followed by MS analysis [39]. Furthermore, Lankisch et al. and Rupp et al. attempted to differentiate the protein profiles of PSC and CC patients using capillary electrophoresis (CE) and two-dimensional difference gel electrophoresis (2D-DIGE) for protein separation [40,41]. In recent years, the use of the “one sample, one-shot” approach has become popular, as such approaches do not require any type of pre-fractionation method. Megger's group created and tested sixteen different protocols that combined different desalting, delipidation, deglycosylation, and tryptic digestion methods in order to determine which protocol provided the best balance of proteome coverage and simplicity. The acetone precipitation protocol combined with in-gel digestion provided the best results in terms of the number of proteins identified. Furthermore, although in-gel and in-solution digestions gave complementary results in protein identification, in-gel digestions performed better due to the lower matrix effect, which adversely affects the chromatographic performance (peak width) [42]. Delipidation and/or deglycosylation steps significantly increased the number of proteins identified in bile only in the case of in-solution digestion. In different study, Ciordia et al. compared the acetone precipitation of normal and delipidated bile with a new protocol based on SDS extraction and protein purification and digestion in “suspension-trapping” (S-TRAP) columns. No significant effect on the number of bile proteins was found by including the delipidation step when compared to acetone precipitation alone. Whereas protein solubilization with SDS followed by purification and digestion in S-TRAP columns increased by 3-fold the number of proteins identified and, simultaneously, it reduced by 85% the amount of sample needed [43].

2.4.3. Metabolomics and lipidomics

Bile is a matrix that is also commonly used in research in other omics sciences, such as metabolomics and lipidomics. The metabolic profiling approach employs information-rich analytical methodologies to generate metabolite profiles. At present, the most prominent of these analytical methodologies are nuclear

Table 2
Bile acid analysis in various biological matrices and their use as a support in the diagnosis of liver dysfunction.

Application	Matrix	Sample preparation	Analytical platform	Utility of bile acids as a biomarker	Author (reference)
Bile acids profiling as a diagnostic tool for several different pediatric diseases liver pathologies	serum	protein precipitation with MeOH, evaporation, reconstitution in MeOH:H_2O (60:40)	LC-MS Column: Kinetex C18 (150 × 4.6 mm, 2.6 μm) A: 2% (v/v) 250 mM ammonium acetate in H_2O B: 2% (v/v) 250 mM ammonium acetate in MeOH	↑ TBA (especially ↑ conjugated primary BAs) in NICCD patients; ↑GCDCA and TCDCA in biliary atresia patients; ↑CDCA/CA ratio in hepatoblastoma patients	Fu et al. [56]
Assessment of bile acid profiles as biomarkers of liver injury and liver disease in humans	serum	protein precipitation with formic acid:ACN (1:99), evaporation, reconstitution in MeOH:H_2O (50:50)	LC-MS Column: Acquity UPLC BEH C18 (100 × 2.1 mm, 1.7 μm) A: 0.05% (v/v) formic acid in H_2O B: 5% (v/v) ACN in MeOH	↑ individual BAs in patients with hepatic impairments, with the exception of DCA (↓)	Luo et al. [57]
Bile acids profiling as potential biomarkers for human liver diseases	plasma	protein precipitation with ACN, evaporation, reconstitution in MeOH:H_2O (30:70)	LC-MS Column: Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm) A: 5 mM ammonium acetate in H_2O B: ACN:MeOH (50:50)	Four potential bile acid biomarkers for human liver diseases (sensitivity %/specificity%): TBA (83.8/85), GBAs (77.8/90), total CDCA (66.7/95), GLCAS + TLCAS (83.3/70)	Shi et al. [58]
Differentiating primary biliary cirrhosis patients from primary sclerosing cholangitis patients	serum	sample dilution with 0.1% formic acid, SPE (60 mg/3 mL Strata-X 33 μm Polymeric Reversed Phase), evaporation, reconstitution in H_2O:MeOH (50:50) containing 5 mM ammonium acetate and 0.01% formic acid	LC-MS Column: Synergie RP Hydro (50 × 3 mm, 2.5 μm) A: H_2O:MeOH (50:50) containing 5 mM ammonium acetate and 0.01% formic acid B: ACN	↑ TCA, TCDCA, TDCA, GDCA, LCAS in PBC patients compared to PSC patients; ↓ GCA in PBC patients compared to PSC patients	Trottier et al. [60]
Evaluation of bile acid profiles in NICCD infants	plasma	sample dilution with H_2O, centrifugation, evaporation, reconstitution in mobile phase A:B (50:50)	LC-MS Column: Symmetry C18 (150 × 2.1 mm, 3.5 μm) A: 5 mmol/l ammonium acetate in H_2O (87.5%) and 0.02% ammonium hydroxide (12.5%) B: 5 mmol/l ammonium acetate in MeOH (87.5%) and 0.02% ammonium hydroxide (12.5%)	Combining primary/secondary BAs ratio (>433) with conjugated/free ratio (>90) allows differentiating infants with NICCD from those with non-NICCD	Yang et al. [66]
Assessment of metabolites of bile acids pathway during liver function recovery after liver transplantation	serum	protein precipitation with MeOH, evaporation, reconstitution in MeOH:H_2O (10:90)	LC-MS Column: Acquity UPLC HSS T3 (50 × 2.1 mm, 1.8 μm) A: H_2O B: MeOH	↑ TCA and TCDCA in preoperative patients compared to normal controls	Sui et al. [67]
Bile acids profiling as potential biomarkers for human liver diseases	urine	SPE (SupelcleanTM LC-18), evaporation, reconstitution in MeOH:H_2O (50:50)	LC-MS Column: Acquity UPLC BEH C18 (150 × 2.1 mm, 1.7 μm) A: 7.5 mM ammonium acetate, adjusted to pH 6.0 using 10 M ammonium hydroxide B: 20% ACN in MeOH	↑ CDCA and CA, ↓ LCA and DCA in patients with liver disease; ↑ CDCA and ↓LCA with the severity of the disease	Bathena et al. [70]
Profiling of sulfated bile acids for the diagnosis and grading of ICP	urine	sample dilution with PBS, SPE (Oasis HLB), evaporation, reconstitution in 0.1% formic acid:MeOH (90:10)	LC-MS Column: Luna C18 (150 × 2.00 mm, 3 μm) A: 2 mmol/L ammonium acetate and 0.1% formic acid in H_2O B: 2 mmol/L ammonium acetate and 0.1% formic acid in ACN	GCAS as a biomarker for the diagnosis of ICP with the sensitivity of 100% and specificity of 95.5%	Li et al. [72]
Bile acid profiling in healthy and cirrhotic patients	feces	lyophilization, resuspension in H_2O, enzymatic deconjugation, extraction with NaOH, SPE (Sep Pak C18), derivatization, SPE (Sep Pak Silica)	HPLC-UV (254 nm) Column: Nova-Pak C18 (300 × 3.9 mm, 4 μm) Mobile phase: 82% MeOH	↑DCA/CA and LCA/CDCA ratios in advanced cirrhosis	Kakiyama et al. [81]
Evaluation of bile acid profiles of adults with NAFLD and NASH	feces	lyophilization, reconstitution in 75% EtOH, homogenization	LC-MS in accordance with Biocrates Life Science Bile Acid Kit manufacturer's instructions	↑TBA, CDCA and primary/secondary BA ratio in patients with NASH compared to HC; ↑ CA in both NASH and NAFL compared to HC	Mouzaki et al. [82]
Investigation bile acids in benign and malignant biliary stenosis	bile	dilution and protein precipitation with ACN, filtration	LC-MS Column: Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm) A: 0.5 mM ammonium acetate and 0.001% acetic acid in H_2O	↑CA in benign compared to malignant biliary stenosis	Rejchrt et al. [85]

(continued on next page)

Table 2 (*continued*)

Application	Matrix	Sample preparation	Analytical platform	Utility of bile acids as a biomarker	Author (reference)
			B: MeOH:ACN (75:25) containing 0.5 mM ammonium acetate and 0.001% acetic acid		
Bile acid profiling in patients with pancreatic cancer and benign disease	bile	protein precipitation with MeOH, dilution	LC-MS Column: Kinetex C18 (50 × 2.1 mm, 1.7 μm) A: MeOH:ACN:H_2O (33.3:16.7:50) with 0.1% formic acid B: MeOH:ACN (63.3:31.7) with 0.1% formic acid	↑TBA, CA, UDCA, CDCA, DCA in adenocarcinoma of the pancreas patients compared to benign disease	Rees et al. [86]

magnetic resonance (NMR) spectroscopy and LC-MS. NMR has been successfully used for the metabolic profiling of bile in patients with CC [44], and in determining changes in bile after decompression therapy in extrahepatic biliary obstructed patients [45]. In contrast, Legido-Quigley et al. used an SPE-UPLC-MS platform to monitor changes in the bile profiles of donors and recipients during successful liver transplants. This study indicated that taurocholic acid (TCA) was significantly increased after transplantation and may be related to liver regeneration and de novo synthesis. A commonality between donors and recipients was an overall increase of the secondary bile acids lithocholic acid (LCA) and ursodeoxycholic acid (UDCA), which are hepatotoxic and chemopreventive, respectively [46]. Supercritical fluid chromatography (SFC) is one notable alternative high-throughput technology for metabolic profiling. The results of one study that examined bile samples obtained from rats and dogs showed that the SFC system's performance was at least as good as that of sub 2 μm particle LC/MS; however, the results also showed that the SFC system consumed 75% less organic solvent than an equivalent reversed-phase separation of similar length. The platform used proved to be suitable for separating compounds present in bile, such as bile acids, and a comparison of QC samples proved that the system was capable of operating without loss of performance for a sample analysis set of 100 samples [47]. In addition, approaches based on the direct introduction of samples to detectors without prior separation of the analytes are also being sought in order to improve analytical throughput. While direct-MS methods have certain limitations compared to LC-MS (e.g., the high possibility of matrix effects; lower certainty in the identification of particular signals, which leads to compromised application in biomarker discovery), they may well prove suitable for the quantitative determination of selected biomarkers when an appropriate normalization method is used or for the rapid analysis of samples to be mapped against a validated model. For instance, one study applied a mass spectrometer equipped with a solids analysis probe (atmospheric solids analysis probe-mass spectrometry (ASAP-MS)) for the high-throughput qualitative analysis of bile samples [48].

Bile is usually only subjected to lipidomic analyses due to its composition. Navaneethan et al. performed lipidomic profiling using Bligh and Dyer extraction followed by analysis on an LC-MS platform [49]. Their results indicated that biliary oxidized phospholipids (oxPLs) may be potentially very valuable in distinguishing patients with CC from those with other etiologies of biliary strictures. The same clinical goal was the subject of multi-omic studies that combined metabolomics—which was mainly focused on bile lipidome (UHPLC-MS and 1H NMR)—and proteomics (S-TRAP-LC-MS) [50]. Most of the cited omics studies emphasize the putative role of bile acids as potential biomarkers of the diseases; therefore, the methods described therein still require further validation if they are to complement current diagnostic techniques, which aim to differentiate pathological changes in the biliary tract.

3. Determination of bile acids in various biological matrices

Bile acids (BAs) are steroidal C24 carboxylic acids that are synthesized in the liver from cholesterol. The two primary bile acids in humans, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized in hepatocytes, where they are conjugated with glycine or taurine and metabolized via hydroxylation, sulfation, and glucuronidation. BAs are secreted into the gallbladder and then released into the small intestine, where CA and CDCA are dehydroxylated by intestinal bacteria to form lipophilic secondary deoxycholic acid (DCA) and LCA. 95% of these compounds are reabsorbed in the distal ileum and return to the liver via enterohepatic circulation. BAs play an essential role in the control of cholesterol homeostasis, and the absorption of lipids and fat-soluble vitamins in the intestinal tract. In addition, BAs alter multiple metabolic pathways in many tissues through several receptors, such as the farnesoid X receptor (FXR), the pregnane X receptor (PXR), the vitamin D receptor (VDR), and one G-protein-coupled receptor (TGR5). As a result, several individual bile acids have been proposed as biomarkers of biliary and liver diseases [51,52].

In the last years, LC-MS platform has become a standard method for the direct and simultaneous quantitative profiling of free and conjugated bile acids in biological samples with high specificity and selectivity. The chromatographic analysis is performed mainly on C18 columns by the reverse phase technique with conventional gradients using water, acetonitrile, and isopropanol and additives such as formic acid, ammonium formate, or ammonium acetate. In the determination of bile acids, electrospray ionization (ESI) source in the negative-ion mode working in the multiple reaction monitoring (MRM) mode is the most commonly used. Fragmentation of BA is characterized by fragmentation of hydroxylic and carboxylic side groups at low collision energies and fragmentation of the steroid backbone at higher collision energies [53].

3.1. Blood analysis

Venous blood is the most frequently used matrix for the determination of bile acids. Due to high concentrations of proteins and phospholipids, as well as the possibility of non-covalent binding of BA to proteins, the preparation of serum or plasma for analysis requires protein denaturation with organic solvents. To this end, recent works have utilized simple PP with ACN [54,55], MeOH [56], or IPA [51] in conjunction with a 96-well high-throughput format [57,58], followed by evaporation and reconstitution. Other studies have used SPE to extract and concentrate BAs, and to clean up the samples prior to analysis [59,60]. Bile acid determination results are also subject to interference due to the presence of phospholipids, which may interact with BAs in a manner similar to proteins. Han et al. attempted to address this issue by developing a phospholipid-depletion SPE (PD-SPE) method and comparing the results obtained via this method to those of PP, reversed-phase SPE, and reversed-phase SPE with high pH (hi-pH SPE). In Han et al.‘s method, the serum samples first undergo centrifugation with ACN before being loaded into a Hybrid®SPE-Phospholipid 96-well plate for elution. This protocol enables the removal of phospholipids based on a mechanism of the Lewis acid-base interactions, thus reducing the matrix effect. Han et al.‘s results indicated that their method was more reproducible than the other three sample preparation approaches, and permitted the quantification of 43 BAs in human serum [52]. Another variant of SPE that can be applied to extract BA from biological fluids is magnetic solid-phase extraction (MSPE). Sun et al. presented a method of preparing magnetic mesoporous epoxy resin (MMRE), wherein epoxy resin is bound to amine-functionalized magnetic nanoparticles for use as an MSPE sorbent. Significantly, the separation step can be performed using an external magnet, which eliminates the need for the filtration and centrifugation steps required in conventional SPE methods, while still providing high extraction ability, a large enrichment factor, and low consumption of organic reagents [61]. In contrast to these methods, de Paiva et al. used hollow fiber liquid-phase microextraction (HF-LPME) to extract bile acids from human plasma. In this technique, a hydrophobic porous capillary membrane is impregnated with an organic extraction solvent, and the lumen is filled with microliters of an acceptor phase. Analyte transfer occurs via passive diffusion between the sample and the acceptor phase, and the application of a pH gradient results in high extraction yields and efficient extraction kinetics. Among the various solvents tested by de Paiva et al., octanol was deemed to be the most optimal acceptor phase for bile extraction due to its superior yields [62].

Despite their differences, all of the above-described methods require the use of an LC-MS platform for the determination of individual BAs. In screening for hepatobiliary diseases, the determination of total bile acids (TBA) is of great diagnostic importance. Fortunately, this does not require advanced instrumental facilities. Danese et al. performed an analytical evaluation of 3 enzyme assays (from Diazyme, Randox, and Sentiel) based on the conversion of BAs and NAD$^+$ to 3-keto-steroids and NADH by 3-α-hydroxysteroid dehydrogenase (3-α-HSD) in order to measure TBA in plasma on a fully-automated analyzer (Roche Cobas 501). Although all assays had optimal linearity between 3 ± 138 μmol/L, optimal inaccuracy, and good correlation with LC-MS data, the BA values in all of the plasma samples were significantly underestimated [63]. The same redox reaction was used by Zhang et al. to develop an electrochemical method for detecting TBA in serum. The emergent NADH was oxidized onto the surface of a screen printed carbon electrode (SPCE), which generated a signal that was collected by differential pulse voltammetry (DPV). This signal was then used to calculate the TBA concentration [64]. The addition of ruthenium (II)-tris(bipyridine) to the above enzymatic reactions made it possible to obtain a novel electrochemical biosensor for the ultrasensitive detection of TBA in serum [65].

The determination of BA in the blood of patients with liver and biliary diseases has been the focus of numerous studies in recent years. The work of Fu et al. suggests that the vast differences in TBA, as well as individual metabolites and subgroups of bile acids, may enable the differentiation of pediatric diseases such as neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), biliary atresia, and hepatoblastoma [56]. Similarly, combining the primary/secondary bile acid ratio (>433) with the conjugated/free ratio (>90) allowed infants with NICCD to be differentiated from those without NICCD with a sensitivity of 100% and a specificity of 100% [66]. Combining bile acid profiles with analyses of bile acid patterns may also enable the differential diagnosis of liver diseases in adults. Luo et al. showed that, compared to healthy subjects, an increased ratio of tauro-conjugates to glyco-conjugates and low DCA concentrations in the serum are characteristic to subjects with liver impairments [57]. Moreover, TBA, glycine-conjugated BAs (GBAs), total CDCA, and the sum of glycolithocholic acid 3-sulfate (GLCAS) and taurolithocholic acid 3-sulfate (TLCAS) have been identified as four potential biomarkers for liver diseases [58]. The work of Sui et al. provides a new perspective on using dynamic changes in the metabolites of metabolic pathways to identify biomarkers during perioperative liver transplantation. Indeed, their findings suggest that TCA and TCDCA may be important metabolites that can be used to evaluate liver function recovery after transplantation [67].

3.2. *Urine analysis*

Urine is a frequently used matrix because it is readily available in large volumes, and does not require the use of invasive collection methods. While the analytical methods used to analyze bile in urine samples vary in terms of their sample preparation approaches, they are all primarily based on LC-MS platforms. The two basic approaches for preparing urine samples prior to quantifying bile acids are the use of SPE cartridges, and evaporating the eluate and reconstituting it in methanol solution [68—70]. Humbert et al. compared several extraction techniques, followed by HPLC-MS/MS, to determine the most effective protocol for profiling bile acids in clinical samples. Their results indicated that the best urine analysis results were obtained using an acetonitrile extraction followed by a clean-up step with an SPE C18 cartridge [71]. The simultaneous determination of bile acids and their sulfate metabolites (LC-MS and commercially available UBASTEC-AUTO kit) is another commonly used approach to urinary BAs quantification, as sulfation is a major metabolic pathway involved in the elimination of bile acids with urine [68,69]. This approach was employed by Bethena et al. to compare the differences between the urinary BA profiles of healthy subjects and those of patients with a variety of liver diseases. The authors quantified the BA profiles using “BA indices” that described the composition, hydrophilicity, and metabolism (including amidation with amino acids and sulfation) of the total and individual BAs. Bethena et al.‘s pilot study demonstrated the advantages of using novel “BA indices”—rather than the current approach of using liver enzymes and proteins—as diagnostic and prognostic biomarkers of hepatobiliary diseases [70]. The profiling of sulfated bile acids (SBAs) in urine has been proposed for the diagnosis and grading of intrahepatic cholestasis of pregnancy (ICP). Since most of the authentic SBAs were not available, each peak captured by multiple reaction monitoring (MRM) was identified via an enhanced product ion (EPI) scan. MRM transitions were used as survey scans, and triggered the acquisition of EPI spectra when the signal intensity of the survey scan exceeded a predefined threshold value. Compared to non-enhanced basic product ion scans, EPI scans deliver high sensitivity, high mass accuracy, and fast scanning. The EPI scan results indicated that glycine cholic acid 3-sulfate (GCAS) is well-suited as a biomarker for the diagnosis of ICP, as it offers a sensitivity of 100% and a specificity of 95.5% [72].

Modern analytical chemistry strives to fully automate the sample preparation process and integrate it directly into instrumental platforms. To this end, Liu et al. developed a large-volume direct injection-online solid phase extraction-ultra high performance liquid chromatography-polarity switching tandem mass spectrometry (LVDI-online SPE-UHPLC-psMS/MS) method to enable the sensitive profiling of various compounds, including bile acids, in urine. A single analytical run was divided into loading and elution phases by alternating the electronic 6-port/2-channel valve. In the loading phase, the autosampler was programmed to inject 500 μL of diluted urine sample onto the SPE cartridge via ten consecutive injections. During the elution phase, the lipophilic compounds that had adsorbed onto the SPE column packing were washed into the analytical column using a programmed gradient elution of formic acid and acetonitrile. Analyte detection was performed using both positive and negative MRM modes [73].

Another proposed approach for determining urinary bile acids combines the enzymatic digestion of conjugated BAs with targeted LC-MS analysis. Fang et al. and Zhu et al. developed several enzyme assays to determine the amount of unconjugated, amidated, sulfated/glucuronated, and TBA [74,75]. In both sets of assays, individual bile acid fractions were analyzed after incubation with sodium acetate buffer, choloylglycine hydrolase, sulfatase and beta-glucuronidase, and a mixture of all enzyme solutions [74,75]. Moreover, numerous metabolomics studies of urine samples have attempted to determine the role of bile acids in the pathomechanism of liver and biliary diseases, such as hepatocellular carcinoma [76], intrahepatic cholestasis of pregnancy [77], and primary biliary cirrhosis [78].

3.3. Feces analysis

Feces is a less frequently utilized matrix for analyses of bile acids, as it contains low levels of bile acids due to enterohepatic circulation. Nevertheless, in recent years, several studies have attempted to determine fecal bile acid concentrations using the LC-MS platform. For example, Humbert et al. compared 4 extraction protocols based on lyophilization and homogenization with a suitable solvent (NaOH, ACN, IPA or water) and SPE step to select the most efficient procedure for extracting bile acids from stool [71]. The advantage of NaOH extraction is that it provides sensitivity for a wide range of analytes, which enables the determination of minor species—aside from the comparatively abundant fecal bile acids—that are potentially relevant as biomarkers. The same protocol was also used in later studies with some modifications [79]. In one such study, Kakiyama's group assessed the bile acid profiles in stool samples taken from patients with cirrhosis; their results indicated that cirrhosis is associated with a decrease in total fecal BA concentration and a decrease in conversion from primary to secondary BAs compared to controls, which worsens with the severity of the disease [80]. The authors measured fecal BAs by developing a simple and accurate HPLC-UV method that utilized the phenacyl derivatives of unconjugated bile acids, producing outcomes that confirmed previously published results. This method, which is also based on lyophilisation and extraction with NaOH and SPE, was validated, and its results were compared to those of the more advanced GC-MS and LC-ESI-MS/MS platforms [81]. Mouzaki et al. proposed using the Biocrates Life Science Bile Acid Kit (Biocrates, Innsbruck, Austria) to quantify BAs in fecal samples taken from patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). Their results showed that the total fecal BAs and the ratio of primary to secondary BAs were higher in patients with NASH compared to healthy control (HC). Levels of unconjugated CA were higher in both the NASH and NAFLD patients than in the HC, while CDCA was higher in the NASH patients than in the HC. Unconjugated primary bile acids were also correlated with serum markers of liver injury, AST, ALT, and triglyceride levels [82]. Another preparation method is to suspend a raw stool sample in ACN solution and use the centrifugation supernatant for LC-MS analysis. This approach has allowed researchers to prove that PSC reduces the concentration of LCA in the feces of people with inflammatory bowel disease (IBD) [83].

3.4. Bile analysis

Bile fluid is the biological matrix with the highest concentrations of BAs. Unfortunately, this matrix is rarely used to profile bile acids for the purpose of diagnosing liver and biliary diseases, as it is quite difficult to access. For instance, bile collection from human subjects is mainly done during endoscopic retrograde cholangiopancreatography (ERCP). In recent years, the LC-MS platform has been the only one used to determine BAs in bile samples. As with blood and urine samples, the bile samples in these studies were prepared using protein precipitation and SPE methods [54,79,84]. However, the high concentration of BAs requires the sample to be diluted. For instance, Rejchrt et al. used dilution factors of 1000x and 10000x to cover a broad range of concentrations in their samples, including major and minor BAs. In their pilot study, they examined 23 BAs in liver bile samples taken from patients with benign and malignant biliary stenosis, and found that CA concentrations differed significantly between the two groups. In addition, their results indicated significant relationships ($r > 0.7$) between several pairs of bile acids [85]. In another study, Rees et al. evaluated the composition of BAs in bile samples taken from patients with adenocarcinoma of the pancreas and benign biliary disease. The results showed an increased concentration of total unconjugated BAs in the malignant group, which indicates that CA is the most important factor differentiating these groups from one another [86].

Moreover, the analysis of bile composition, including BA, collected during machine perfusion to assess liver function and bile duct condition before transplantation seems to be promising. Unfortunately, literature on the topic describes determination of TBA rather than individual bile acids profiles. Linares-Cervantes et al. determined TBA in bile samples produced by the organ with varying ischemia degrees before and after normothermic ex vivo liver perfusion (NEVLP) using an enzymatic method with colorimetric detection. TBA levels tended to decrease with the degree of ischemia, but normothermic perfusion resulted in increase of TBA in all groups, except for the group with the longest ischemic time [87]. Similarly, Boehnert et al. compared bile composition after reperfusion of liver subjected previously to NEVLP or static cold storage (SCS). Bile acids were significantly higher in normothermic perfused grafts versus short or prolonged cold static preserved livers [88]. These results indicate the usefulness of determination of BA in assessing organ quality. Moreover, profiling of individual BAs may reveal the importance of biochemical changes occurring in the organ.

4. Conclusion

Bile has been the subject of many studies aimed at explaining the pathomechanisms of diseases of the liver and bile ducts, as the composition of bile is significantly influenced by the physiological state of these organs. This review has presented a wide selection of medical and environmental research that has utilized bile fluids, and it has summarized the various sample preparation and instrumental methods that have enabled the use of this complex matrix in fields such as microbiology, pharmacokinetics, and omics sciences.

However, bile acids can be found in various biological matrices as result of enterohepatic circulation and urinary excretion. The development of new, highly sensitive methods for the quantitative determination of bile acids may contribute to a better understanding of many pathophysiological processes and serve as tools that can support traditional diagnostic tests for liver dysfunction and biliary diseases in the future.

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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5.4. Mikroekstrakcja do fazy stałej – P.2.

Mikroekstrakcja do fazy stałej (SPME, ang. solid-phase microextraction) jest metodą przygotowania próbek do analiz instrumentalnych, która łączy pobieranie próbek, preparatykę i ekstrakcję w jednym kroku. SPME jest techniką ekstrakcji niewyczerpującej, opartą na równowadze, która upraszcza przygotowanie próbek do jednego prostego kroku, eliminując konieczność filtrowania, wirowania, homogenizacji tkanek oraz użycia dużych ilości rozpuszczalników organicznych wymaganych w tradycyjnych metodach preparatyki próbek. Choć początkowo metoda ta była szeroko stosowana do analizy lotnych związków w środowisku i żywności, obecnie znajduje coraz szersze zastosowanie w badaniach biomedycznych, umożliwiając ekstrakcję bezpośrednio z kompleksowych matryc, takich jak osocze, mocz, surowica, ślina, pełna krew, żółć czy tkanki (tzw. biopsja chemiczna). Taka wszechstronność wykorzystania SPME wynika z możliwości wykorzystania różnorodnych geometrii, które pozwalają sprostać wielu wymaganiom analitycznym, których nie spełniają inne techniki przygotowania próbek. Najczęściej stosowanymi geometriami są sondy SPME – cienkie włókna o średnicy ok. 250 µm pokryte fazą ekstrakcyjną lub stalowe blaszki pokryte sorbentem, czyli tzw. mikroekstrakcja cienkowarstwowa (TFME, ang. thin-film microextraction). W zależności od zastosowanego podejścia, unikalne cechy SPME, takie jak szeroki zakres polarności ekstrahowanych metabolitów, niskoinwazyjność, brak fizycznego zużycia próbki, możliwość ekstrakcji nietrwałych metabolitów, wysokoprzepustowość analiz sprawiają, że technika ta została zaproponowana w różnych badaniach metabomicznych jako skuteczne narzędzie do oznaczania związków istotnych w ocenie funkcji przeszczepu oraz do profilowania chemicznego narządów poddawanych różnym protokołom konserwacji.

Zagadnienia związane z wykorzystaniem SPME oraz badań omicznych jako narzędzia do monitorowania jakości przeszczepu zostały opisane w pracy poglądowej pt. „Solid Phase Microextraction—A Promising Tool for Graft Quality Monitoring in Solid Organ Transplantation” (Separations, 2023, 10, 153) będącej częścią cyklu publikacji prezentowanych w niniejszej rozprawie doktorskiej (publikacja P.2.).

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Review

Solid Phase Microextraction—A Promising Tool for Graft Quality Monitoring in Solid Organ Transplantation

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Abstract: Solid organ transplantation is a life-saving intervention for patients suffering from end-stage organ failure. Although improvements in surgical techniques, standards of care, and immunosuppression have been observed over the last few decades, transplant centers have to face the problem of an insufficient number of organs for transplantation concerning the growing demand. An opportunity to increase the pool of organs intended for transplantation is the more frequent use of organs from extended criteria and the development of analytical methods allowing for a better assessment of the quality of organs to minimize the risk of post-transplant organ injury and rejection. Therefore, solid-phase microextraction (SPME) has been proposed in various studies as an effective tool for determining compounds of significance during graft function assessment or for the chemical profiling of grafts undergoing various preservation protocols. This review summarizes how SPME addresses the analytical challenges associated with different matrices utilized in the peri-transplant period and discusses its potential as a diagnostic tool in future work.

Keywords: solid-phase microextraction; SPME; transplantation; graft quality assessment; biomarkers

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1. Introduction

Solid organ transplantation is a life-saving intervention for patients suffering from end-stage organ failure. The transplantation of livers, kidneys, hearts, and lungs has become a routine part of clinical care worldwide, and has contributed to higher survival rates and greater quality of life among patients [1]. Over the last few decades, advancements in surgical techniques, standards of care, and immunosuppression have significantly improved transplantation outcomes. However, the number of patients on waiting lists for all solid organs has rapidly increased, resulting in an ever-growing disparity between organ availability and demand [2]. Furthermore, this shortage of donor organs is exacerbated by the fact that many organs are ultimately deemed unsuitable for transplantation due to strict criteria and are discarded as a result [3]. Indeed, it is estimated that only 20% of lungs and less than 40% of hearts are considered suitable for transplantation [4,5].

Given this scarcity of viable organs, strategies aimed at increasing the donor pool, reducing patient wait times, and decreasing the number of unnecessarily discarded organs are crucial. Among such strategies, one of the most notable is the use of organs from expanded criteria donors (ECD) and donors after circulatory death (DCD) [6,7]. However, it is well known that compared with standard criteria donors (SCD), marginal grafts tend to have worse outcomes, including an increased risk of delayed graft function (DGF) and primary nonfunction incidence (PNF) [8,9]. Hence, there is a great need for accurate methods of assessing graft quality and estimating donor risk, especially in relation to marginal grafts. Currently, an organ's suitability for transplantation is determined based on detailed parameters, including the donor's medical history and examination results. Depending on the organ, different parameters will be considered. The factors taken into account when assessing organ quality include, among others: smoking history, chest X-rays, oxygenation, active infections, hemodynamic stability, electrocardiograms, echocardiograms, serum bilirubin, AST, ALT, creatinine concentration, visual assessment of the organ, and biopsy [7,10–13]. Unfortunately, the decision-making process for accepting an organ for transplantation is always subjective to some extent and often lacks sufficient overall predictive power. Furthermore, many of the evaluated parameters cannot be used as independent predictors due to their low specificity.

The transplant team's visual assessment of donor organs is often fundamental in decision-making. Although a general inspection can help to identify tumors and anatomical changes, this method is subjective and depends on the transplant team's experience level [12]. In the case of heart transplants, several studies have indicated that elevated troponin levels in donor serum may be related to primary graft dysfunction (PGD). However, higher troponin levels in serum from donors who have experienced brain death may be a result of the brain's degradation rather than an indication of myocardial damage [7,14]. Similarly, left ventricular dysfunction is another common reason for discarding potential transplant organs, but this condition may be caused by a potentially reversible sequela of brain death, rather than a defective heart [14]. For lung transplants, non-smokers are ideal donors, as a donor history of smoking has been related to PGD. Nonetheless, findings have shown that recipients who received a graft from an actively smoking donor have significantly lower mortality compared to patients who remained on the waiting list [15]. In the case of kidney and liver transplantation, pre-transplant biopsies remain the gold standard for diagnosing organ injury. However, histological assessments are usually applied selectively, predominantly in ECD and DCD grafts, and the frequency of performed biopsies varies between medical facilities and countries. Although biopsies can provide comprehensive information relating to organ status, the role of a pre-transplant biopsy in graft evaluation is intensely debated. The use of biopsies to evaluate graft quality is hampered by two major limitations: the low reproducibility of results between on-call pathologists, and their time-consuming nature. Previous studies have analyzed the consistency of scores assigned to kidney donor biopsies by different on-call pathologists and the retrospective evaluation of the same sections performed by the renal pathologist. Interestingly, a correlation between donor histology and graft outcome was observed in the evaluations performed by the renal pathologists, but not in the evaluations performed by the on-call pathologists [16]. Thus, histological evaluation remains only one component of graft assessment and should not be the sole determinant in deciding whether to accept an organ for transplantation or to discard it. All of the above examples illustrate the comprehensiveness and complexity of organ-quality assessment and the decision-making processes. In an attempt to improve and standardize the evaluation process, researchers have developed several scoring systems aimed at providing an objective estimate of post-transplant survival based on donors' and/or recipients' characteristics. Two of these systems, the Kidney Donor Risk Index (KDRI) and the Liver Donor Risk Index (LDRI), are among the most established metrics for estimating graft survival; however, new solutions are still being proposed [17]. Although risk indices can facilitate and improve the decision-making process, they are not intended to serve as the only metric for determining donor suitability; rather, they should be utilized as part of a comprehensive evaluation along with other factors. Furthermore, while donor age is one of the most influential factors in calculating most risk indexes, it is not well-established whether these indexes can be applied to elderly and pediatric donors [18,19].

Another way to increase the donor pool is the implementation of new organ preservation methods. At present, static cold storage (SCS) is the standard technique for organ preservation in clinical practice due to its readily available logistics and low cost. The hypothermic conditions of SCS reduce the organ's metabolic activity and oxygen demand, thereby reducing ischemic injury. However, ECD grafts appear to be more prone to prolonged ischemia, resulting in increased morbidity and mortality in recipients. Thus, the technique of preserving these types of organs is crucial [20,21]. In recent years, numerous preclinical and clinical studies have sought to develop and optimize alternative organ-preservation methods in an attempt to improve the outcomes of ECD graft transplantation. Currently, the proposed machine perfusion methods can be divided into three categories based on the pumping temperature of the preservation solution and the oxygen supply (i.e., with or without oxygen): normothermic (35–37 °C), sub-normothermic (20–25 °C), and hypothermic (4–10 °C) [22]. These methods are advantageous because they minimize the risk of organ damage and early allograft dysfunction (EAD), in addition to allowing the administration of additional pharmacological agents for protective ischemic postconditioning and for assessing organ function immediately before transplantation [4,5]. Unfortunately, although machine perfusion enables the monitoring of perfusion parameters that can predict organ viability after transplantation, there is still a lack of representative methods for assessing graft quality.

Solid-phase microextraction (SPME) is a widely accepted analytical method in bioanalysis. Undoubtedly, the analysis of volatile compounds in the headspace of a given sample is both the original and most studied application of SPME. However, in recent years, the direct extraction of non-volatile compounds from complex matrices has emerged as a very attractive alternative to conventional sample preparation methods in bioanalysis. While SPME fibers—which are probes with a small diameter (ca. 250 nm)—are the most well-known geometry, thin-film devices are also frequently used, as their larger coating area and use of higher sorbent volumes enables greater sensitivity. The literature contains numerous reviews detailing developments in and applications of SPME [23–30]. In addition, there is an ever-growing trend showing that the SPME technique is increasingly used for sample preparation prior to analysis in recent years (Figure 1).

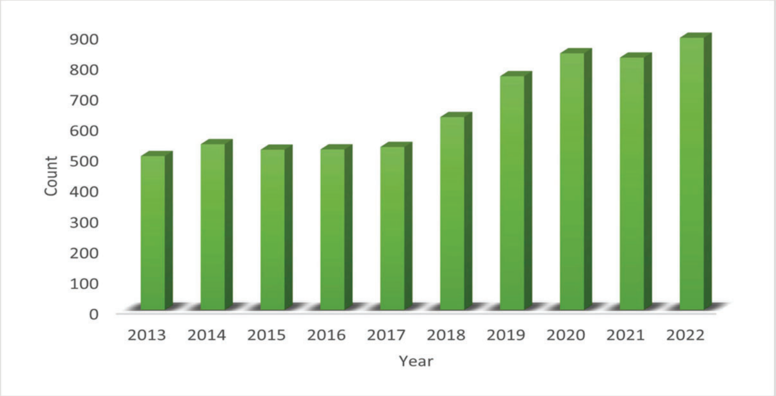

Figure 1. The number of articles that include SPME as a sample preparation method. Numbers are based on PubMed searching for "SPME" or "solid-phase microextraction" in titles and/or abstracts.

In brief, SPME is an equilibrium-based technique that extracts via free concentration. In complex matrices containing macromolecules (e.g., proteins), small species equilibrate with the macromolecules so that they are partially bound and partially free. In general, the more hydrophobic the compound, the higher the binding and the lower the free concentration; therefore, one would expect SPME to perform poorly in recovering these species. However, the coatings most commonly used in bioanalysis, such as C18 or mixed-mode coatings, have a high affinity toward hydrophobic compounds, thus compensating for the aforementioned phenomenon. The opposite situation is observed for hydrophilic metabolites, thereby allowing SPME to provide balanced coverage of the compounds. Another feature of SPME that should be emphasized is its capacity for capturing unstable species, which is made possible by its ability to extract small compounds while excluding large molecules. The chemistry and porosity of commonly used SPME coatings restrict the penetration of macromolecules into the sorbent, which protects the absorbed metabolites from enzymatic degradation. Moreover, due to the small size of probes (e.g., fibers), SPME affords minimal invasiveness and allows the same organ to be sampled several times without damaging the tissue. Conventional sample preparation methods demand the collection and homogenization of tissue, followed by the extraction of analytes with aqueous and organic solvents. Such techniques are widely used; however, their complex protocols make them unsuitable for on-site extraction. Additionally, tissue collection usually restricts analysis to a single sampling because repeated biopsy carries the risk of tissue damage and other side effects [31]. Because of SPME's unique features—which include, but are not limited to, balanced analyte coverage, minimal invasiveness [32], lack of physical sample consumption [33], the ability to extract labile metabolites and determine free (i.e., active) concentrations of metabolites [34], and the biocompatibility of SPME probes—new applications are continually being tested and proposed. As mentioned above, one of the major challenges in transplant surgery is assessing graft quality and function in order to minimize the risk of post-transplant organ injury and rejection. Given the above-discussed possibilities, SPME has been proposed in various studies as an effective tool for determining compounds of significance during graft function assessment, or for the chemical profiling of grafts undergoing various preservation protocols. The recent literature contains numerous papers detailing the use of SPME and omics approaches to assess the quality of organs intended for transplantation. As such, this review summarizes how solid-phase microextraction addresses the analytical challenges associated with different matrices utilized in the peri-transplant period (Figure 2) and discusses its potential as diagnostic tool in future work. The main assumptions of the cited studies are summarized in Table 1.

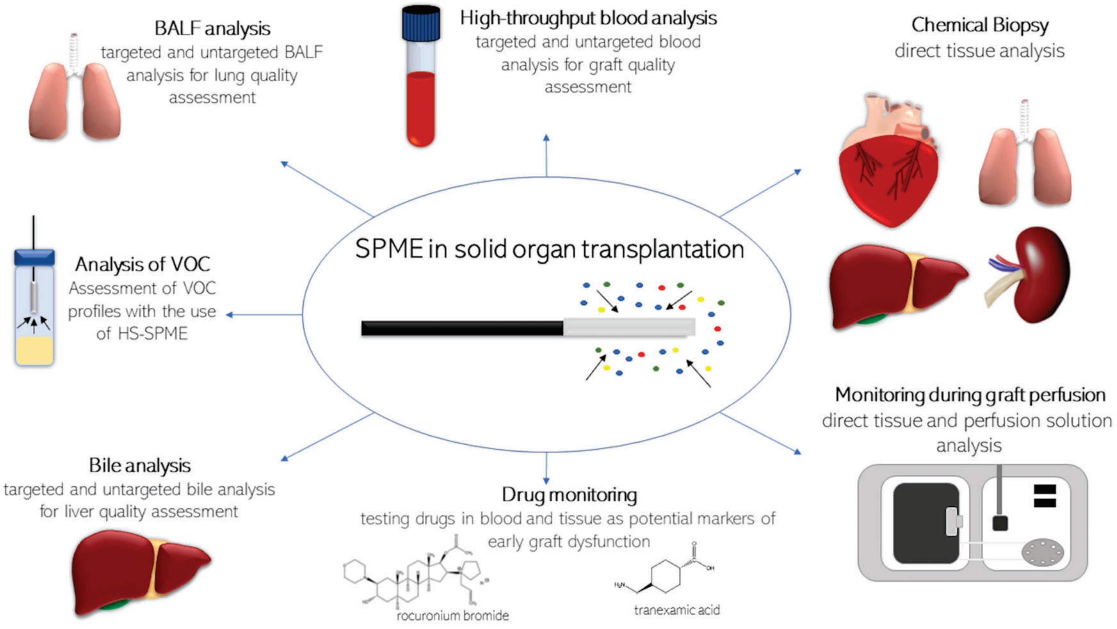

Figure 2. Exemplary application of solid-phase microextraction in solid organ transplantation. Details are discussed in the text.

Table 1. Applications of solid-phase microextraction in solid organ transplantation.

Monitored Organ	Biological Specimen	Sampling Mode (Ex Vivo/In Vivo; Head-Space/Direct Immersion HS/DI)	Device Geometry and Coating Chemistry	Target Analyte	Comments	Reference
lung	bronchoalveolar lavage fluid (BALF) (human)	ex vivo; DI	TFME, PAN-C18	cholic acid (CA), deoxycholic acid (DCA)	• comparison of the results with total bile acids concentration performed by enzymatic assay	[35]
lung	bronchoalveolar lavage fluid (BALF) (human)	ex vivo; DI	fiber, mix-mode	untargeted profiling	• separation of patients with TBA < and > TBA > 0.05 mmol/L driven by bile acids, steroid, fatty aldehydes, fatty alcohol, prenol lipids, fatty amide, fatty acids suggested re-consideration TBA cut-off value for “healthy/sick” BALF samples	[35]
lung	bronchoalveolar lavage fluid BALF and blind bronchial aspirate (BBA) (human)	ex vivo; HS	fiber, DVB/CAR/PDMS	untargeted profiling	• 20 compounds selected with support vector machine (SVM) model resulted in a sensitivity of 0.63, specificity of 0.94, positives and negatives predictive value of 0.87 and 0.80, respectively; • no relationship between the type of donation, cause of death or time of ischemia and VOC profile was statistically significant; • donor BMI correlated with the VOC pattern	[36]
liver	plasma (human)	ex vivo; DI	TFME, PAN-WCX	rocuronium bromide (ROC), tranexamic acid (TXA)	• testing drugs as potential markers of early graft dysfunction using living donors (LD) and heart beating brain dead donors (HBD); • ROC showed lower liver clearance in HBD group; • TXA showed no difference in renal clearance in HBD and LD, but in both cases it was lower than normal; supported by analysis of known biomarkers of liver injury	[37]
liver	plasma (human)	ex vivo; DI	TFME, HLB	untargeted profiling	• searching biomarkers of early graft dysfunction; • HBD and LD groups discriminated by oxidized lipids and bile acids	[37]
liver	bile (pig)	ex vivo; DI	TFME, HLB	untargeted profiling	• evaluation of the metabolomic profile of bile from livers preserved via SCS and NEVLP: Bile produced in the SCS-preserved livers was characterized by increased levels of metabolites such as CDCA, AA, and 5S-HETE, as well as saturated and monounsaturated LPCs. • assessment of the effect of the organ’s ischemic time on the composition of bile: changes mainly in amino acids and lipids, including bile acids and steroid compounds, along with their derivatives	[38]

Table 1. *Cont.*

Monitored Organ	Biological Specimen	Sampling Mode (Ex Vivo/In Vivo; Head-Space/Direct Immersion HS/DI)	Device Geometry and Coating Chemistry	Target Analyte	Comments	Reference
liver	bile (pig)	ex vivo; DI	TFME, C18	glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), taurolithocholic acid (TLCA), glycoursodeoxycolic acid (GUDCA), tauroursodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), hyocholic acid (HCA), taurochenodeoxycholic acid (TCDCA), ursodeoxycholic acid (UDCA)	• evaluation of individual bile acid profile of bile samples in the peri-transplant period: the conjugated forms of bile acids were significantly predominant in the bile samples compared to unconjugated forms; • high concentration of TCA was characterized the perfusion period and they remained high during reperfusion in the 90′DCD group; • prolonged ischemia caused an increase in TDCA and GDCA levels in the first days after transplantation compared to HBD group	[39]
lung; liver	tissue (pig)	ex vivo, in vivo; DI	fiber, mix-mode	untargeted profiling	• evaluation of the method’s efficiency, the utilized storage and transportation conditions; • in the extracts from ex vivo sampling, the absence of some labile metabolites (e.g., itaconic acid, hypoxanthine, lactaldehyde or hydroxyacetone), but the presence of metabolites recognized as the products of the primary metabolite degradation (e.g., diacylglycerol) were observed; • in vivo sampling followed by on-fiber storage was the most convenient strategy; • the best sensitivity was observed for the longer coating, while reproducibility was better for the two shorter coatings, which was likely due to the heterogeneity of the liver	[40]
lung; liver	tissue (pig)	ex vivo, in vivo; DI	fiber, mix-mode	methylprednisolone (MP)	• monitoring drugs routinely used during the transplantation procedure as markers of changes in liver function; • methylprednisolone was only present in non-ischemic organs	[41]
lung; liver	tissue (pig), perfusate	ex vivo, in vivo; DI	fiber, mix-mode	untargeted profiling	• evaluation of changes of the metabolic profiling of lungs at different stages of their transplantation procedure; • monitoring metabolites in perfusate circulating throughout the graft in a closed circuit as a non-invasive approach for monitoring graft function during perfusion	[41]

Table 1. *Cont.*

Monitored Organ	Biological Specimen	Sampling Mode (Ex Vivo/In Vivo; Head-Space/Direct Immersion HS/DI)	Device Geometry and Coating Chemistry	Target Analyte	Comments	Reference
kidney	tissue (rabbit)	ex vivo; DI	fiber, mix-mode	untargeted profiling	• assessment of the effect of prolonged ischemia and the accompanying oxidative stress affected the condition of kidneys subjected to static cold storage; • a significant change in the metabolic profile of the kidney over the first 4 h of storage; • the most pronounced alterations were observed in the levels of essential amino acids, which changed depending on exposure to the ischemic milieu, and purine nucleosides, which are associated with various metabolic pathways involved in the production of reactive oxygen species (ROS)	[42]
kidney	tissue (pig, human)	ex vivo, in vivo; DI	fiber, mix-mode	untargeted profiling	• presentation of a metabolomic and lipidomic protocol for untargeted analyses for graft quality assessment	[43]
kidney	tissue (pig)	ex vivo, in vivo; DI	fiber, mix-mode	untargeted profiling	• evaluation of kidney quality during the transplantation procedure with particular emphasis on HBD and DCD donors; • revealed changes in purine and LPC levels between the HBD and DCD groups; • level of certain amino acids and LPC were correlated with the organ's warm ischemia duration; • the decreased PC/PE ratio in the DCD organs during reperfusion	[44]
kidney	tissue (pig)	ex vivo, in vivo; DI	fiber, mix-mode	untargeted profiling	• comparison of the effects of SCS, NEVKP, and HMP on the lipidomic profile of a kidney graft; • the preservation temperature had a more significant impact on the lipidomic profile than its mechanical characteristics; • higher levels of CARs, PCs and PEs (including ether-linked), PIs, TGs, most LPCs, and LPEs were observed in the hypothermic preservation group; • lower accumulation of pro-inflammatory lipids during NEKVP method	[45]
heart	tissue (pig), perfusate	ex vivo, in vivo; DI	fiber, mix-mode, C18, HLB	untargeted profiling	• assessment of protocol feasibility for metabolomic and lipidomic profiling of changes associated with declines in myocardial function during ESHP; • comparison of the results obtained with SPME and those obtained via solid-liquid extraction (SLE) performed on biopsies	[5]
heart	tissue (human), perfusate	ex vivo; DI	fiber, mix-mode, C18	untargeted profiling	• validation of the experimental protocol and the metabolomic findings during ESHP	[5]

2. Monitoring Graft Function in Donor Specimens

One of the first protocols involving both thin-film and solid-phase microextraction explored the diagnostic value of bronchoalveolar lavage fluid (BALF) in lung transplant patients [35]. The presence of bile acids (BAs) and pepsin in BALF is a marker of the aspiration of gastric components, which carries a risk of obliterative bronchiolitis and allograft dysfunction. In routine clinical practice, the total concentration of bile acids is measured using an enzymatic assay; however, this method is not specific and might be associated to cross-reactions. Mass spectrometry coupled to liquid chromatography is capable of successfully measuring individual bile acids. As such, Bessonneau et al. combined this approach with PAN-C18-coated thin-film microextraction (TFME) blades for the determination of cholic acid (CA) and deoxycholic acid (DCA), which served as representatives of di- and trihydroxy bile acids, respectively. Their method-validation results showed good figures of merit, with an RSD < 4% and an LOQ of 0.02 μmol/L. In addition, the results obtained for the clinical samples revealed that the proposed method was able to detect the investigated bile acids at concentrations below the LOQ. The summary concentration of the two acids ranged from 0.02 to 0.06 μmol/L and was correlated with the results of the enzymatic assay (TBA 0.01 to 0.24 μmol/L). While no correlation was found (R2 = 0.11) when all of the studied samples were included in the comparison, the removal of one patient's data resulted in a correlation coefficient of 0.92. BALF samples were also extracted using fibers with a mix-mode coating, followed by untargeted profiling on a high-resolution orbitrap mass spectrometer. Here, the results revealed that of the few thousand detected features, three belonged to bile acids, with 7-ketolithocholic acid as the predominant type (ca. 94% of all BAs). The summary peak area of the identified bile acids correlated with the TBA enzymatic assay data (R2 = 0.86). Interestingly, the principal component analysis plot showed clear separation between two groups of patients, which was characterized by the TBA value < and > 0.05 μmol/L. This separation was driven by the 13 metabolites in the "TBA > 0.05 μmol/L" group but was absent in the other. Among these compounds were three bile acids, one steroid, three fatty aldehydes, one fatty alcohol, two prenol lipids, one fatty amide, and two fatty acids (p-value <0.01 and fold change $\geq$5). These results highlighted the importance of re-considering the TBA cut-off value for "healthy/sick" BALF samples, which is currently set for 0.2 μmol/L. Furthermore, Bessonneau et al.'s work also demonstrated the potential diagnostic value of metabolites apart from BAs in assessing the risk of obliterative bronchiolitis and lung allograft dysfunction. Elsewhere, Stefanuto et al. proposed a different SPME-based approach for identifying PGD after lung transplantation in BALF and blind bronchial aspirate (BBA) samples [36]. To this end, the authors employed 2 cm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibers to extract volatile compounds from the headspace of 20 mL headspace vial that contained 500 μL of lung fluid samples and was sealed with a PTFE/silicone cap. The extracted analytes were then analyzed via two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer (HS-SPME-GC × GC-TOFMS). In the first step, samples obtained at the end of transplant surgery were profiled and the VOC patterns of patients who later developed severe PGD and those who either did not develop PGD, or who only developed low-grade PGD (0-2), were compared. In the next step, a support vector machine (SVM) with a linear kernel on the training set model was built to select features discriminating the studied groups at the first 6 h after transplantation. For 20 selected compounds, the SVM model provided sensitivity of 0.63, specificity of 0.94, and positive and negative predictive value of 0.87 and 0.80, respectively. The majority of the detected compounds were up-regulated in grade 3 PGD (Figure 3).

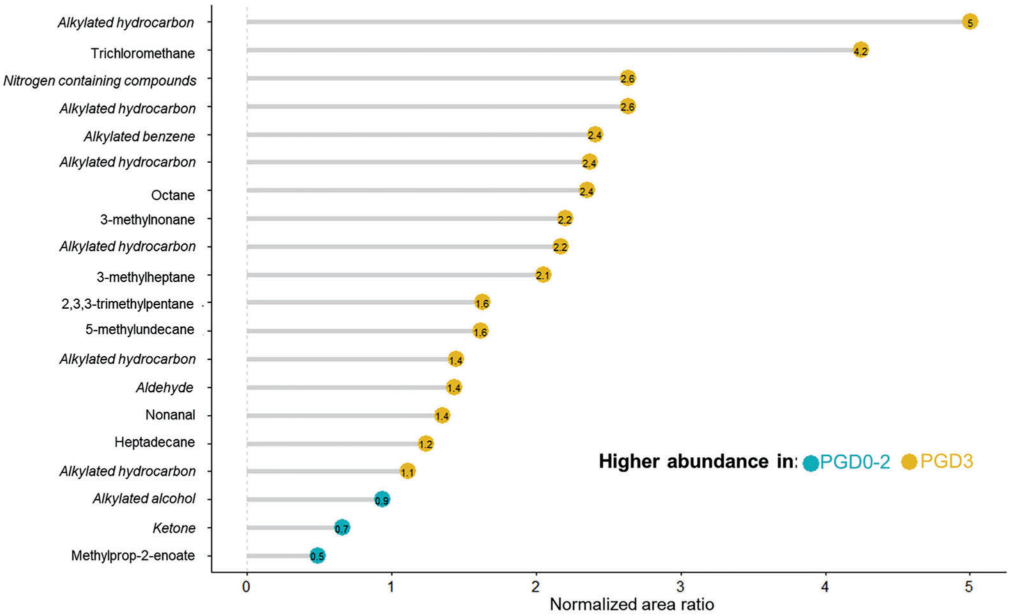

Figure 3. Ratio of the normalized areas of the 20 selected features. Reprinted from [36] with permission under a Creative Commons Attribution 4.0 International License.

Given the complexity of the factors influencing the VOC profiles, the authors employed a multivariate analysis of variance (MANOVA) to assess how clinical risk factors influenced the VOC patterns. The results showed no statistically significant relationships between the VOC profile and the type of donation, cause of death, or time of ischemia. Instead, the donor's body mass index (BMI) was found to be most strongly correlate with the VOC pattern ($p < 0.05$). From a biochemical point of view, the results indicated the potential importance of lipid peroxidation in the development of PGD; from a diagnostic point of view, the findings demonstrated that the analysis of volatile compounds in lung fluids may be applicable in clinical practice.

Yang et al. tested a completely different hypothesis in liver transplant patients [37]. In this work, they sought to determine the concentrations of two drugs (i.e., rocuronium bromide (ROC) and tranexamic acid (TXA)) routinely used during transplant surgery in order to evaluate liver and kidney performance in patients receiving grafts from living donors (LD) or heart beating brain dead donors (HBD). Although the transplanted grafts in living donors were smaller (<50%) compared to those from the HBDs (full size), the latter group of grafts may result in worse outcomes due to the longer ischemia times required for graft preservation. Rocuronium bromide is primarily eliminated through hepatobiliary excretion (up to 90%), with the kidneys excreting only about 10%. In contrast, the kidneys are the main route through which TXA is eliminated (>95%). Therefore, the simultaneous monitoring of these two drugs can potentially provide insight into the development of multiorgan dysfunction in the neohepatic phase. The extractions were performed using high-throughput TFME with stainless steel blades coated with polyacrylonitrile (PAN) and weak cation exchange (WCX) particles according to a previously published method [46]. The WCX particles were selected to compensate for the characteristics of ROC; specifically, as a quaternary ammonium compound, sample preparation for ROC can be challenging due to its propensity to interact with glass surfaces. Moreover, prior studies have reported that due to its instability in collected blood samples, ROC analysis requires sample acidification, which may affect the analysis of other metabolites and drugs from the same sample. The method proposed by Gorynski et al. [46] enabled the effective extraction of both analytes of interest without the need for matrix modification. The obtained data was further used to build pharmacokinetic profiles of the targeted drugs using a two-compartment model. In the case of ROC, liver clearance was lower in the HBD group, while the LD group showed similar clearance to that observed in people with normal liver function. While the comparison of TXA renal clearance did not reveal any significant differences between the HBD and LD cohorts, it was found to be slightly lower than normal in both cases. In addition to targeted drug analysis, TFME coated with hydrophilic-lipophilic balance (HLB) particles was applied for the metabolomic profiling of plasma. The HBD and LD groups formed separate clusters, with oxidized lipids and bile acids being identified as the main discriminating metabolites. The SPME-LC-MS measurements were supplemented by determining the relative expression of miR-122, miR-148a, and γ-glutamyltranspeptidase (GGT), a known biomarker of liver injury. Indeed, GGT levels were significantly higher in HBD patients, suggesting that this increase may have been the result of cold ischemia time (CIT) and may result in injury to the sinusoidal epithelial cells. The study's findings demonstrated that ROC analysis may be a good indicator of early liver function, while TXA clearance may be a promising marker of the granular filtration rate and, consequently, chronic or acute renal injury, which may lead to early graft dysfunction. On the other hand, metabolomic profiling may help to develop a more detailed understanding of the mechanisms underlying the development of graft injury, in addition to identifying potential diagnostic biomarkers.

Łuczykowski et al. developed a high-throughput sample-analysis protocol based on TFME to evaluate the metabolomic profiles of bile samples from porcine model donors with mild (HBD) and moderate warm ischemia (DCD) grafts that had been subjected to SCS or normothermic ex vivo liver perfusion (NEVLP) prior to transplantation [38]. Extraction was performed using steel blades coated with an HLB sorbent, and each sample consisted of 10 µL of bile diluted in phosphate-buffered saline (1:99). The extraction and desorption times were 120 min, and an ACN:H_2O (80:20, *v*:*v*) mixture was selected as the desorption solution. The findings showed that the bile produced in the SCS-preserved livers was characterized by increased levels of chenodeoxycholic acid, arachidonic acid, and 5S-hydroxyeicosatetraeonic acid, as well as saturated and monounsaturated LPCs, which may be due to changes in the bile acid synthesis pathways and organ inflammation. In addition, the metabolomic profile of bile produced by the SCS-preserved livers changed after a short-term (30 min) ischemia (22 and 7 statistically significant differentiating compounds for reperfusion and POD, respectively), while significant changes only appeared in the NEVLP group after 90 min. These metabolites primarily consisted of amino acids and lipids, including bile acids and steroid compounds, along with their derivatives. Moreover, a linear relationship was observed between levels of lipids from the LPC group and the organ ischemia time. Ultimately, Łuczykowski et al. identified a number of metabolites worth considering in future targeted and fully validated analyses as potential markers of changes occurring in preserved grafts. Based on the above experiment, Łuczykowski et al. conducted a subsequent pilot study to assess whether BA concentrations in bile are correlated with changes in the transplanted organ [39]. Here, sample preparation was performed via TFME using C18 sorbent as the extraction phase and methanol as the desorption solvent. Due to the high concentration of BA in this biological matrix, dilution factors of 100x and 20,000x were used to cover a wide range of primary and secondary BA concentrations. Conjugated forms of bile acids (with taurine or glycine) were significantly predominant in the bile samples compared to unconjugated forms. High concentrations of taurocholic acid characterized the perfusion period and they remained high during reperfusion in the $90'$DCD group. Furthermore, prolonged ischemia caused an increase in taurodeoxycholic (15,87 ± 8,22 vs. 2,83 ± 0,57 ug/mL) and glycodeoxycholic acid (135,82 ± 78,5 vs. 18,56 ± 4,57 ug/mL) levels in the first days after transplantation compared to the HBD group.

3. Monitoring Graft Function by Direct Analysis of Graft

As noted above, the wide range of SPME applications is largely attributable to the tremendous variability of the devices. One unique feature making SPME ideal for in vivo and in situ analyses of intact tissues and organs is its low invasiveness when using the fiber geometry. There are other restrictions and requirements related to in vivo sampling, particularly when human subjects are involved in the studies, such as biocompatibility, single use of the devices, and sterilizability in the conditions established in the given hospital (in most cases, via autoclaving or ethylene oxide sterilization). As previously detailed, SPME is a safe and minimally invasive tissue sampling technique capable of providing coverage for a broad spectrum of metabolites. Moreover, the miniaturized nature of the device enables simultaneous analysis in different areas of the organ and more importantly, multiple analyses of the same organ over time.

The very first trials to use SPME for the direct extraction of metabolites from organ grafts focused on evaluating not only the method's efficiency, but also the utilized storage and transportation conditions [40]. For the latter objective, the authors used pig liver grafts undergoing NEVLP. Six 7 mm mix-mode fibers were used to perform extractions on-site during graft perfusion, while three identical fibers were used to perform extractions from liver fragments, which had been collected at the beginning of the medical procedure and stored at −80 °C after being transported to the laboratory in dry ice. Immediately after sampling, the six fibers were divided into two groups. The first group was subjected to desorption on-site, with the resultant extracts being transported to the laboratory and stored using the same conditions described for the tissue, while the second group of fibers was secured in the empty vials for transportation and storage. As expected, the results revealed marked differences between the three approaches. The authors suggested that the discrepancies between on-fiber storage and in vivo sampling followed by extract storage may have been due to the partial evaporation of the desorption solvent, and thus, the preconcentration of the metabolites. To address this issue, complete evaporation and reconstitution in lab would be advisable. On the other hand, the in vivo vs. ex vivo sampling was characterized by differences in the presence of metabolites. For instance, in the extracts from ex vivo sampling, the authors observed the absence of some labile metabolites (e.g., itaconic acid, hypoxanthine, lactaldehyde or hydroxyacetone), but the presence of metabolites recognized as the products of the primary metabolite degradation (e.g., diacylglycerol). Although the untargeted nature of these studies precluded the strict monitoring of the extracted metabolites' stability, the results indicated that in vivo sampling followed by on-fiber storage was the most convenient strategy. With regards to method performance, the authors tested three fiber coating lengths (i.e., 4, 7, and 15 mm) for 30 min extractions from liver tissue. The best sensitivity was observed for the 15 mm coating, while reproducibility was better for the two shorter coatings, which was likely due to the heterogeneity of the liver. When selecting the optimum sorbent length, it is very important to carefully consider all aspects of the experiment, including: the main goal of the experiment (spatial resolution studies e.g., characterization of the specific region such as malignant lesion vs. obtaining average information about the studied area); characteristics of the sampled tissue (homo- vs. heterogenous); size of the studied area; and the properties and concentrations of the compounds of interest. Furthermore, in clinical environments, particularly during surgical procedures, restrictions related to sampling time are very challenging from an analytical perspective. Specifically, while the method's overall sensitivity largely depends on the coating length and extraction time, both parameters must be as small as possible to ensure that the method is minimally invasive and disturbs the subject as little as possible. For that reason, the authors of the above study selected probes with a 7 mm coating for use in subsequent experiments. Another practical aspect discussed in [40] was the option of using needle-assembled fibers, which enable penetration through harder tissues such as muscles or barriers (e.g., pleura). Although this approach is slightly more invasive, it is still less so than a regular biopsy, while continuing to allow for sampling with no physical tissue consumption. The extraction coverage results showed that the proposed method provided coverage ranging from 239 molecular features in negative ionization mode for lung sampling to 1580 in positive ionization mode for liver samples, thus confirming the excellent efficiency reported previously for SPME. As emphasized in previous metabolomics studies using SPME, the number and range of the metabolites extracted via SPME is lower compared to conventional sample preparation methods based on tissue collection, homogenization, and multi-solvent extraction, because SPME is an equilibrium technique that extracts via free fraction of metabolites. Thus, only metabolites present in their free form are available for extraction. This feature can be either an advantage or a disadvantage depending on the biological information the researcher wants to obtain from their study. If the main goal is to identify the active molecules involved in a given mechanism, then SPME is a good choice; however, if the objective is to characterize all molecules in the studied system independently based on their biological activity, then more conventional strategies should be considered. The optimized protocol described in [40] was subsequently used in pilot studies conducted on liver and lungs, wherein sampling times of 20 and 30 min were used, respectively [41]. Similar to the previously described studies on ROC and TXA [37], these studies showed that monitoring drugs routinely used during the transplantation procedure may shed light on changes in liver function. The authors found that methylprednisolone was only present in non-ischemic organs, while no drug metabolites were detected in livers subjected to 38 and 78 min of warm ischemia time (WIT), thus suggesting impaired organ function. This hypothesis was supported by observed alterations in endogenous compounds from the Krebs, pentose cycles, and TCA pathway. The metabolic profiling of lungs at different stages of their transplantation procedure (i.e., CIT, ex vivo perfusion (EVLP) and after revascularization) revealed clear differences between the subsequent groups of samples and enabled the tentative identification of metabolites that have been up- and down-regulated under different conditions and factors. In addition to organ sampling, the authors proposed monitoring metabolites in perfusate circulating throughout the graft in a closed circuit as a non-invasive approach for monitoring graft function during perfusion. In addition, this experiment bypassed the need for sample withdrawal, as extraction was performed by exposing the SPME needle-assembled fibers to the perfusate by inserting them into the three-way stopcock—which was equipped with a Luer-lock connected to the perfusate line—for 2 min. In this study, all sampling was conducted in triplicate. Unsupervised analysis of the data showed clustering of the samples at different perfusion time points, indicating the occurrence of alterations in the studied organs, mainly involving amino acids, fatty acids, and their derivatives.

Stryjak et al. conducted a proof-of-concept study to demonstrate this technology's suitability for characterizing changes during the cold preservation of kidneys [42]. Specifically, Stryjak et al. assessed how prolonged ischemia and the accompanying oxidative stress affected the condition of kidneys subjected to static cold storage using a rabbit model. Extraction was performed by inserting duplicate SPME probes (4 mm length mixed-mode extraction phase) into each kidney cortex for 30 min at 5 different time points: immediately following the removal of kidneys, and after 2, 4, 6, and 21 h of cold preservation. The results revealed a significant change in the metabolic profile of the kidney over the first 4 h of storage. The most pronounced alterations were observed in the levels of essential amino acids, which changed depending on exposure to the ischemic milieu, and purine nucleosides, which are associated with various metabolic pathways involved in the production of reactive oxygen species (ROS). Among the identified metabolites, the presence of adenosine and adenosine monophosphate was notable, as this indicated the adenosine-dependent nature of the mechanisms underlying preconditioning and protection against renal ischemia. Other notable identified metabolites also included inosine, hypoxanthine, and xanthine. Furthermore, the short half-life of some of the identified metabolites highlights this analytical approach's usefulness for capturing unstable and short-lived compounds. Moreover, Stryjak et al. extended the existing protocol for use in lipidomic studies and optimized it for in vivo studies on a porcine model and in human subjects [43].

Since biological applications necessitate a compromise between the method's sensitivity and repeatability and the restrictions inherent to medical procedures, it is necessary to determine the optimal extraction phase length and extraction time. Although equilibrium extraction provides the highest sensitivity, the authors decided to use pre-equilibrium conditions for safety reasons, as this would avoid affecting the operation's total duration. The findings confirmed that a 10-min extraction using probes coated with 7 mm of mix-mode (MM) sorbent provided sufficient sensitivity for a broad spectrum of metabolites, enabling the effective profiling of the renal cortex during ex vivo kidney perfusion, in vivo prior to organ harvest, and in vivo after revascularization. The developed protocol used 100 µL of desorption solutions consisting of ACN:H_2O (80:20, *v*:*v*) and IPA:MeOH (50:50) for the metabolomic and lipidomic analyses, respectively. The SPME method's potential for evaluating kidney quality during the transplantation procedure was demonstrated in a pig autotransplantation model, with particular emphasis on HBD and DCD donors [44]. For both donor types, extractions were performed in vivo prior to retrieval; after 1 h, 3 h, 5 h, and 7 h of perfusion; and again in vivo immediately after revascularization. Additionally, the DCD kidneys were also sampled after 45 min and 2 h of warm ischemia. Direct tissue sampling was carried out for 30 min using probes coated with 7 mm of MM extraction phase. The utilized metabolomic and lipidomic platforms enabled the identification of a set of metabolites that may have diagnostic value in monitoring organ function before transplantation. The metabolomic data revealed changes in purine levels between the HBD and DCD groups. The accumulation of adenosine observed during perfusion in the HBD group may be associated with its protective effects against ischemia-reperfusion injury IRI, while the reduced levels of inosine observed during reperfusion in the DCD group may be associated with a worse prognosis after transplantation. In addition, changes in the levels of certain amino acids were found to be correlated with the organ's warm ischemia duration. For instance, increased levels of alanine and valine are associated with impaired organ function, can be a sensitive discriminator of ischemia, and may lead to less favourable outcomes. On the other hand, the decrease in histidine concentrations during WIT may have resulted from ongoing inflammatory processes and the development of oxidative stress. The lipidomic portion of the work revealed differences in LPC levels between the HBD and DCD organs and an upward trend in the level of these metabolites during WIT. Although the exact mechanisms governing the effects of LPC on the graft remain unclear, this group of lipids may be associated with increased oxidative stress, increased inflammatory responses, and the remodelling of cell membranes. Furthermore, the decreased PC/PE ratio in the DCD organs during reperfusion may be associated with impaired liver regeneration, alterations in energy metabolism, increased cell leakage, and endoplasmic reticulum stress. This research was continued by Warmuzińska et al., who used an SPME chemical biopsy to compare the effects of SCS, normothermic ex vivo kidney perfusion (NEVKP), and hypothermic machine perfusion (HMP) on the lipidomic profile of a graft in a renal DCD autotransplantation porcine model [45]. Similar to Stryjak et al.'s study, 7 mm length MM fibre probes were employed to perform 30 min direct extractions from the kidney cortex. The samples were harvested in vivo before retrieval; after 1 h and 2 h of warm ischemia; after 1 h, 3 h, 5 h, and 7 h of perfusion; in vivo immediately after reperfusion; and in vivo under deep anesthesia at the time of sacrifice on postoperative day 3. The findings indicated that the preservation temperature had a more significant impact on the lipidomic profile than the mechanical character of perfusion. Higher levels of CARs, PCs and PEs (including ether-linked), PIs, TGs, most LPCs, and LPEs were observed in the hypothermic preservation group, which may be related to IRI, mitochondrial dysfunction, and oxidative stress. The obtained results also revealed that the NEKVP method may have a beneficial effect on graft function. Specifically, NEKVP-perfused kidneys showed lower accumulation of pro-inflammatory lipids, which contributes to improved graft function after perfusion compared to hypothermic preservation methods.

In one of the most recent studies on this topic, SPME was applied to sample myocardium during prolonged ex situ heart perfusion (ESHP) [5]. In this work, a porcine model was used for protocol development and exploratory analysis, while two explanted human hearts that had been declined for transplantation and used for scientific purposes were treated as validatory grafts. In the porcine model, sampling was conducted at various in vivo and ex vivo stages (i.e., heart beating before harvesting the organ and during perfusion, respectively). The human hearts were only monitored during the ESHP procedure. The untargeted screening covered both the polar metabolome and the lipidome, thereby offering a comprehensive insight into the molecular changes taking place within the examined organs. From a biological perspective, increased metabolite dysregulation in response to prolonged organ preservation indicated the presence of inflammatory processes, the progression of mitochondrial oxidative stress, the disturbance of mitochondrial bioenergetics, and the dysregulation of many lipids, with increases in the level of species negatively impacting the tissue. An especially interesting component of the study was the authors' comparison of the results obtained with SPME and those obtained via solid-liquid extraction (SLE) performed on biopsies collected from the same hearts that had been sampled with SPME probes. The results confirmed that SLE enables more comprehensive analyte coverage, particularly with respect to lipid species that are integrated components of cell membranes, and that are not present in free form or are present at concentrations that are too low to be detected by SPME. In contrast to SLE, SPME proved to be more effective in detecting unstable and intermediate compounds and in offering better sample clean-up, which enabled the detection of low abundance species and a greater variety of lipid subclasses. In SLE, sample homogenization followed by solvent extraction releases and dissolves compounds bound to macromolecules located in different organelles and fractions of the cells and tissues, thus providing higher sensitivity; however, this improved sensitivity comes at the cost of compromised extract clean-up and the production of matrix effects.

4. Conclusions

This review's up-to-date survey of SPME-based studies focusing on graft monitoring in the peri-transplant period demonstrates this technology's profound potential for use in the diagnostic process. The ability to process different biofluid samples regardless of their complexity can be effectively exploited to monitor the state of the graft after surgery when access to the organ is very limited or impossible. On the other hand, the minimally invasive nature of SPME fibres creates the unique opportunity to directly monitor biochemical changes occurring in the graft over the entire period of transplantation (i.e., "from donor to recipient"). Furthermore, untargeted studies, particularly on well-controlled animal models, can lead to a better understanding of the mechanisms underlying organ injury and rejection, in addition to identifying metabolites with potential diagnostic value. Considering the fact that SPME devices have been directly coupled with stand-alone instruments (e.g., mass spectrometers) in numerous studies, it can be envisioned that such strategies can be used for determination of discovered biomarkers. Given SPME's short sample preparation time and ability to perform on-site extractions without the need for sample consumption, combined with its ability to provide the rapid, quantitative, and sensitive determination of target analytes, the use of this technology to obtain results in close to real time appears to be an attainable goal.

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6. Cel rozprawy doktorskiej

Tematyka poruszana w niniejszej rozprawie doktorskiej wpisuje się w trend rozwijania nowych metod konserwacji narządów oraz rozwiązań analitycznych poszukujących parametrów lub związków umożliwiających bardziej efektywną ocenę jakości narządów przed transplantacją. Celem rozprawy była ocena wpływu różnych metod prezerwacji wątroby oraz stopnia niedokrwienia organu na zmiany profilu metabolomicznego żółci z wykorzystaniem modelu zwierzęcego, co pozwoliłoby na wytypowanie potencjalnych biomarkerów zmian zachodzących w konserwowanych narządach w okresie okołotransplantacyjnym. Żółć będąca bezpośrednim produktem aktywności wątroby i dróg żółciowych wydaje się być obiecującym płynem biologicznym odzwierciedlającym zachodzące w nich procesy. W związku z tym, podjęto próbę opracowania wysokoprzepustowego protokołu przygotowania próbek opartego na mikroekstrakcji cienkowarstwowej (TFME, ang. thin-film microextraction) i połączeniu go z analizą metabolomiczną wykorzystując chromatografię cieczową wraz ze spektrometrią mas (LC-MS, ang. liquid chromatography-mass spectrometry).

Ponadto, zważywszy na fakt, że kwasy żółciowe są głównym składnikiem żółci niniejsza rozprawa doktorska miała na celu optymalizację i pełną walidację celowanej metody oznaczania tych metabolitów z wykorzystaniem platformy TFME-LC-MS. Dodatkowo, zaplanowano opracowanie szybkiej i ekologicznej analizy kwasów żółciowych poprzez bezpośrednie sprzężenie SPME ze spektrometrem mas z pominięciem separacji chromatograficznej, jako odpowiedź na wyzwania stawiane przez zasady zielonej chemii.

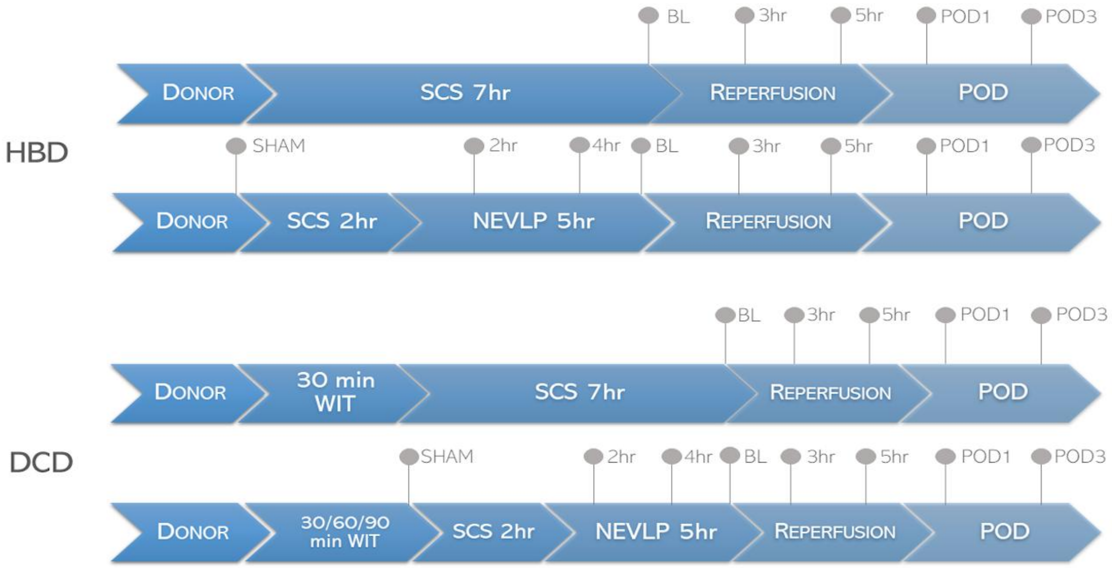
7. Wyniki badań

7.1. Profilowanie metabolomiczne żółci w celu oceny metabolizmu wątroby i funkcji dróg żółciowych w zależności od metody konserwacji narządów oraz stopnia niedokrwienia na modelu wieprzowym – P.3.

Opis dotyczy pracy: **K. Łuczykowski**, N. Warmuzińska, D. Kollmann, M. Selzner, B. Bojko: ***Biliary Metabolome Profiling for Evaluation of Liver Metabolism and Biliary Tract Function Related to Organ Preservation Method and Degree of Ischemia in a Porcine Model.*** Int. J. Mol. Sci., 2023, 24, 2127

Niniejsza praca opisuje możliwość wykorzystania profilowania metabolomicznego żółci do oceny jakości wątroby i dróg żółciowych w okresie okołotransplantacyjnym. W obliczu rosnącej liczby pacjentów wymagających przeszczepu oraz ograniczonej liczby dawców, konieczne staje się jak najlepsze wykorzystanie narządów od dawców ECD oraz opracowanie nowych rozwiązań analitycznych, pozwalających na bardziej efektywną ocenę jakości narządu przed transplantacją. Mimo, że już sama produkcja żółci podczas perfuzji normotermicznej stanowi istotny wskaźnik żywotności wątroby, to określenie zmian w składzie tego płynu biologicznego, może dostarczyć dodatkowych informacji o stanie narządu.

Eksperymenty przeprowadzono na modelu wieprzowym wykorzystując dwa rodzaje dawców wątroby: dawców z bijącym sercem (HBD, ang. heart beating donor) i dawcy po śmierci krążeniowej (DCD, ang. donation after circulatory death), którzy imitowali odpowiednio dawców ze standardowych i rozszerzonych kryteriów. Badanie miało na celu porównanie wpływu ciepłego niedokrwienia organu oraz zastosowanych metod konserwacji narządu: SCS i normotermicznej perfuzji wątroby *ex vivo* (NEVLP, ang. normothermic ex vivo liver perfusion) na profil metabolomiczny żółci. Próbki pobierano po SCS oraz podczas NEVLP w określonych punktach czasowych, tj. przed pobraniem narządów, podczas perfuzji (w przypadku NEVLP), reperfuzji i pierwszych kilku dniach po przeszczepieniu (Ryc. 2.).



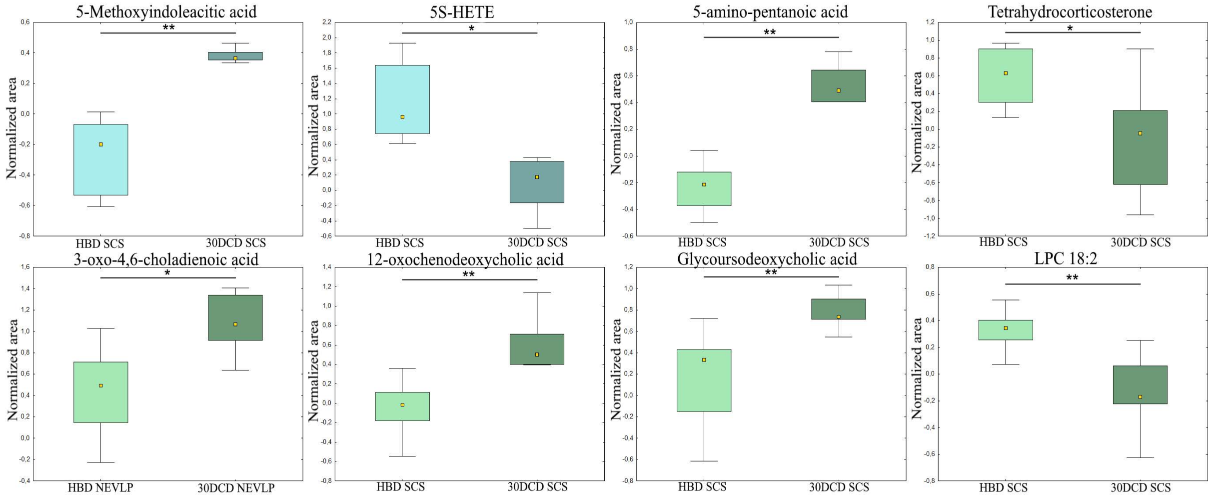
Rycina 2. Projekt eksperymentu z uwzględnieniem grup badanych. *Przed pobraniem narząd poddawano 0 min (HBD; dawcy z bijącym sercem), 30 min, 60 min i 90 min ciepłemu niedokrwieniu imitując donację po śmierci krążeniowej (DCD). Szare kropki oznaczają punkty czasowe pobierania próbek żółci. SHAM — przed pobraniem narządu; BL — początek reperfuzji; WIT — czas ciepłego niedokrwienia.*

Analizy metabolomiczne próbek żółci zostały przeprowadzone na platformie LC-MS po uprzednim przygotowaniu ich za pomocą TFME. Według naszej wiedzy jest to pierwsza praca dotycząca oceny metabolomu żółci wykorzystująca SPME w badaniach biomedycznych z zakresu diagnostyki okołotransplantacyjnej.

Przeprowadzone analizy wykazały, że profil metabolomiczny żółci zmienia się w okresie okołotransplantacyjnym w zależności od przedziału czasowego, w którym zostały pobrane. Zaobserwowano, że próbki pobrane podczas perfuzji, charakteryzowały się najbardziej odmiennym metabolomem w porównaniu do pozostałych próbek, gdyż jako jedyne zostały pobrane w warunkach *ex vivo*. W próbkach tych stwierdzono wyższe poziomy aminokwasów i peptydów oraz niższe stężenia poszczególnych nieskoniugowanych pierwotnych i wtórnych kwasów żółciowych. Zmiany takie mogą być wynikiem zmian w metabolizmie wątrobowym, dodatkową suplementacją oraz chwilowym brakiem krążenia jelitowo-wątrobowego.

Kolejnym krokiem było określenie wpływu stosowanej metody konserwacji oraz czasu ciepłego niedokrwienia na profil metabolomiczny produkowanej żółci. Próbki pochodzące z organów poddanych uprzednio SCS wykazywały wyższe poziomy poszczególnych kwasów żółciowych, m.in. chenodeksycholowego, tauroursodeoksycholowego czy glikohyocholowego, a także licznych lipidów należących do grupy lizofosfatydylocholin

(LPC, ang. lysophosphatidylcholines) i lizofosfatydyloetanoloamin (LPE, ang. lysophosphatidylethanolamines). Wymienione metabolity były już opisywane w literaturze w kontekście chorób wątroby i pierwotnego stwardniającego zapalenia dróg żółciowych. Ponadto, w pracy wykazano, że już krótkie niedokrwienie narządu (30 min) powoduje zmiany profilu metabolomicznego żółci konserwowanej za pomocą SCS w czasie reperfuzji (Ryc. 3.).



Rycina 3. Wybrane metabolity różnicujące grupy HBD i 30’DCD. *Wykresy pudełkowe przedstawiają znormalizowane powierzchnie pików w postaci mediany wraz z rozstępem kwartylowym i wartościami odstającymi. *—p < 0,05; **—p < 0,01; niebieski – początek reperfuzji; zielony – reperfuzja.*

Dla odmiany, zastosowanie metody NEVLP ograniczyło negatywny wpływ niedokrwienia na funkcję narządu, gdyż znaczące zmiany w składzie żółci były widoczne dopiero w przypadku organów poddanych 90 minutowemu niedokrwieniu. Różnicującymi w tych porównaniach metabolitami były m.in. kwasy żółciowe i ich utlenione formy, amidy kwasów tłuszczowych oraz LPC, których poziomy dodatkowo liniowo korelowały ze stopniem niedokrwienia narządu. Różnice w poziomach LPC można przypisać zmianom w aktywności acylotransferaz lizofosfatydylocholiny w przekształcaniu LPC do fosfatydylocholiny, natomiast różnice w homeostazie kwasów żółciowych mogą wynikać z wpływu stanu zapalnego na klasyczne i alternatywne ścieżki ich syntezy.

Podsumowując, w powyższej pracy udało się zaproponować protokół analizy próbek o wysokiej przepustowości oparty na TFME i z powodzeniem zastosować go do przygotowania próbek żółci. Badanie zidentyfikowało metabolity warte rozważenia jako potencjalne markery zmian zachodzących w konserwowanych przeszczepach w przyszłych analizach przeprowadzonych na ludzkich graftach wątroby.

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Article

Biliary Metabolome Profiling for Evaluation of Liver Metabolism and Biliary Tract Function Related to Organ Preservation Method and Degree of Ischemia in a Porcine Model

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Abstract: The development of surgical techniques, immunosuppressive strategies and new organ preservation methods have meant that transplant centers have to face the problem of an insufficient number of organs for transplantation concerning the constantly growing demand. Therefore, using organs from expanded criteria donors and developing new analytical solutions to find parameters or compounds that would allow a more efficient assessment of organ quality before transplantation are options for meeting this challenge. This study proposed bile metabolomic analysis to evaluate liver metabolism and biliary tract function depending on the organ preservation method and degree of warm ischemia time. The analyses were performed on solid-phase microextraction-prepared bile samples from porcine model donors with mild (heart beating donor [HBD]) and moderate warm ischemia (donation after circulatory death [DCD]) grafts subjected to static cold storage (SCS) or normothermic ex vivo liver perfusion (NEVLP) before transplantation. Bile produced in the SCS-preserved livers was characterized by increased levels of metabolites such as chenodeoxycholic acid, arachidonic acid and 5S-hydroxyeicosatetraeonic acid, as well as saturated and monounsaturated lysophosphatidylcholines (LPC). Such changes may be associated with differences in the bile acid synthesis pathways and organ inflammation. Moreover, it has been shown that NEVLP reduced the negative effect of ischemia on organ function. A linear relationship was observed between levels of lipids from the LPC group and the time of organ ischemia. This study identified metabolites worth considering as potential markers of changes occurring in preserved grafts.

Keywords: bile; liver transplantation; solid-phase microextraction (SPME); organ preservation; graft quality assessment; NEVLP; metabolomics

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1. Introduction

Transplantation is a very effective treatment for various forms of end-stage liver failure. Thanks to developments in surgical techniques, immunosuppressive strategies and patient-management approaches, the number of candidates eligible for liver transplantation is constantly growing; unfortunately, however, the number of donors remains constant and insufficient [1]. Transplantation using organs from expanded criteria donors (ECD) is one potential option for meeting this increasing demand. Although the use of ECD organs can significantly reduce wait times for transplantation, these organs also carry an increased risk of dysfunction or rejection [2,3]. Thus, the greatest challenge in contemporary transplantation is the development of an organ-preservation method that not only enables the organ to be maintained in optimal condition, but also allows its function to be assessed prior to surgery.

Due to its readily available logistics and low cost, static cold storage (SCS) is currently the standard technique for organ preservation in clinical practice. The low temperatures used during SCS delay metabolic processes, thus reducing ischemic injury [4,5]. However, ECD grafts appear to be more susceptible to prolonged ischemia, increased morbidity and mortality in post-liver-transplantation recipients [3]. Normothermic ex vivo liver perfusion (NEVLP) has emerged as an intriguing alternative to SCS, as it allows active metabolism to be maintained during the storage period. On the one hand, this approach results in increased energy consumption and oxygen demand, which makes the preservation procedure much more complex compared to cold preservation; on the other hand, it also minimizes the risk of liver damage and early allograft dysfunction (EAD) by reducing the exposure of grafts to static cold ischemia [6]. Since the liver exhibits metabolic activity during normothermic perfusion, one key advantage of this approach is that it enables the assessment of organ viability before transplantation. The literature contains numerous studies wherein liver function during perfusion has been assessed through the analysis of tissues, perfusate and bile [6–8].

In the present study, solid-phase microextraction (SPME) is used as a sample-preparation method to evaluate the metabolomic profile of bile from livers preserved via SCS and NEVLP. SPME has already been applied successfully in metabolomics studies, and its usefulness has been reported in many applications, including differentiating patients with bladder cancer [9], profiling brain tumors [10] and assessing the quality of organs intended for transplantation [11,12]. In addition, the effect of the organ's ischemic time on the composition of bile was assessed to monitor changes in liver metabolism and biliary functions.

2. Results

Principal component analysis (PCA) was performed to evaluate the instrument's stability during the analyses. The compact clustering observed among the QC samples confirmed the quality of the obtained results (Figure S1A,B). Furthermore, the two-dimensional score plots (PC1 vs. PC2) showed an apparent separation indicating differences in the metabolomic patterns of the samples based on the time interval at which they were collected. The PCA plot for the bile samples revealed a clear separation between perfusion and the other samples along PC 1 and less separation between reperfusion and the POD sample groups along PC 2 (Figure 1A,B). ANOVA tests were used to identify the top 30 differentiating metabolites in the study group, with the overall changes in these compounds being visualized as heatmaps (Figure 2A,B). These metabolites primarily consisted of amino acids and lipids, including bile acids and steroid compounds, along with their derivatives. The results revealed that the perfusion samples—which, unlike the other samples, were collected under ex vivo conditions—possessed a different metabolic profile compared to the other samples.

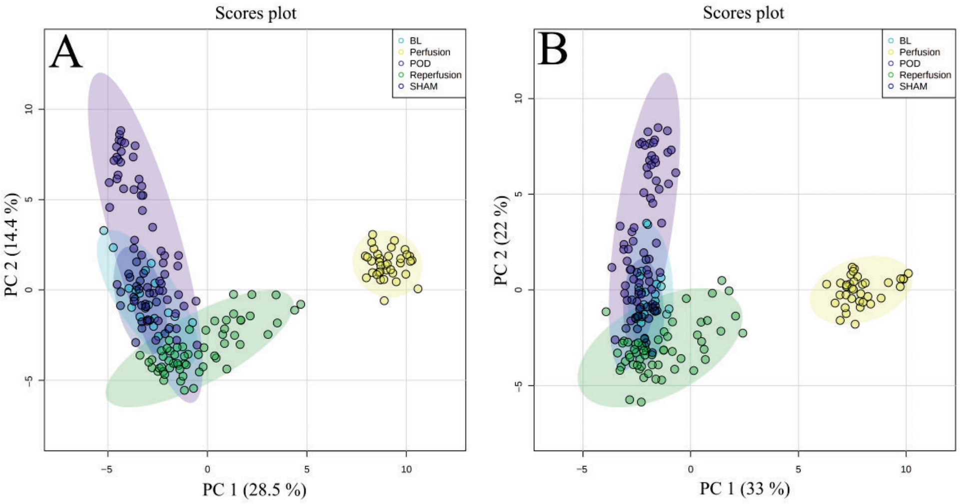

Figure 1. Score plots (PCA) showing separation indicating differences in the metabolomic patterns of the samples based on the time interval at which they were collected. (**A**) positive ionization mode, (**B**) negative ionization mode.

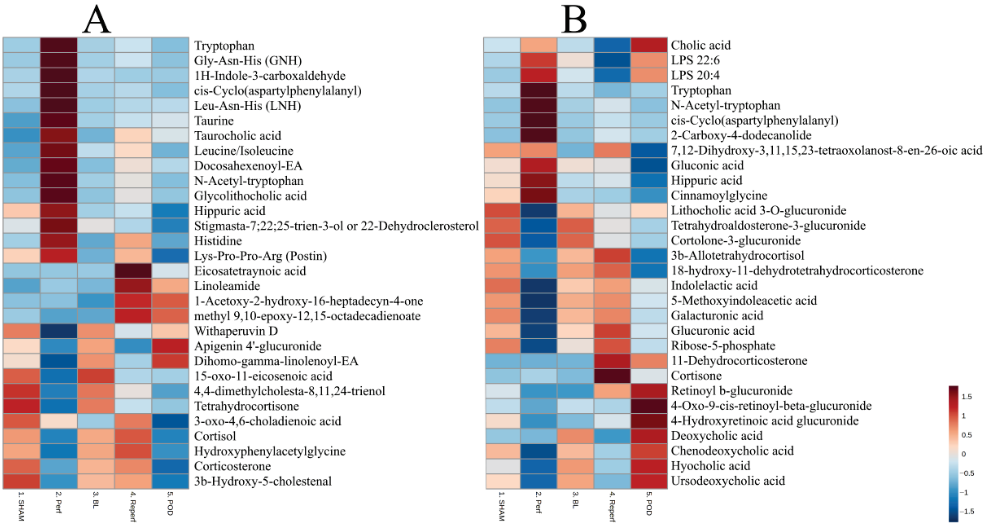

Figure 2. Heatmap showing the overall levels of the metabolites differentiating the samples collected at each time interval (Ward's clustering algorithms, Euclidean distances). (**A**) Positive ionization mode, (**B**) negative ionization mode. The dark red squares on the heat map indicate a high abundance of that feature in a specific group of samples, whereas dark blue indicates a low abundance.

A further, more detailed analysis of the results was subsequently conducted. This analysis was divided into three parts: (i) assessing changes in the metabolomic profile of bile after transplantation based on the method of organ preservation (i.e., SCS or NEVLP); (ii) determining how 30 min warm ischemia affected the metabolomic profile of bile after transplantation; and (iii) examining how the degree of warm ischemia influenced the bile metabolome in the peri-transplant period during the application of NEVLP.

2.1. Evaluation of the Metabolomic Profile of Bile in the Post-Transplant Period after Employing SCS and NEVLP

The Kruskal—Wallis test and multiple (post-hoc) mean-rank comparisons were employed to determine how SCS and NEVLP methods affected the bile metabolome. Analyses were performed for the HBD (heart beating donor) and 30′DCD (donation after circulatory death) groups in the samples collected after organ implantation. Samples collected during reperfusion (3 h and 5 h) and post-operative days 1 and 3 (POD1 and POD3, resepectively) were combined into larger groups to increase the readability and statistical significance of the obtained results. No significant differences in the composition of the bile samples collected immediately after transplantation (baseline) were observed among the studied groups. Rather, changes only appeared during ongoing reperfusion or a few days after surgery, affecting organs from both the HBD and 30′DCD donors. Samples that had been preserved via SCS were characterized by higher levels of lysophosphatidylcholines (LPC) and lysophosphatidylethanolamines (LPE). Additionally, the bile samples from the SCS group also contained higher levels of glycocholic acid (GCA), glycoursodeoxycholic acid (GUDCA), glycohyocholic acid (GHCA) and tauroursodeoxycholic acid (TUDCA) during reperfusion, and higher concentrations of chenodeoxycholic acid (CDCA) post-operation. In contrast, the organs from the NEVLP group showed significantly increased taurocholic acid (TCA) secretion into the bile during reperfusion. The differences in metabolite levels based on the preservation method used for the HBD and 30′DCD groups are summarized in Tables 1 and 2 for positive and negative ionization mode, respectively. The largest number of differentiating compounds were identified during reperfusion, with 39 and 17 being isolated for the HBD and 30′DCD groups, respectively. By contrast, only 14 (HBD) and 6 (30′DCD) were identified post-operation. A high degree of change characterized not only bile acids, but also amino acids and their derivatives. Among the differentiating lipids, LPCs constituted the largest group.

2.2. Effect of 30 Min Ischemia on the Metabolomic Profile of Bile after Transplantation

Next, we examined how a 30 min warm ischemia affected the composition of bile produced by the transplanted organ. To this end, analyses were performed separately for the SCS and NEVLP groups, with the changes in the levels of the selected metabolites being shown in Figure 3. The obtained results showed that a 30 min ischemia had a greater impact on the function of the livers subjected to SCS. In this group, changes in metabolites became apparent immediately after transplantation. At the remaining time points, changes in the levels of numerous metabolites were observed in the SCS group, while differences were only observed in single compounds in the NEVLP group. In the SCS group, the bile secreted by the liver undergoing a short ischemia period was characterized by decreased levels of LPCs and increased levels of fatty amides and oxidized bile acids such as 3-oxo-4,6-choladienoic acid and 12-oxochenodeoxycholic acid. Moreover, the bile samples from the 30′DCD group exhibited lower levels of 5S-hydroxyeicosatetraeonic acid (5S-HETE) at baseline and tetrahydrocorticosterone during reperfusion, and elevated levels of 5-methoxyindoleacetic acid at baseline and 5-amino-pentanoic acid during reperfusion. A complete list of metabolites exhibiting differences due to undergoing ischemia is provided in Tables S1 and S2 for positive and negative ionization modes, respectively.

Table 1. Statistically significant compounds identified in SCS- and NEVLP-preserved organs from HBD and 30′DCD donors at particular time points. Data were acquired in positive ionization mode. The Kruskal—Wallis test and multiple (post-hoc) mean-rank comparisons were used to select the metabolites statistically significantly different from the SCS and NEVLP methods. Results are presented as fold change.

Name	**Molecular Weight**	**RT**	**The Effect of the Organ Preservation Method (SCS vs. NEVLP)**					
			Baseline (*n* = 5)		**Reperfusion (*n* = 10)**		**POD (*n* = 10)**	
			$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$
			FC	**FC**	**FC**	**FC**	**FC**	**FC**
Taurine	125.01	10.71	−1.18	2.19	−3.13 *	−1.19	−1.35	−1.39
4-Methylene-2-pyrrolidinecarboxylic acid	127.06	7.26	−2.50	1.09	−2.00 *	−1.75	−2.50	1.20
Pyroglutamic acid	129.04	1.50	−2.94	1.01	−2.5 *	−1.25	−1.61	1.75
Leucine/Isoleucine	131.09	8.13	−1.19	−1.11	1.33	1.73 *	1.18	−1.69
1H-Indole-3-carboxaldehyde	145.05	11.26	1.16	1.01	−1.52 *	1.16	1.01	−1.08
Tryptophan	204.09	11.25	1.14	−1.04	−7.14 **	−2.86	1.06	−1.10
2,5-Dichloro-4-oxohex-2-enedioate	225.94	1.96	1.02	−1.14	−1.37 **	1.10	−1.35 *	−1.49
11-hydroxy-9-tridecenoic acid	228.17	14.80	1.05	−1.43	−1.69 **	−1.11	−1.43	−1.85
N-Acetyl-tryptophan	246.10	11.26	−1.12	−1.16	−10.00 **	−4.76	−1.04	−1.14
4,9-heptadeca-1,4,9-trien-6-yn-3-ol	246.20	20.46	1.18	−1.39	−1.41	−1.08	−1.20	−2.27 **
Asn-Arg	288.16	8.72	−1.16	−1.39	−1.52 **	−1.04	−1.25	−2.00
3-keto stearic acid	298.25	19.49	−1.10	−1.35	−1.47 *	−1.15	−1.30	−2.13
Arachidonic acid	304.24	21.14	1.90	1.14	2.12 *	1.27	4.15	−1.25
methyl 9,10-epoxy-12,15-octadecadienoate	308.24	20.99	−3.23	−1.19	−3.13 *	−1.52	−1.49	−4.76
1-Acetoxy-2-hydroxy-16-heptadecyn-4-one	324.23	18.22	−1.18	−1.37	−1.09	2.08 *	1.04	−1.22
3-hydroxy-4-isopropylbenzyl alcohol 3-glucoside	328.15	21.03	−2.22	−1.43	−2.56 **	−1.22	−1.02	−1.96
Docosapentaenoic acid methyl ester	344.27	15.41	1.66	−1.52	1.98 *	−1.32	1.04	−1.28
Tetracosahexaenoic acid	356.27	14.69	1.78	−1.03	−1.20	−2.78 *	−1.19	−1.28
Docosahexenoyl-EA	371.28	13.55	−1.59	1.23	−11.11 **	−7.14 **	−2.70 **	−1.18

Table 1. *Cont.*

Name	Molecular Weight	RT	The Effect of the Organ Preservation Method (SCS vs. NEVLP)					
			Baseline (n = 5)		**Reperfusion (n = 10)**		**POD (n = 10)**	
			$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$
			FC	**FC**	**FC**	**FC**	**FC**	**FC**
Leu-Asn-His (LNH)	382.20	19.77	1.25	−1.52	−1.52 *	1.00	−1.15	−1.79
9′-Carboxy-alpha-chromanol	390.28	15.78	1.69	1.71	2.46	1.34	12.83 *	−1.75
22-Dehydroclerosterol or Stigmasta-7,22,25-trien-3-ol	410.36	18.93	1.02	−1.10	−2.00	−3.57 **	−1.64	−1.12
Tricosanoylglycine	411.37	22.62	1.04	−1.56	−1.56 *	−1.15	−1.49	−2.13 *
LPE 14:0	425.26	13.98	−1.18	2.14	−1.28	1.65	−2.27	2.08 *
(3b,5a,6a,9a,22,24)-Ergosta-7,22-diene-3,5,6,9-tetrol	446.34	25.12	1.17	−1.61	−1.56 **	1.02	−1.28	−1.61
Glycoursodeoxycholic acid	449.31	13.44	1.65	1.75	1.21	2.25 *	1.99	2.48
Glycohyocholic acid	465.31	12.71	2.30	2.05	−1.01	4.46 **	−1.08	1.42
LPC 14:0	467.30	16.60	1.62	1.05	2.21 *	1.68	3.20 **	−1.1
LPC 15:0	481.32	17.18	1.18	−1.35	2.82 *	2.29	4.12 **	−1.54
LPC 16:1	493.32	17.00	1.89	−1.12	2.96 *	1.90	3.48 *	−1.23
LPC 16:0	495.33	17.74	1.61	−1.27	2.67 *	2.14	3.16 *	−1.69
Tauroursodeoxycholic acid	499.30	11.26	1.28	1.09	−1.05	1.86 *	1.20	1.17
LPE 20:4	501.28	17.92	1.56	−1.67	1.01	1.28	3.37 *	−1.69
PE 20:1	507.33	17.38	1.64	−1.54	2.16 *	1.71	4.42	−1.35
LPC 17:0	509.35	18.29	1.65	−1.43	2.30	1.97	5.23 **	−2.00
Sulfoglycolithocholic acid	513.28	10.21	−1.10	2.69	−4.35 *	−2.70	−1.67	1.49
Taurocholic acid	515.29	10.84	−2.38	1.47	−50.00 **	−25.00 *	−4.35 *	−1.49
Taurohyocholic acid	515.29	10.71	−1.30	1.68	−4.76 **	−1.56	−1.32	−1.33
Tauro-b-murocholic acid	515.29	10.28	3.06	1.23	−2.70	3.56 *	−1.75	−2.00

Table 1. *Cont.*

Name	Molecular Weight	RT	The Effect of the Organ Preservation Method (SCS vs. NEVLP)					
			Baseline (*n* = 5)		**Reperfusion (*n* = 10)**		**POD (*n* = 10)**	
			$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$
			FC	**FC**	**FC**	**FC**	**FC**	**FC**
LPC 18:0	523.37	18.84	1.53	−1.30	2.20 *	1.86	3.13 *	−2.22
LPS 20:4	545.28	16.28	4.56	1.21	2.42	3.07 *	−1.25	1.01
LPC 22:1	577.41	18.15	1.23	−1.19	1.15	2.29 *	1.88	6.90
SM 16:1;O2/18:0	702.57	22.64	1.19	1.20	−1.64 *	−1.59	1.26	1.05
PC 32:2	729.53	24.01	−1.04	−1.12	−1.79 *	1.01	−1.12	−1.08
PE 36:4	741.53	24.23	−1.54	1.57	−1.82 *	−1.02	−1.41	1.30
PE 38:4	767.55	24.94	−1.75	1.21	−1.75 *	−1.28	−1.23	1.10

− denotes an increased level in the group located in the denominator of the fraction; FC—fold change; *—$p < 0.05$; **—$p < 0.01$.

Table 2. Statistically significant compounds identified in SCS- and NEVLP-preserved organs from HBD and 30′DCD donors at particular time points. Data were acquired in negative ionization mode. The Kruskal—Wallis test and multiple (post-hoc) mean-rank comparisons were used to select the metabolites statistically significantly different from the SCS and NEVLP methods. Results are presented as fold change.

Name	Molecular Weight	RT	The Effect of the Organ Preservation Method (SCS vs. NEVLP)					
			Baseline (*n* = 5)		**Reperfusion (*n* = 10)**		**POD (*n* = 10)**	
			$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$
			FC	**FC**	**FC**	**FC**	**FC**	**FC**
Glucuronic acid	194.04	1.17	−2.70	1.35	−2.44 *	−1.10	−5.56	3.61
Cinnamoylglycine	205.07	11.46	1.79	1.19	−1.14	−2.04 *	1.56	1.58
Indolelactic acid	205.07	13.65	−1.82	1.14	−1.85 *	−1.56	−1.85	1.43
Ribose-5-phosphate	230.02	1.17	−2.70 *	1.27	−2.17 *	−1.12	−4.55	2.84
Tridecanedioic acid	244.17	15.26	1.30	−1.47	−1.37	1.03	1.00	−2.22 *

Table 2. *Cont.*

Name	Molecular Weight	RT	The Effect of the Organ Preservation Method (SCS vs. NEVLP)					
			Baseline (n = 5)		Reperfusion (n = 10)		POD (n = 10)	
			$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$	$\frac{HBDSCS}{HBDNEVLP}$	$\frac{30'DCDSCS}{30'DCDNEVLP}$
			FC	**FC**	**FC**	**FC**	**FC**	**FC**
N-Acetyl-tryptophan	246.10	11.23	1.32	1.00	−5.56 **	−2.17	1.46	1.37
5S-HETE	320.24	18.94	8.33	−2.04	11.36 **	2.35	2.04	−5.56
Chenodeoxycholic acid	392.29	16.28	2.08	1.13	1.86	1.04	3.24 *	−5.00
N-Palmitoyl tyrosine	419.30	15.97	6.13	−1.23	7.33 **	−2.08	5.38	−4.17
Glycoursodeoxycholic acid	449.31	13.44	1.50	1.50	1.28	1.80 *	2.52	2.72
3a,7a,12a-trihydroxy-5b-cholestan-26-oic acid	450.34	16.06	2.16	2.36	1.94 *	2.03 *	−1.96	−1.59
Glycocholic acid	465.31	13.19	−1.03	1.68	−1.30	1.99 *	−1.28	−1.54
Cholesterol sulfate	466.31	12.49	2.07	1.24	−1.75	2.77 *	−1.30	1.03
Retinoyl b-glucuronide	476.24	16.60	23.25	−1.18	6.30 *	−2.13	−1.72	3.50 *
1-O-all-trans-retinoyl-beta-glucuronic acid	476.24	16.94	3.97	−1.45	5.36	1.27	−2.78	5.50 *
LPE 18:0	481.32	18.62	1.38	−2.63	1.93	2.02	3.90 *	−2.00
4-Hydroxyretinoic acid glucuronide	492.24	13.62	13.7	1.45	9.45 **	−2.44	−1.92	2.30
LPC 17:1	507.33	18.11	1.61 *	−1.67	1.20	1.46	3.02	−1.10
LPC 17:0	509.35	18.29	1.75	−1.20	2.84	2.47	3.85 *	−2.50
Taurohyocholic acid	515.29	10.71	1.27	2.65	−5.26 *	−1.89	−1.15	−1.39
Taurocholic acid	515.29	10.84	−1.30	1.75	−50.00 **	−20.00 *	−2.63	−1.43
LPS 20:4	545.28	16.29	5.10	1.07	2.56 *	4.57	−1.02	1.03

− denotes an increased level in the group located in the denominator of the fraction; FC—fold change; *—$p < 0.05$; **—$p < 0.01$.

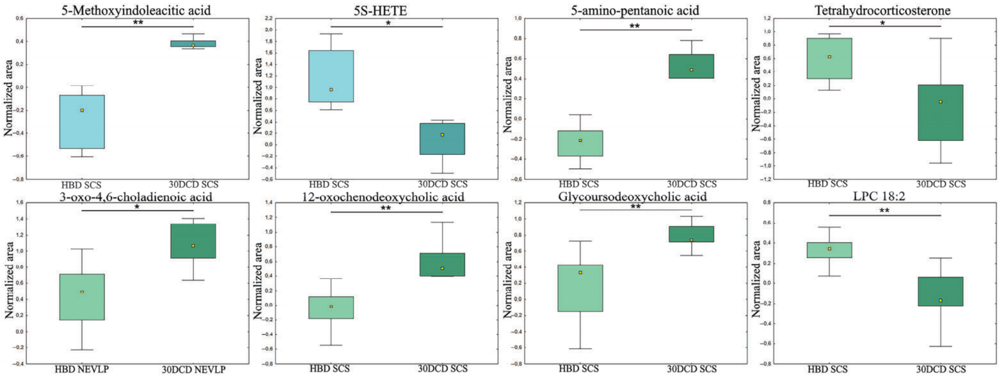

Figure 3. Selected metabolites differentiating the HBD and 30′DCD groups. The boxplots display the normalized peak areas, while the height of the rectangle represents the peak areas in the interquartile range (Q1 and Q3). The upper whisker denotes the largest data point excluding any outliers, and the lower whisker indicates the lowest data point excluding any outliers. The median normalized peak area of each group is indicated with a yellow square. *—$p < 0.05$; **—$p < 0.01$; blue = baseline; green = reperfusion.

2.3. *Influence of Degree of Ischemia on the Bile Metabolome in the Peri-Transplant Period during NEVLP*

Tests were conducted to assess how gradually increasing organ warm ischemia time affected the bile metabolome. To this end, chemometric analysis was performed to visualize the data and investigate differences in the metabolomic profiles of bile samples collected from livers in the HBD, 30′DCD, 60′DCD and 90′DCD groups prior to organ harvest (SHAM samples). The PCA results for the analyzed groups (Figure 4) show that prolonging the ischemia time affects the bile metabolome. Next, Spearman's rank correlation was used to select compounds with levels that correlated linearly (R > 0.7) to organ ischemia time. The identified metabolites mainly consisted of LPCs, and their biliary levels were found to be negatively correlated with ischemia time (Figure 5). Detailed data relating to these correlations, including correlation coefficients and significance levels, are presented in Table S3.

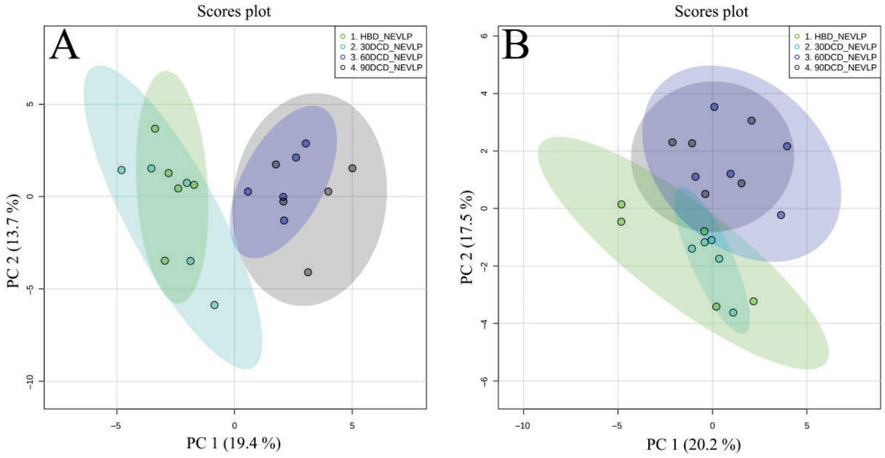

Figure 4. Score plots (PCA) showing the separation between groups with different ischemic times prior to organ harvest. (**A**) Positive ionization mode, (**B**) negative ionization mode.

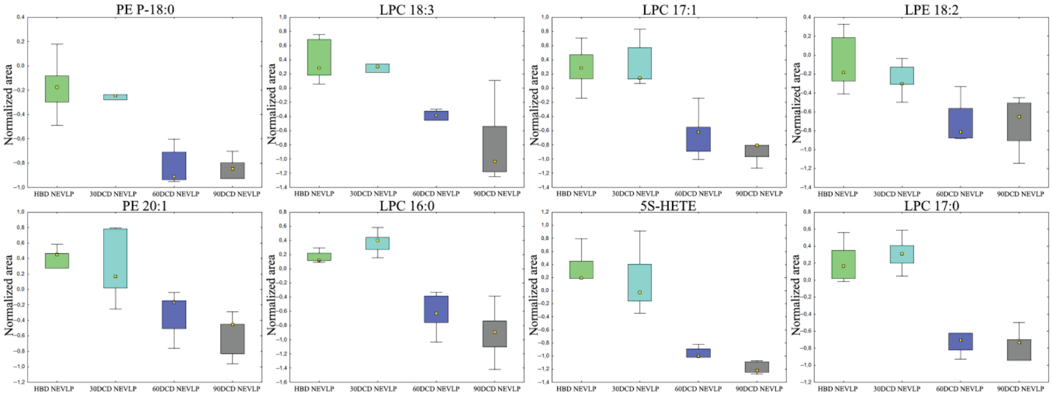

Figure 5. Selected compounds with levels linearly correlated with ischemia time prior to organ harvest. The boxplots show the normalized peak areas, and the height of the rectangles represent the peak areas in the interquartile range (Q1 and Q3). The upper whiskers indicate the largest data point, excluding any outliers, while the lower whiskers indicate the lowest data point, excluding any outliers. The median normalized peak area of each group is indicated with a yellow square.

Additionally, individual ischemic groups were compared with the HBD group using a Volcano plot, assuming differences with a fold change > 2 and $p < 0.05$ with false discovery rate (FDR) correction as statistically significant. The data capturing the number of metabolites differentiating particular groups at specific time intervals indicate that ischemia lasting up to 60 min does not significantly affect bile composition, and thus, only causes slight metabolic changes within the organ. However, more extensive changes in the bile metabolome were observed after 90 min of ischemia. These two groups (90′DCD vs. HBD) were differentiated by the highest number of metabolites during the perfusion period, with visible normalization at further time points. Table 3 shows the number of metabolites differentiating the moderate ischemic groups from the HBD group, while Figure S2 shows the identified metabolites distinguishing the 90′DCD group from the HBD group.

Table 3. Number of metabolites (pos/neg) differentiating the moderated ischemia (30′DCD, 60′DCD and 90′DCD) groups from the HBD group at different phases of the peri-transplant period. The number of metabolites exhibiting significant changes was determined based on a Volcano plot (fold change > 2, $p < 0.05$ with FDR correction). ↓ indicates down-regulation and ↑ indicates up-regulation after ischemia.

	Perfusion (*n* = 5)		**Reperfusion (*n* = 10)**		**POD (*n* = 10)**	
	↑	↓	↑	↓	↑	↓
30′DCD vs. HBD	0/1	0/0	0/0	0/0	1/0	0/0
60′DCD vs. HBD	12/5	12/9	4/1	11/2	0/0	5/0
90′DCD vs. HBD	44/17	26/10	22/11	21/19	6/2	18/5

In addition to examining the effect of ischemia on metabolite concentrations in bile, levels of individual compounds were monitored throughout the peri-transplant period by taking samples at specific time points. Individual metabolites showed different concentration trends between organ harvest and transplantation. LPCs were consistently present at lower levels in bile produced by livers that had undergone prolonged ischemia. The most significant differences in the levels of the remaining metabolites, including bile acids (e.g., TCA, THCA, GLCA-sulfate), amino acids, peptides and others, were observed during perfusion and reperfusion with gradual equalization occurring post-operation. Selected compounds showing differences at individual phases of the peri-transplant period are illustrated in Figure 6.

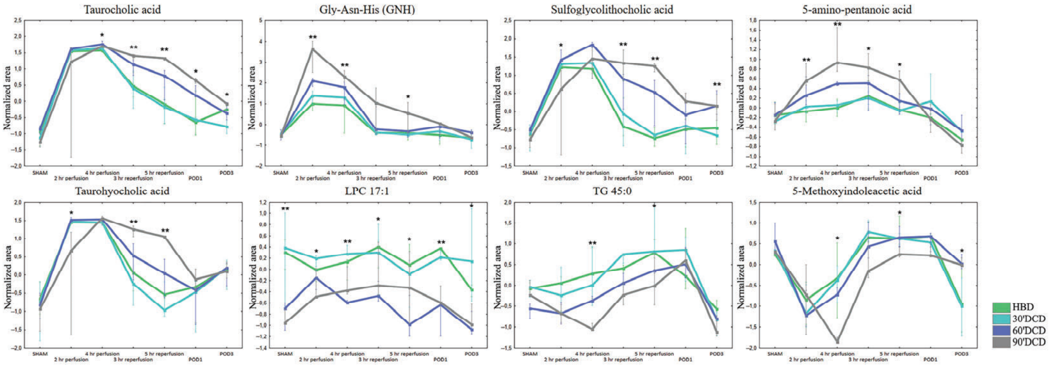

Figure 6. Changes in the levels of selected metabolites in the peri-transplant period. The plots display the medians of the normalized peak areas. The upper whisker denotes the largest data point, excluding any outliers, while the lower whisker indicates the lowest data point, excluding any outliers. *—$p < 0.05$; **—$p < 0.01$.

3. Discussion

This study aimed to develop a new organ-preservation method that also allows for the assessment of graft quality prior to transplantation. To this end, metabolomic analysis of bile produced by livers in the peri-transplant period was conducted. Bile is not commonly used in scientific research, as it is difficult to prepare due to its consistency (density, thickness), high lipid content and high variability of physicochemical properties. However, the literature contains reports detailing the high scientific value of bile analysis, particularly as a matrix in proteomic and metabolomic analysis in patients with biliary tract diseases [13–16]. Furthermore, it has been proven that bile production during NEVLP is an important indicator of liver viability. Moreover, it is believed that, along with secretion levels, the composition of bile may also be of high importance [17]. Unfortunately, the literature on this topic predominantly focuses on the determination of total bile acids rather than individual bile acid profiles, and does not consider the other components of the bile metabolome. To the best of our knowledge, this is the first bile metabolome study to use SPME to compare different liver preservation methods and to evaluate how varying degrees of ischemia times impact bile composition.

The analysis of the metabolomic profiles of bile at particular time intervals revealed significant differences among the samples collected during perfusion (Figure 1). In particular, these samples contained higher concentrations of amino acids, including tryptophan, leucine, isoleucine and histidine, as well as some peptides (Figure 2). It is known that the administration of amino acids during transplantation has a nutritional and hepatoprotective effect on the transplanted organ [18]. Therefore, elevated levels of these metabolites in bile may be due to hepatic metabolism, or related only to the increased supply of amino acids in the perfusion fluid, as it is impossible to distinguish the origin of these compounds. In addition, the perfusion samples had a slightly different bile acid profile compared to the in vivo samples, especially in the post-transplant period, while the ex vivo samples showed significantly lower concentrations of individual unconjugated primary and secondary bile acids compared to the samples collected after transplantation. Primary bile acids are excreted into the bile mainly as taurine or glycine conjugates. After entering the intestine, they are deconjugated by the bile salt hydrolase (BSH) produced by bacteria, and some are converted to secondary bile acids and passively absorbed into the hepatic portal circulation [19]. Depending on their structure, they are then re-secreted into the bile unchanged or they undergo glucuronidation in the endoplasmic reticulum [20]. Therefore, the pool of these newly formed bile acids increases after organ implantation due to the restoration of enterohepatic circulation.

3.1. Evaluation of the Metabolomic Profile of Bile in the Post-Transplant Period after Employing SCS and NEVLP

In recent years, normothermic machine perfusion (NMP) has been increasingly used to preserve the liver before transplantation [21]. NMP's advantages over routine static cold storage have been summarized in numerous papers [2,4,22]. For instance, Op den Dries et al. compared NMP and conventional SCS with respect to their effects on liver function and bile duct preservation in both DCD and non-DCD livers. Their study, which utilized a rat model, showed that NEVLP mitigates hepatic injury and provides better bile duct protection compared to SCS, especially in DCD livers [23]. A similar study by Kollmann et al. investigated the impact of both preservation methods on platelet aggregation and platelet-mediated sinusoidal endothelial cell (SEC) injury during liver transplantation. Their findings indicated that NEVLP is a useful strategy for reducing platelet-mediated ischemia-reperfusion injury (IRI) during and after LT [24]. Unfortunately, these studies were exclusively based on biochemical panels, and there continues to be a lack of data in the literature regarding how preservation methods affect low-molecular-weight compounds in body fluids. This is a notable gap, as such information could shed new light on the metabolic processes in the organ. In comparing the composition of bile produced by the liver after NEVLP and SCS, we observed changes in the concentrations of some bile acids during reperfusion. Specifically, increased concentrations of GHCA, TUDCA, coprocholic acid and CDCA acid were observed in the bile from the SCS-treated livers, while increased concentrations of TCA and taurohyocholic acid were detected in the bile from the livers preserved via NEVLP (Tables 1 and 2). Previous studies have reported significantly elevated levels of GHCA and TUDCA in bile and serum samples from patients with liver disease and primary sclerosing cholangitis (PSC) [25], as well as increased serum TUDCA levels in patients with primary biliary cirrhosis (PBC) [26]. TUDCA is a hydrophilic bile acid that protects against cholestatic and hepatocellular injury by enhancing the secretory capacity of the cholestatic liver cells and through its cytoprotective action against hydrophobic bile salts. In summarizing ischemia-reperfusion injury and ischemic-type biliary lesions following liver transplantation, Cursio and Gugenheim note that cytokines secreted by Kuppfer cells in the rejected organ contribute to the loss of Na (+), K (+)-ATPase activity, which plays an important role in bile secretion. Reduced activity of Na (+), K(+)-ATPase, a cotransporter for hepatocyte taurocholate uptake, leads to the impairment of TUDCA secretion and may result in subsequent cholestatic injury [27]. Our results did not reflect this assumption, as the bile samples from the SCS group were characterized by higher concentrations of TUDCA, which indicates that post-reperfusion graft injury after the use of SCS probably does not result from the presented mechanism. Moreover, increased concentrations of CDCA in biological materials have also been reported in liver diseases. For instance, Bathena et al. observed increased concentrations of CDCA in urine samples from patients suffering from various liver diseases, finding that levels of this bile acid were positively correlated with the stage of the disease [28]. Additionally, Mouzaki et al. found higher levels of CDCA in stool samples from non-alcoholic steatohepatitis (NASH) patients [29]. In the present study, the bile samples from the SCS group were characterized by a slightly higher concentration of CDCA compared to the NEVLP group and, as in the case of NASH patients, this finding may be due to the dominant role played by the alternative bile acid synthesis pathway. It has been suggested that the decreased expression of sterol 12-α-hydroxylase (CYP8B1) and the concomitant overexpression of oxysterol 7-α hydroxylase (CYP7B1) leads to increased CDCA synthesis, although the mechanism driving this relationship remains unclear [30]. In addition, arachidonic acid (AA) and 5-hydroxyeicosatetraenoic acid (5-HETE), two metabolites that may be associated with organ inflammation, were present in higher concentrations in bile samples collected from the SCS group during POD and reperfusion, respectively. AA is released from cell membranes by cytoplasmic phospholipase A2 alpha (cPLA2α), which can be activated during IRI [31,32]. Professor Kojima's group demonstrated that AA suppresses the growth of hepatic cells by inducing the production of reactive oxygen species (ROS) and the activation of mediated cellular (mostly nuclear) transglutaminase 2 (TG2). The nuclear accumulation of TG2 induces hepatocyte apoptosis via Sp1 transcription factor cross-linking and inactivation, resulting in the inhibition of the c-Met expression required for hepatic cell viability [33,34]. In addition, arachidonate 5-lipoxygenase metabolizes AA to 5-hydroperoxyeicosatetraenoic acid, which is used to synthesize leukotrienes or is converted to 5-HETE with the release of ROS [31,35].

The analysis of how SCS and NEVLP affected the metabolic profile of bile further revealed differences in the levels of numerous lipids belonging to the LPC group (Tables 1 and 2). Recent studies have found that changes associated with LPC 16:0 and 18:0 levels may play a role in signalling liver tissue damage and EAD in patients undergoing liver transplantation. For example, Xu et al. observed increased levels of these lipids in biopsy samples from patients with EAD [36], while Tsai et al. found lower levels of LPC 16:0 in plasma samples from patients with EAD [37]. It has been shown that LPCs can produce either pro- or anti-inflammatory effects. Pro-inflammatory effects, such as the expression of adhesion molecules, the release of chemotactic factors or an increase in ROS production, have mainly been attributed to saturated LPCs (LPC 16:0 and LPC 18:0) and monounsaturated LPC 18:1. On the other hand, polyunsaturated LPC species, such as LPC 22:4 and LPC 22:6, have been found to be anti-inflammatory and capable of neutralizing the in vivo inflammatory effect induced by saturated LPC 16:0 [38]. In our study, the bile samples from the SCS group were characterized by higher levels of saturated and monounsaturated LPCs compared to the NEVLP group, which suggests differences in the metabolic processes occurring in organs subjected to different preservation methods. Unfortunately, many of the processes occurring in the organ during the peri-transplant period—which in turn influence bile composition—remain unclear. Nonetheless, the metabolites identified in this comparison indicate that the type of preservation influences both the degree of damage to the bile ducts and the organ's condition.

3.2. Effect of 30 Min Ischemia on the Metabolomic Profile of Bile after Transplantation

Analysis of the effect of short-term (30 min) graft ischemia on the composition of bile secreted after transplantation revealed that the most significant changes occur during the reperfusion of the organ following SCS (Tables S1 and S2). Notably, Op den Dries et al. obtained similar results in their comparison of non-DCD and DCD groups subjected to both preservation methods in rat models. In addition to an increase in markers of biliary epithelial cell injury (LDH and GGT) in the bile samples from the SCS group, Op den Dries et al. observed that ultrastructural changes were most prominent in the SCS-preserved DCD livers after reperfusion [23]. Some of the metabolites identified in our study have already been reported in previous studies as being associated with lesions in the organ. Patterson et al. found increased levels of oxidized bile acid in the plasma of HCC patients compared to a cancer group without hepatic involvement, as well as decreased LPC levels compared to a group of healthy volunteers [39]. Although relatively little is known about fetal 3-oxo-4,6-choladienoic acid in adults, increased levels of this compound have been observed in plasma samples from cirrhosis patients, which may be related to deterioration in the activity of Δ4-3-oxo-steroid 5β-reductase [40]. Moreover, it has been shown that 5-methoxyindoleacetic acid, produced by Lactobacillus sp., is transported from the intestine to the liver, where it participates in activating nuclear factor erythroid 2-related factor, which helps to protect against oxidative damage to the liver [41].

3.3. Influence of Degree of Ischemia on the Bile Metabolome in the Peri-Transplant Period during NEVLP

The analysis of ischemia's impact on the function of livers subjected to NEVLP showed that 90 min of ischemia resulted in the most metabolic changes in the organ, which accounts for the differences in the bile's metabolomic profile compared to the HBD group (Table 3). As indicated above, compared to NEVLP, the use of SCS resulted in increased LPC levels in the bile samples acquired during reperfusion. However, the levels of LPCs were observed to decrease in the case of worsening ischemia (Figure 5). As previously mentioned, Tsai et al. found that reduced levels of LPC lipids in plasma correlate with the occurrence of EAD in patients [42]. Furthermore, Tanaka et al. demonstrated a decrease in LPC levels in serum samples in a mouse NASH model and the disruption of bile acid homeostasis manifested by an increase in tauro-β-muricholate and TCA levels. Differences in LPC levels can be attributed to changes in the activity of lysophosphatidylcholine acyltransferases (LPCATs) in converting LPC to PC, while differences in bile acid homeostasis may be due to the influence of inflammation on the classical and alternative pathways of its synthesis [43].

Although this study yielded promising results, it has some limitations. First, the analysis in this work focused exclusively on bile samples. To better understand the mechanisms driving the observed metabolic changes, it would be helpful to simultaneously analyze the graft tissue and blood samples as well. Another limitation is that the obtained results were not compared with routinely assessed clinical parameters; such a comparison would help to ensure a comprehensive evaluation of the results. Moreover, the analysis presented herein was non-targeted, which means that it requires confirmation via a fully validated targeted method. Given these limitations, subsequent experiments have already been planned to simultaneously analyze tissue, bile, perfusate and blood samples from liver patients in order to develop targeted approaches for potential biomarkers.

4. Materials and Methods

4.1. Animals

Male Yorkshire pigs weighing 29–35 kg were used for this study. The experiments in this work were approved by the Animal Resource Centre from University Health Network. All animals used in this work received humane care in accordance with the "Guide for the Care of Laboratory Animals" published by the National Institutes of Health.

4.2. Study Design

The analyses were performed on bile samples from porcine model donors with mild (heart beating donor [HBD]) and moderate warm ischemia (donation after circulatory death [DCD]) grafts. The obtained livers were subjected to 7 h SCS or NEVLP before transplantation. The SCS group consisted of two subgroups (5 animals in each group): HBD (HBD-SCS) livers and DCD livers with 30 min ischemia time (30′DCD-SCS). The livers from the NEVLP groups (HBD/30′DCD/60′DCD/90′DCD-NEVLP (5 animals in each group)) were stored at 4 °C in histidine-tryptophan-ketoglutarate (HTK) solution during the back-table preparation for ex vivo perfusion and were subsequently subjected to 5 h NEVLP at 37 °C. The livers from the SCS and NEVLP groups were subjected to a preservation time of 7 h, followed by transplantation into the recipient pigs. The recipient pigs were followed for a survival period of 4 days. Bile samples were collected during the peri-transplant period at the time points shown in Figure 7. The research material was provided by scientists from the Department of Surgery at the Toronto General Hospital (University Health Network, Toronto, ON, Canada).

The HBD pigs received heparin at 500 international units/kg of body weight 5 min prior to cross-clamping and cold flushing. In the case of the DCD grafts, the donor pigs received the same dose of heparin 5 min prior to the induction of cardiac arrest, which was accomplished by the intracardiac infusion of potassium chloride (20 mEq). After the induction of cardiac arrest, the desired warm ischemia time was awaited according to the protocol for the respective DCD model (30 min, 60 min or 90 min). Subsequently, all livers were flushed with a total volume of 3 L cold (4 °C) Custodiol-HTK (Essential Pharmaceuticals, LCC, Ewing, NJ, USA) through the aorta and portal vein. In the SCS groups, the livers were packed in bags filled with Custodiol-HTK and then stored in an icebox (4 °C) for 7 h; in the NEVLP-groups, the livers were cannulated and prepared for perfusion on ice (4 °C). To ensure the preservation time was comparable for all experiments, livers from the NEVLP groups were stored on ice for 2 h before being perfused for 5 h at 37 °C. After 5 h of NEVLP, the livers were flushed with cold (4 °C) Custodiol-HTK and then stored on ice before implantation was performed. Following SCS and NEVLP, the grafts were transplanted into recipient pigs using the method described in [44,45]. Animals were euthanized under deep anesthesia on postoperative day 4.

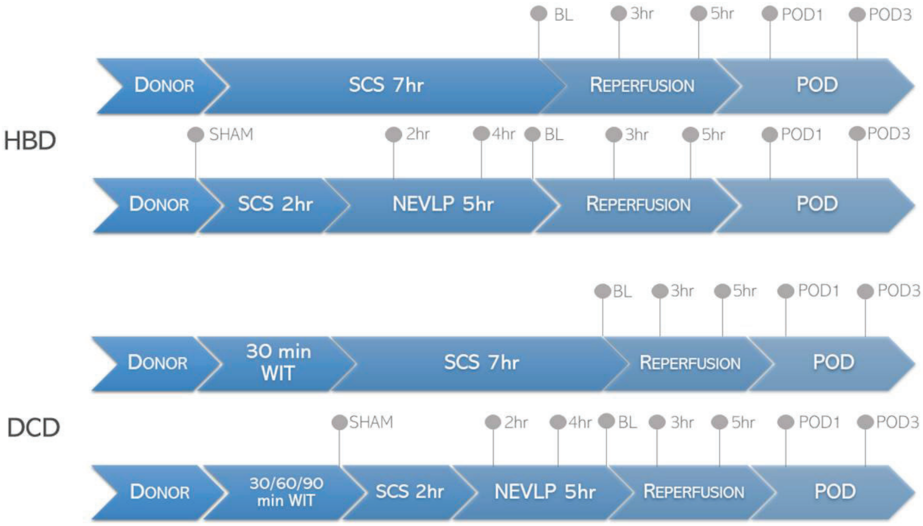

Figure 7. Experimental groups and design. Prior to procurement, animals were subjected to 0 min (HBD; heart beating donors), 30 min, 60 min and 90 min of warm ischemia to mimic donation-after cardiac death (DCD). Gray dots represent bile sampling time points. SHAM—prior to organ harvest; BL—baseline (start of reperfusion); WIT—warm ischemia time.

4.3. Normothermic Ex Vivo Liver Perfusion Setup

The ex vivio perfusion circuit was composed of a centrifugal pump (Rotaflow centrifugal pump), 2 hard shell reservoirs (Maquet, Hirrlingen, Germany), a leukocyte filter and a hollow-fiber dialyzer (NR16, Fresenius, Bad Homburg, Germany). The setup was similar to the OrganOx Metra that has recently been published in human clinical trials and has been described previously [46–48]. The hepatic artery pressure was set to 50–60 mm Hg, resulting in a flow of up to 400 mL/h. A second reservoir was used to regulate the portal vein pressure, which was intended to reach 2–4 mm Hg and a flow of 900–1400 mL/h. Porcine blood was obtained from the donor animal shortly before liver retrieval and erythrocytes were passed through leukocyte filters. For the perfusate, 1.5 L of Steen Solution (XVIVO Perfusion, Goteborg, Sweden) was mixed with the washed porcine erythrocytes (around 400 g) to achieve a final hematocrit of 15% with a hemoglobin level of 45 mg/dL. During the priming of the circuit, Heparin (10,000 international units (IU), Sandoz Canada, Quebec, QC, Canada), amino acid concentrate (Travasol 4.25%, 50 mL Bolus, Baxter, Hamilton, ON, Canada), sodium bicarbonate (20 mmol), calcium chloride (9.2 mmol/L) and antibiotics (Cefazolin—1 g, Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada and Metronidazole—500 mg, Baxter, Mississauga, ON, Canada) were added. Additionally, amino acid concentrate (Travasol 4.25%, 8 mL/h, Baxter, Hamilton, ON, Canada), insulin (125 IU/h, NovoRapid, Novo Nordisk, Mississauga, ON, Canada), 2% taurocholic acid

(7 mL/h, Sigma-Aldrich, St. Louis, MO, USA infused as a precursor for bile production) and prostaglandin E1 (500 µg/3 h, Pfizer, Kirkland, QC, Canada) were continuously administered during the perfusion. The surgical protocol for the donor liver retrieval and the liver transplantation has been described in more detail previously [24,44,45].

4.4. Sample Preparation

LC-MS-grade water, methanol, acetonitrile and formic acid were purchased from Merck (Poznań, Poland). The phosphate-buffered saline solution (PBS; pH 7.4) was prepared using analytical-grade sodium chloride, potassium chloride, potassium phosphate monobasic, sodium phosphate dibasic, hydrochloric acid and sodium hydroxide obtained from Merck (Poznań, Poland).

Sample preparation was performed via SPME, with each step of the process being performed on a high-throughput 96-manual thin-film microextraction (TFME) system (Professional Analytical System (PAS) Technology, Magdala, Germany). The use of this system allowed all of the samples to be analyzed simultaneously [9]. Extractions were performed using steel blades coated with a Hydrophilic-lipophilic balanced (HLB) N-vinylpyrrolidone-divinylbenzene copolymer sorbent (Alchem, Toruń, Poland). The coating preparation procedure was based on the spraying method described by Mirnaghi et al. [49]. The steel blades were purchased form Professional Analytical System (PAS) Technology (Magdala, Germany), while the polypropylene Nunc 96 DeepWell plates were purchased from Merck (Poznań, Poland).

Before beginning the extractions, the SPME blades were conditioned for 30 min in 1.0 mL of methanol:water (50:50; *v*/*v*) solution in 96-well-plates with agitation at 1000 rpm. After conditioning, a 10 s wash step was performed. Extractions were performed from 1.0 mL of diluted bile (1:99 in PBS) with the addition of glycodeoxycholic-2,2,4,4-d_4 acid (Merck, Poland) as an internal standard for 1 h (1000 rpm). After extraction, the blades were placed in 1 mL of nanopure water for 10 s. Following the wash step, desorption was conducted in 1 mL of ACN:H_2O (80:20; *v*/*v*) with agitation (1000 rpm) for 2 h.

4.5. LC-MS Analysis

Chromatographic separation was performed on a Dionex UHPLC system. Bile extracts, obtained per the method described above, were injected at a volume of 10 µL on a reversed-phase pentafluorophenyl (PFP) column (Discovery HS F5 100 × 2.1 mm, 3 µm). The autosampler and column temperatures were set to 4 °C and 25 °C, respectively, and the flow rate was set to 0.3 mL/min. Mobile phase A consisted of water with formic acid (99.9:0.1; *v*/*v*) and mobile phase B comprised acetonitrile and formic acid (99.9:0.1; *v*/*v*). The total analysis time for each sample was 40 min. The starting mobile phase conditions were as follows: 0% B from 0 to 3.0 min; a linear gradient to 90% B from 3.0 to 25.0 min; an isocratic hold at 90% B from 25.0 min to 34.0 min; and finally, a 6 min column re-equilibration time [50].

The analyses were performed in both positive and negative electrospray ionisation modes in separate runs on a Q Exactive Focus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). In positive ionization mode, the following HESI ion source parameters were used: spray voltage—1500 V; capillary temperature—300 °C; sheath gas—40 a.u.; aux gas flow rate—15 a.u.; probe heater temperature—300 °C; and S-Lens RF level—55%. For negative ionization mode, the HESI ion source parameters were as follows: spray voltage—2500 V; capillary temperature—256 °C; sheath gas—48 a.u.; aux gas flow rate—11 a.u.; probe heater temperature—413 °C; and S-Lens RF level—55%. The scan range was set to m/z 80–1000 with a resolution of 70,000. The instrument was calibrated using external calibration immediately before the analysis and every 48 h thereafter, resulting in a mass accuracy of <2 ppm. Data acquisition was performed using Xcalibur software v. 4.2 and Free Style 1.4 (Thermo Fisher Scientific, San Jose, CA, USA).

All samples were analysed in one randomised sequence and QC samples were run periodically (8–10 injections) to verify the instrument's performance. QC samples were prepared by mixing 20 µL of each of the bile extracts.

The putative identification of compounds was confirmed based on Full MS/dd-MS2 mode. The fragmentation parameters were as follows: mass resolution—35,000 full width at half maximum (FWHM); AGC target—2×10^4; minimum AGC—8×10^3; intensity threshold—auto; maximum IT—auto; isolation window—3.0 m/z; stepped collision energy—10 V, 20 V, 40 V; loop count—2; and dynamic exclusion—auto. Fragmentation spectra were confirmed using online databases such as LIPID MAPS, HMDB, METLIN and mzCloud.

4.6. Data Processing and Statistical Analysis

The raw data were processed using Compound Discoverer 3.1 (Thermo Fisher Scientific, San Jose, CA, USA) software, with the aim of identifying metabolites present in the samples. Detected metabolites with a signal-to-noise ratio > 3 and a peak intensity > 100,000 were subjected to further analysis. The intensity tolerance was set at 30%, and the RT tolerance was set at 0.2 min. The QC-based area was used for correction (min 80% coverage, max 30% RSD in QC). After peak alignment, gap filling was applied to fill in the missing values via a very small peak at the level of spectrum noise for the compound. The effect of the extraction process on the results was normalized by internal standard. After confirming the identification of the compounds, the results were summarized in a table and entered into MetaboAnalyst 5.0 software (accessed on 25 November 2022). Data were normalized by median, log-transformation and Pareto scaling. PCA was used to visually assess the separation between the sample groups. The differences among groups were evaluated using the Kruskal—Wallis test and multiple (post-hoc) mean-ranks comparisons using Statistica 13.3 PL software (StatSoft, Inc., Tulsa, OK, USA). Differences were deemed to be significant if $p < 0.05$. If only two groups were compared, the results were illustrated using a Volcano plot, which combines results from Fold Change (FC > 2) analysis and Wilcoxon rank-sum test ($p < 0.05$) with FDR correction into one single graph. Partial least squares discriminant analysis (PLS-DA) was used to assess the separation between groups and the model was cross-validated using leave-one-out cross validation. Finally, a pattern-matching method based on Spearman's rank correlation coefficient was implemented to search for compounds present at levels that were linearly correlated with organ ischemia time.

5. Conclusions

This study had two main goals: to evaluate how different preservation methods and ischemia impact the metabolome of bile produced by livers in the peri-transplant period, and to identify compounds that can be used as indicators of organ quality. To this end, high-throughput sample-analysis protocol based on TFME was developed and successfully applied to prepare bile samples—a matrix with a high content of lipid components and high variability with respect to its physicochemical properties. The analysis of the impact of preservation methods revealed that the first changes in the bile metabolome appeared only during the ongoing reperfusion. Bile produced in the SCS-preserved livers was characterized by increased levels of metabolites such as CDCA, AA and 5S-HETE, as well as saturated and monounsaturated LPCs. Such changes may be associated with differences in the bile acid synthesis pathways and organ inflammation. The study showed that short ischemia of the organ prior to bile collection led to changes in the metabolome of the bile produced by SCS-preserved organs during reperfusion. In contrast, the use of NEVLP reduced the negative effect of ischemia on organ function, as significant changes in bile composition were only visible after 90 min of ischemia. Notably, a linear relationship was observed between levels of lipids from the LPC group and the time of organ ischemia. Thus, this study identified metabolites worth considering as potential markers of changes occurring in preserved grafts in future analyses of larger study groups.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24032127/s1.

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Biliary Metabolome Profiling for Evaluation of Liver Metabolism and Biliary Tract Function Related to Organ Preservation Method and Degree of Ischemia in a Porcine Model

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Supplementary Material

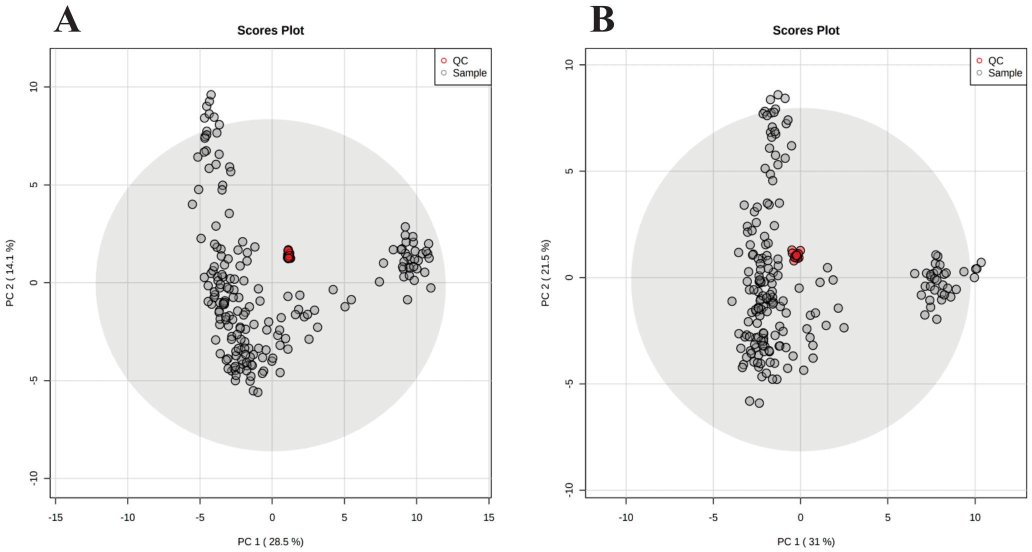


Figure S1. Principal component analysis (PCA) plots of all analyzed samples and extraction quality control (QC) samples for positive ionization mode (A) and negative ionization mode (B)

Table S1. The list of statistically significant compounds in comparison HBD versus 30'DCD for SCS and NEVLP donors in particular time points. Data for positive ionization mode.

Name	Molecular weight	RT	Effect of 30-minute ischemia (HBD vs 30'DCD)					
			Baseline (n=5)		Reperfusion (n=10)		POD (n=10)	
			HBD SCS / 30′DCD SCS	HBD NEVLP / 30′DCD NEVLP	HBD SCS / 30′DCD SCS	HBD NEVLP / 30′DCD NEVLP	HBD SCS / 30′DCD SCS	HBD NEVLP / 30′DCD NEVLP
			FC	FC	FC	FC	FC	FC
Indole	117.06	13.82	-1.25	-1.32	**-1.89****	1.02	-1.12	1.04
5-amino-pentanoic acid	117.08	1.63	-1.25	-1.49	**-2.08****	1.05	-1.19	-1.69
4-Methylene-2-pyrrolidinecarboxylic acid	127.06	7.26	**-3.45***	-1.25	-1.41	-1.23	-3.23	-1.05
Pyroglutamic acid	129.04	1.50	-2.70	1.08	**-2.22***	-1.11	**-2.63***	1.06
1H-Indole-3-carboxaldehyde	145.05	11.26	-1.11	-1.28	**-1.54***	1.15	-1.08	-1.16
Phenylalanine	165.08	9.43	-1.59	-1.25	**-1.64***	1.00	-1.52	-2.22
2,5-Dichloro-4-oxohex-2-enedioate	225.94	1.96	-1.19	-1.39	**-1.33***	1.14	-1.05	-1.18
Palmitamide	255.26	20.25	-1.25	-1.79	**-1.39***	-1.09	-1.20	-1.72
Linoleamide	279.26	19.88	-1.08	1.01	**-2.17***	-1.47	-2.17	-3.57
Stearamide	283.29	21.38	-1.28	-1.67	**-1.47****	-1.02	-1.30	-1.85
N-isobutyl-2,4-octadecadienoyl amine	335.32	21.89	-1.39	-1.64	**-2.00***	-1.39	-1.59	-1.92
Docosanamide	339.35	23.35	-1.39	-1.69	**-1.49***	-1.15	-1.32	-1.61
Tetrahydrocorticosterone	350.25	15.99	2.29	1.66	**2.37***	2.55	-1.06	1.45
3-Oxo-4,6-choladienoic acid	370.25	16.08	-7.69	1.95	-2.17	**-2.94***	-1.96	-1.10
12-Oxochenodeoxycholic acid	406.27	13.99	-2.44	-1.06	**-3.33****	**-1.54***	-1.75	-1.09

N-Docosahexaenoyl GABA	413.29	13.82	-1.3	-1.32	**-1.85****	1.08	-1.04	1.03
LPE 14:0	425.26	13.98	-1.85	1.36	-1.79	1.19	**-2.22***	**2.14***
Glycoursodeoxycholic acid	449.31	13.44	-1.23	-1.18	**-2.38****	-1.28	-1.22	1.02
Glycocholic acid	465.31	13.20	-1.08	1.19	**-1.61***	1.44	-1.05	-1.03
Glycohyocholic acid	465.31	12.71	1.55	1.38	**-2.44***	1.86	-1.27	1.21
PE O-18:0	481.32	18.62	2.11	-2.04	1.03	1.07	**4.36***	-1.75
LPC 15:0	481.32	17.18	1.75	1.10	1.47	1.19	**3.41***	-1.85
LPC 17:0	509.35	18.29	2.29	-1.03	1.60	1.37	**5.50***	-1.89
LPC 18:2	519.33	17.29	1.52	-1.10	**1.76****	1.81	1.77	1.29
LPC 18:0	523.37	18.84	1.72	-1.16	1.27	1.08	**3.34***	-2.08
PC 32:2	729.53	24.01	1.24	1.15	-1.01	**1.79***	1.52	1.58
PC 32:1	731.55	24.90	1.43	-1.59	-1.04	**1.62***	1.39	1.31
PE 36:4	741.53	24.23	-1.61	1.49	**-1.82***	-1.01	1.07	1.98

- denotes an increased level in the group located in the denominator of the fraction; *- p<0,05; **- p<0.01

Table S2. The list of statistically significant compounds in comparison HBD versus 30'DCD for SCS and NEVLP donors in particular time points. Data for negative ionization mode.

Name	Molecular weight	RT	Effect of 30-minute ischemia (HBD vs 30'DCD)					
			Baseline (n=5)		Reperfusion (n=10)		POD (n=10)	
			$\frac{HBD\ SCS}{30'DCD\ SCS}$	$\frac{HBD\ NEVLP}{30'DCD\ NEVLP}$	$\frac{HBD\ SCS}{30'DCD\ SCS}$	$\frac{HBD\ NEVLP}{30'DCD\ NEVLP}$	$\frac{HBD\ SCS}{30'DCD\ SCS}$	$\frac{HBD\ NEVLP}{30'DCD\ NEVLP}$
			FC	FC	FC	FC	FC	FC
Glucuronic acid	194.04	1.17	**-3.03***	1.21	-2.38	-1.05	-11.11	1.76
5-Methoxyindoleacetic acid	205.07	14.18	**-2.7****	1.22	-1.16	-1.06	-2.00	1.13
Ribose-5-phosphate	230.02	1.17	**-2.86***	1.21	-1.96	-1.01	-7.69	1.64
Tridecanedioic acid	244.17	15.26	1.01	-1.89	-1.32	1.08	-1.01	**-2.27***
5S-HETE	320.24	18.94	**9.46***	-1.79	2.15	-2.27	2.41	-4.55
N-Palmitoyl tyrosine	419.30	15.97	6.28	-1.20	**5.00***	-3.13	5.74	-4.00
Cortisol 21-sulfate	442.17	9.20	-1.61	-1.01	**2.74***	-1.12	-1.33	-1.05
Retinoyl b-glucuronide	476.24	16.60	9.68	-2.78	5.06	-2.63	-1.12	**5.34***
1-O-all-trans-retinoyl-beta-glucuronic acid	476.24	16.94	4.43	-1.30	4.70	1.12	-2.22	**6.94****
alpha-Ionol O-[arabinosyl-(1->6)-glucoside]	488.26	14.05	2.31	1.70	**5.41***	2.76	-2.04	1.80
4-Hydroxyretinoic acid glucuronide	492.24	13.62	6.29	-1.49	6.50	**-3.45***	-1.30	3.39
N-[(3a.5b.7a.12a)-3.7-dihydroxy-24-oxo-12-(sulfooxy)cholan-24-yl]-Glycine	545.27	10.62	1.08	1.48	-2.17	1.28	**-2.78****	-1.20

- denotes an increased level in the group located in the denominator of the fraction; *- p<0.05; **- p<0.01

Table S3. Detailed data of linear correlation coefficients and significance levels for metabolite correlated with organ ischemia.

Name	Molecular Weight	RT [min]	Correlation	Raw p-value	FDR adjusted p-value
Positive ionization mode					
PE P-18:0	481.32	18.62	-0.80659	1.7404e-05	0.0013468
LPC 18:3	517.32	16.92	-0.79883	2.405e-05	0.0013468
LPC 17:1	507.33	17.56	-0.79883	2.405e-05	0.0013468
LPE 18:2	477.29	17.29	-0.78332	4.4148e-05	0.0018542
PE 20:1	507.33	17.38	-0.77557	5.8753e-05	0.0018563
LPC 20:4	543.33	17.56	-0.76781	7.7345e-05	0.0018563
LPC 22:6	567.33	17.64	-0.76781	7.7345e-05	0.0018563
LPC 16:1	493.32	17.00	-0.76006	0.00010079	0.0021166
LPC 16:0	495.33	17.74	-0.7523	0.0001301	0.0021857
LPC 14:0	467.30	16.60	-0.7523	0.0001301	0.0021857
LPC 18:0	523.37	18.84	-0.74454	0.00016644	0.002542
LPC 18:1	535.33	15.07	-0.72903	0.00026575	0.003189
LPC 18:2	519.33	17.29	-0.72903	0.00026575	0.003189
LPC 15:0	481.32	17.18	-0.72903	0.00026575	0.003189
Arachidonic acid	304.24	21.14	-0.72128	0.00033198	0.0037181
Negative ionization mode					
5S-HETE	320.24	18.94	-0.85617	1.4664e-06	0.00012171
LPC 17:0	509.35	18.86	-0.82093	9.1997e-06	0.0002914
LPE 18:2	477,29	17.29	-0.81799	1.0533e-05	0.0002914
LPE 18:1	479,30	17,91	-0.80122	2.1807e-05	0.0004525
LPC 17:1	507.33	17.56	-0.79525	2.7795e-05	0.00046139
LPE 20:4	501,29	17,39	-0.73915	0.00019656	0.0027191
9,10,13-TriHOME	330,24	13,64	-0.7339	0.00023022	0.0027297

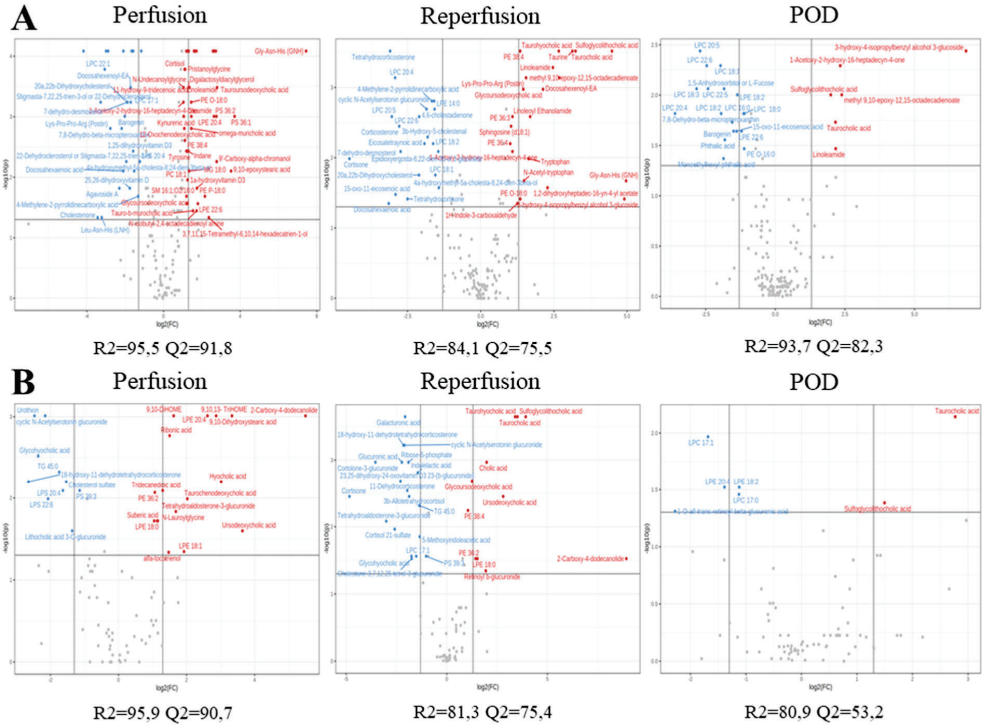


Figure S2. Volcano plots showing the number of metabolites differentiating the 90'DCD group compared to the HBD group in particular time intervals (fold change >2. p< 0.05 with FDR correction) for positive ioniazation mode (A) and negative ionization mode (B). red color - increased level after ischemia. blue color - decreased level after ischemia

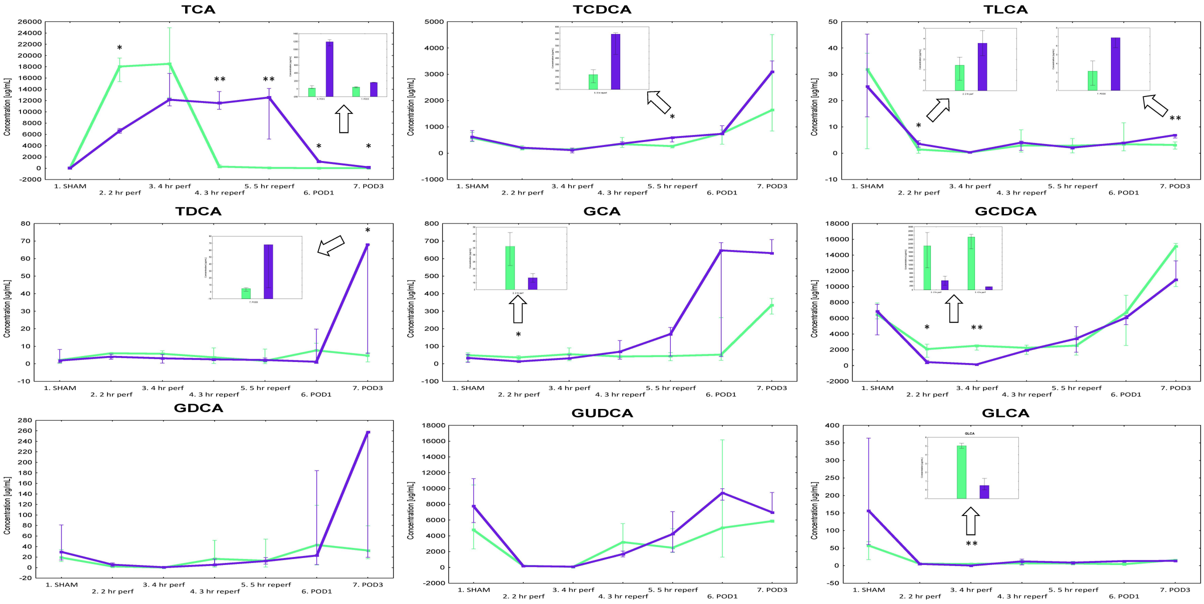
7.2. Najnowsze podejścia analityczne oparte na mikroekstrakcji do fazy stałej do profilowania kwasów żółciowych w ocenie przeszczepów wątroby poddanych normotermicznej perfuzji wątroby ex vivo – P.4.

Opis dotyczy pracy: **K. Łuczykowski**, N. Warmuzińska, K. Jaroch, D. Kollmann, M. Selzner, B. Bojko: ***Recent solid-phase microextraction-based analytical approaches for the profiling of biliary bile acids in pre-transplant assessments of liver grafts subjected to normothermic ex vivo liver perfusion.*** Anal. Chim. Acta, 2024, 1318, 342954

Niniejsza praca jest kontynuacją niecelowanych metabolomicznych badań żółci mającą na celu opracowywanie nowych metod analitycznych oraz poszukiwanie potencjalnych biomarkerów funkcji i żywotności wątroby oraz dróg żółciowych w okresie okołotransplantacyjnym. Poprzednie badania wykazały, że poziom poszczególnych indywidualnych kwasów żółciowych w żółci może zależeć od sposobu konserwacji narządu oraz stopnia jego niedokrwienia. Metabolity te są głównym organicznym składnikiem żółci, a oprócz roli w podtrzymywaniu funkcji trawiennych i wchłanianiu składników odżywczych, odgrywają również rolę sygnalizacyjną w regulacji procesów metabolicznych. Ocena profilu kwasów żółciowych w próbkach żółci może zatem dostarczyć cennych informacji na temat procesów patofizjologicznych narządu i służyć jako potencjalny wskaźnik dysfunkcji wątroby i dróg żółciowych.

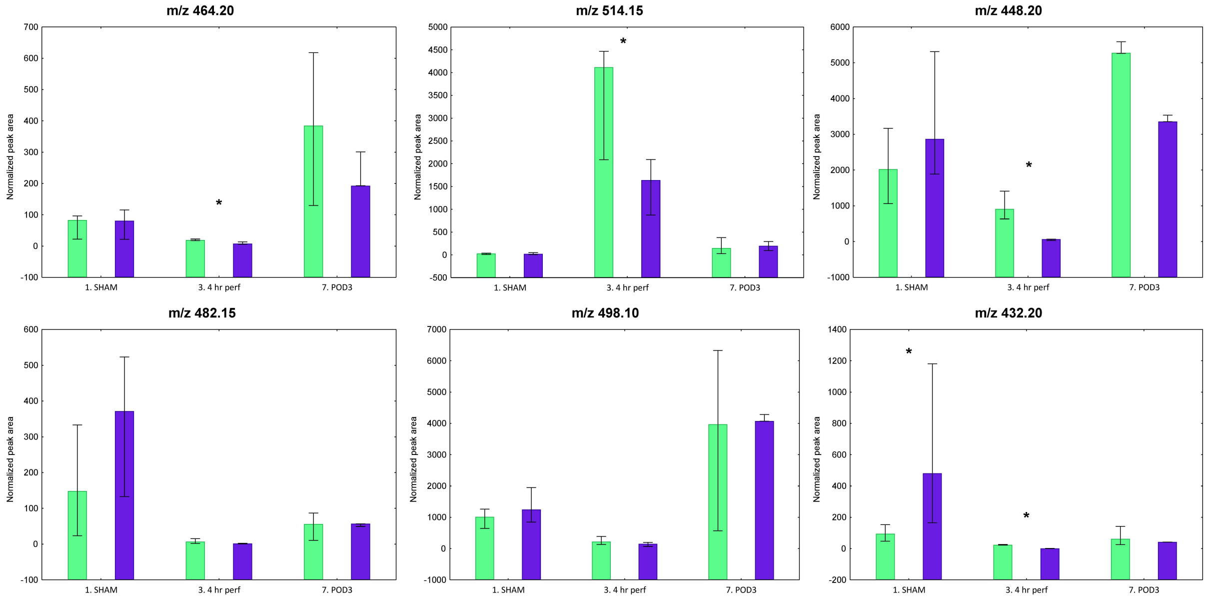
Przedstawione w pracy badania miały na celu opracowanie oraz pełną walidację wysokoprzepustowej celowanej metody oznaczania wybranych kwasów żółciowych w próbkach żółci wykorzystując platformę TFME-LC-MS. Eksperymenty optymalizacyjne dotyczyły doboru najefektywniejszego roztworu do desorpcji oraz czasów ekstrakcji i desorpcji. Opracowany protokół analityczny został zwalidowany zgodnie z wytycznymi dla metod bioanalitycznych określonymi przez amerykańską Agencję ds. Żywności i Leków (FDA, ang. Food and Drug Administration). Ponadto, w pracy zaproponowano metodę bezpośredniej analizy kwasów żółciowych z żółci bez rozdziału chromatograficznego, wykorzystując bezpośrednie sprzężenie SPME ze spektrometrem masowym za pośrednictwem otwartego interfejsu mikroprzepływowego (MOI, ang. microfluidic open interface). Podejście to skraca czas analizy do zaledwie kilku minut, upraszcza przygotowanie próbki i znacznie minimalizuje użycie odczynników organicznych, co wpisuje się w trend rozwijania ekologicznych metod tzw. „zielonej chemii”.

Obie opracowane metody zostały zastosowane do analizy próbek żółci zebranych podczas eksperymentu oceniającego wpływ niedokrwienia na przeszczep wątroby z wykorzystaniem NEVLP na modelu wieprzowym. Analiza z wykorzystaniem TFME-LC-MS wykazała zmiany profilu kwasów żółciowych w całym okresie okołoprzeszczepowym (Ryc. 4.).



Rycina 4. Zmiany stężeń poszczególnych kwasów żółciowych w okresie okołoprzeszczepowym. *Zielony – grupa HBD; niebieski – grupa 90’DCD.*

Niedokrwienie narządu spowodowało istotne różnice w poziomach kwasu taurocholowego, glikocholowego oraz glikochenodeoksycholowego, które mogą być rozważane jako obiecujący wskaźnik jakości narządu. Ze względu na bliskie podobieństwo strukturalne izomerów kwasów żółciowych oraz brak separacji chromatograficznej, podejście analityczne z wykorzystaniem platformy SPME-MOI-MS obejmowało analizę wspólnych sygnałów m/z pochodzących od określonych grup izomerów. Próbki perfuzyjne z grupy niedokrwiennej charakteryzowały się niższymi sygnałami dla m/z 464,20, 514,15, 448,20 i 432,20, co można tłumaczyć różnicą w poziomach odpowiednio kwasu glikocholowego, taurocholowego, glikochenodekosycholowego oraz glikolitocholowego oznaczonych metodą TFME-LC-MS (Ryc. 5.).



Rycina 5. Wynik analizy poszczególnych izobarów kwasów żółciowych metodą SPME-MOI-MS. *Zielony – grupa HBD; niebieski – grupa 90'DCD.*

Wyniki uzyskane za pomocą obu platform wykazywały wysoki współczynnik korelacji. Zaproponowane metodologie wydają się być obiecujące w określaniu profili kwasów żółciowych w różnych matrycach biologicznych w dysfunkcjach wątroby i dróg żółciowych.

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Recent solid-phase microextraction-based analytical approaches for the profiling of biliary bile acids in pre-transplant assessments of liver grafts subjected to normothermic ex vivo liver perfusion

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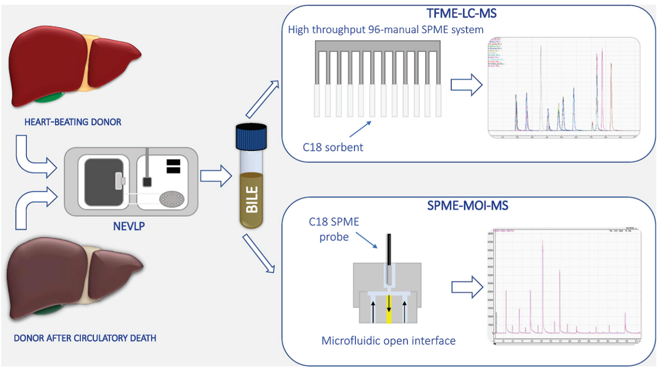
HIGHLIGHTS

- Validation of TFME-LC-MS method for bile acids determination in bile.
- SPME-MOI-MS method for rapid direct analysis of bile acid isobar groups.
- Assessment of the bile acid profile in the peritransplantation period.
- NEVLP in tandem with bile acid analysis methods for assessments of organ viability.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
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Solid-phase microextraction (SPME)
Microfluidic open interface (MOI)
Liver transplantation
Warm ischemia time

ABSTRACT

Background: Liver transplantation is the definitive treatment for end-stage liver failure, but the scarcity of donor organs remains a significant challenge. Leveraging organs from extended criteria donors (ECD) offers a potential avenue to address worldwide shortages, though these organs are more susceptible to post-reperfusion injury. This study explores the use of normothermic ex vivo liver perfusion (NEVLP) as a method for organ preservation – an approach that sustains liver metabolism and facilitates pre-transplant assessments of organ viability via bile analysis. The focal point of this study revolves on the development of analytical methods for determining the bile acid profile throughout the peritransplantation period as a potential indicator of liver function and viability.

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Results: The study optimized and validated a high-throughput analytical method to quantify selected bile acids in bile samples using a thin-film microextraction-liquid chromatography-mass spectrometry (TFME-LC-MS) platform. Furthermore, it introduced a solid-phase microextraction-microfluidic open interface-mass spectrometry (SPME-MOI-MS) method for rapid direct analysis of bile acid isobar groups. In the animal study, discernible variations in the concentrations of specific bile acids were observed between donors after circulatory death (DCD) and heart-beating donors (HBD), particularly following normothermic perfusion and reperfusion. Noteworthy fluctuations in individual bile acid concentrations were observed throughout the entire organ transplantation process, with taurocholic acid (TCA), glycocholic acid (GCA), and glycochenodeoxycholic acid (GCDCA) emerging as promising indicators of organ quality. The efficacy of the SPME-MOI-MS platform in corroborating these trends highlights its potential for real-time bile acid analysis during liver transplantation procedures.

Significance: Our findings underscore the efficacy of NEVLP in tandem with advanced bile acid analysis methods as a reliable strategy for pre-transplant assessments of organ viability, potentially increasing the use of ECD organs and reducing organ shortages. The ability to monitor bile acid profiles in real-time provides crucial insights into liver function and ischemic injury, making significant strides in improving transplant outcomes and patient survival rates.

Abbreviations

ACN	acetonitrile
BA	bile acid
BSH	bile salt hydrolase
C18	octadecyl silica
CA	cholic acid
CDCA	chenodeoxycholic acid
CV	coefficient of variation
DCA	deoxycholic acid
DCD	donor after circulatory death
ECD	extended criteria donors
FDA	US Food and Drug Administration
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GDCA	glycodeoxycholic acid
GHCA	glycohyocholic acid
GLCA	glycolithocholic acid
GUDCA	glycoursodeoxycholic acid
HBD	heart-beating donor
IPA	isopropanol
IRI	ischemia-reperfusion injury
LLE	liquid–liquid extraction
LLOQ	lower limit of quantification
MeOH	methanol
MRM	multiple reaction monitoring
NEVLP	normothermic ex vivo liver perfusion
PBS	phosphate-buffered saline
QC	quality control
SCS	static cold storage
SPME-MOI-MS	solid-phase microextraction-microfluidic open interface-mass spectrometry
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic
TDCA	taurodeoxycholic acid
TFME-LC-MS	thin-film microextraction-liquid chromatography-mass spectrometry
TLCA	taurolithocholic acid
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid
WIT	warm ischemia time

1. Introduction

Liver transplantation is a highly effective treatment and often the only option for patients with end-stage liver failure. Continuous advancements in surgical techniques, immunosuppression, and patient management have expanded the eligibility criteria, enabling more patients to qualify for the procedure. However, the primary obstacle remains the shortage of available organs, which significantly delays care and results in the deaths of many patients on waiting lists worldwide [1,2]. One approach to address this imbalance is to utilize organs from extended criteria donors (ECD), which include donors over 65-years old, organs presenting steatosis, donations after circulatory death (DCD), and organs subjected to prolonged cold ischemia [3]. Unfortunately, these organs are more susceptible to post-reperfusion damage, presenting significant challenges in contemporary transplantation. To overcome these challenges, there is a pressing need for new preservation strategies to replace static cold storage (SCS) and for the development of advanced methods to assess organ viability before transplantation [1,4]. As a rapidly advancing preservation procedure, normothermic ex vivo liver perfusion (NEVLP) has gained popularity due to its enhanced preservation abilities. NEVLP maintains normothermic conditions, supports active liver metabolism during storage, and reduces the risk of liver damage and early allograft dysfunction post-transplantation [5]. A significant advantage of NEVLP is that it facilitates organ quality assessments prior to transplantation by preserving the liver's metabolic activity. Thus, this procedure can provide a significantly clearer picture of viability during perfusion, as organ function can be evaluated through analysis of collected tissue, the perfusion solution, and the bile consistently produced by the liver [5].

Bile is a fluid produced by hepatocytes and concentrated in the gallbladder, playing a crucial role in the digestion and absorption of fats when secreted into the small intestine. Hepatic bile is primarily composed of water (∼95 %), with the remaining 5 % consisting of bile acids, bilirubin, lipids, small amounts of proteins, and other metabolites [6,7]. Analysis of this fluid has garnered significant interest from scientists and clinicians, as the production and composition of bile samples collected during normothermic perfusion can serve as valuable indicators of graft viability and the secretory function of hepatocytes and cholangiocytes [8]. As such, recent studies have focused on the analysis of bile acids, the primary organic component of bile. Beyond their role in supporting digestive functions and nutrient absorption, bile acids also play a signaling role in the regulation of metabolic processes by activating the farnesoid X nuclear receptor and the membrane G protein-coupled bile acid receptor-1 [9,10]. Thus, evaluating acid profiles in bile samples can provide valuable insights into the organ's pathophysiological processes, and serve as a potential indicator of liver and bile duct dysfunction during the peritransplant period.

The current study proposed protocols for the determination of selected bile acids in bile using solid-phase microextraction (SPME). The primary objective was to optimize and validate a protocol for thin-film microextraction-liquid chromatography-mass spectrometry (TFME-LC-MS) to enable high-throughput analysis of these analytes in bile samples. Moreover, as a proof-of-concept study, the study presented a method for rapid direct analysis of bile acid isobar groups without chromatographic separation using the solid-phase microextraction-microfluidic open interface-mass spectrometry (SPME-MOI-MS) platform. These methods were employed to compare the effect of warm ischemia on the bile acid profile in bile samples from two types of porcine model donors: heart-beating donors (HBD), and DCD subjected to NEVLP.

2. Materials and methods

2.1. Materials

All employed LC-MS grade chromatographic solvents, namely water, methanol, acetonitrile, and formic acid, as well as all bile acid standards, including cholic acid (CA), d_4-cholic acid (d_4-CA), chenodeoxycholic acid (CDCA), d_4-chenodeoxycholic acid (d_4-CDCA), deoxycholic acid (DCA), d_4-deoxycholic acid (d_4-DCA), ursodeoxycholic acid (UDCA), d_4-ursodeoxycholic acid (d_4-UDCA), glycocholic acid (GCA), d_4-glycocholic acid (d_4-GCA), glycochenodeoxycholic acid (GCDCA), d_4-glycochenodeoxycholic acid (d_4-GCDCA), glycodeoxycholic acid (GDCA), d_4-glycodeoxycholic acid (d_4-GDCA), glycoursodeoxycholic acid (GUDCA), d_4-glycoursodeoxycholic acid (d_4-GUDCA), glycolithocholic acid (GLCA), d_4-glycolithocholic acid (d_4-GLCA), taurocholic acid (TCA), d_4-taurocholic acid (d_4-TCA), taurochenodeoxycholic acid (TCDCA), d_9-taurochenodeoxycholic acid (d_9-TCDCA), taurodeoxycholic acid (TDCA), d_4-taurodeoxycholic acid (d_4-TDCA), tauroursodeoxycholic acid (TUDCA), d_4-tauroursodeoxycholic acid (d_4-TUDCA), taurolithocholic acid (TLCA), d_4-taurolithocholic acid (d_4-TLCA), were purchased from Merck (Poznań, Poland). Phosphate-buffered saline (PBS pH 7.4) powder used to prepare solutions and activated charcoal were purchased from Merck (Poznań, Poland). The octadecyl silica (C18) sorbent was purchased from Alchem (Toruń, Poland).

2.2. Preparation of stock solution, working solutions and quality control samples

A stock solution of all bile acids was prepared at a concentration of 100 μg mL^{-1} in methanol. Working solutions were prepared at 12 different levels (10 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 500 ng mL^{-1}, 1 μg mL^{-1}, 2.5 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1}, 25 μg mL^{-1}, 50 μg mL^{-1}, 75 μg mL^{-1} and 100 μg mL^{-1}) by diluting the stock solution with methanol. 10 μl of each working solution were freshly added to blank bile matrix, resulting in calibrators ranging from 0.1 to 1000 ng mL^{-1}. The internal standards mix solution was made in methanol, with each deuterated bile acid at a concentration of 5 μg mL^{-1}. Quality control samples for bile acids were prepared at four different final concentrations: lower limit of quantification (LLOQ), low (3 times of LLOQ), medium (400 μg mL^{-1}), and high (800 μg mL^{-1}).

2.3. Sample preparation

Preparation of samples was carried out using SPME, with each step of the process conducted on a high throughput 96-manual SPME system (Professional Analytical System (PAS) Technology, Magdala, Germany), enabling simultaneous analysis of all samples (Fig. S1). Steel blades coated with a 10 mm octadecyl silica (C18) sorbent were prepared for extraction following the spraying method described by Mirnaghi et al. [11]. Steel blades were purchased from PAS Technology (Magdala, Germany) and polypropylene Nunc 96 DeepWell plates were purchased from Merck (Poznań, Poland).

2.4. Selection of desorption solvent

A study to determine the appropriate desorption solvent for the presented application was carried out as a first step in method development. Eight different mixtures were prepared, each containing different proportions of acetonitrile (ACN), methanol (MeOH), isopropanol (IPA), and water (H_2O). Extractions were performed from PBS spiked with 100 ng mL^{-1} of standards. Both extraction and desorption steps were performed for a period of 120 min each under agitation at 1000 rpm at room temperature.

2.5. Extraction and desorption time profile

Desorption time was investigated as an initial step in the optimization of the time profile. For this purpose, extractions were performed from PBS spiked with 100 ng mL^{-1} of standards over a duration of 120 min, with desorption times evaluated at intervals of 5, 15, 30, 45, 60, 90, and 120 min. Following the determination of optimal desorption time, an analogous experiment was carried out to optimize the extraction time. Extraction periods of 5, 15, 30, 45, 60, 90, and 120 min were assessed, with desorption subsequently carried out for 45 min. Both extraction and desorption steps were carried out under 1000 rpm agitation at room temperature.

2.6. SPME procedure

Prior to extraction, SPME blades were conditioned for 30 min in 1.0 mL of a methanol: water (50:50; v/v) solution in 96-well-plates, with agitation at 1000 rpm at room temperature. This was followed by a 10-s wash step. Subsequently, extractions were carried out using 1 mL of diluted bile (100-fold and 10000-fold in PBS) with an internal standard (IS) added to achieve a final concentration of 50 ng mL^{-1}, under the same conditions, for 60 min. The concentration of organic solvents in the studied samples did not exceed 1 % (v/v), thereby ensuring optimal extraction efficiency [12]. Post-extraction, the blades were washed in 1 mL of nanopure water for 10 s. Desorption was subsequently performed in 1 mL of methanol with agitation at 1000 rpm for 45 min at room temperature.

2.7. Method validation

The method was validated in accordance with the bioanalytical method validation guidelines outlined by the US Food and Drug Administration (FDA) in 2018 [13]. In order to create a stripped matrix for validation purposes, bile samples diluted in PBS (100-fold) were subjected to incubation with 100 mg mL^{-1} of activated charcoal for 1 h, with agitation at 1000 rpm. Subsequently, this process was replicated for the supernatant obtained post-centrifugation [14].

2.7.1. Linearity range

A 980 μl volume of the blank bile was spiked with 10 μl of calibration standard containing all measured bile acids (BAs) and 10 μl of internal standards (IS) to construct a calibration curve with a minimum of 6 calibration points. Linearity was investigated using the least-squares linear regression method. Calibration curves were constructed by plotting peak area ratios of the analyte to the internal standard against the nominal concentration of the analyte in bile. Acceptance criteria included deviations within ±15 % of the nominal concentration (except for the lower limit of quantitation (LLOQ), which was set at ±20 %) for at least six non-zero calibrator levels, covering at least over 75 % of all samples in each validation run.

2.7.2. Accuracy and precision

The accuracy and precision of intra- and inter-day analyses were evaluated across three independent runs utilizing four quality control (QC) levels: LLOQ, low (L, three times the LLOQ), medium (M), and high (H), each containing five replicates. Accuracy was reported as the percentage of the nominal concentrations, with the mean concentration required to fall within 15 % of the nominal values for all QC levels (LLOQ: ±20 %). Precision was expressed as the coefficient of variation (CV), and the acceptance criteria mirrored those of the accuracy assessment.

2.7.3. *Recovery and matrix effect*

Five replicates of low, medium, and high concentrations of QC samples were utilized to assess recovery and matrix effect. Recovery was calculated by comparing the peak areas of analytes from extracted samples versus those from blank bile spiked with analyte post-extraction. The matrix effect was evaluated by comparing post-extraction spiked bile against the neat desorption solvent of the same concentration.

2.7.4. *Sample stability*

The stability of the samples was assessed with respect to four distinct parameters: autosampler stability, bench-top stability, long-term stability, and freeze-thaw stability. In order to assess the stability of the samples in the autosampler, samples were analyzed before and after being stored in the autosampler at 4 °C for 24 h. Bench-top stability was assessed by keeping samples at room temperature for 3 h, while long-term stability was evaluated by storing samples at −80 °C for a period of 4 weeks. Freeze-thaw stability was tested over three cycles of freezing at −80° followed by completely thawing at room temperature. In accordance with FDA guidelines, all stability experiments were performed at low and high concentrations of QC samples, each in three replicates.

2.8. *LC-MS analysis*

Chromatographic separation was performed on the Nexera UHPLC system. Bile extracts were injected in a volume of 5 μL on an ACQUITY UPLC CSH C18 Column (1.7 μm, 2.1 mm × 100 mm, Waters). The autosampler and column temperatures were maintained at 4 °C and 55 °C, respectively, throughout the analysis. The flow rate was set to 0.4 mL min^{-1}. Mobile phase A consisted of water with formic acid (99.9:0.1; v/v), while mobile phase B was composed of acetonitrile with formic acid (99.9:0.1; v/v). The total analysis time per sample was 13 min. The initial mobile phase conditions were set at 40 % B from 0 to 4.0 min, followed by a linear gradient to 70 % B from 4.0 to 10.0 min, an isocratic hold at 100 % B from 10.1 to 11.0 min, and a 2-min column re-equilibration time with 40 % B.

The analyses were performed on a LC–MS 8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), equipped with an electrospray ionization source operating in negative-ion mode and utilizing multiple reaction monitoring (MRM). The MRM transitions, along with the collision energies for individual bile acids, are detailed in Table S1. The ESI source parameters were set as following: interface voltage at 3.0 kV, interface temperature at 300 °C, DL temperature at 250 °C, heat block temperature at 450 °C, nebulizing gas flow at 3.0 L min^{-1}, drying gas flow at 10.0 L min^{-1}, and heating gas flow at 10.0 L min^{-1}.

2.9. *Solid phase microextraction - microfluidic open interface – mass spectrometry (SPME-MOI-MS) analysis*

A rapid analysis of bile acids without chromatographic separation was performed using a microfluidic open interface (MOI) coupled directly to a mass spectrometer (Fig. S2). Analytes were extracted from bile samples using 8 mm C18 SPME fibers. Prior to sampling, fibers were preconditioned for 60 min in a methanol:water (50:50 v/v) solution and then rinsed with purified water. Extractions were carried out from 1 mL of diluted bile samples (1:99 in PBS) spiked with CA-d$_4$ to a final concentration of 50 ng mL^{-1}, for 15 min under agitation at 1000 rpm. Following extraction, SPME fibers were quickly rinsed with ultrapure water for 5 s to remove residual salts and non-specific bile components loosely adhered to the sorbent surface. Individual fibers were then placed in the desorption chamber of the MOI for 10 s for desorption, and subsequently removed from the chamber. The resulting solution containing the desorbed bile acids was transferred from the MOI to the MS, and ionized by electrospray, with a dwell time of 10 msec. The construction and functional principles of the MOI device are described in detail elsewhere [15,16]. The desorption solution, consisting of MeOH with formic acid (99.9:0.1; v/v), was delivered to the MOI device by a Harvard Apparatus Pump 11 Elite pump (Holliston, Massachusetts, USA). The desorption step was performed by equilibrating the pump flow rate, set at 165 μL min^{-1}, with the electrospray ionization (ESI) aspiration. Similar to the LC-MS analysis described above, a triple quadrupole mass spectrometer operating in negative ionization mode was utilized, employing previously optimized MRM settings for bile acids. The MS parameters were configured as follows: interface voltage at 3.0 kV, interface temperature at 300 °C, DL temperature at 250 °C, heat block temperature off, nebulizing gas flow at 3.0 L min^{-1}, drying gas flow at 10.0 L min^{-1}, and heating gas flow at 10.0 L min^{-1}.

2.10. *Animal study*

The study utilized bile samples from two types of porcine model donors (Yorkshire pigs weighing 29–35 kgs): HBD, and DCD. The experiments were approved by the Animal Resource Centre at the University Health Network, and all animals were treated humanely in accordance with the National Institute of Health's "Guide for the Care of Laboratory Animals". The research material was provided by the Department of Surgery at Toronto General Hospital (University Health Network, Toronto). Bile samples were collected at specific time points during NEVLP and after liver transplantation: before organ procurement, during perfusion, 3 and 5 h after reperfusion, and on days 1 and 3 post-transplantation (Fig. 1). For the DCD group, a model with 90 min warm ischemia time was used (n = 5 in each group). The setup for ex vivo normothermic liver perfusion has been previously described in more detail [6].

2.11. *Data processing and statistical analysis*

Data acquisition was performed by LabSolutions software v. 5.97 (Shimadzu, Kyoto, Japan). Subsequent analysis utilized Microsoft Excel and Statistica software v. 13. Statistical analyses between the two groups were conducted using the Mann-Whitney *U* test. Spearman correlation analysis was employed to evaluate the association between the LC-MS and MOI analytical platforms [17]. Statistical significance was defined as $p < 0.05$. The Chloroform-oriented Toxicity Estimation Scale (ChlorTox Scale) was used to assess the overall risk to health and the environment associated with the use of chemicals of both developed analytical methods [18].

3. Results and discussion

3.1. *Method development*

Optimization of the sample preparation method included the selection of three basic parameters: the most effective desorption solution, and optimal desorption and extraction times. The initial step was to identify the ideal desorption solution. For this purpose, a comparative analysis of the effectiveness of 8 different variations of solution mixtures commonly employed in metabolomics and lipidomics studies using SPME [19,20] was carried out. These solutions included MeOH, ACN: H_2O (50:50), ACN: H_2O (80:20), IPA: MeOH (50:50), MeOH: ACN (50:50), ACN, MeOH: ACN: H_2O (45:45:10) and MeOH: ACN (80:20). Comparative analysis revealed similar recovery rates for bile acids

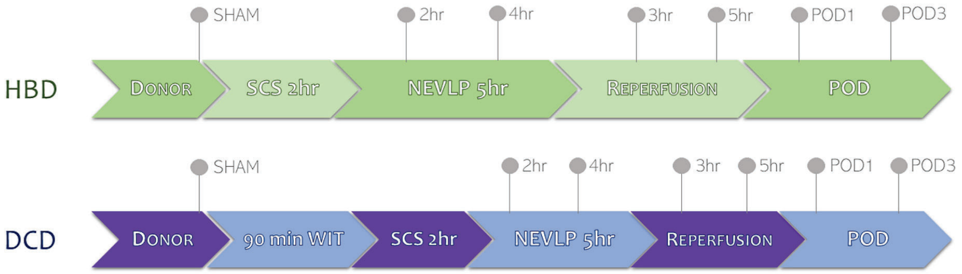

Fig. 1. Experimental groups and design. Prior to procurement, animals were subjected to 0 min (HBD; heart beating donors), or 90 min of warm ischemia to simulate donation after circulatory death (DCD). Gray dots represent the time points for bile sampling. SHAM—prior to organ procurement; WIT—warm ischemia time.

across most solutions, except for acetonitrile, which exhibited significantly lower rates. Additionally, the isopropanol: methanol mixture caused distorted peak shapes due to its incompatibility with the employed mobile phases (Fig. S3). Therefore, our focus shifted to addressing the suboptimal recovery of TLCA, which ranged from 45 % to 61 % across different solutions (Fig. 2). Notably, pure methanol consistently delivered the best results, aligning with its well-documented, widespread use in preparing various biological samples for bile acid analysis [21–23].

Methanol was used in subsequent optimizations of desorption and extraction times. Considering the need for an efficient and expedient preparation method, it was determined that these times should not exceed 2 h. Given the similar properties of all analyzed analytes, they unsurprisingly displayed analogous trends in recovery rates with respect to desorption and extraction time. Our findings revealed that while extending the desorption time did not significantly impact analyte recovery, significantly lower RSDs were attained after the 45-min mark, making 45 min an ideal desorption time for our method (Table S2). For extraction time, prolonging it increased recovery until equilibrium was reached between the concentrations of analytes in the liquid phase and the solid phase of the SPME sorbent [24,25]. Consequently, a 60-min point was selected due to its near-equilibrium recoveries, and superior repeatability of results (Fig. 3). Utilization of a high-throughput TFME system capable of simultaneously analyzing up to 96 samples under the developed protocol enables a total preparation time per sample of 1.5 min, inclusive of the time required for the conditioning of the stationary phase, extraction, and desorption of analytes. This underscores SPME as a simple, fast, and effective sample preparation method that can effectively substitute traditionally employed methods such as SPE, LLE, or protein precipitation.

3.2. *Method validation*

The selectivity and specificity of the proposed method were ensured by employing multiple reaction monitoring mode during MS acquisition along with the inclusion of distinct IS for each bile acid. This approach additionally facilitated the confirmation of the retention times of each individual bile acid. Unspiked blank bile samples exhibited no interferences at the retention times of the analytes and their respective internal standards. Moreover, analyses of blank samples immediately following the ULOQ QC sample (1000 ng mL^{-1}) also did not indicate the presence of the analyzed compounds, which indicated the absence of a carry-over effect.

The linearity of the developed TFME-LC-MS method for all bile acids was assessed across a dynamic concentration range of 0.1–1000 ng mL^{-1}. The LLOQ ranged from 1 to 10 ng mL^{-1} depending on the specific bile acid, as detailed in Table 1. The coefficients of determination (R^2) for all bile acids exceeded 0.99, indicating high linearity. Accuracy and repeatability, expressed as inter- and intra-day precision, were evaluated by analyzing four different concentration levels of the target compounds: LLOQ (1, 5 or 10 ng mL^{-1}), low (3, 15 or 30 ng mL^{-1}), medium (400 ng mL^{-1}), and high (800 ng mL^{-1}). The obtained accuracy and precision values ranged from 82 to 114 % and 1.22–18 % for intra-assay, and 85–118 % and 0.29–17 % for inter-assay. These results met the criteria outlined in the FDA guidelines and are summarized in Table 2.

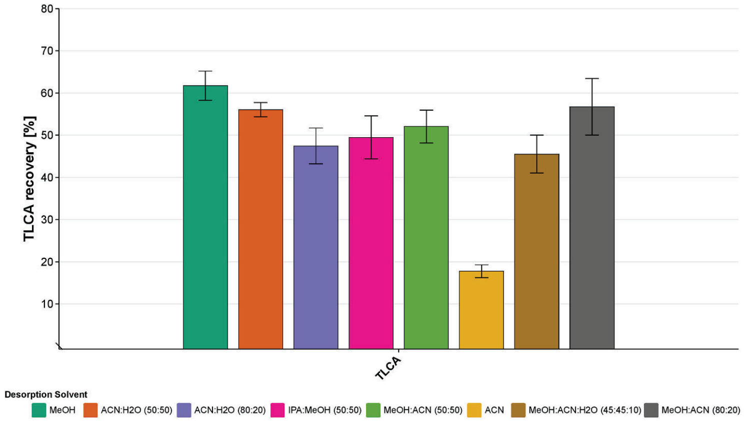

Fig. 2. Efficiency of desorption solutions for TLCA.

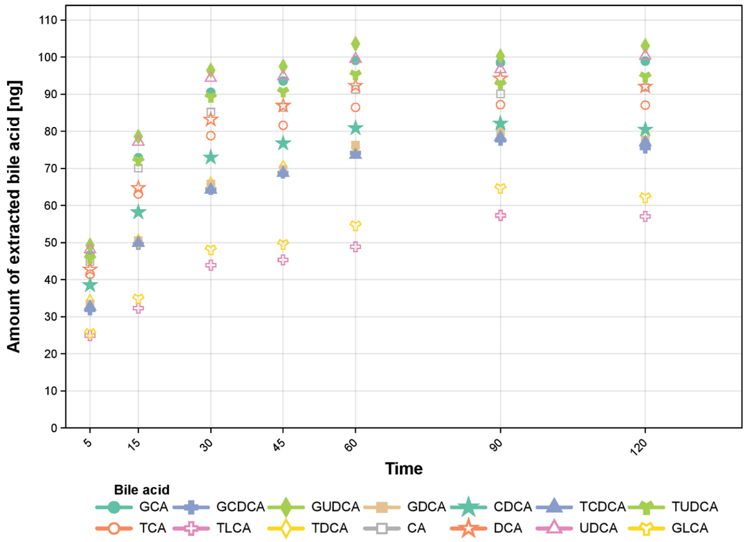

Fig. 3. Extraction time profiles for the analyzed bile acids.

Table 1
List of analyzed bile acids with linearity results.

Analyte	Rt [min]	M [g/mol]	Linearity [ng/mL]	R^2
GCA	1.99	465.62	5–1000	0.9987
GUDCA	2.05	449.62	1–1000	0.9987
TUDCA	2.88	499.70	10–1000	0.9984
TCA	2.94	515.70	5–1000	0.9990
CA	3.87	408.57	5–1000	0.9985
GCDCA	4.43	449.62	5–1000	0.9987
UDCA	4.47	392.57	10–1000	0.9979
GDCA	5.16	449.62	1–1000	0.9980
TCDCA	5.62	499.70	10–1000	0.9982
TDCA	6.28	499.70	1–1000	0.9986
CDCA	7.30	392.57	5–1000	0.9981
DCA	7.60	392.57	10–1000	0.9981
GLCA	7.96	433.32	5–1000	0.9980
TLCA	8.81	483.71	5–1000	0.9980

Extraction recoveries and the presence of a matrix effect were subsequently assessed, with the findings detailed in Table 3. As previously discussed, the proposed TFME protocol yielded the lowest average recovery for TLCA (41–71 %), while UDCA exhibited the highest recovery rates (82–90 %) among the analyzed compounds. Although a slight ion suppression associated with the matrix effect was observed for each bile acid, this effect remained insignificant, not exceeding 12 %.

A comprehensive assessment of the stability of the analyzed bile acids under various conditions was conducted. This included short-term storage of samples at room temperature, covering the entire sample preparation procedure, as well as storage during a 24-h period under autosampler conditions. Additionally, the stability of samples was evaluated following long-term storage at −80 °C, and after undergoing three cycles of thawing and refreezing. The resulting stability values ranged from 86 to 109 %, as detailed in Table 4.

3.3. Application in animal study

The developed method was employed to analyze bile acids in porcine bile samples collected during an experiment focused on liver transplantation utilizing NEVLP for organ preservation. The study compared bile samples from two types of donors: HBD, and DCD subjected to 90 min of warm ischemia. Conjugated primary and secondary bile acids were predominantly identified in all samples. Unconjugated bile acids were detected in trace amounts only in samples taken a few days post-transplantation. This finding aligns with observations from our pilot experiment involving untargeted metabolomic analysis of bile samples, and may stem from the restoration of enterohepatic circulation [6]. Primary bile acids are excreted into the bile after conjugation with taurine or glycine. Within the intestinal lumen, they are deconjugated by bile salt hydrolase (BSH) produced by bacteria, with some converting to secondary bile acids and being passively absorbed into the hepatic portal circulation [26]. Depending on their structure, bile acids are then secreted back into the bile unchanged or undergo glucuronidation in the endoplasmic reticulum [27]. Si et al. also observed the absence of free bile acids in porcine bile when analyzing bile acid profiles across various mammalian species [28]. In the analyzed samples, UDCA and TUDCA concentrations were below the quantification limit and could not be quantified. The bile acid profile of samples varied slightly according to the time intervals in which the samples were collected. In most samples, the predominant acids were GUDCA, GCDCA, and TCDCA, except in perfusion samples, where TCA was notably high owed to its continuous administration during perfusion as a precursor of bile production. Elevated TCA levels were also observed during reperfusion in samples from the 90′DCD donors. The average percentage of bile acids in bile samples collected at different time intervals is shown in Fig. 4., while Fig. S4 details the bile acid content for HBD and 90′DCD donors separately.

Subsequent data analysis focused on tracking the trends in changes in the concentrations of individual bile acids in bile over time, from organ procurement to the third day post-transplantation. The effect of organ warm ischemia time on individual bile acid levels was assessed by comparing bile acid phenotype profiles from livers of HBD and 90′DCD groups. No statistically significant differences in bile acid concentrations were observed between the two study groups for samples taken immediately before organ procurement (SHAM samples). However, notable differences were observed for GUDCA (7762.18 vs. 4756.8 μg mL^{-1}, $p = 0.22$) and GLCA (156.86 vs. 57.45 μg mL^{-1}, $p = 0.09$), with the 90′DCD group exhibiting higher levels. Regardless of organ ischemia duration, 2 h of normothermic ex vivo perfusion decreased the concentration of all

Table 2
Summarized intra-day and inter-day accuracy and precision of bile acids.

	Intra-day								Inter-day							
	LLOQ		Low		Medium		High		LLOQ		Low		Medium		High	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
GCA	93.32	2.47	89.16	4.26	99.38	2.37	96.74	2.24	102.32	0.90	109.84	7.03	99.17	5.41	103.39	1.60
GUDCA	97.24	6.64	85.95	2.40	93.86	1.88	91.03	2.41	99.62	3.90	108.06	4.82	105.57	3.95	109.10	0.29
TUDCA	91.95	3.54	87.59	1.33	93.34	4.15	89.48	2.13	113.68	6.33	110.34	5.16	110.89	6.86	113.96	4.08
TCA	103.91	4.92	85.86	3.93	91.24	1.66	88.25	3.36	94.61	3.93	111.86	5.33	106.32	3.96	110.11	2.23
CA	87.24	3.23	99.47	3.70	111.29	2.87	106.27	1.22	113.64	4.04	102.97	7.13	91.86	7.21	96.77	3.07
GCDCA	112.20	2.82	99.24	5.78	105.53	2.80	102.24	3.09	87.18	2.69	104.12	1.92	94.79	4.27	100.84	2.62
UDCA	95.34	2.46	88.94	8.39	88.26	3.51	88.76	2.19	102.05	7.15	111.27	2.38	112.31	4.18	112.01	2.09
GDCA	114.47	18.75	95.9	12.97	111.81	6.65	104.52	3.90	94.17	17.19	91.48	3.18	86.64	4.12	85.45	3.51
TCDCA	82.21	2.51	92.05	1.87	86.85	2.13	90.68	2.17	114.62	10.73	114.61	2.64	110.22	4.14	111.79	3.55
TDCA	84.11	2.89	85.94	10.03	92.96	2.38	85.31	1.47	114.10	3.73	114.29	4.22	112.86	11.65	113.15	2.10
CDCA	95.83	14.16	95.74	6.57	106.03	3.41	102.43	2.91	100.99	6.77	107.47	8.09	95.76	5.14	100.53	0.99
DCA	89.76	3.87	86.27	4.67	90.57	2.34	86.70	2.16	111.38	2.50	110.72	5.93	109.83	8.55	110.14	4.61
GLCA	99.65	7.28	92.31	2.87	109.59	2.49	104.81	2.86	96.93	5.38	110.79	1.46	90.11	5.80	97.39	3.00
TLCA	85.94	5.38	102.54	4.38	103.47	6.19	104.13	6.15	118.31	2.11	113.85	7.24	102.37	5.04	108.16	2.39

bile acids except TCA and TDCA. Additionally, by the end of the experiment (POD3), the ischemic group was characterized by higher concentrations of all bile acids except GCDCA and GLCA.

Bile acids are increasingly the subject of research in the context of liver and biliary tract diseases, with serum or plasma typically used as matrix [29–32]. Due to the invasive nature of bile collection and its challenging physicochemical properties, which translate into difficulties in sample preparation, bile is rarely used as material for biomarker research in organ-related changes. Despite these challenges, there is growing recognition of the potential for using this biological material for research, including in assessments of bile acid profiles as indicators of graft quality in the peritransplant period [33,34]. Most existing studies, however, often concern the analysis of bile samples taken from a single time point, limiting our understanding of the dynamics of changes in individual acids during organ preservation and transplantation.

In our work, we aimed to track the trend of changes in bile acids in bile by taking samples at several hour-intervals throughout the peritransplant period. Based on the results obtained, it can be concluded that individual bile acids exhibit distinct trends in their levels, reflecting the different stages of the transplantation process and the impact of ischemia (Fig. 5). This approach provides a more comprehensive understanding of the dynamic changes in bile acid levels during transplantation. In the samples collected at the onset of perfusion, the ischemic group exhibited higher TLCA (3.57 vs. 1.44 μg mL^{-1}, $p = 0.02$) and lower TCA (6643.21 vs. 18051.8 μg mL^{-1}, $p = 0.02$), GCA (13.50 vs. 36.18 μg mL^{-1}, $p = 0.02$), and GCDCA (441.31 vs. 2091.2 μg mL^{-1}, $p = 0.02$) concentrations compared to the HBD group. However, over the course of perfusion, these differences became statistically insignificant except for GCDCA (150.12 vs. 2516.63 μg mL^{-1}, $p = 0.01$). Previous reports in the literature have linked this bile acid with necrotic liver cell death, oxidative stress, and induction of liver fibrosis in vitro [35–38]. The levels of this bile acid have also been reported to increase in the serum [39,40] but decrease in the bile [41] of patients with cholestatic lesions of the bile ducts and liver. These findings suggest that cholestatic changes may influence GCDCA concentrations, although further research is needed to correlate its concentration in body fluids and fully understand its role in liver disorders.

In our level assessment of bile acids in bile samples collected at the end of reperfusion, slight differences were noted between the BA profiles of HBD and 90′DCD groups. This stage is critical during organ transplantation due to the risk of ischemia-reperfusion injury (IRI). Bile acids that were elevated in the 90′DCD group compared to the HBD group and increased between the third and fifth hours after reperfusion included TCA (12559.6 vs. 72.22 μg mL^{-1}, $p = 0.008$), TCDCA (592.64 vs. 268.38 μg mL^{-1}, $p = 0.03$), and GCA (170.16 vs. 44.54 μg mL^{-1}, $p = 0.15$). The profile of TCA secretion into bile notably distinguishes the two groups. During reperfusion, the ischemic organ continued to secrete large amounts of this bile acid into the bile even after administration was stopped, possibly indicating increased synthesis or accumulation of TCA by the organ with delayed secretion into the bile. Although there are no direct reports on the involvement or changes in the levels of TCA and TCDCA in body fluids with respect to mechanisms related to IRI, increased concentrations of these bile acids have been associated with liver cirrhosis and cholangitis [31,42]. In contrast, the elevated biliary GCA level of the 90′DCD group persisted for several days after transplantation. This was accompanied by significant increases in the concentrations of the secondary bile acids TLCA (6.91 vs. 3.15 μg mL^{-1}, $p = 0.008$) and TDCA (67.95 vs. 4.70 μg mL^{-1}, $p = 0.02$). In a recent study by Rogino et al. on changes in the BA pool induced by IRI in porcine model [34], increased biliary GCA levels were associated with a higher degree of liver damage and correlated positively with biochemical markers of cellular injury.

3.4. A proof-of-concept study using SPME-MOI-MS for bile acid analysis

This work introduced a method for direct analysis of bile acids from

Table 3
Recovery and matrix effect results for bile acids.

	Low		Medium		High	
	Recovery (CV%)	ME (CV%)	Recovery (CV%)	ME (CV%)	Recovery (CV%)	ME (CV%)
GCA	81.94 (1.63)	108.89 (3.02)	81.09 (5.44)	107.02 (3.81)	90.31 (2.30)	109.55 (1.45)
GUDCA	83.42 (4.74)	112.43 (6.49)	82.93 (3.74)	107.13 (3.30)	91.09 (1.16)	107.62 (1.04)
TUDCA	88.30 (3.76)	110.32 (2.17)	82.01 (4.89)	107.77 (3.07)	90.15 (1.37)	109.75 (1.37)
TCA	82.16 (4.97)	109.03 (3.42)	80.79 (5.68)	107.08 (3.15)	90.33 (1.59)	109.42 (0.87)
CA	86.26 (2.55)	109.18 (2.64)	81.06 (4.69)	107.64 (3.53)	90.67 (1.88)	108.05 (1.25)
GCDCA	68.53 (6.41)	109.65 (2.36)	74.72 (7.79)	107.50 (3.64)	86.88 (2.45)	109.59 (1.18)
UDCA	88.92 (3.33)	108.24 (2.40)	82.38 (3.46)	107.46 (2.77)	90.15 (0.80)	108.81 (1.79)
GDCA	66.26 (6.87)	112.22 (3.00)	74.29 (7.61)	107.60 (3.28)	87.12 (2.46)	109.24 (1.00)
TCDCA	66.49 (8.76)	109.97 (1.59)	72.72 (8.24)	108.19 (3.58)	86.29 (2.76)	109.72 (1.13)
TDCA	62.42 (8.73)	111.26 (8.42)	72.54 (7.75)	107.27 (3.71)	86.18 (3.14)	108.70 (1.08)
CDCA	74.62 (9.12)	105.37 (2.27)	77.62 (7.05)	105.01 (3.92)	87.72 (1.88)	106.54 (2.43)
DCA	77.04 (4.40)	109.57 (2.91)	77.41 (7.19)	106.75 (3.67)	88.01 (3.80)	108.87 (2.11)
GLCA	44.95 (11.67)	111.29 (2.42)	58.90 (11.58)	106.78 (2.95)	74.80 (4.96)	108.80 (1.57)
TLCA	41.26 (14.19)	111.84 (3.35)	55.14 (11.78)	106.49 (3.84)	71.66 (6.38)	108.22 (1.95)

Table 4
Stability results for bile acids.

	Room temperature (3hr)		Autosampler (4 °C)		4 weeks (−80 °C)		Freeze-thaw (3 cycles)	
	Low (%CV)	High (%CV)	Low (%CV)	High (%CV)	Low (%CV)	High (%CV)	Low (%CV)	High (%CV)
GCA	96.24 (2.37)	97.63 (2.71)	105.24 (1.32)	102.85 (0.99)	94.51 (4.70)	108.27 (3.32)	104.66 (5.29)	105.14 (3.52)
GUDCA	99.95 (5.37)	100.82 (0.39)	101.39 (2.69)	102.36 (0.39)	102.04 (3.91)	108.36 (5.18)	105.40 (9.15)	104.75 (4.13)
TUDCA	91.48 (3.23)	98.42 (3.76)	90.43 (2.90)	92.86 (0.95)	102.27 (3.63)	110.10 (3.87)	107.66 (4.71)	105.24 (3.41)
TCA	95.64 (4.18)	99.12 (1.97)	97.72 (3.22)	100.36 (1.56)	94.53 (4.02)	107.67 (5.06)	103.85 (6.34)	104.18 (4.02)
CA	95.66 (1.70)	98.98 (2.00)	103.20 (0.52)	105.85 (0.80)	99.02 (2.26)	109.53 (4.69)	106.05 (5.34)	105.15 (4.85)
GCDCA	95.04 (2.20)	98.68 (3.08)	98.84 (3.38)	101.94 (1.34)	86.37 (3.33)	108.39 (4.23)	101.47 (9.76)	102.53 (3.74)
UDCA	94.71 (7.74)	94.50 (1.11)	92.87 (4.80)	89.31 (1.95)	102.35 (3.07)	108.50 (4.86)	106.79 (6.38)	105.46 (4.43)
GDCA	99.20 (2.33)	100.25 (2.74)	100.41 (0.73)	100.98 (3.00)	90.98 (8.44)	109.00 (4.59)	102.20 (6.74)	102.83 (3.85)
TCDCA	98.64 (2.83)	97.77 (2.62)	94.86 (8.76)	95.09 (8.24)	86.14 (4.39)	107.80 (4.05)	99.10 (11.25)	101.47 (4.39)
TDCA	97.77 (4.17)	96.58 (2.23)	96.78 (13.27)	94.23 (0.39)	89.65 (9.27)	107.79 (4.74)	104.49 (14.62)	101.62 (3.55)
CDCA	100.32 (7.05)	98.91 (0.54)	98.33 (3.05)	102.25 (1.64)	87.70 (7.89)	108.67 (5.20)	109.29 (3.25)	103.99 (6.16)
DCA	95.77 (2.41)	100.64 (4.22)	101.48 (2.50)	109.42 (1.24)	95.23 (9.76)	108.92 (5.33)	102.91 (7.97)	103.33 (4.37)
GLCA	96.20 (1.84)	97.72 (1.32)	102.27 (1.92)	102.29 (0.11)	85.93 (14.28)	103.11 (3.78)	90.27 (10.60)	99.28 (2.89)
TLCA	98.02 (2.94)	98.34 (2.66)	97.89 (1.25)	96.66 (0.58)	86.65 (3.50)	101.95 (4.51)	87.93 (14.31)	98.36 (3.37)

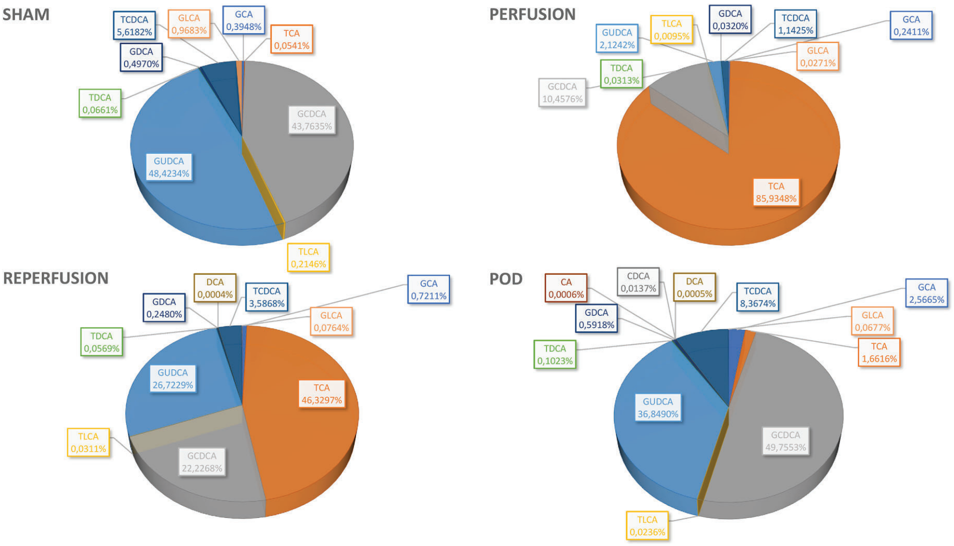

Fig. 4. Percentage composition of individual bile acids in bile samples across different time intervals.

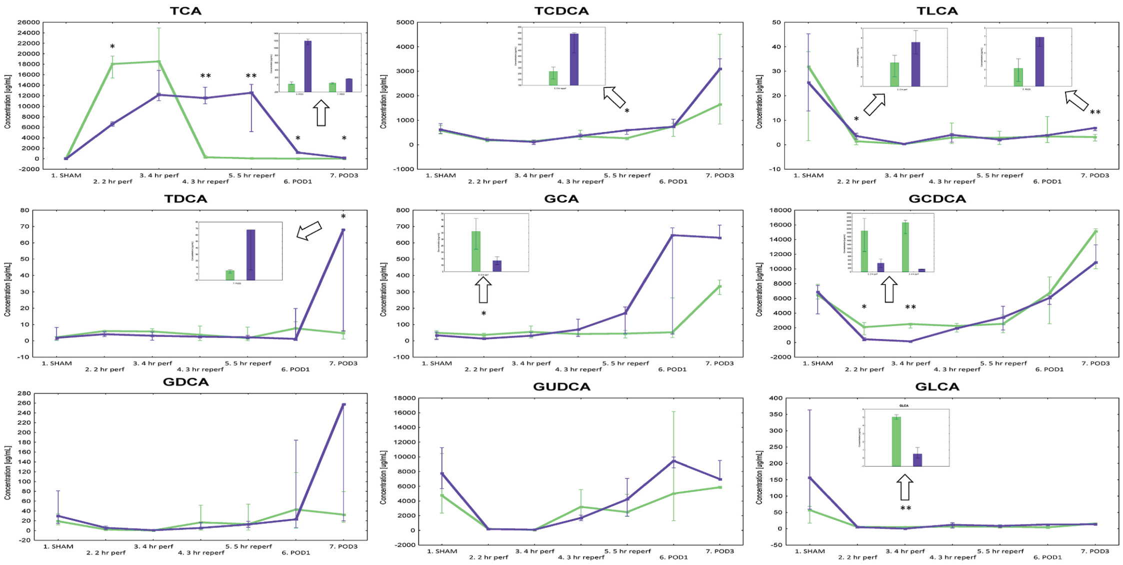

Fig. 5. Variations in individual bile acid concentrations throughout the peritransplantation period.

bile without chromatographic separation by using direct coupling of SPME with a mass spectrometer via the MOI interface. This approach minimizes the use of organic reagents, simplifies sample preparation, and reduces analysis time to mere minutes, enabling near real-time results, which is in line with current trends in green chemistry. Although the TFME-LC-MS method is characterized by a low chemical risk value (1.81 according to the ChlorTox Scale), the direct SPME-MOI-MS analysis approach reduces this risk by almost five times (0.39 according to the ChlorTox Scale) (Table S3). While bypassing chromatographic separation results in simultaneous signals from all analytes, the employment of MRM mode slightly compensates for this by aligning signals with specific analytes. However, the close structural similarity of isobaric BA isomers in biological samples [43] poses challenges, limiting the analysis to common signals (*m/z*) for specific groups of isomers. Consequently, our study targeted specific *m/z* precursors: 464.20 (GCA), 514.15 (TCA), 448.20 (GUDCA, GCDCA, GDCA), 482.15 (TLCA), 498.10 (TUDCA, TCDCA, TDCA) and 432.20 (GLCA). However, it is important to note that other isobars present in bile acid, such as glycohyocholic acid (GHCA), which shares an *m/z* with GCA, were not analyzed in the LC-MS platform. Although this approach does not enable the analysis of

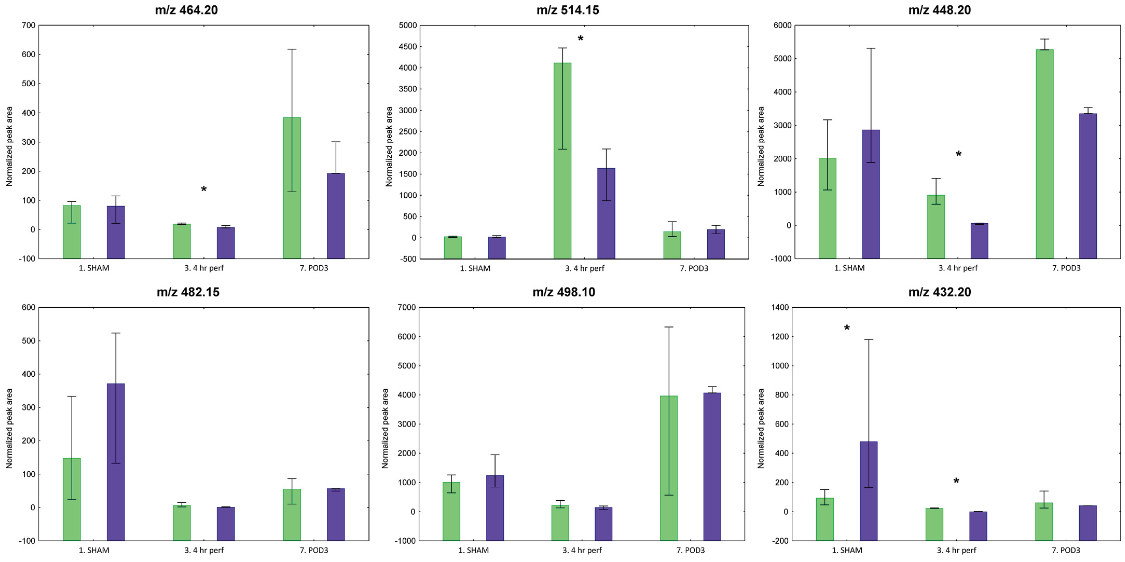

Fig. 6. Result of analysis of individual bile acid isobars using the SPME-MOI-MS method.

specific individual bile acids, it effectively complements the determination of total bile acids widely used in research [44–46].

An analysis of bile samples collected before organ retrieval, after 4 h of perfusion, and 3 days after transplantation was performed, and the results obtained are presented in Fig. 6. A statistically significant difference between the HBD and 90′DCD groups in samples collected before organ retrieval was only noted for *m/z* 432.20. This result is similar to the GLCA determination on the LC-MS platform, differing only in the significance level. In a comparison of perfusion sample profiles, a distinct correlation between the SPME-MOI-MS and LC-MS platforms in the HBD group was observed. Notably, HBD group profiles were characterized by higher signals for *m/z* 464.20, 514.15, 448.20, and 432.20, which corresponded to GCA, TCA, GCDCA, and GLCA. However, an analysis of samples collected a few days after organ transplantation did not reveal discrepancies in *m/z* signals that could differentiate the two tested groups of donors.

The correlations between results obtained via the direct MOI method and the LC-MS platform were assessed. For this purpose, values corresponding to each *m/z* were compared with the sum of the concentrations of the corresponding bile acids. All analyzed *m/z* signals attained via the MOI method correlated with the concentrations of bile acids obtained by LC-MS, with an $R > 0.87$, except for *m/z* 464.20, which had $R = 0.64$ (Fig. 7). The low correlation of this *m/z* with GCA may be attributed to the significant contribution of GHCA, which was not analyzed on the LC-MS platform. These results demonstrated that MOI-based determinations can be effectively utilized in experiments requiring rapid results, such as in assessments of graft quality during the peritransplant period. Full optimization and validation of this quantitative method for various metabolites, including but not limited to bile acids, could pave the way for future clinical applications.

Despite the promising results of the presented study, several limitations should be noted. Firstly, the concentration of TCA in some perfusion samples and that of GCDCA at POD3 were slightly outside the assumed linear range. Given that the differences in concentrations between the studied groups were either insignificant or distinctly different, and did not impact the overall interpretation of experimental results, extrapolated values were adopted to conserve biological samples. The biological part of the project relied on a porcine model with a relatively small sample size ($n = 5$ for each group). Only bile samples were analyzed, thus concurrent analysis of transplanted tissue would be beneficial to better understand the mechanisms driving the observed changes. Another limitation is the lack of comparison between the obtained results and routinely assessed clinical parameters, which would provide a more comprehensive evaluation of the results. Currently, there are no published results on bile analysis covering the entire transplantation process, making it challenging to draw definitive conclusions when comparing results from short organ ischemia donors with those from chronic liver disease studies. Nonetheless, this study suggests that changes in bile acid concentrations may reflect underlying processes taking place in the organ. In light of these limitations, we are performing further experiments aimed at the concurrent analysis of tissue, bile, perfusate, and blood samples from liver transplant patients. This approach is expected to enhance our current understanding and help develop methods to identify potential biomarkers of organ quality.

4. Conclusions

In this study, a method for determining selected bile acids in bile samples based on the TFME-LC-MS platform was optimized and validated. The developed method was applied to investigate changes in the bile acid profile occurring during the peritransplant period, with focus on the effects of organ ischemia. The study demonstrated that individual bile acids exhibit changes in their concentrations throughout the entire organ transplantation process, with some, such as TCA, GCA, and GCDCA, showing promise as indicators of organ quality. Moreover, the study introduced a rapid assessment method for individual bile acid isobars using the innovative SPME-MOI-MS platform. These methodologies hold promise for future applications in the determination of bile acid profiles across various biological samples related to liver and biliary tract diseases.

CRediT authorship contribution statement

Kamil Łuczykowski: Writing – original draft, Writing – review & editing, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Natalia Warmuzińska:** Writing –

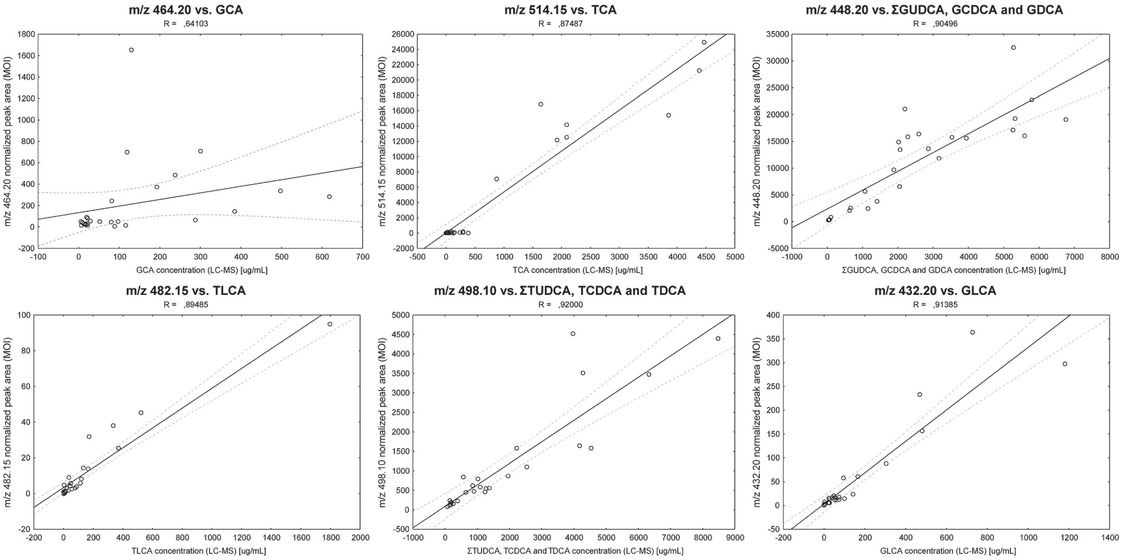

Fig. 7. Correlation between the results obtained in direct MOI analysis and the determination of bile acids on the LC-MS platform.

review & editing, Visualization, Formal analysis. **Karol Jaroch:** Methodology. **Dagmar Kollmann:** Writing – review & editing, Methodology, Conceptualization. **Markus Selzner:** Writing – review & editing, Resources, Methodology, Conceptualization. **Barbara Bojko:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kamil Łuczykowski reports financial support was provided by National Science Centre Poland. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.342954.

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Supplementary information

Profiling of biliary bile acids for pre-transplant assessments of liver grafts subjected to normothermic ex vivo liver perfusion (NEVLP). Thin film microextraction-liquid chromatography-mass spectrometry and solid phase microextraction-microfluidic open interface-mass spectrometry study

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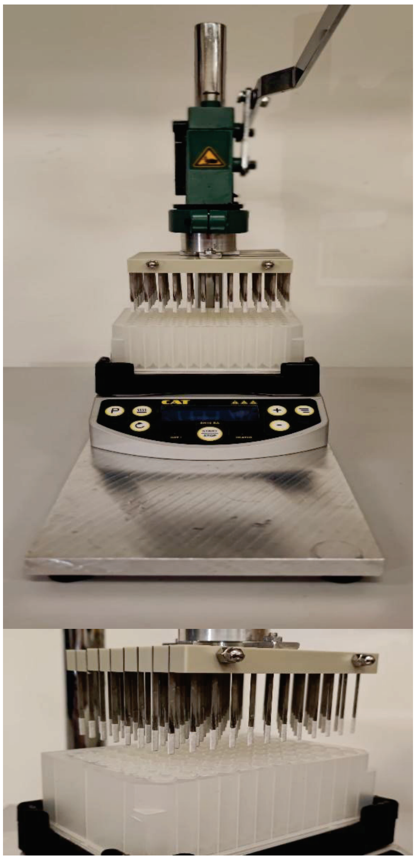

Fig. S1. A high throughput 96-manual SPME system.

Table. S1. MRM transitions with collision energy for individual bile acids

Analyte	M [g/mol]	MRM transition (m/z)	CE
GCA	465.62	464.20→73.85	39
		464.20→402.15	36
		464.20→410.10	46
GUDCA	449.62	448.20→73.85	36
		448.20→386.25	36
		448.20→384.10	40
TUDCA	499.70	498.10→124.00	54
		498.10→107.00	55
		498.10→79.95	55
TCA	515.70	514.15→123.80	54
		514.15→106.80	55
		514.15→280.90	30
CA	408.57	453.10→407.15	22
		453.10→343.20	42
		453.10→353.35	41
GCDCA	449.62	448.20→73.85	37
		448.20→386.40	35
		448.20→330.05	47
UDCA	392.57	437.10→391.35	24
		437.10→44.80	41
GDCA	449.62	448.20→73.85	39
		448.20→404.15	33
		448.20→402.25	38
TCDCA	499.70	498.10→123.75	54
		498.10→106.80	54
		498.10→80.05	55
TDCA	499.70	498.10→123.80	53
		498.10→106.80	54
		498.10→80.05	55
CDCA	392.57	437.10→391.15	24
		437.10→44.95	45
		437.10→373.20	43
DCA	392.57	437.10→391.20	25
		437.10→345.15	41
		437.10→343.20	48
GLCA	433.32	432.20→74.00	35
		432.20→388.40	34
		432.20→386.40	40
TLCA	483.71	482.15→123.80	51
		482.15→106.80	54
		482.15→80.05	55

Analyte	M [g/mol]	MRM transition (m/z)	CE
d_4-GCA	469.65	468.30→74.00	45
		468.30→406.45	37
		468.30→404.45	41
d_4-GUDCA	453.65	452.20→74.00	37
		452.20→390.50	37
		452.20→408.50	32
d_4-TUDCA	503.71	502.50→124.10	54
		502.50→107.00	55
		502.50→79.95	55
d_4-TCA	519.70	518.40→124.10	55
		518.40→106.95	55
		518.40→79.95	55
d_4-CA	412.57	457.30→411.40	27
		457.30→347.40	44
		457.30→349.50	40
d_4-GCDCA	453.62	452.30→74.00	40
		452.30→389.50	36
		452.30→390.50	37
d_4-UDCA	396.57	441.30→395.45	26
		441.30→377.40	45
		441.30→45.00	46
d_4-GDCA	453.62	452.25→73.55	42
		452.25→406.20	38
		452.25→408.25	34
d_9-TCDCA	508.70	507.50→124.10	55
		507.50→107.00	55
		507.50→79.95	55
d_4-TDCA	503.70	502.50→124.05	55
		502.50→107.00	55
		502.50→79.90	55
d_4-CDCA	396.57	441.30→395.45	25
		441.30→356.60	43
		441.30→45.00	49
d_4-DCA	396.57	441.30→395.50	25
		441.30→349.45	41
		441.30→351.45	48
d_4-GLCA	437.32	436.30→74.00	36
		436.30→392.55	36
		436.30→414.00	13
d_4-TLCA	487.71	486.40→124.05	52
		486.40→106.95	55
		486.40→79.90	55

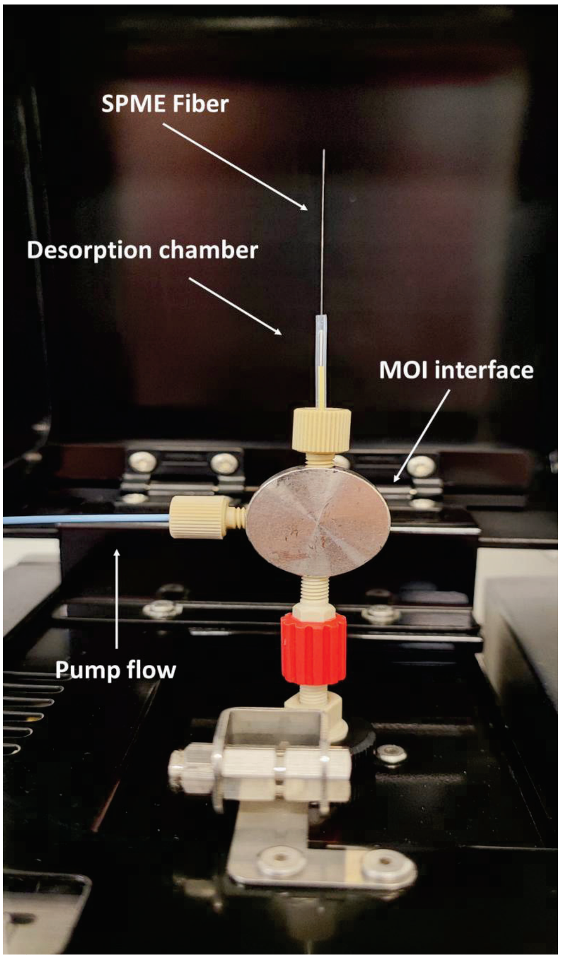

Fig. S2. Microfluidic open interface

Tab. S2. Correlation between the amount of extracted bile acids and the desorption time.

Extracted amount [ng]								RSD [%]							
	5	15	30	**45**	60	90	120		5	15	30	**45**	60	90	120
GCA	82.98	78.68	86.86	**83.50**	90.50	91.77	97.32	GCA	6%	3%	6%	**1%**	4%	4%	5%
TCA	70.82	67.14	74.55	**70.42**	76.94	75.92	83.14	TCA	8%	5%	6%	**2%**	5%	3%	6%
GCDCA	58.72	51.48	61.55	**54.46**	62.22	60.94	65.69	GCDCA	11%	6%	9%	**2%**	7%	6%	10%
TLCA	43.57	34.21	41.59	**35.42**	41.90	38.31	44.49	TLCA	17%	5%	10%	**1%**	11%	6%	12%
GUDCA	88.15	87.66	92.09	**91.86**	96.53	98.62	107.10	GUDCA	3%	2%	4%	**2%**	3%	1%	2%
TDCA	60.40	52.09	62.33	**54.80**	63.06	60.03	66.54	TDCA	12%	6%	9%	**1%**	8%	5%	10%
GDCA	61.51	54.54	64.33	**57.16**	66.65	63.36	69.74	GDCA	11%	5%	8%	**1%**	9%	5%	9%
CA	78.36	76.60	81.44	**79.73**	85.08	85.73	92.80	CA	3%	1%	2%	**1%**	2%	3%	3%
CDCA	65.54	59.23	67.35	**62.33**	69.56	68.07	74.60	CDCA	6%	2%	6%	**4%**	5%	5%	7%
DCA	75.57	68.62	78.81	**73.57**	81.58	79.77	86.17	DCA	7%	2%	5%	**2%**	4%	5%	7%
TCDCA	59.13	50.51	60.28	**52.85**	61.51	58.48	63.86	TCDCA	13%	6%	8%	**2%**	7%	5%	11%
UDCA	82.89	84.12	87.04	**85.87**	91.48	92.77	100.19	UDCA	2%	1%	2%	**1%**	2%	2%	2%
TUDCA	81.06	80.77	84.86	**83.81**	89.23	89.01	97.68	TUDCA	3%	2%	3%	**2%**	3%	2%	3%
GLCA	43.23	33.12	42.35	**34.33**	42.43	39.08	44.68	GLCA	18%	8%	13%	**1%**	15%	7%	15%

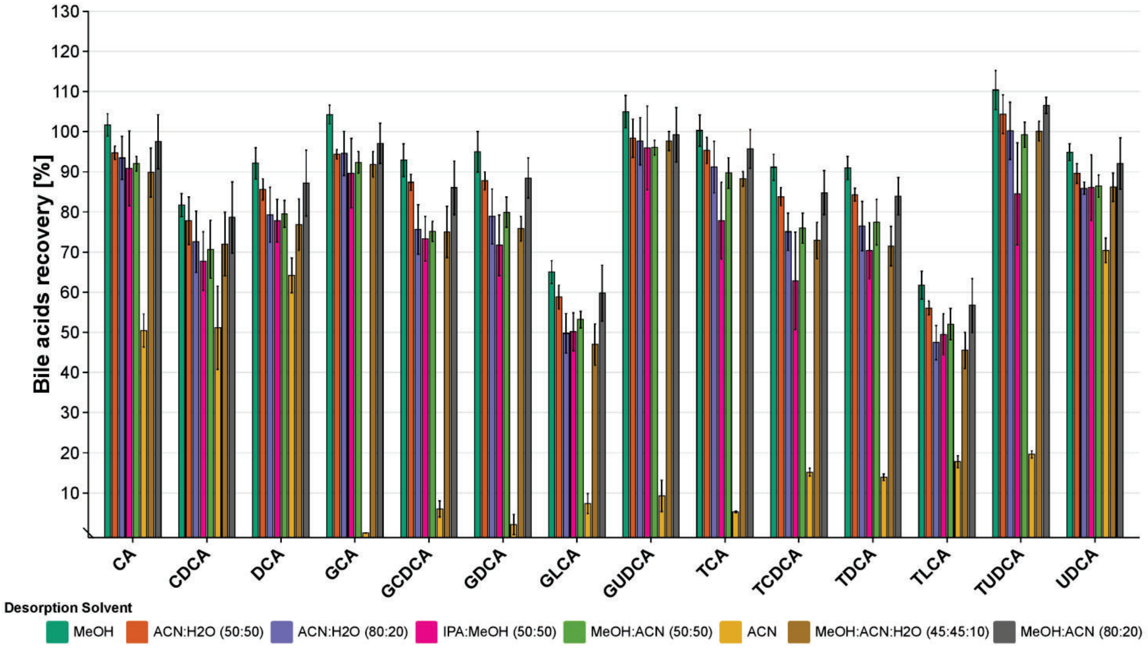

Fig. S3. Efficiency of desorption solutions for BA

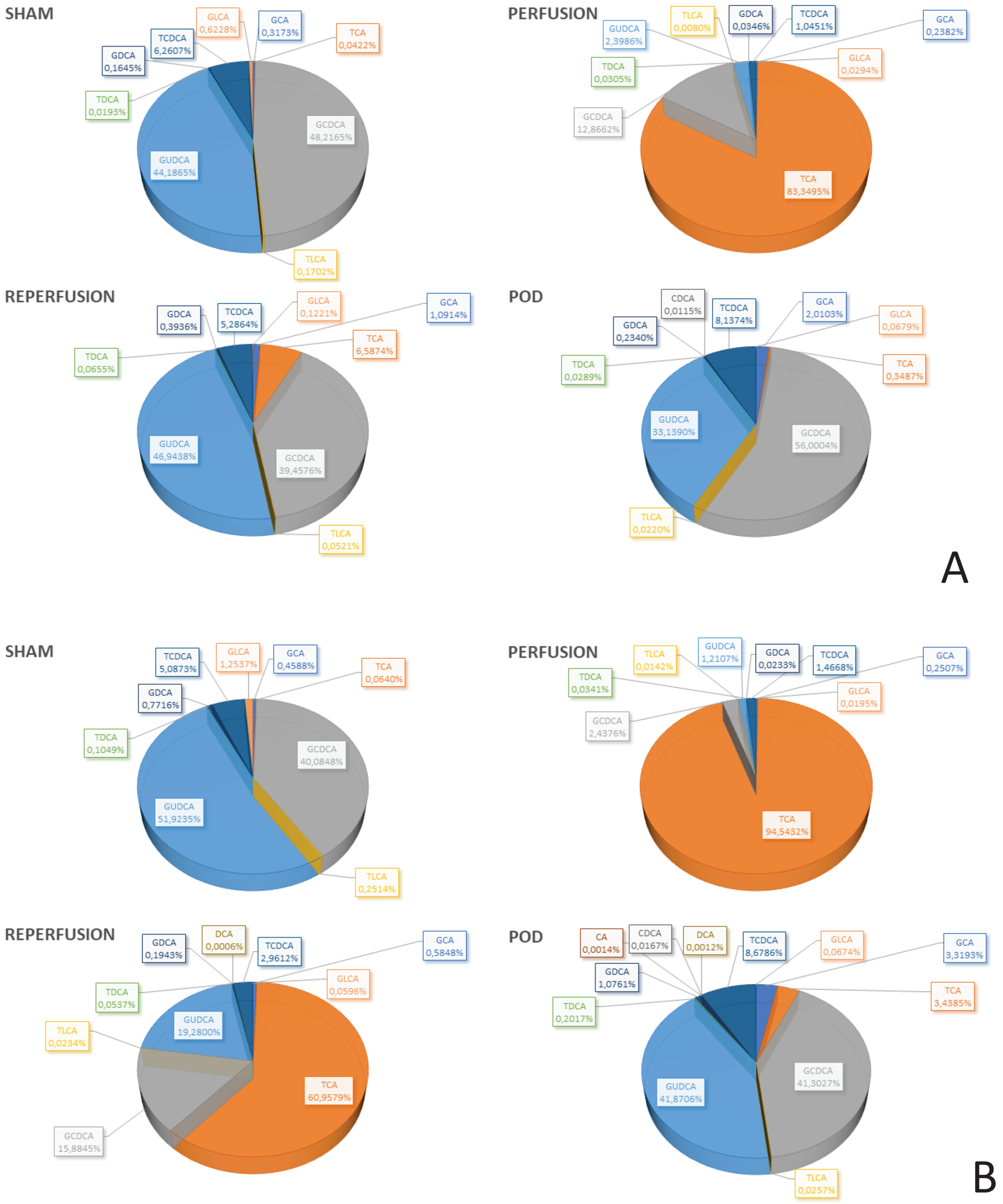

Fig. S4. The percentage of individual bile acids in bile samples depending on the time interval for HBD (A) and 90’DCD (B) donors.

Tab. S3. Comparison of chemical risk of TFME-LC-MS and SPME-MOI-MS methods in terms of the ChlorTox values.

Technique	**Reagents**	**CAS**	**Amount [g] per 1x measurement**	**CHsub**	**Chsub/CHref**	**ChlorTox [g]**	**Total Chlor Tox [g]**
TFME-LC-MS	Methanol	67-56-1	1.19441	4.81	0.82504	0.98544	**1.80510**
	Acetonitrile	75-05-8	2.11434	2.25	0.38593	0.81600	
	Formic acid	64-18-6	0.00656	3.25	0.55746	0.00366	
SPME-MOI-MS	Methanol	67-56-1	0.47856	4.81	0.82504	0.39483	**0.39517**
	Formic acid	64-18-6	0.00060	3.25	0.55746	0.00034	

8. Wnioski

Wyniki badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej pozwoliły na sformułowanie następujących wniosków:

- technika mikroekstrakcji do fazy stałej może być z powodzeniem stosowana do przygotowania próbek żółci — matrycy o dużej zmienności właściwości fizykochemicznych oraz wysokiej zawartości składników lipidowych,
- normotermiczna perfuzja maszynowa ograniczała negatywny wpływ niedokrwienia na funkcje narządu, co potwierdza korzystny wpływ stosowania tej metody na jakość organu po przeszczepie w porównaniu do hipotermii prostej. Wybór tej metody konserwacji może przyczynić się do zwiększenia wykorzystywania organów pochodzących od dawców o rozszerzonych kryteriach wpływając na ogólną pulę dostępnych narządów,
- analiza metabolomiczna żółci pozwoliła na wyselekcjonowanie potencjalnych biologicznych markerów jakości narządów, których poziomy korelowały m.in. ze stopniem niedokrwienia wątroby. Możliwość monitorowania stężeń tych metabolitów w okresie okołotransplantacyjnym może być źródłem dodatkowych informacji na temat zmian zachodzących w konserwowanym narządzie i przekładać się na podejmowane decyzje kliniczne,
- profil kwasów żółciowych w produkowanej przez wątrobę żółci zmienia się w ciągu całego okresu okołotransplantacyjnego. Analiza próbek perfuzyjnych wykazała różnice w poziomach wybranych kwasów żółciowych pomiędzy grupą kontrolną i niedokrwienną. Ponadto, zaproponowana metoda SPME-MOI-MS pozwala na uzyskiwanie wyników w czasie niemal rzeczywistym, oferując obiecujące narzędzie do oceny funkcji narządów i wczesnej predykcji ewentualnych powikłań po przeszczepie.

9. Dorobek naukowy doktoranta

9.1. Publikacje naukowe

- J. Bogusiewicz, K. Burlikowska, B. Kupcewicz, K. Łuczykowski, K. Jaroch, P. Goryńska, K. Goryński, M. Birski, J. Furtak, D. Paczkowski, M. Harat, J. Pawliszyn, B. Bojko: ***Analiza lipidomiczna w diagnostyce guzów mózgu.*** Wiadomości Chemiczne, 2025, 79, 21-35; punktacja MNiSW: 20, IF: -
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- I. Stryjak, N. Warmuzińska, **K. Łuczykowski**, K. Jaroch, P. Urbanellis, M. Selzner, B. Bojko: ***Metabolomic and lipidomic landscape of porcine kidney associated with kidney perfusion in heart beating donors and donors after cardiac death.*** Transl Res., 2024, 267:79-90; punktacja MNiSW: 140, IF: 6,400

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- N. Warmuzińska, **K. Łuczykowski**, B. Bojko: ***A review of current and emerging trends in donor graft-quality assessment techniques***. J. Clin. Med., 2022, 11, 487; punktacja MNiSW:140, IF: 3,900
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Łączna punktacja MNiSW: 1920, IF: 93,255

9.2. Rozdziały w monografii naukowej

- A. Roszkowska, **K. Łuczykowski**, N. Warmuzińska, B. Bojko, ***Chapter 13: SPME and Related Techniques in Biomedical Research w książce Evolution of Solid Phase Microextraction Technology***; The Royal Society of Chemistry 2023, ISBN 9781839166808; punktacja MNiSW dla wydawnictw publikujących recenzowane monografie naukowe: 20
- A. Roszkowska, N. Warmuzińska, **K. Łuczykowski**, B. Bojko, ***Chapter 17: SPME in Lipid Analysis*** w książce Evolution of Solid Phase Microextraction Technology; The Royal Society of Chemistry 2023, ISBN 9781839166808 punktacja MNiSW dla wydawnictw publikujących recenzowane monografie naukowe: 20

9.3. Wystąpienia konferencyjne

- K. Piechowska, M. Mizerska-Kowalska, B. Zdzisińska, J. Cytarska, A. Baranowska-Łączkowska, K. Jaroch, **K. Łuczykowski**, W. Płaziński, B. Bojko, S. Kruszewski, K. Misiura, K. Łączkowski: ***Tropinone-thiazole derivati ves ss anticancer agents.*** 3rd International Conference "Chemistry fo beauty and health", 13.06.2024-15.06.2024, Kraków, Polska
- J. Bogusiewicz, K. Burlikowska, B. Kupcewicz, **K. Łuczykowski**, K. Jaroch, P. Z. Goryńska, K. Goryński, M. Birski, J. Furtak, D. Paczkowski, M. Harat, J. Pawliszyn, B. Bojko: ***Analiza lipidomiczna w diagnostyce guzów mózgu.*** XXVII Konferencja „Nowoczesne Metody Instrumentalne w Analizie Śladowej", 09.12.2024-10.12.2024, Łódź, Polska
- N. Warmuzińska, K. Łuczykowski, I. Stryjak, D. Kollmann, P. Urbanellis, M. Selzner, B. Bojko: Mikroekstrakcja do fazy stałej jako nowa alternatywa diagnostyczna w transplantologii. 65. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, 18.09.2023-22.09.2023, Toruń, Polska
- S. Gaździński, J. Bogusiewicz, K. Burlikowska, **K. Łuczykowski**, R. Rola, Ł. Dziuda, B. Kossowski, M. Birski, J. Furtak, M. Harat, J. Pawliszyn, B. Bojko: ***Preliminary in-vivo characterization of healthy gray and white matter of patients treated for brain neoplams using chemical biopsy and 1H-MRS.*** 39th Annual Scientific Meeting, 04.10.2023-07.10.2023, Online, Niemcy
- P. Cuevas-Delgado, N. Warmuzińska, **K. Łuczykowski**, B. Bojko, C. Barbas: ***Untargeted metabolomics sample treatment strategies for renal tissue: a comparative study of solid phase microextraction (SPME) and homogenization-solid liquid extraction (Homo-SLE).*** 3rd Nordic Metabolomics Conference, October 18.10.2023-20.10.2023, Trondheim, Norwegia
- N. Warmuzińska, **K. Łuczykowski**, I. Stryjak, P. Urbanellis, M. Selzner, B.Bojko: ***Comparison of the impact of normothermic and hypothermic preservation methods on kidney lipidomic profile using spme chemical biopsy.*** 21st Congress of the European Society for Organ Transplantation, 17.09.2023-20.09.2023, Ateny, Grecja
- **K. Łuczykowski**, N. Warmuzińska, D. Kollmann, M. Selzner, B. Bojko: ***Analysis of the bile metabolome to search for potential biomarkers of quality of the liver with***

particular emphasis on bile acids. 21st Congress of the European Society for Organ Transplantation, 17.09.2023-20.09.2023, Ateny, Grecja

- N. Warmuzińska, **K. Łuczykowski**, I. Stryjak, D. Kollmann, P. Urbanellis, M. Selzner, B. Bojko: ***Solid phase microextraction: new diagnostic tool in transplant surgery.*** 33rd International Symposium on Pharmaceutical and Biomedical Analysis (PBA 2023), 02.07.2023-06.07.2023, Ankara, Turcja
- J. Bogusiewicz, K. Burlikowska, **K. Łuczykowski**, K. Jaroch, J. Furtak, M. Birski, M. Harat, J. Pawliszyn, B. Bojko: **Chemical biopsy as a new tool in profiling of the human brain in vivo.** The 18th International Conference "Monitoring Molecules in Neuroscience", 29.06.2022-02.07.2022, Lyon, Francja
- **K. Łuczykowski**, N. Warmuzińska, I. Stryjak, D. Kollmann, M. Selzner, B. Bojko: ***Application of thin-film solid-phase microextraction to bile analysis: bile fingerprint as a prognostic marker during liver transplantation.*** 20th Biennial European Society for Organ Transplantation (ESOT) Congress, 29.08.2021-01.09.2021, Mediolan, Włochy
- N. Warmuzińska, I. Stryjak, **K. Łuczykowski**, M. Hamar, P. Urbanellis, M. Selzner, B. Bojko: ***Low invasive SPME tissue sampling as a new tool, for graft quality assessment.*** 20th Biennial European Society for Organ Transplantation (ESOT) Congress, 29.08.2021-01.09.2021, Mediolan, Włochy
- N. Warmuzińska, I. Stryjak, K. Łuczykowski, M. Hamar, P. Urbanellis, M. Selzner, B. Bojko: Identification of lipidomic changes by SPME-LC-MS as a graft quality assessment in kidney transplantation. 9th International Singapore Lipid Symposium "Lipidomic technologies and applications", 01.03.2021-05.03.2021, Singapur, Republika Singapuru
- A. Roszkowska, J. Bogusiewicz, P. Z. Goryńska, **K. Łuczykowski**, K. Burlikowska, K. Jaroch, K. Goryński, M. Birski, D. Paczkowski, J. Furtak, M. Harat, T. Baczek, J. Pawliszyn, B. Bojko: ***SPME-based sample preparation strategies in clinical and biomedical analysis.*** 23rd International Symposium on Advances in Extraction Technologies, 30.06.2021-02.07.2021, Online, Hiszpania
- J. Bogusiewicz, K. Burlikowska, **K. Łuczykowski**, K. Jaroch, J. Furtak, M. Birski, M. Harat, J. Pawliszyn, B. Bojko: ***Spatially resolved profiling of human brain in vivo:***

chemical biopsy as a new tool in neuroscience research. 7th Scientific Conference of Polish Metabolomics Society, 04.11.2020-06.11.2020, Białystok, Polska

- B. Bojko, D. Paczkowski, J. Furtak, J. Pawliszyn, J. Bogusiewicz, **K. Łuczykowski**, K. Jaroch, K. Burlikowska, K. Goryński, M. Birski, M. Harat, P. Goryńska: ***Chemical biopsy based on solid phase microextraction : why should it be of scientists and clinicals interest?.*** PITTCON 2020 Conference & Expo. The Clear Advantage, 01.03.2020-05.03.2020, Chicago, Stany Zjednoczone
- **K. Łuczykowski**, N. Warmuzińska, I. Stryjak, D. Kollmann, M. Selzner, B. Bojko: ***Analysis of changes in bile acids concentration in bile in response to the degree of liver ischemia and the method of organ preservation.*** MSACL 2019 EU. The 6th European Congress & Exhibits : Mass Spectrometry: Applications to the Clinical Lab., 22.09.2019-26.09.2019, Salzburg, Austria
- I. Stryjak, N. Warmuzińska, **K. Łuczykowski**, M. Hamar, M. Selzner, B. Bojko: ***Solid phase microextraction (SPME) in kidney examination : LC-MS/MS-based identification of potentially significant metabolites in graft quality assessment.*** MSACL 2019 EU. The 6th European Congress & Exhibits : Mass Spectrometry: Applications to the Clinical Lab., 22.09.2019-26.09.2019, Salzburg, Austria
- N. Warmuzińska, I. Stryjak, **K. Łuczykowski**, J. Bogusiewicz, M. Hamar, M. Selzner B. Bojko: ***Graft quality assessment in kidney transplantation by monitoring lipidomic changes in the organ during transplantation using solid phase microextraction (SPME).*** MSACL 2019 EU. The 6th European Congress & Exhibits: Mass Spectrometry: Applications to the Clinical Lab., 22.09.2019-26.09.2019, Salzburg, Austria
- J. Bogusiewicz, P. Z. Goryńska, **K. Łuczykowski**, K. Burlikowska, K. Jaroch, K. Chmara, K. Goryński, D. Paczkowski, J. Furtak, M. Harat, J. Pawliszyn, B. Bojko: ***Application of solid phase microextraction to in vivo brain and brain tumor analysis.*** XX Euroanalysis, 01.09.2019-05.09.2019, Stambuł, Turcja
- K. Jaroch, J. Bogusiewicz, P. Z. Goryńska, K. Goryński, K. Burlikowska, **K. Łuczykowski**, D. Paczkowski, J. Furtak, M. Harat, E. Boyaci, J. Pawliszyn, B. Bojko: ***From in vitro cell line to in vivo brain and brain cancer studies: SPME: one technique that fits it all.*** 48th International Symposium on High-Performance

Liquid Phase Separations and Related Techniques, 16.06.2019-20.06.2019, Mediolan, Włochy

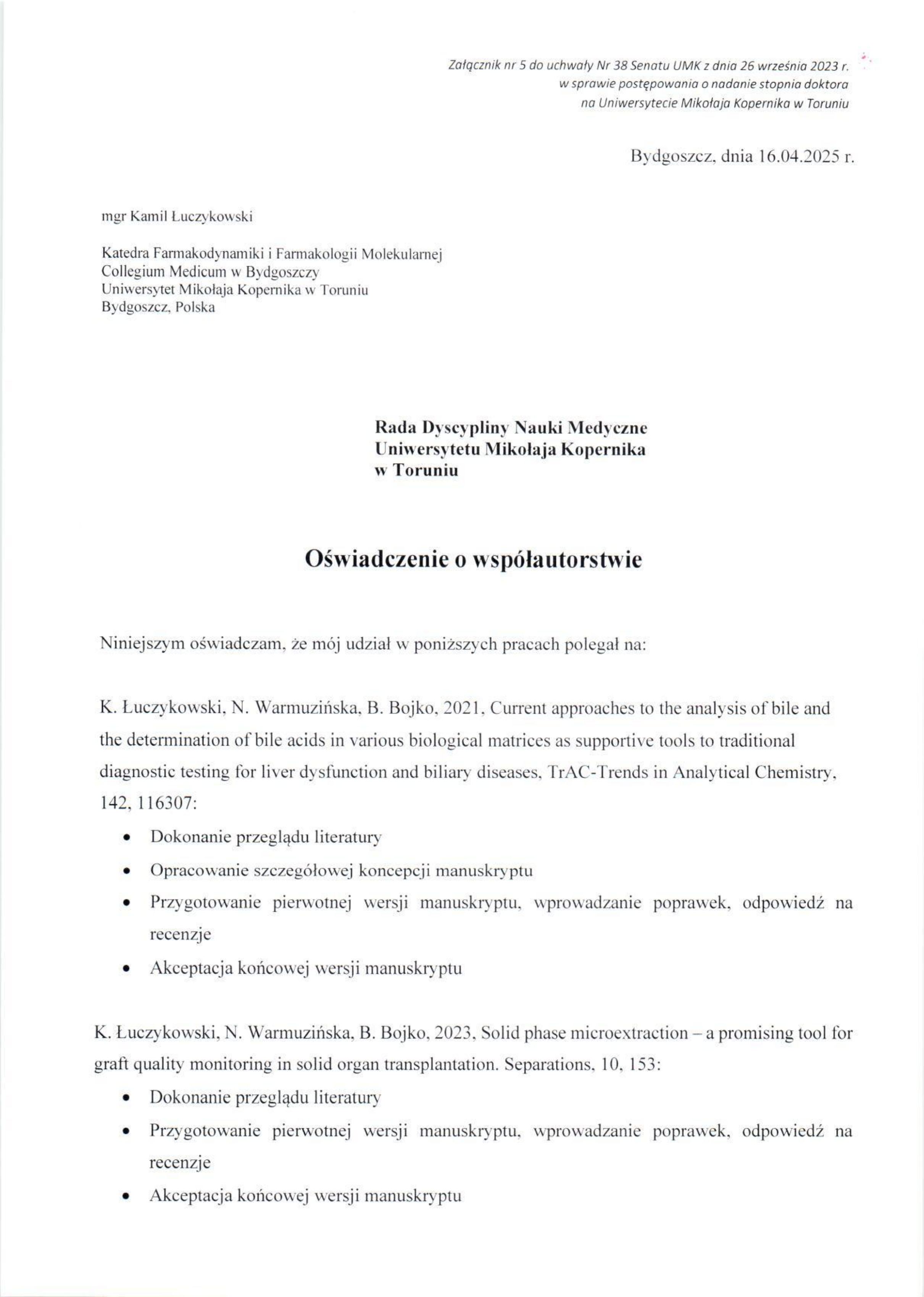
- **K. Łuczykowski**, N. Warmuzińska, I. Stryjak, B. Bojko: ***Profilowanie metabolomiczne żółci z wykorzystaniem mikroekstrakcji do fazy stałej (SPME): określenie zmian zachodzących w czasie perfuzji wątroby podczas zabiegu transplantacji.*** XI Interdyscyplinarna Konferencja Naukowa TYGIEL 2019 "Interdyscyplinarność kluczem do rozwoju", 23.03.2019-24.03.2019, Lublin, Polska
- I. Stryjak, N. Warmuzińska, **K. Łuczykowski**, B. Bojko: ***Mikroekstrakcja do fazy stałej (SPME) w badaniach nerek : identyfikacja metabolitów o potencjalnym znaczeniu diagnostycznym.*** XI Interdyscyplinarna Konferencja Naukowa TYGIEL 2019 "Interdyscyplinarność kluczem do rozwoju", 23.03.2019-24.03.2019, Lublin, Polska
- N. Warmuzińska, **K. Łuczykowski**, J. Bogusiewicz, B.Bojko: ***Lipidomika w badaniach in vivo: jak przygotować włókna SPME?.*** XI Interdyscyplinarna Konferencja Naukowa TYGIEL 2019 "Interdyscyplinarność kluczem do rozwoju", 23.03.2019-24.03.2019, Lublin, Polska

9.4. Projekty naukowe

- „Nowe narzędzia analityczne oparte na mikroekstrakcji cienkowarstwowej do poszukiwania i oznaczania żółciowych biomarkerów wczesnego uszkodzenia przeszczepu wątroby” - NCN Preludium 21 - **kierownik projektu**
- „Analiza zmiany stężeń kwasów żółciowych w żółci jako wskaźnik wydajności funkcji wątroby” - grant młodych naukowców MN- SDF/5/WF/2019 - **kierownik projektu**
- „Profilowanie metabolomiczne i lipidomiczne substancji białej i szarej kory mózgowej z wykorzystaniem nowoczesnej technologii opartej na biopsji chemicznej połączonej ze spektrometrią mas” - KOŚCIUSZKO, II edycja; - **wykonawca projektu**
- „Nowe rozwiązania analityczne w onkologii: od badań podstawowych do szybkiej diagnostyki śródoperacyjnej” - NCN Harmonia 7 - **wykonawca projektu**

- „Szybka i nieinwazyjna ocena okołotransplantacyjna ludzkich graftów wątroby poddanych perfuzji hipo- i normotermicznej” - NCN OPUS 21 - **wykonawca projektu**

10. Oświadczenie autora rozprawy doktorskiej



Załącznik nr 5 do uchwały Nr 38 Senatu UMK z dnia 26 września 2023 r.
w sprawie postępowania o nadanie stopnia doktora
na Uniwersytecie Mikołaja Kopernika w Toruniu

Bydgoszcz, dnia 16.04.2025 r.

mgr Kamil Łuczykowski

Katedra Farmakodynamiki i Farmakologii Molekularnej
Collegium Medicum w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu
Bydgoszcz, Polska

Rada Dyscypliny Nauki Medyczne
Uniwersytetu Mikołaja Kopernika
w Toruniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że mój udział w poniższych pracach polegał na:

K. Łuczykowski, N. Warmuzińska, B. Bojko, 2021, Current approaches to the analysis of bile and the determination of bile acids in various biological matrices as supportive tools to traditional diagnostic testing for liver dysfunction and biliary diseases, TrAC-Trends in Analytical Chemistry, 142, 116307:

- Dokonanie przeglądu literatury
- Opracowanie szczegółowej koncepcji manuskryptu
- Przygotowanie pierwotnej wersji manuskryptu, wprowadzanie poprawek, odpowiedź na recenzje
- Akceptacja końcowej wersji manuskryptu

K. Łuczykowski, N. Warmuzińska, B. Bojko, 2023, Solid phase microextraction – a promising tool for graft quality monitoring in solid organ transplantation. Separations, 10, 153:

- Dokonanie przeglądu literatury
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K. Łuczykowski, N. Warmuzińska, D. Kollmann, M. Selzner, B. Bojko, 2023, Biliary Metabolome Profiling for Evaluation of Liver Metabolism and Biliary Tract Function Related to Organ Preservation Method and Degree of Ischemia in a Porcine Model. Int. J. Mol. Sci., 24, 2127:

- Przeprowadzenie analizy instrumentalnej, analizy statystycznej uzyskanych wyników oraz ich interpretacji
- Przygotowanie pierwotnej wersji manuskryptu, wprowadzanie poprawek, odpowiedź na recenzje
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K. Łuczykowski, N. Warmuzińska, K. Jaroch, D. Kollmann, M; Selzner, B. Bojko, 2024, Recent solid-phase microextraction-based analytical approaches for the profiling of biliary bile acids in pre-transplant assessments of liver grafts subjected to normothermic ex vivo liver perfusion. Anal. Chim. Acta, 1318, 342954:

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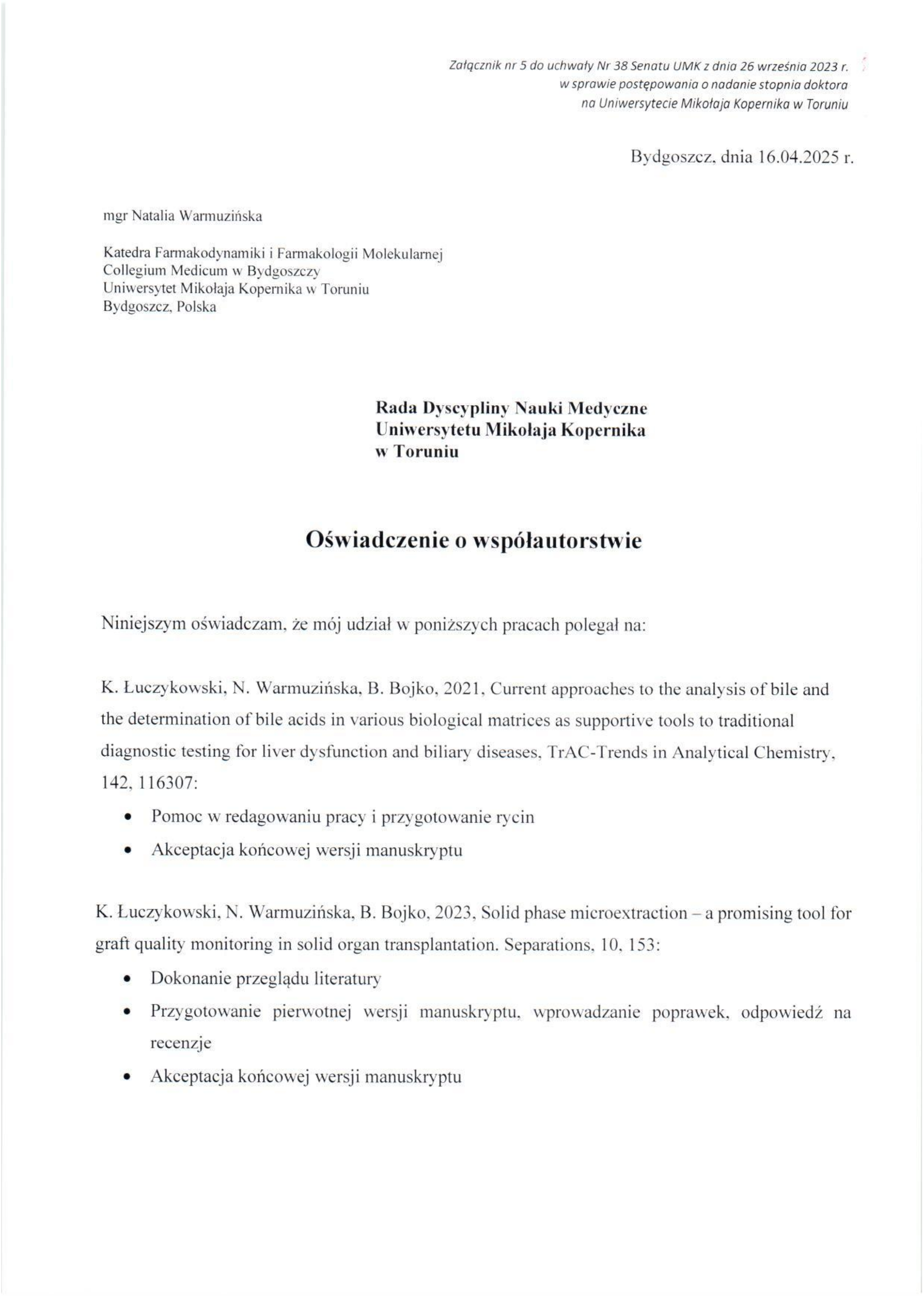
- Akceptacja końcowej wersji manuskryptu

Łuczykowski
(podpis)

11. Oświadczenia współautorów



*Załącznik nr 5 do uchwały Nr 38 Senatu UMK z dnia 26 września 2023 r.
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mgr Natalia Warmuzińska

Katedra Farmakodynamiki i Farmakologii Molekularnej
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K. Łuczykowski, N. Warmuzińska, B. Bojko, 2023, Solid phase microextraction – a promising tool for graft quality monitoring in solid organ transplantation. Separations, 10, 153:

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K. Łuczykowski, N. Warmuzińska, D. Kollmann, M. Selzner, B. Bojko, 2023, Biliary Metabolome Profiling for Evaluation of Liver Metabolism and Biliary Tract Function Related to Organ Preservation Method and Degree of Ischemia in a Porcine Model. Int. J. Mol. Sci., 24, 2127:

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Natalia Warmuzińska
....
(podpis)

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dr hab. Karol Jaroch, prof. UMK

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Uniwersytet Mikołaja Kopernika w Toruniu
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- Pomoc w opracowaniu metodyki do bezpośredniej analizy kwasów żółciowych SPME-MOI-MS
- Akceptacja końcowej wersji manuskryptu

……………
(podpis)

Bydgoszcz, dnia 16.04.2025 r.

prof. dr hab. Barbara Bojko

Katedra Farmakodynamiki i Farmakologii Molekularnej
Collegium Medicum w Bydgoszczy
Uniwersytet Mikołaja Kopernika w Toruniu
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Rada Dyscypliny Nauki Medyczne
Uniwersytetu Mikołaja Kopernika
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Kierownik
Katedry Farmakodynamiki i Farmakologii Molekularnej
prof. dr hab. Barbara Bojko
……………………
(podpis)

Załącznik nr 5 do uchwały Nr 38 Senatu UMK z dnia 26 września 2023 r.
w sprawie postępowania o nadanie stopnia doktora
na Uniwersytecie Mikołaja Kopernika w Toruniu

Vienna, 14.04.2025 r.

Dagmar Kollmann, PhD

Department of General Surgery,
Medical University of Vienna
Vienna, Austria

Council for the Discipline of Medical Sciences
Nicolaus Copernicus University in Toruń

Declaration of Contribution

I declare that my contribution to the following papers was as follows:

K. Łuczykowski, N. Warmuzińska, D. Kollmann, M. Selzner, B. Bojko, 2023, Biliary Metabolome Profiling for Evaluation of Liver Metabolism and Biliary Tract Function Related to Organ Preservation Method and Degree of Ischemia in a Porcine Model. Int. J. Mol. Sci., 24, 2127:

- Conceptualization of the experiment and methodology
- Performed animal procedures and sample collection
- Reviewed and approved the final version of the manuscript

K. Łuczykowski, N. Warmuzińska, K. Jaroch, D. Kollmann, M; Selzner, B. Bojko, 2024, Recent solid-phase microextraction-based analytical approaches for the profiling of biliary bile acids in pre-transplant assessments of liver grafts subjected to normothermic ex vivo liver perfusion. Anal. Chim. Acta, 1318, 342954:

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Kollmann D.
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(signature)

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Toronto, 14.04.2025 r.

Markus Selzner, MD

Department of Surgery
Ajmera Transplant Centre
Toronto General Hospital, University Health Network
Toronto, Canada

Council for the Discipline of
Medical Sciences
Nicolaus Copernicus University in
Toruń

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- Conceptualization of the experiment and methodology
- Performed animal procedures and sample collection
- Reviewed and approved the final version of the manuscript

Markus Selzner
……………………….
(signature)

12. Spis rycin

13. Piśmiennictwo

1. Whitson, B.A.; Black, S.M. Organ Assessment and Repair Centers: The Future of Transplantation Is Near. *World J. Transplant.* **2014**, *4*, 40–43, doi:10.5500/wjt.v4.i2.40.
2. Saidi, R.F.; Kenari, S.K.H. Challenges of Organ Shortage for Transplantation: Solutions and Opportunities. *Int. J. Organ Transplant. Med.* **2014**, *5*, 87–96.
3. Sutton, M.E.; Op den Dries, S.; Karimian, N.; Weeder, P.D.; de Boer, M.T.; Wiersema-Buist, J.; Gouw, A.S.H.; Leuvenink, H.G.D.; Lisman, T.; Porte, R.J. Criteria for Viability Assessment of Discarded Human Donor Livers during Ex Vivo Normothermic Machine Perfusion. *PLoS One* **2014**, *9*, e110642, doi:10.1371/journal.pone.0110642.
4. Terrault, N.A.; Francoz, C.; Berenguer, M.; Charlton, M.; Heimbach, J. Liver Transplantation 2023: Status Report, Current and Future Challenges. *Clin. Gastroenterol. Hepatol.* **2023**, *21*, 2150–2166, doi:10.1016/j.cgh.2023.04.005.
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