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VYUŽITÍ KAPILÁRNÍ ELEKTROFORÉZY V ANALÝZE SUCHÝCH KREVNÍCH SKVRN
APPLICATION OF CAPILLARY ELECTROPHORESIS FOR DRIED BLOOD SPOTS ANALYSIS

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

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Využití kapilární elektroforézy v analýze suchých krevních skvrn

Zadání dizertační práce:

Cílem disertační práce je vývoj nových postupů a metod využívajících kapilární elektroforézu (CE) pro analýzu suchých krevních skvrn (DBS).

1. Vývoj CE metod a postupů pro přímé sledování charakteristik a vybraných modelových analytů v DBS.
2. In–line spojení CE metod s technikami mikroextrakce pro analýzu DBS a přímé dávkování analytů z DBS eluátů do separační kapiláry.
3. Zjednodušení odběrů vzorků, extrakcí, analýz a účinná eliminace negativních vlivů při odběru vzorků krve (např. vliv hematokritu).
4. Vývoj elučních postupů pro přímý nástřik DBS eluátu do separační kapiláry pro analýzu aminokyselin.
5. Reálné aplikace vyvinutých metod pro stanovení koncentrací acidických a bazických léčiv a terapeutické monitorování léčiv.
6. Vývoj nových rozpustných vzorkovacích materiálů pro kvantitativní analýzy DBS.

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ABSTRAKT

Krevní vzorky jsou standardně analyzovány ve formě plazmy nebo séra. Jako alternativa pro vzorkování a skladování krve se stále častěji uplatňují suché krevní skvrny (DBS). V předložené disertační práci jsou DBS zpracovány a analyzovány kapilární elektroforézou (CE), která byla v minulosti pro analýzu DBS využívána velmi zřídka. Nicméně CE nabízí mnohé výhody, dané vývojem této techniky v poslední době, kterými standardní analytické metody nedisponují. Přímé spojení CE s mikroextrakčními technikami a simultánní stanovení analytů přítomných v komplexních matricích vede k minimalizaci nebo eliminaci nedostatků DBS analýz, jako je například vliv matrice, vliv hematokritu a nehomogenní distribuce analytů v DBS. V nejjednodušším možném uspořádání je dosaženo úpravy, dávkování a analýzy DBS vzorků výhradně komerční CE přístrojem. Vývojem nových plně rozpustných vzorkovacích materiálů pro DBS je dosaženo lepších extrakčních vlastností a kvantitativních výsledků. Vyvinuté postupy zahrnují nové metody pro účinnou úpravu DBS vzorků a jejich přímou analýzu bez nutnosti zásahu operátora a dosahují dostatečnou selektivitu a citlivost pro stanovení významných analytů nejenom v DBS, ale i v dalších vzorcích se složitými matricemi.

KLÍČOVÁ SLOVA

Suché krevní skvrny, kapilární elektroforéza, přímé spojení, aminokyseliny, acidická a bazická léčiva, mikroextrakční techniky, rozpustné polymerní pěny.

ABSTRACT

Blood samples are normally analyzed in the form of plasma or serum. As an alternative for blood sampling and storage, dry blood spots (DBS) have been increasingly used in recent years. In the actual dissertation thesis, DBS are processed and analyzed by capillary electrophoresis (CE), which has been very rarely used for DBS analysis in the past. However, CE offers several features, given by the recent development of this technique, not available for standard analytical methods. Direct coupling of microextraction techniques to CE and simultaneous determination of analytes present in complex matrices leads to minimization or elimination of deficiencies in DBS analysis, for example the effect of sample matrix, hematocrit effect, and inhomogeneous distribution of analytes in DBS. In the simplest possible arrangement, sample treatment, injection, and analysis of DBS samples are achieved exclusively by a single commercial CE instrument. Application of newly developed fully soluble materials for DBS sampling offers better extraction efficiency and more accurate quantitation. The developed concepts include new methods for efficient treatment of DBS samples and their direct analysis without the need for operator intervention and they provide sufficient selectivity and sensitivity for the determination of important analytes not only in DBS but also in other complex samples.

KEYWORDS

Dried blood spots, capillary electrophoresis, in-line coupling, amino acids, acidic and basic drugs, microextraction techniques, soluble polymer foams.

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Prohlášení

Prohlašuji, že jsem dizertační práci vypracovala samostatně, a že všechny použité literární zdroje jsem citovala správně a úplně. Dizertační práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího dizertační práce a děkana FCH VUT.

……………………

podpis studentky

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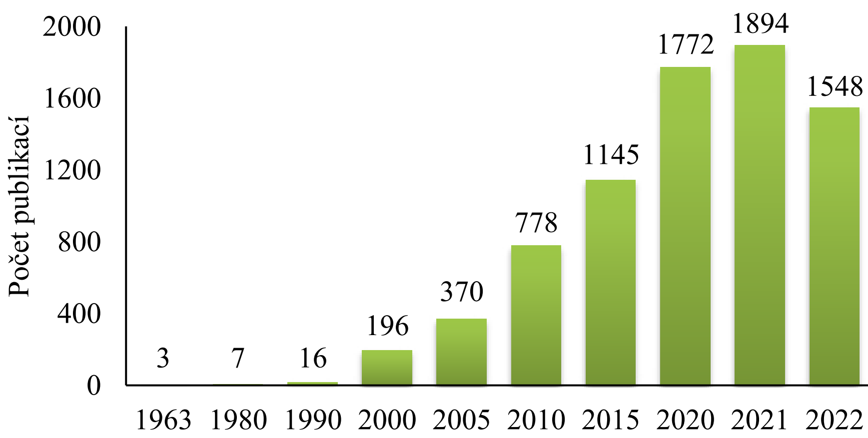
1 ÚVOD

Krevní vzorky se v klinické praxi standardně odebírají a zpracovávají v „mokrém“ stavu, tedy ve formě krevní plazmy nebo séra získaného odběrem venózní krve. Alternativní technikou může být vzorkování kapilární krve ve formě suchých krevních skvrn (DBS) [1]. Odběr a analýza vzorků krve ve formě DBS se stávají v oblasti analytické chemie populární technikou (viz obr. č. 1). Poprvé v roce 1963 R. Guthrie ukázal, že odběr malého objemu krve z paty nebo bříška prstu na kousek filtračního papíru, který byl poté několik dní skladován, by mohl poskytnout spolehlivá analytická měření pro klinické studie [2]. Od té doby je odběr DBS široce využíván pro screeningové vyšetření vrozených metabolických poruch u novorozenců [3]. V posledních letech se DBS uplatňuje také v oblasti farmakokinetiky, toxikologie [4], preklinickém a klinickém vývoji léčiv [5, 6] a při terapeutickém monitorování léčiv [7, 8, 9]. Nicméně je dlouhá cesta k tomu, aby byla tato technika vzorkování plně využívána, jak ve výzkumu, tak i v klinické praxi zejména kvůli problémům s nehomogenitou výsledného vzorku a kvantitativním stanovením.

Překvapivé je, že v širokém portfoliu analytických technik používaných při vývoji vzorkování a analýz DBS je kapilární elektroforéza (CE) málo používanou technikou. CE je totiž předurčena k analýze vzorků s minimálními objemy, tudíž nedochází k velkému zředění analytů eluovaných z DBS. Tato separační technika také umožňuje v krátkém čase současně stanovit více složek vzorku. CE instrumentaci je možné přímo spojit s různými technikami mikroextrakce, které umožňují přečištění vzorku a případně i zakoncentrování analytů [9, 10].



Obr. č. 1: Publikační činnost v jednotlivých letech v období 1963–2022 v oblasti použití DBS. (Extrahováno z databáze WOS použitím klíčového slova „dried blood spot“).

2 ŘEŠENÁ PROBLEMATIKA SUCHÝCH KREVNÍCH SKVRN

2.1 Metody přípravy DBS

Ve srovnání s konvenčním vzorkováním žilní krve je vzorkování DBS příjemnější a jednodušší metodou odběru vzorků s větším komfortem pro pacienta. Odběr DBS má řadu výhod a nevýhod oproti konvenčnímu odběru vzorků ze žíly. Hlavní výhodou DBS je méně invazivní odběr vzorku. Místo bolestivé venepunkce s následným odebráním venózní krve v řádech mililitrů se malé množství kapilární krve (obvykle menší než 50 µl) odebírá z vpichu lancetou z paty (u kojenců), z bříška prstu (u dospělých) nebo z ocasu (u zvířat). Odběr vzorků je tedy vhodný pro všechny skupiny pacientů, kde může být venepunkce příliš invazivní [1]. Odběr DBS je díky jednoduchosti možno provést samotnými pacienty.

Odebraná krev se nanese na vzorkovací kartu a ponechá se 2–3 hodiny volně sušit na okolním vzduchu [8]. Velkou výhodou DBS je dlouhodobá stabilita analytů ve srovnání se vzorky krevní plazmy nebo séra, protože při sušení vzorků krve je řada látek, např. enzymů, deaktivována. Vzorkovací karty mohou být použity pro sběr, skladování a analýzu i jiných biologických materiálů, než je krev (např. moč, plazma, sérum). Pro transport do laboratoře jsou karty zabaleny do uzavíratelného plastového sáčku s vysoušedlem a jsou poslány poštou nebo jinými přepravními společnostmi. V laboratoři jsou buď podrobeny analýze, nebo jsou uskladněny pro další použití [10].

Problematika nedostatků při vzorkování a analýze DBS je komplexně zkoumána. Odběr malého objemu krve v řádech µl je jedna z hlavních výhod, ale paradoxně i nevýhod, protože je možné provést zpracování vzorku před vlastní analýzou jen jednou a není k dispozici náhradní vzorek. Odběr vzorků DBS není vhodný pro analyty citlivé na vzduch a pro těkavé analyty [1, 9]. Koncentrace analytů v kapilární krvi se mohou lišit od koncentrací ve venózní krvi a musí být stanovena korelace mezi těmito dvěma matricemi před jakoukoli farmakokinetickou a toxikokinetickou analýzou nebo před terapeutickým monitorováním léčiv. Jedním z nejdůležitějších parametrů, ovlivňujících odběr DBS a následnou kvantitativní analýzu, je však hodnota hematokritu v krvi [9, 11]. Hematokrit udává poměr objemu erytrocytů k celkovému objemu krve a pohybuje se mezi 0,37 a 0,51 (tj. 37 % a 51 %) u zdravého dospělého jedince [12]. Hematokrit je za fyziologických okolností vyšší u novorozenců (45 – 60 %) a u populace žijících ve vysokých nadmořských výškách [13, 14]. Hematokrit má přímý vliv na velikost krevní skvrny nanesené na vzorkovací DBS kartu. Například vyšší

hodnota hematokritu značí vyšší viskozitu krve, což má za následek menší plochu krevní skvrny na vzorkovací DBS kartě. Naopak krev s nízkou hodnotou hematokritu poskytne větší plochu krevní skvrny [11]. Různá hodnota hematokritu odebraných vzorků také indukuje nehomogenitu krevní skvrny na DBS kartě. Dochází k tzv. „halo efektu“, kdy krevní buňky zůstávají na středu nanesené krevní skvrny, zatímco plazma migruje k periferii. V případě, že se pro analýzu použije jen výřez různých částí DBS může vliv hematokritu mít negativní dopad na kvantitativní analýzu DBS [9, 15, 16].

2.1.1 Vzorkovací karty

Pro odběr vzorků krve ve formě DBS se používají různé komerční DBS karty podle typu analytických požadavků. Karty Whatman 903 se běžně používají pro screeningové vyšetření novorozenců. Karty FTA DMPK typu A, B, C se používají pro farmakokinetické nebo toxikokinetické studie a karty FTA Elute jsou určeny především k purifikaci DNA. Pro odběr krve byly legislativními orgány schváleny karty Whatman 903, Ahlstrom GenSaver 2.0 a Perkin Elmer 226 [8].

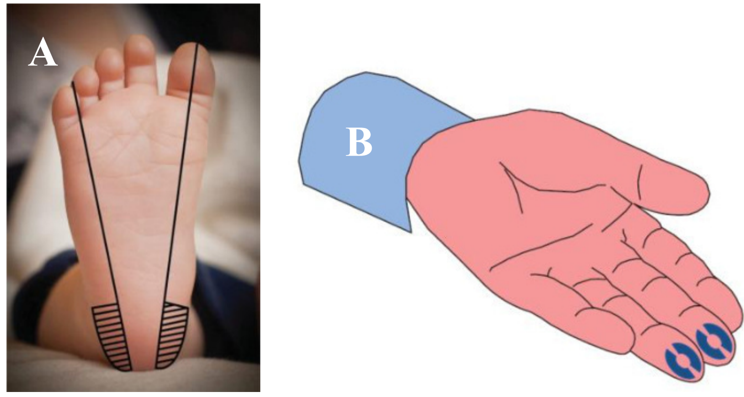
Odběrové karty pro vzorkování DBS jsou vyrobeny z celulózy. Na kartě jsou vyznačené oblasti (viz obr. č. 2), do kterých se nadávkuje vzorek a nechá se po dobu 2–3 hodin schnout. Karty jsou skladovány v uzavíratelných plastových obalech s vysoušedlem [17, 18].

Obr. č. 2: Příklad odběrové karty se čtyřmi odběrovými zónami (Whatman 903).

2.1.2 Odběr krve a vzorkovaní na DBS karty

Způsoby odběru krve pro vzorkování ve formě DBS jsou stále řešenou problematikou, protože mají určitá omezení, která brání jejich širšímu využití. Před samotným odběrem kapky krve je nutné místo vpichu prohřát (teplou vodou po dobu 5 minut pro zvýšení prokrvenosti)

a vydesinfikovat. Pro odběr DBS se kapilární krev získává z vpichu automatickou lancetou do paty (obr. č. 3 - A) nebo bříška prstu (obr. č. 3 - B). Krev se nevymačkává, ale okolí místa vpichu se mírně masíruje pro podporu průtoku krve [15]. První kapka je setřena sterilním tamponem, protože obsahuje více tkáňové tekutiny a mohla by mít vliv na výslednou analýzu. Druhá kapka je odebrána na vzorkovací kartu s přesně definovanou savostí přiložením k vyznačené hranici, která vymezuje množství odebrané krve. Je kladen důraz na to, aby krev vyplnila celou vyznačenou odběrovou zónu na DBS kartě. Místo vpichu je po odběru vydesinfikováno [17].

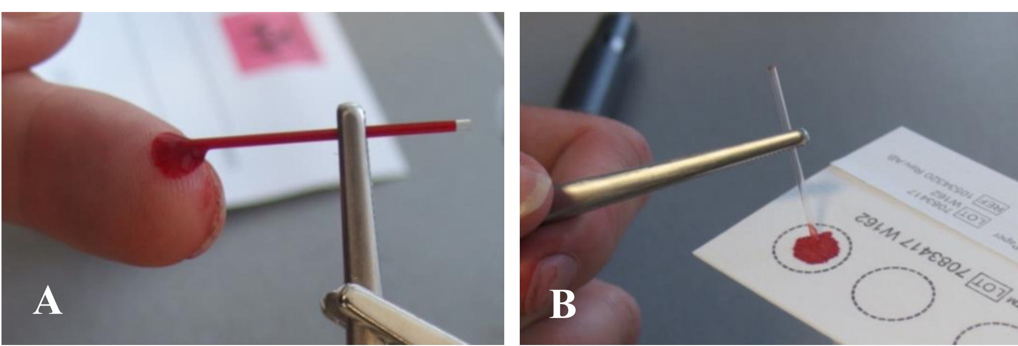


Obr. č. 3: Nejvhodnější zóny odběru pro DBS vzorky, A – z paty novorozence, B – z bříška prstu [17].

DBS se v minulosti používaly především pro kvalitativní a semikvantitativní analýzu. S vývojem analytických přístrojů se metodika DBS začala uplatňovat i pro kvantitativní analýzu, kdy je nutné znát přesný objem vzorku, který byl odebrán. Pro kvantitativní analýzu je metoda odběru z kapky krve limitována z hlediska odebrání opakovaného a přesného objemu vzorku také dochází k nerovnoměrné distribuci krve na odběrové DBS kartě vlivem hematokritu – viz kapitola 2.1 [17, 15].

Jedním z přístupů, jak je možné zjistit objem krve nanesený na DBS kartě, je měření specifické vodivosti eluátu. Obsah hlavních složek krve určujících specifickou vodivost je pro zdravou populaci konstantní, proto lze objem krve odebraný na DBS kartu stanovit měřením vodivosti vodného DBS eluátu [19]. Podobně lze pro korelaci objemu krve naneseného na DBS kartu použít koncentraci některých anorganických iontů (např. sodíku a draslíku) v DBS eluátu [20, 21]. Dalším zpřesněním může být odebrání přesného objemu pomocí pipety nebo jednorázové skleněné kapiláry diskutované zde volumetrických odběrových zařízení (diskutovaných v kapitole 2.1.3 a 2.1.4) kalibrovaných na přesný objem v řádech µl [15].

Z odběrového místa se krev odebere do kalibrované skleněné kapiláry (obr. č. 4 – A). Následně se naplněná kapilára přiloží k odběrové kartě (obr. č. 4 – B) až do úplného přenosu krve na kartu. Velká pozornost musí být věnována odběru a sušení vzorku, protože nedostatečné množství odebrané krve nebo případně málo usušený vzorek může znehodnotit výsledky analýzy [15].



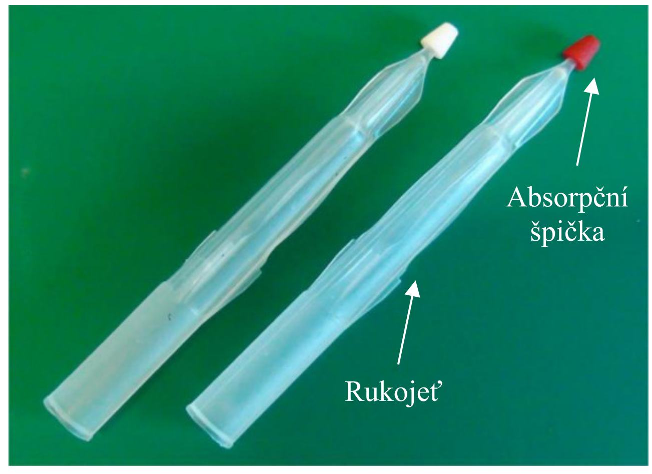
Obr. č. 4: Odběr a vzorkování kapilární krve pomocí skleněné kapiláry na vzorkovací DBS kartu; A – odběr kapilární krve z bříška prstu do skleněné kapiláry, B – přenos odebrané krve na DBS kartu.

Vzorkovací DBS karta s odebranými skvrnami se ponechá 2–3 hodiny schnout ve stojanu při laboratorní teplotě. Proces schnutí nesmí být urychlen působením horkého vzduchu ani ponecháním na slunci [17]. Takto připravené karty s DBS vzorky je možné skladovat zabalené v uzavíratelném plastovém obalu s vysoušedlem, nebo je lze poslat k analýze do laboratoře. Odběrové karty DBS jsou legislativními orgány považovány za materiál bez biologického nebezpečí, a proto mohou být transportovány běžnou přepravní službou do laboratoře. Metoda vzorkování kapilární krve v podobě DBS je díky jednoduchosti odběru a transportu vhodná pro odběr vzorků v domácnosti nebo v místech s omezenými možnostmi odebírání vzorků plazmy nebo séra [10].

2.1.3 VAMS (Volumetric absorptive microsampling)

Tato technika odměrného vzorkování mikro-objemů se používá k získání suchých krevních vzorků nebo jiných biologických matric pro řadu bioanalytických analýz [22]. Rozvíjí techniku odběru krve na odběrové DBS karty, která se široce používá v posledních 40 letech. VAMS je technika, která významně eliminuje některé nevýhody dosavadní metody odběru DBS (zpřesňuje odebraný objem a eliminuje vliv hematokritu, tzv. „halo efekt“) [22, 23].

Zařízení VAMS obsahuje plastovou rukojeť s absorpční špičkou vyrobenou z hydrofilního polymeru (viz obr. č. 5). Podle velikosti polymerní špičky je absorbováno do pórů 10 µl, 20 µl nebo 30 µl krve [22, 24].



Obr. č. 5: Odběrové zařízení VAMS na objem 10 µl, nalevo – VAMS bez krve, napravo – VAMS s odebranou krví [22].

Odběr technikou VAMS se provádí přiložením špičky ke kapce krve, přičemž je nutné, aby krev nepřišla do kontaktu s plastovou rukojetí a nedošlo tak k přeplnění odběrového zařízení. Odběr kapilární krve z bříška prstu pomocí VAMS se provádí pod úhlem 45°, jak je definováno výrobcem (viz obr. č. 6A). Poté se vzorek nechá ve stojanu uschnout (viz obr. č. 6B) a takto připravený vzorek je možné skladovat nebo upravit pro analýzu v laboratoři (viz obr. č. 6C) [24].

Obr. č. 6: Odběr vzorku kapilární krve pomocí VAMS; A – krevní kapka se získává z vpichu do bříška prstu lancetou a poté se první kapka setře sterilním tamponem. Dotykem polymerní špičky pod úhlem 45° se z druhé kapky odebere vzorek, B – odebraný vzorek se suší ve stojanu 2–3 hodiny, C – pro analýzu je polymerní špička odlomena do elučního rozpouštědla [24].

Polymerní špičky byly navrženy jako alternativní vzorkovací materiál vzhledem k jejich předdefinovaným konstantním rozměrům a absorpčním vlastnostem, což umožňuje odebrat přesné objemy vzorků nezávisle na obsahu hematokritu v odebrané krvi. VAMS je stále více uznávanou alternativou ke konvenčním odběrům pomocí DBS odběrových karet [24, 25].

2.1.4 Další komerční odběrové zařízení pro vzorkování kapilární krve

Vzorkování na odběrové DBS karty je prezentováno jako neinvazivní odběr kapilární krve. DBS poskytuje kvantitativní informaci v případě, že je na odběrovou kartu odebrán přesný objem vzorku. Odběr přesného objemu vzorku kapilární krve na kartu ve vybavené laboratoři s vyškoleným personálem je jednoduše proveditelný, ale pro klinické studie při odběru vzorku mimo laboratoř je odběr přesného objemu obtížný.

V posledních letech byly navrženy různé strategie pro eliminaci tohoto problému (např. VAMS viz kapitola 2.1.3). Dalším z nich je mikrofluidní zařízení pro odběr krve na DBS kartu (Hemaxis, Capitainer) [26, 27, 28]. Zařízení obsahuje mikrofluidní kanálky s předdefinovaným objemem 5 a 10 µl, které jsou integrovány s odběrovou kartou. Další komerční odběrové zařízení je na bázi skleněných kapilár (hemaPEN), krev je vzorkována na předem vyříznutý papírový disk [15]. I když jsou popsané postupy pro odběr kapilární krve realizovány odlišně, jejich cílem je odběr přesného objemu vzorku na odběrové médium, což je klíčové pro přesnou kvantifikaci.

2.1.5 Alternativní materiály pro vzorkování kapilární krve

V posledních letech byly zkoumány různé materiály pro odběr DBS, které v některých aspektech překonávají standardní odběrová média. A. Gjelstad a její kolektiv představili alternativní alginátové a chitosanové materiály, které jsou při eluci rozpustné, na rozdíl od standardních celulózových vzorkovacích DBS karet a polymerních VAMS [18]. Alginát a chitosan jsou biopolymery, které se mohou vyrábět ve formě vláken, pěn nebo gelů a jsou komerčně používané v oblastech hojení ran, tkáňového inženýrství a při řízeném uvolňování léčiv [29, 30]. Krevní skvrna je nanesena na alginátový nebo chitosanový materiál a usušena. Následně je skladována nebo podrobena analýze stejným způsobem jako u vzorků odebraných standardně na DBS karty. Při eluci se materiál rozpustí v elučním roztoku a výsledný eluát se přímo používá pro další zpracování a analýzu. Skutečnost, že se během eluce rozpustí celý odběrový materiál, a tím pádem také celá DBS, může zajistit zlepšení výtěžnosti pro sledované analyty. Nicméně, protože se vzorkovací sorbenty DBS rozpustí, zvýší se komplexnost eluátu, které proto nejsou přímo kompatibilní s následnou analýzou a je nutná jejich úprava. U komerčně dostupných DBS karet byly výtěžky až 5krát nižší, než v případě vzorkování na alginátový nebo chitosanový materiál, kde výtěžky u vybraných analytů dosahovaly

až 100 %. Zvýšené výtěžnosti představují obrovský potenciál pro vývoj a aplikaci alternativních materiálů, protože dosud tyto materiály nejsou běžně používané [18]. V budoucnu by tyto rozpustné materiály mohly najít uplatnění u VAMS technologií, které doposud používají pro vzorkování polymerní špičku z nerozpustného materiálu.

2.2 Analýza DBS vzorků

Připravené DBS na odběrových médiích (viz kapitola 2.1) mohou být v laboratoři buď skladovány nebo podrobeny analýze. Pro analýzu je nutné z DBS karty vyříznout vhodnou část skvrny. U odběru z kapky krve, která nemá přesně definovaný objem vzorku na odběrové kartě, dochází k nerovnoměrné distribuci krve kvůli různým hladinám hematokritu (viz kapitola 2.1). Dochází tak ke snížení přesnosti a opakovatelnosti kvantitativních analýz [15] zvláště, když jsou pro analýzu použity disky vyražené z různých míst odebrané DBS [9]. Vhodnějším přístupem pro eliminaci negativního vlivu hematokritu je vzorkování přesného objemu krve a následně vyřezání celé DBS [11] (viz kapitola 2.1.2).Vzorkování krve na perforovanou zónu nebo předem vyražený disk z DBS karty se provádí proto, aby všechna krev byla zachycena pouze v definované oblasti [11, 15, 31, 32].

Standardní postup zpracování odběrových médií je proces náročný na čas a na posloupnost pracovních úkonů. Při eluci se vyříznutý disk se vzorkem suché krve umístí do vialky a převrství se vhodným rozpouštědlem. Podle typu zvoleného rozpouštědla se mohou do eluátu uvolnit různé analyty a složky krve. Vzniklý kapalný eluát se odstředí nebo odpaří do sucha a rekonstituuje vhodným rozpouštědlem [18]. Běžně se pro eluci používá deionizovaná voda, případně slabá kyselina, zásada nebo je možné krevní skvrnu eluovat směsí vody a metanolu (či acetonitrilu) nebo čistým organickým rozpouštědlem. Organická rozpouštědla se pro eluci používají v případě, že je pro analýzu nezbytně nutné vysrážet proteiny ze vzorku nebo pro eluci nepolárních látek. Při eluci dochází u stanovovaných analytů ke snížení jejich koncentrace v porovnání s původní kapilární krví, proto musí být pro jejich stanovení použity citlivé analytické metody [1, 18].

Pro některé metody jsou nutné ještě další kroky úpravy vzorku jako je derivatizace (pro zvýšení citlivosti detekce nebo těkavosti stanovovaných analytů), případně působení ultrazvuku (pro zlepšení extrakční nebo eluční výtěžnosti). Proces úpravy je zvolen podle stanovovaných analytů a použité analytické metody. Příklady různých instrumentálních analytických metod použitých k analýze vzorků DBS jsou uvedeny v tab. č. 1. Byly vybrány

tak, aby znázornily rozsah použitých analytických metod v závislosti na jejich dosažených limitů detekce (LOD) [1].

Tab. č. 1: Vybrané příklady analytických metod použitých na vzorky DBS [1, 3].

Metoda stanovení	Zpracování DBS vzorku	LOD [µg/ml]	Aplikace*
GC-MS	Derivace/eluce části skvrny	1,0	NBS
GC-MS/MS	Derivatizace/eluce	$2{,}0 \cdot 10^{-5}$	TDM
LC-UV	Eluce DBS disku	$1{,}5 \cdot 10^{-3}$	TDM / NBS
LC-fluorescence	Eluce DBS disku, derivatizace	$5{,}0 \cdot 10^{-3}$	TDM
LC-MS	Eluce DBS disku	$1{,}0 \cdot 10^{-3}$	TDM / NBS / DMPK
LC-MS/MS	Eluce DBS disku	$2{,}0 \cdot 10^{-4}$	TDM / NBS / DMPK
LC-HRMS	Eluce DBS disku	0,1	TDM
DESI-MS	Přímý nástřik vzorku	0,01	NBS / TDM
DART-MS	Přímý nástřik vzorku	0,3	TDM
ICP-MS	Eluce DBS disku	0,03	Elementární analýza
CE-ESI-MS	Eluce DBS disku	0,03	NBS

* DART-MS = hmotnostní spektrometrie s přímou analýzou v reálném čase, DESI-MS = hmotnostní spektrometrie s desorpční elektrosprejovou ionizací, DMPK = metabolismus léčiv / farmakokinetika, GC = plynová chromatografie, HRMS = hmotnostní spektrometrie s vysokým rozlišením, ICP-MS = hmotnostní spektrometrie s indukčně vázaným plazmatem, LC = kapalinová chromatografie, MS = hmotnostní spektrometrie, NBS = novorozenecký screening, TDM = terapeutické monitorování léčiv

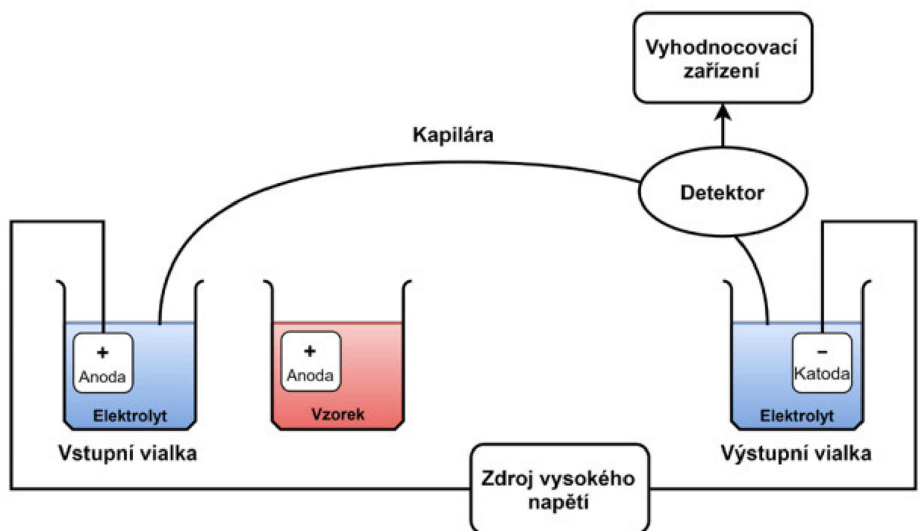
Je docela překvapivé, že v širokém portfoliu analytických technik používaných pro analýzu DBS je CE velmi málo používanou analytickou koncovkou. Ve srovnání s jinými analytickými technikami aplikovanými pro analýzu analytů z DBS eluátu je CE instrumentace mnohem jednodušší a finančně méně náročná. Dosud byla CE v této oblasti využita pouze pro analýzu biochemicky významných sloučenin [33, 34] a aminokyselin [3]. CE je však předurčena pro analýzy minimálního objemu vzorků. Navíc je to technika pro rychlé separace umožňující současné monitorování více složek vzorku najednou v krátkém časovém intervalu. Tyto charakteristiky CE mohou být užitečné při analýze různých analytů v DBS eluátu.

Navíc instrumentace CE je ve srovnání s jinými analytickými technikami mnohem jednodušší a umožňuje přímé propojení s různými technikami mikroextrakce [35, 36].

2.3 Instrumentace kapilární elektroforézy

Schematický nákres CE instrumentace je znázorněn na obr. č. 7. Skládá se ze zdroje vysokého napětí (0–30 kV), dvou platinových elektrod, separační kapiláry z křemenného skla s vnější vrstvou polyimidu (vnitřní průměr kapiláry 10–100 µm, délka kapiláry 30–70 cm), dvou vialek s roztokem elektrolytu (BGE), detektoru a systému pro sběr dat připojeného k počítači [36].

Nabité analyty jsou separovány v kapiláře na základě jejich různé elektroforetické mobility, velikosti a směru elektroosmotického toku. Ionty migrují separační kapilárou různou rychlostí a oddělí se do jednotlivých zón, které jsou detekovány v různých časech při průchodu detektorem. Jednotlivé zóny jsou následně vizualizovány ve formě elektroforeogramu, kde jednotlivé píky odpovídají zónám iontů se stejnou elektroforetickou mobilitou [37]. Výhodou CE je, že může být provedena v mnoha operačních módech, které umožňují separace kladně a záporně nabitých stejně tak jako neutrálních analytů.



Obr. č. 7: Schematický nákres kapilární elektroforézy [36].

CE je jednou z nejrychleji vyvíjejících se analytických technik [38], existuje pro to několik důvodů. Jedním z nich je jednoduchost CE instrumentace a s tím spojené nízké náklady na spotřební materiál (kapilára aj.), dále také vysoká separační účinnost, minimální spotřeba roztoků a vzorků. Nevýhodou CE instrumentace je nižší dlouhodobá stabilita analytického sytému ve srovnání s chromatografickými technikami a nižší citlivost detekce při použití UV-VIS detektoru.

Nižší stabilita systémů CE je způsobena malými změnami elektroosmotického toku, ke kterému dochází vlivem postupného opotřebení vnitřního povrchu separační kapiláry a tím jsou způsobeny změny v migračních časech analytů a účinnosti jejich separace [39]. Pro zlepšení stability elektroosmotického toku CE systému se mohou do BGE přidat vysokomolekulární sloučeniny (např. deriváty celulózy a polymerní roztoky), které ovlivní povrchový náboj na vnitřní stěně kapiláry a potlačí vliv elektroosmotického toku [36].

Dávkované množství vzorku (standardně v řádu pl až nl) do CE je limitováno objemem separační kapiláry. Celková efektivní dráha optických detektorů v CE je dána vnitřním průměrem separačních kapilár. Krátká optická dráha detektoru je spolu s malým objemem dávkovaného vzorku důvodem nižší citlivosti CE analýz, proto byly zkoumány různé přístupy pro zlepšení detekčních limitů. Nejjednodušší alternativou je použití selektivnějších a citlivějších detekčních zařízení ve spojení s CE, například hmotnostní spektrometrie (MS) [40] nebo laserem indukovanou fluorescence [41]. Detekční citlivost může být také zvýšena použitím vhodných procesů úpravy vzorků (viz kapitola 2.4), které umožní při spojení s komerční CE přečištění, zakoncentrování a následnou analýzu analytů (viz kapitola 2.5) [36].

2.4 Mikroextrakční techniky

Analýza DBS není triviálním úkolem z důvodů komplexního složení matrice a nízké koncentrace většiny stanovovaných analytů. Kapilární krev obsahuje krevní elementy, vysoké koncentrace proteinů (~70 g/l), malé anorganické ionty (~ 300 mM), lipidy (~8 g/l) a také další biochemicky významné látky, jako je glukóza, močovina, aminokyseliny a mastné kyseliny. Některé matriční složky (obzvláště makromolekulární) mohou ulpívat na vnitřních strukturách analytických systémů, zejména kolon (v kapalinové a plynové chromatografii) a separačních kapilár (v CE), což může vést k nižší analytické účinnosti nebo může dojít až k poškození analytického systému [36].

Přímé dávkování komplexních krevních vzorků do analytického systému bez předchozí úpravy je možné jen výjimečně. Tradičně se pro úpravu používají techniky založené na extrakci kapalina-kapalina (LLE) a extrakci na pevné fázi (SPE). Techniky LLE vyžadují velké objemy organických rozpouštědel a vzorků. SPE pracuje s velkými objemy vzorků, na druhou stranu ji lze snadno miniaturizovat a automatizovat, proto může být používána v klinické praxi [42, 43]. V minulosti byly k tradičním extrakčním technikám navrženy alternativní přístupy, které kladou stále větší důraz na úsporu času, snížení pořizovacích a provozních nákladů,

zjednodušení a automatizaci celého procesu a také na snížení spotřeby organických činidel. Dalším cílem vývoje nových extrakčních technik byla také snaha o přímé spojení s analytickým systémem. Jednou z dalších motivací pro vývoj byla i možnost izolace sledovaných sloučenin přímo v místě odběru [36, 44]. Zvláštní pozornost je v poslední době věnována mikroextrakčním technikám založených na přenosu analytů přes membránu, mezi které patří mikrodialýza [45], extrakce přes dutá vlákna [46-48], extrakce přes kapalnou membránu na pevném nosiči (SLM) [49-51] a elektromembránové extrakce [51, 52].

Po více než tři desetiletí je značný zájem o vývoj mikroextrakčních technik [53-58] a zejména o mikroextrakci přes SLM [46, 49-51, 53, 57, 59, 60]. V extrakcích přes SLM jsou cílové analyty přeneseny z mililitrového objemu donoru do SLM a následně do mikrolitrového objemu akceptoru na opačné straně SLM. SLM je obvykle vytvořena jako tenká vrstva rozpouštědla nemísitelného s vodou ukotveného v nosném materiálu a působí jako selektivní bariéra pro přenos cílových analytů. Složky matrice vzorku (krevní elementy, solné ionty, proteiny a další interferující složky) jsou účinně zadržovány SLM, která zajistí přečištění vzorku. Navíc, objem akceptoru je nižší než objem vzorku, a proto dochází k zakoncentrování cílových analytů v akceptoru, který může být použit pro opakované analýzy na CE systémech. Mikroextrakční techniky tak mohou být řešením pro CE analýzy vzorku obsahujících nízké koncentrace analytů, které by jinak byly pod detekčními limity použité CE metody [36, 61].

2.5 Spojení CE s mikroextrakčními technikami

Mikroextrakční techniky se většinou používají v off-line uspořádání, kde je krok úpravy prováděn odděleně a výsledný extrakt je manuálně přenesen do analytického systému. Existují však aplikace, u kterých je tento off-line přenos vzorků nevhodný z důvodu možné kontaminace při ruční manipulaci nebo nutnosti bezobslužných analýz. Automatizace extrakčního a analytického procesu je v popředí zájmu. V minulosti byla publikována originální uspořádání pro přímé spojení různých technik membránové extrakce k běžně dostupným analytickým metodám (převážně k separačním metodám) [35, 49, 62]. Instrumentace rutinně používaných analytických metod (HPLC, MS) jsou složité a nákladné, proto je nutný další vývoj, zejména s ohledem na kvantitativní analýzu [63, 64].

Narozdíl od výše uvedených metod využívá CE výrazně jednodušší instrumentaci a nižší dávkované objemy. CE v poslední době ukazuje vysoký potenciál také pro klinickou analýzu [65-67] a je velmi atraktivní pro analýzu komplexních vzorků [68, 69]. Přímého spojení CE

s mikroextrakce do kapalné fáze (LPME) bylo dosaženo zmenšením extrakční jednotky [70], a tím i snížením dávkovaného objemu vzorku do kapiláry [71] nebo dávkováním pouze části akceptorového roztoku [72]. Při vývoji přímého spojení LPME a CE byla pro jednoduchou kontrolu přenosu analytů mezi extrakčním systémem a separační kapilárou použita laboratorně sestavená CE. Použití laboratorně sestavené CE instrumentace ale vyžadovalo ruční manipulaci s kapilárou a úplná automatizace LPME/CE nebyla možná. Později bylo přímé spojení LPME s komerční CE dosaženo pomocí na míru vyrobeného mikroextrakčního zařízení kompatibilního s CE vialkami. Výsledný vodný akceptorový roztok obsahující analyty, které byly během extrakčního procesu transportovány přes membránu, byl přímo automaticky analyzován stávající komerční CE instrumentací [36].

Pro účely extrakce lze použít membrány s různou selektivitou, jako jsou dialyzační membrány, polymerně inkluzní membrány nebo SLM. Použití membrán s různými vlastnostmi zajistí vysokou variabilitu nastavení extrakce DBS s CE, které umožní přímou analýzu různých klinicky důležitých analytů. Selektivita pro přenos specifických analytů může být dále upravena složením membrány, jak bylo nedávno uvedeno pro SLM [73, 74] a polymerně inkluzní membrány [61].

2.6 Využití DBS

DBS se obvykle používají pro novorozenecký screening vrozených dědičných metabolických poruch [3]. Postupem času se analýza DBS rozšířila i na mikrobiologické a epidemiologické sledování chorob [60]. Mezi další důležité aplikace DBS patří molekulární metody pro stanovení DNA nebo RNA, imunologické studie a testování infekčních chorob u kojenců, dětí i dospělých [75]. Další uplatnění DBS je v oblasti toxikokinetických a farmakokinetických studií [4], terapeutického monitorování léčiv [7] a forenzních analýz [76]. Kromě toho se DBS používají pro stanovení mnoha metabolických meziproduktů, jako jsou žlučové kyseliny, karnitin, kreatinin, homocystein a varianty hemoglobinu [77].

2.6.1 Dědičné poruchy metabolismu aminokyselin

Dědičné metabolické poruchy jsou heterogenní skupinou zhruba devíti set vzácných onemocnění s vrozenou genetickou mutací, která je způsobena enzymovým deficitem nebo disfunkcí strukturálního či transportního proteinu v organismu. Následkem toho dochází k narušení biochemické reakce látek, které se mohou v organismu hromadit. Včasná diagnostika těchto poruch

je důležitá pro jejich léčbu a většinou rozhoduje o míře poškození pacienta [78]. Mezi nejčastější dědičné poruchy metabolismu aminokyselin v České republice patří fenylketonurie, hyperfenylalaninemie, alkaptonurie a tyrozinémie. [79, 80].

Fenylketonurie a hyperfenylalaninemie jsou vrozené poruchy metabolismu aminokyseliny fenylalaninu. Příčinou onemocnění je mutace genu, která způsobuje deficit fenylalaninhydroxylázy (PAH), která hydroxyluje fenylalanin na tyrosin. Fenylalanin je aminokyselina, která je obsažena v bílkovinách rostlinných i živočišných organismů. U hyperfenylalaninemie je plazmatická koncentrace fenylalaninu zvýšená (viz tab. č. 2). U nemocných pacientů s koncentrací fenylalaninu v plazmě vyšší než 600 μmol/l organismus neprodukuje PAH nebo má enzym jen velmi nízkou aktivitu a dochází k hromadění fenylalaninu, které vede k poruchám centrální nervové soustavy (mentálnímu postižení až k demenci) [3, 78].

Tab. č. 2: Klasifikace poruch metabolismu podle hladiny fenylalaninu a aktivity PAH v krvi [43].

Název onemocnění	Plazmatická koncentrace fenylalaninu před terapií [μmol/l]	Reziduální aktivita PAH [%]
Hyperfenylalaninemie	120–600	> 5
Mírná fenylketonurie	600–1200	1–5
Fenylketonurie	1200	<1

Dalšími dědičnými poruchami metabolismu aminokyselin, projevující se již v novorozeneckém věku, jsou tyrozinémie a alkaptonurie, které jsou způsobené poruchou metabolismu aminokyseliny tyrosinu. Tyrosin je významný pro proteosyntézu neurotransmiterů (dopaminu, adrenalinu, noradrenalinu), melaninu a hormonu thyroxinu. Metabolismus fenylalaninu a tyrosinu a jejich metabolické poruchy jsou zobrazeny na obr. č. 8 [78].

Obr. č. 8: Metabolismus fenylalaninu a tyrosinu a jejich metabolické poruchy [81].

Dědičné metabolické poruchy zmíněných aminokyselin se projevují už po narození. Jakmile začne novorozenec pít mateřské mléko, hladina aminokyselin v jeho krvi začne stoupat a v případě fenylketonurie dochází v důsledku zvýšeného fenylalaninu postupně k rozvoji mentální retardace, která dále progreduje ve středně těžkou až těžkou. Z tohoto důvodu je nutná včasná diagnostika ještě před klinickými projevy. Pokud se onemocnění jedince potvrdí, je nutné zahájit léčbu pomocí speciální potravinové diety s nízkým obsahem fenylalaninu ve stravě. Výsledkem nízko-bílkovinové diety u dědičných poruch metabolismu aminokyselin fenylalaninu a tyrosinu by mělo být udržení optimální stálé koncentrace fenylalaninu v krvi, kterou je třeba pravidelně kontrolovat. Nadměrné snížení hladiny fenylalaninu se projevuje nechutenstvím, únavou, anémií a průjmy. Důležitou součástí nízkobílkovinové diety je podávání aminokyselinových léčebných přípravků bez obsahu fenylalaninu, které se přizpůsobuje věku a potřebám nemocného. Dále je důležitý neomezený příjem potravin s velmi nízkým obsahem fenylalaninu, např. cukr, med, máslo, některé druhy zeleniny (salátová okurka, hlávkový salát) nebo ovoce (jablko, hruška). Vyloženě nevhodné pro pacienty trpící fenylketonurií jsou potraviny s vysokým obsahem bílkovin a tím i fenylalaninu (maso, vejce, ořechy, obiloviny, mléko), ať již živočišného nebo rostlinného původu. Správně by měl být obsah fenylalaninu na všech potravinách uveden v rámci jejich složení [78]. Dieta by měla být naordinována už v novorozeneckém věku, tedy v období vývoje mozku. Po ukončení vývoje mozku se snižuje i riziko jeho poškození.

Důležitost dodržování dietního plánu v období dospělosti je zatím předmětem diskusí odborníků a je velmi individuální [82].

2.6.1.1 Screening dědičných poruch metabolismu u novorozenců

Celoplošný novorozenecký screening se provádí z DBS odebrané mezi 48. a 72. hodinou po narození. Rozmanitost dědičných metabolických poruch vyžaduje širokou škálu analytických separačních metod pro jejich diagnostiku. Separační a detekční postupy od jednoduchých aplikací až po složité instrumentální metody se volí podle fyzikálně-chemických vlastností stanovovaných analytů. Zvolená metoda také musí eliminovat vliv biologické matrice, obsahující velké množství interferujících látek, mnohdy v koncentracích vysoce převyšující stanovované analyty. Analyty obsažené v malém množství vzorku jsou obvykle zastoupeny v koncentraci nmol/l až mmol/l, proto je nutné volit metodu s velmi citlivou detekcí. V této oblasti se nejčastěji využívá kombinace chromatografických systémů s vysoce selektivní a citlivou MS. S příchodem MS se analýzy DBS staly velmi populární po celém světě a očekává se, že s dalším rozvojem a rozšiřováním DBS vzorkování dojde k revoluci v oblasti klinické diagnostiky. S nástupem elektrosprejové ionizační techniky (ESI-MS) se provádí rozšířený novorozenecký screening dědičných metabolických poruch pomocí kvantitativní analýzy aminokyselin a acylkarnitinů z DBS [3, 83].

Navzdory úspěšné aplikaci ESI-MS existuje stále řada významných analytických výzev, hlavně při komplikovaném zpracování vzorků. Jako vhodná alternativa může být zmíněno spojení CE-ESI-MS, které nabízí přímou analýzu aminokyselin, acylkarnitinů a jejich stereoizomerů z DBS bez nutnosti předchozí derivatizace a dalších úprav vzorků [83].

2.6.2 Farmaceutická perspektiva DBS

Analýza DBS se používá při novorozeneckém screeningu po celá desetiletí, ale v poslední době se stále více uplatňuje i v oblasti analýzy léčiv pro vývoj nových léků. Ve srovnání s konvenčním odběrem žilní krve nabízí technika odběru vzorků DBS praktické, klinické a finanční výhody (viz kapitola 2.1) [84, 85]. V posledních letech bylo publikováno několik metod pro kvantifikaci léčiv z DBS (například antiepileptik, imunosupresiv, antiretrovirotik, léčiv pro kardiovaskulární terapii, psychoaktivních látek a antibiotik) [1, 9] [13]. A. J. Wilhelm a kol. [9] shrnuli využití DBS v terapeutickém monitorování léčiv.

2.6.2.1 Interakce léčiv s potravinami

Léky mohou pomoci eliminovat mnoho zdravotních problémů. Aby byla zajištěna jejich bezpečnost a dostatečná účinnost, musí být podány správně. Léky by měly mít mimořádně specifický a předvídatelný účinek pro všechny pacienty, neměly by interagovat s jídlem nebo jinými léky, měly by vykazovat lineární účinnost a být zcela netoxické v jakékoli dávce. Nicméně tento ideální lék ještě nebyl objeven [86].

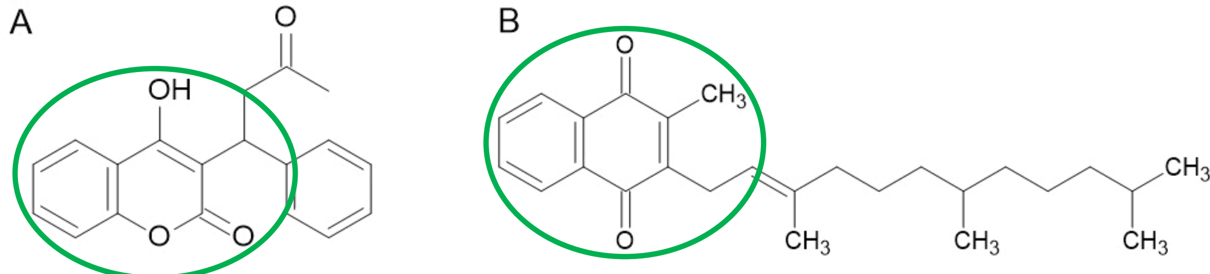
Interakcí se označují procesy, při kterých dochází ke změně aktivity léčiva. Nejčastější jsou vzájemné interakce mezi léčivy, ale může také docházet k interakcím mezi léčivem a potravinami nebo doplňky stravy. Strava a životní styl mají na užívání léků významný vliv. Pro každý lék jsou doporučené jiné podmínky užívání, je tedy nutné pacienty s těmito pravidly seznámit a informovat je, zda je nutné lék užívat před jídlem, po jídle nebo současně s ním [87].

Interakce léčiv s potravinami vede buďto ke snížení účinku a tím pádem k selhání terapie, nebo ke zvýšení účinku s následným rizikem vzniku ireversibilního toxického působení. Na rozdíl od snadného přístupu k informacím o vzájemných interakcích mezi léčivy, nejsou informace o interakcích léčiv s potravinami vždy snadno dostupné. Jedním z léčiv interagujících s potravinami je například warfarin. Přesné stanovení účinků potravy na konkrétní lék je obtížným a složitým procesem, proto je nutné hledat jednoduché a finančně dostupné analytické metody pro stanovení terapeutické koncentrace léčiv z DBS [86, 87].

2.6.2.1.1 Interakce warfarinu s potravinami

Warfarin (4-hydroxy-3-(3-oxo-1-fenylbutyl) kumarin) řadíme mezi léčiva, která potřebují zvláštní dietní opatření. Významně interaguje s celou řadou potravin a tím může způsobit závažné zdravotní komplikace. Jeho účinek musí být pravidelně monitorován. Warfarin patří mezi nejčastěji užívaná perorální antikoagulancia. Účinek warfarinu je založený na blokování vitamínu K, který aktivuje protrombin (faktor II) a triádu srážlivých faktorů VII, IX a X. Hlavním zdrojem vitamínu K (fylochinon) pro lidský organismus je konzumace zelených rostlin. Vitamín K je nezbytným pro γ-karboxylaci glutamátových zbytků na koagulačních faktorech II, VII, IX a X, které jsou syntetizovány játry v biologicky neaktivní formě. Karboxylace těchto faktorů přímo souvisí s oxidací vitamínu K na jeho epoxidovou formu. Warfarin má antikoagulační účinek díky své strukturální podobnosti s vitamínem K (zobrazeno na obr. č. 9) se může vázat na enzym epoxidovou reduktázu vitamínu K, který katalyzuje přeměnu epoxidu vitamínu K zpět na vitamín K [88]. Na základě složitosti hemostatického

mechanismu a farmakokinetiky warfarinu existuje celá řada potenciálních interferencí s potravinami [89].



Obr. č. 9: Strukturální podobnost mezi warfarinem (A) a vitamínem K (B) [89].

Pokud je příjem vitamínu K nestálý a jeho hladina v krvi kolísá, dochází k neustálým změnám srážlivosti krve, což může pacienta léčeného warfarinem ohrozit na životě [90]. Je prokázáno, že k interakci s warfarinem dochází u 58 různých druhů rostlin. Za potraviny a byliny, které v největší míře ovlivňují účinky warfarinu jsou označeny ženšen, česnek, ginkgo biloba, třezalka tečkovaná, zázvor, šalvěj a sójové boby. Mezi potraviny a fytofarmaka, které zvyšují účinek antikoagulačních léků, řadíme papáju, hřebíček, heřmánek, rebarboru, černý rybíz, borůvky, celer a mnoho dalších. Naopak snížení účinků warfarinu způsobuje aloe vera, sójové boby, zelený čaj, vojtěška, psillium a potraviny bohaté na vitamín K, mezi které se řadí především brokolice, kapusta, hlávkové zelí, špenát, kuřecí a krůtí maso, játra, olivový a sójový olej, maliny, ostružiny, mango a kiwi [89].

Vzhledem k hemoragickým nebo trombotickým rizikům spojených s antikoagulační terapií warfarinem je nutné neustále sledovat srážlivost krve pomocí protrombinového času vyjádřeného jako mezinárodní normalizovaný poměr (INR). Sledování pomocí standardních koagulačních testů srážlivosti vyžadují časté invazivní odběry krve, obvykle každý den při zahájení léčby a jednou za každé dva týdny nebo jednou za měsíc při dlouhodobém užívání stabilní dávky warfarinu. Hlavním problémem stanovení INR jako standardního koagulačního testu jsou sociální a ekonomické náklady spojené s častými návštěvami pacientů odběrových a antikoagulačních klinik. Stanovení koncentrace warfarinu a jeho aktivních metabolitů v DBS by mohlo poskytnout užitečné informace doplňující test INR pro méně invazivní a pohodlnější nastavení antikoagulační terapie. Dosud byla koncentrace warfarinu stanovována pomocí ultra-vysokoúčinné kapalinové chromatografie spojené s elektrosprejovou ionizací-tandemovou hmotnostní spektrometrií (UHPLC-ESI-MS/MS) [85].

3 CÍL PRÁCE

Cílem dizertační práce je vývoj nových postupů a metod využívající CE pro analýzu DBS. Práce je také zaměřena na vývoj metodiky pro přímé dávkování vzorku a účinnou eliminaci negativních vlivů vznikajících v důsledku vzorkování, extrakce a eluce DBS. Vyvinuté postupy umožní účinnou úpravu komplexních vzorků a jejich přímou bezobslužnou analýzu a zajistí dostatečnou selektivitu a citlivost pro stanovení významných analytů ve vzorcích se složitými matricemi. Analyzovány budou relevantní analyty v DBS (např. aminokyseliny, acidická a bazická léčiva). Aminokyseliny budou analyzovány jako markery metabolických poruch, z nichž některé mohou souviset přímo s výživou nebo naopak poukazovat na nutnost její úpravy pro kompenzaci těchto poruch. Metody analýzy léčiv budou aplikovány pro jejich kvantitativní stanovení a zjednodušení terapeutického monitorování. Vyvinuté postupy tak mohou představovat účinné nástroje pro potravinářsko-biomedicínské analýzy.

Řešené dílčí úkoly:

- vývoj CE metod a postupů pro přímé sledování charakteristik a vybraných modelových analytů v DBS,
- in-line spojení CE metod s technikami mikroextrakce pro analýzu DBS a přímé dávkování analytů z DBS eluátu do separační kapiláry,
- zjednodušení odběrů vzorků, extrakcí, analýz a účinná eliminace negativních vlivů při odběru vzorků krve (např. vliv hematokritu),
- vývoj elučních postupů pro přímý nástřik DBS eluátu do separační kapiláry pro analýzu aminokyselin,
- reálné aplikace vyvinutých metod pro stanovení koncentrací acidických a bazických léčiv a terapeutické monitorování léčiv,
- vývoj nových rozpustných odběrových vzorkovacích materiálů pro kvantitativní analýzy DBS.

4 DISKUZE

Tato kapitola podrobně diskutuje experimentální výsledky, které již byly v průběhu doktorandského studia publikovány ve vědeckých časopisech. Přiložené články a doplňující podkladové informace samozřejmě také obsahují podrobné popisy metod a výsledků dosažených během studia. Všechny články a doplňující informace jsou k této práci připojeny jako přílohy (viz kapitola 9), jejich seznam se stručným popisem příspěvku autorky této práce je uveden v kapitole 8.

Předložená práce se zaměřuje na využití CE pro analýzu DBS. Krevní vzorky jsou standardně analyzovány ve formě plazmy nebo séra. V mnoha aplikacích se nově stále častěji uplatňují také DBS. V rámci předložené disertační práce jsou DBS analyzovány pomocí CE, která byla v minulosti při řešení DBS problematiky využívána velmi zřídka. CE nicméně nabízí mnohé výhody, dané vývojem této techniky a instrumentálního uspořádání v poslední době, kterými zavedené analytické metody nedisponují (viz kapitola 2.5). Využití přímého spojení CE s mikroextrakčními technikami (kapitola 4.1) a simultánní CE stanovení analytů a vybraných matričních komponent může vést k minimalizaci a eliminaci některých nedostatků při analýze DBS. Práce je zaměřena na vývoj metodiky pro přímé dávkování a analýzy relevantních analytů v DBS (kapitola 4.2). Těmi mohou být například markery metabolických poruch, z nichž některé mohou souviset přímo s výživou nebo naopak poukazovat na nutnost úpravy výživy pro kompenzaci těchto poruch. Zpracování a analýza DBS metodou CE se tak může stát účinným nástrojem v potravinářsko-medicínských analýzách i laboratorní praxi. V popředí zájmu práce je i účinná eliminace negativních vlivů vznikajících v důsledku vzorkování a eluce DBS (kapitola 4.2.1), a s tím související zjednodušení odběru DBS vzorků pro možný samoodběr přesně definovaného objemu kapilární krve (kapitola 4.2.3). Práce se také zabývá vývojem plně automatizovaného analytického zpracování a analýzy DBS vzorků pomocí CE-UV (kapitola 4.2.2 a 4.2.4) a dále se zaměřuje na vývoj nových rozpustných odběrových materiálů pro kvantitativní analýzu DBS (kapitola 4.2.5).

4.1 Spojení CE s mikroextrakčními technikami pro analýzu komplexních vzorků

Komplexní biologické vzorky většinou nelze přímo analyzovat pomocí separačních technik, protože jejich matrice obsahují vysoký podíl proteinů a dalších interferujících látek. Tyto látky

mohou interferovat se sledovanými analyty, které jsou vzhledem k ostatním složkám v matrici přítomny ve stopovém množství. Je tedy nezbytné, aby biologické vzorky byly před analýzou přečištěny od nežádoucích látek a případně aby analyty byly zakoncentrovány. Úprava vzorku před vlastní analýzou má vliv na všechny následující kroky a současně je kritickým a časově nejnáročnějším procesem celého analytického postupu. Ve vysoké míře ovlivňuje výslednou přesnost a správnost stanovení. V minulosti se používaly pro úpravu vzorků tradiční extrakční techniky (LLE [91], SPE [92]), ke kterým byly navrženy alternativní přístupy a jejich vývoj pokračuje i v současnosti. Tyto extrakční metody, obecně nazývané mikroextrakční techniky, obvykle nabízejí srovnatelnou úpravu vzorku s výhodou podstatného snížení nákladů, dopadu na životní prostředí a spotřeby biologických vzorků, použitých roztoků i rozpouštědel. Ve většině mikroextrakčních technik je také výrazně zkrácena doba úpravy, nicméně toto není obecné pravidlo. Mezi nejpoužívanější patří například mikroextrakce z jedné kapky (SDME [93]), HF-LPME [53] a SLM extrakce [49-51].

Extrakce se většinou provádějí off-line v na míru vyrobených mikroextrakčních zařízeních a výsledné extrakty se přenesou do vhodné analytické instrumentace, kde je provedena jejich analýza. SLM extrakce mohou být spojeny přímo s CE bez nutnosti manuálního přenosu výsledných extraktů. Vývoj a principy přímého spojení extrakce přes SLM s CE jsou popsány v kapitole 4.1.1. V kapitole 4.1.2 je prezentováno nové uspořádaní mikroextrakčního zařízení, které umožňuje spojení HF-LPME s CE za účelem účinného přečištění a prekoncentrace cílových analytů z komplexních vzorků pro jejich přímou analýzu.

4.1.1 Přímé spojení SLM extrakce s CE

SLM extrakce byly přímo spojeny s HPLC, GC a dalšími analytickými technikami, ale přímé spojení s CE představuje atraktivní alternativu [49, 50, 94]. Dávkované objemy v CE jsou výrazně nižší než u jiných analytických technik a jsou dobře kompatibilní s mikrolitrovými objemy akceptorových roztoků vzniklých extrakcí přes SLM. Spojení SLM extrakčního zařízení s CE bylo dosaženo přímým kontaktem separační kapiláry s povrchem membrány. Analyty přenesené přes membránu (SLM) byly dávkovány z difuzní vrstvy fázového rozhraní nacházející se těsně u povrchu membrány [36].

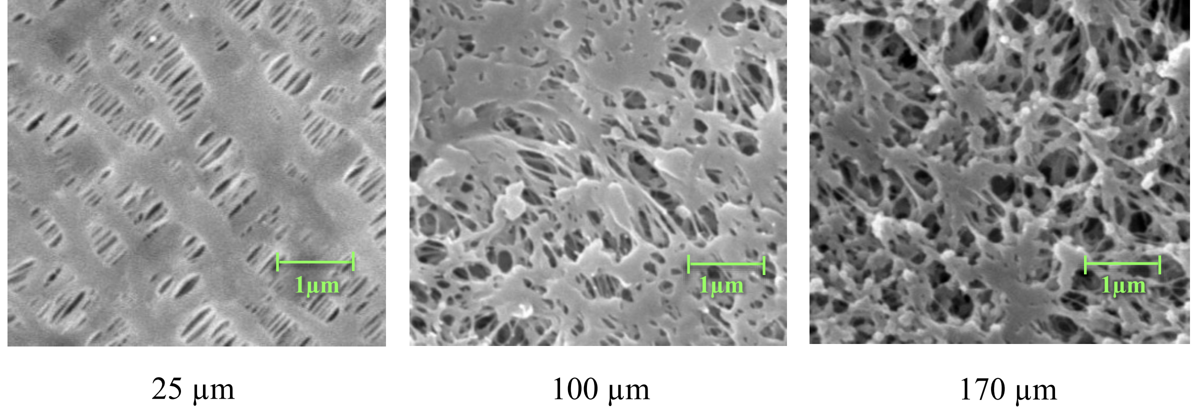
V *příloze I* byly zkoumány tři různé planární polypropylenové (PP) membrány, aby se komplexně prozkoumal vliv tloušťky nosného materiálu na účinnost extrakce přes SLM přímo spojené s komerční CE. Charakteristické parametry vybraných tří PP membrán jsou

shrnuty v tab. č. 3 a snímky membrán elektronovým rastrovacím mikroskopem (ESM) jsou znázorněny na obr. č. 10.

Tab. č. 3: Parametry PP membrán.

Typ membrány	Celgard® 2500	Accurel PP 1E R/P	Accurel PP 2E-HF R/P
Tloušťka	25 µm	100 µm	170 µm
Porozita	55 %	n/a	n/a
Velikost pórů	0,064 µm	0,1 µm	0,2 µm

n/a – data nebyla výrobci uvedena

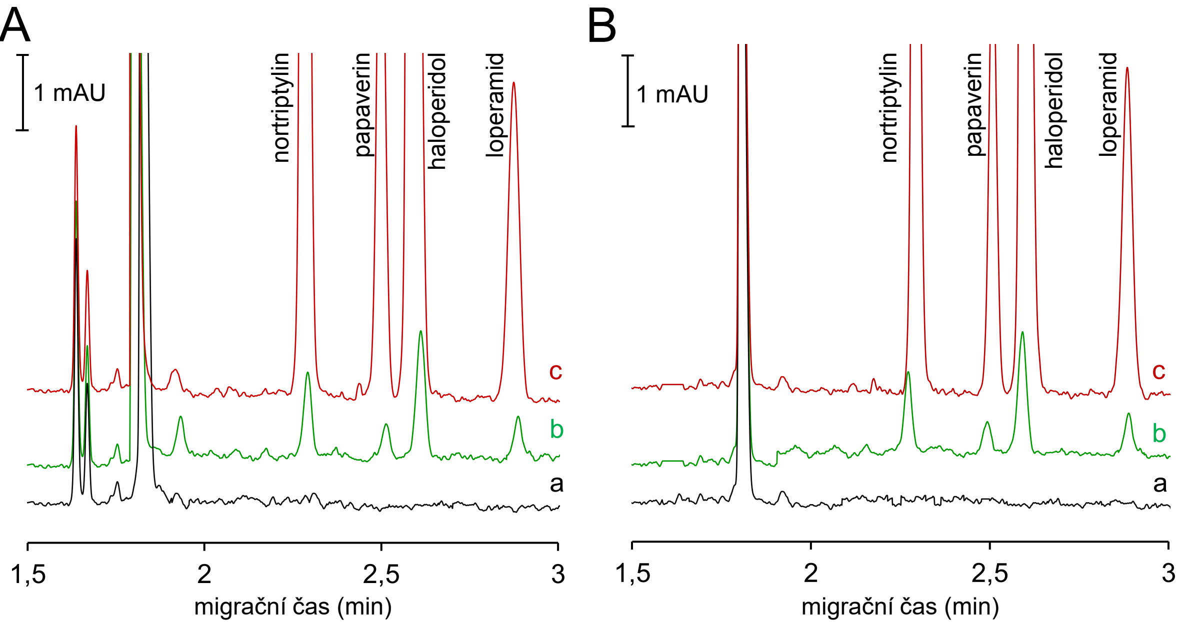


Obr. č. 10: Snímky tří PP membrán s různou tloušťkou získané pomocí ESM.

Na počátku bylo nutné připravit mikroextrační zařízení kompatibilní s přístrojem Agilent 7100. Zařízení se skládalo z donorové a akceptorové jednotky, které byly odděleny PP membránou impregnovanou organickým rozpouštědlem. Analyty přítomné v donorovém roztoku byly difuzně přeneseny přes SLM na základě jejich distribučních koeficientů souvisejících s organickým rozpouštědlem impregnovaným v pórech membrány. Přenosy modelových bazických léčiv přes porézní PP membrány s různou tloušťkou byly významně ovlivněny tloušťkou membrány a nejúčinnější přenosy byly získány pro nejtenčí (25 µm) membránu. Rovnováhy extrakčního procesu bylo navíc dosaženo nejrychleji a spotřeba organických rozpouštědel pro impregnaci byla nejnižší pro 25 µm PP membránu. Na extrakci byl nutný pouze 1 µl organického rozpouštědla, což bylo 5krát a 7krát méně než u 100 a 170 µm PP membrán. Mechanická stabilita SLM nebyla ovlivněna tloušťkou membrány a bylo možné vícenásobné dávkování z povrchu SLM, které vyžadovalo přímý kontakt separační kapiláry s membránou. Nežádoucí složky matrice byly účinně eliminovány z neředěných tělních tekutin

(moč a plazma), což ukazuje užitečnost tenkých SLM v přímých CE analýzách komplexních biologických vzorků. Tato základní studie experimentálně ověřila, že tloušťka membrány hraje klíčovou roli v přenosech modelových analytů přes SLM a potvrdila dříve navržené principy extrakce pomocí SLM.

Výsledné elektroferogramy demonstrující přímé spojení extrakce přes SLM s CE-UV pro analýzu lidské moči bez obsahu stanovovaných analytů a lidské moči obohacené čtyřmi bazickými léčivy v koncentracích 0,5 a 10 µg/ml jsou znázorněny na obr. č. 11 A a pro analýzu lidské plazmy bez obsahu stanovovaných analytů a lidské plazmy obohacené bazickými léčivy o koncentracích 0,5 a 10 µg/ml jsou znázorněny na obr. č. 11 B. Výsledky studie experimentálně ověřily schopnost extrakce přes SLM přečistit komplexní vzorky lidské plazmy a moči od nežádoucích složek, proto je možné tuto metodu použít i pro jiné komplexní vzorky jako jsou např. DBS. Limitujícím faktorem pro využití této techniky je eluce DBS, při které dochází ke zředění vzorku a pro dosažení dostatečné citlivosti je nutné zakoncentrování analytů.

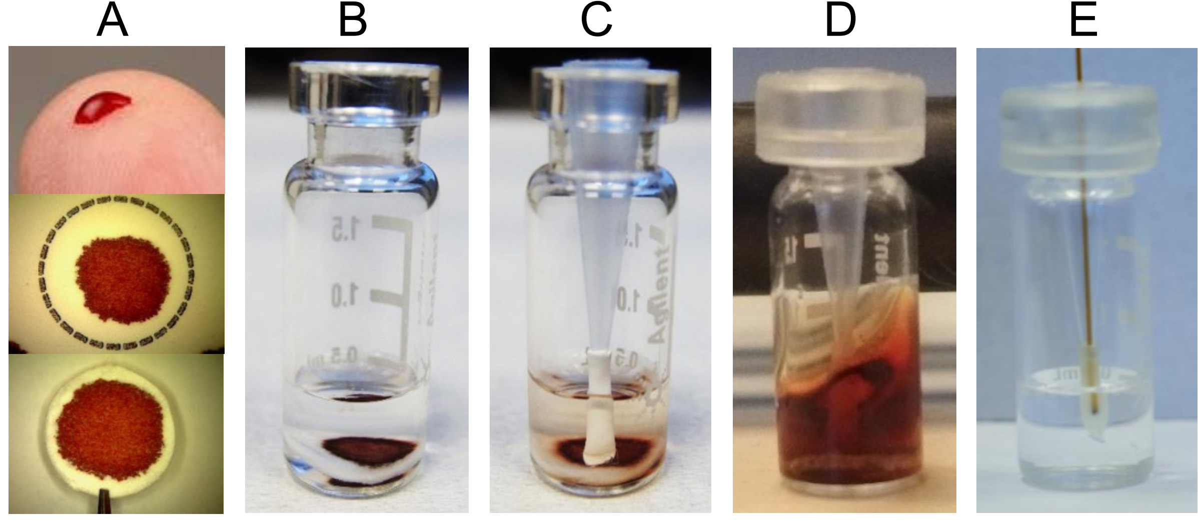


Obr. č. 11 - In-line spojení extrakce přes SLM s CE-UV pro analýzu bazických léčiv v lidské moči (A) a lidské plazmě (B). (a) moč/plazma v 10mM NaOH, (b) moč/plazma s přídavkem 0,5 µg/ml čtyř bazických léčiv v 10mM NaOH, (c) moč/plazma s přídavkem 10 µg/ml čtyř bazických léčiv v 10mM NaOH; akceptor, 10mM HCl.

4.1.2 Přímé spojení HF-LPME s komerční CE

Úprava komplexní matrice (DBS, kapilární krev) a navíc také zakoncentrování analytů je možné pomocí přímého spojení mikroextrakční metody HF-LPME s CE, postupný vývoj

a aplikace jsou uvedeny v *příloze III, V a VI*. Pro extrakci DBS pomocí HF-LPME byl ze vzorkovací karty vyražen disk o průměru 10 mm (obsahující celou DBS) (obr. č. 12 A). Vyražený disk byl umístěn na dno vialky a převrstven 550 µl elučního roztoku (obr. č. 12 B). Porézní duté vlákno (HF) bylo nasazeno na držák vytvořený z PP pipetovací špičky a bylo impregnováno mikrolitrovým objemem rozpouštědla nemísitelného s vodou, jeho vnitřní prostor byl naplněn 5 µl akceptoru a celé mikroextrakční zařízení bylo umístěno do CE vialky s 550 µl elučního roztoku (obr. č. 12 C). Eluce kapilární krve z DBS a HF-LPME probíhala současně během třepání vialky (obr. č. 12 D). Výsledný akceptorový roztok byl analyzován přímým dávkováním z vlákna, bez další ruční manipulace s výjimkou vložení CE vialky do autosampleru (znázorněné na obr. č. 12 E, (eluát je pro ilustraci nahrazen DI vodou)). Eluát a DBS disk nejsou před dávkováním z vialky odstraněny.

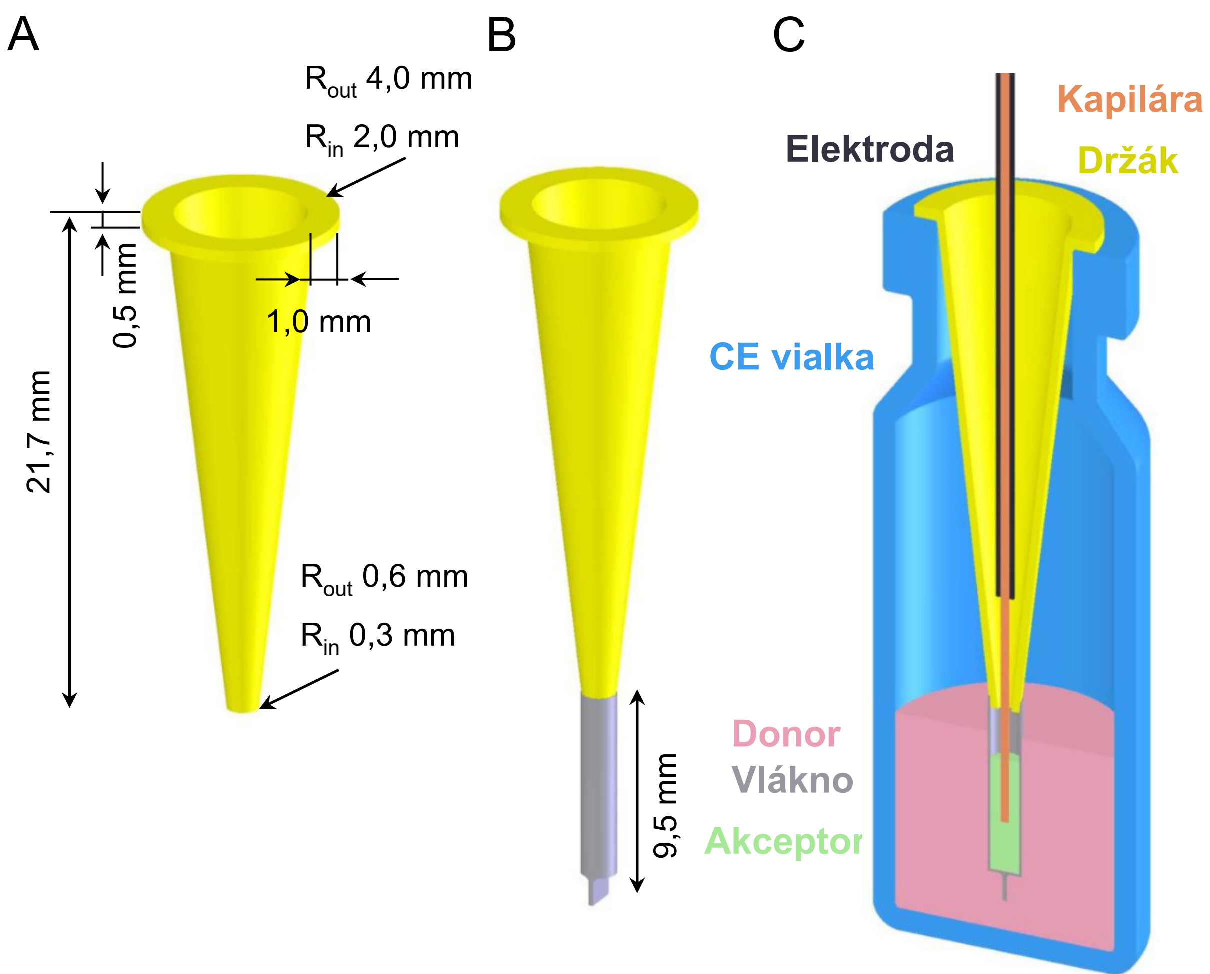


Obr. č. 12: Schéma procesu přípravy (A) a eluce DBS (B) spojené s HF-LPME (C, D) a přímé dávkování do CE (E).

Přenos analytů do akceptorového roztoku je řízen gradientem pH a selektivitou membrány. Analyty jsou účinně prekoncentrovány, zatímco složky matrice jsou eliminovány membránou. Po dosažení rovnováhy na obou stranách membrány je koncentrace analytů v akceptorovém roztoku po dlouhou dobu konstantní a umožňuje automatizované dávkování přečištěných a prekoncentrovaných vzorků do CE. Navržené uspořádání bylo aplikováno pro přímé stanovení modelových bazických léčiv v lidské moči a vzorcích DBS (*příloha III*).

V *příloze V* byla extrakce provedena pomocí vyvinutého jednorázového mikroextrakčního zařízení, které se skládalo z 3D tištěného držáku, na který bylo nasazeno HF. Držák s nasazeným vláknem byl umístěn do vialky kompatibilní s přístroji Agilent CE

pro automatizovanou analýzu (obr. č. 13). 3D tištěný držák udržuje konstantní polohu vlákna v CE vialce během extrakce a současně směřuje separační kapiláru do vlákna během dávkování vzorku.

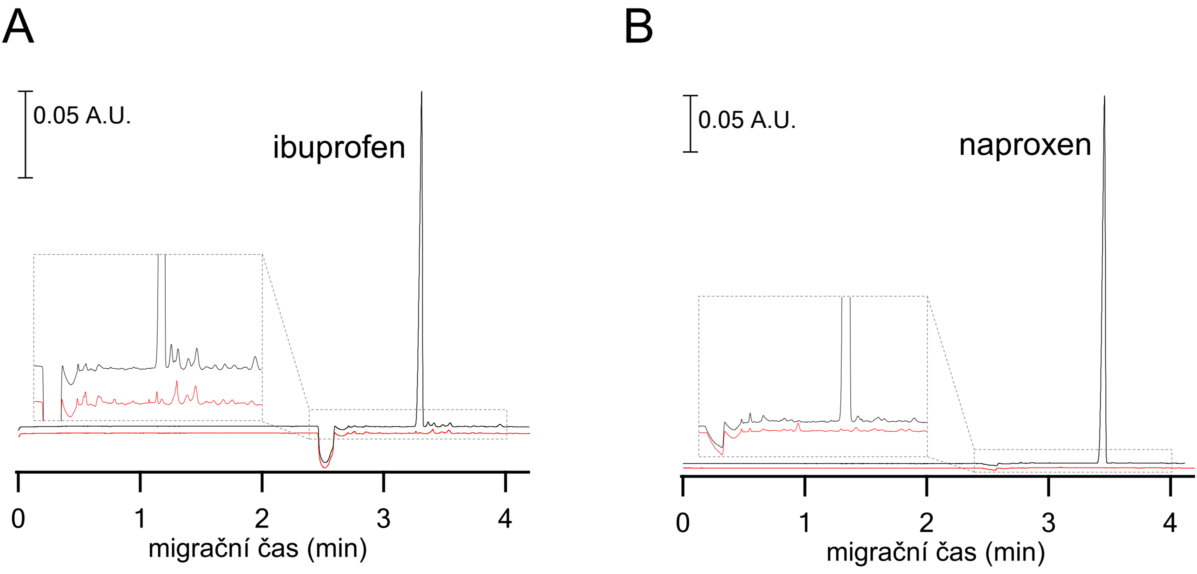


Obr. č. 13: A – Schéma a rozměry 3D tištěného držáku, B – schéma 3D tištěného držáku s nasazeným dutým vláknem, C – schéma 3D tištěného držáku s dutým vláknem uvnitř CE vialky znázorňující tubulární elektrodu a separační kapiláru.

Vyvinutá metoda byla použita pro analýzu vybraných acidických léčiv v DBS a odpadních vodách. Při zpracování je nutná ruční manipulace pouze na počátku při vyražení DBS disku. Reprezentativní elektroferogramy stanovení ibuprofenu a naproxenu v DBS eluátech (po orálním podání tabletky Ibalgin 400 mg a Nalgesin-S 275 mg) jsou na obr. č. 14 .

Univerzální charakter HF-LPME a CE by navíc mohl rozšířit použitelnost navrhovaného uspořádání na širokou škálu analytů a matric v kombinaci s MS. CE-UV analýzy při přečištění vzorků odpadních vod byly nedostačující v aspektech citlivosti i selektivity, proto byla HF-LPME spojena s metodou kapilární izotachoforézy využívající hmotnostní spektrometrii s elektrosprejovou ionizací (ITP-ESI-MS). V HF-LPME/ITP-ESI-MS byl

akceptorový roztok z dutého vlákna dávkován přímo za vedoucí elektrolyt a ITP separace byla realizována umístěním dávkovacího konce kapiláry do koncového elektrolytu.



Obr. č. 14: Přímé HF-LPME/CE-UV stanovení ibuprofenu (A) a naproxenu (B) v DBS eluátu.

4.2 Kvantitativní analýza DBS

Při odběru DBS se mikrolitrový objem kapilární krve odebírá na papírovou odběrovou kartu z paty nebo z prstu. Odběr DBS vzorků je tak vhodný pro všechny jedince včetně kojenců a starších lidí, pro které může být venepunkce příliš invazivní [1]. Krevní skvrna se po odběru suší 2-3 hodiny na okolním vzduchu za vzniku DBS. Karta se po usušení vloží do plastového sáčku s vysoušedlem a DBS se takto může skladovat případně použít pro analýzu. Vzhledem k jednoduchosti odběru lze DBS také odebírat doma, což výrazně zvyšuje ochotu pacientů účastnit se klinických studií. Vzorkovací karty s DBS jsou považovány za materiál, který není biologicky nebezpečný, a jejich přepravu do analytických laboratoří mohou provádět běžné poštovní a balíkové doručovací společnosti [1, 8]. Většina analytů v DBS je stabilní při okolní teplotě, a proto DBS umožňují jednoduchý odběr a transport krevních vzorků ze vzdálených oblastí nebo oblastí s omezenými zdroji, kde by odběr a transport plazmy nebo séra nebyly prakticky ani ekonomicky proveditelné. Dalšími výhodami odběru DBS vzorků jsou všeobecné schválení legislativními orgány, lepší stabilita analytů v suchých než v „mokrých“ vzorcích krve, výrazné snížení celkových nákladů a kompatibilita DBS eluátů se standardními analytickými technikami [1, 8, 9, 83].

Kromě výhod, uvedených v předchozím odstavci, čelí vzorkování DBS také několika výzvám. Snad nejdůležitějším parametrem ovlivňujícím odběr DBS a následnou kvantitativní analýzu je hladina hematokritu v krvi. Hladina hematokritu má přímý vliv na velikost DBS a tím i na množství krve obsažené ve vyražených dílčích částech DBS. Různé hodnoty hematokritu vyvolávají odlišnou distribuci analytů na vzorkovací kartě v důsledku nehomogenity DBS, což může mít další nepříznivé dopady na kvantitativní analýzu (viz kapitola 2.1) [9, 14].

4.2.1 Eliminace negativních vlivů

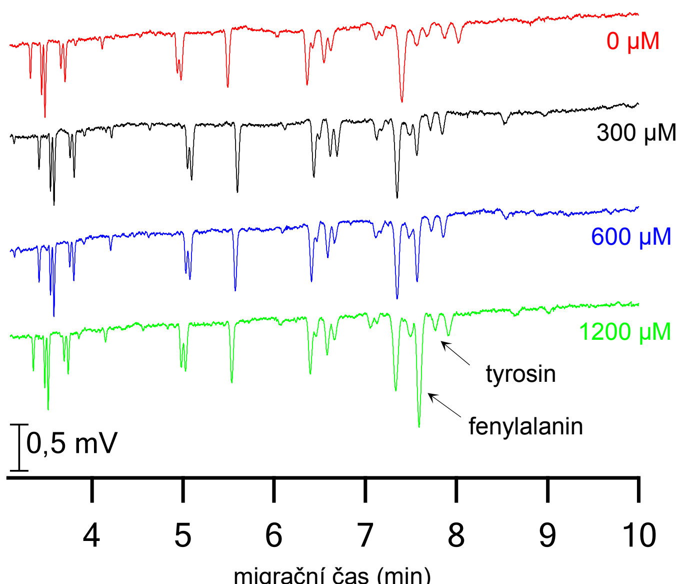
Byly popsány různé přístupy pro korekci nebo eliminaci výše uvedených nepříznivých dopadů na kvantitativní analýzu DBS. Ty zahrnovaly například vyražení celé DBS místo dílčích částí DBS [9] a použití předřezaných DBS karet [11, 31]. Výše uvedené přístupy jsou však zatíženy nutností odebírat přesný objem krve na DBS kartu pomocí kalibrovaných pipet v laboratorním prostředí. Nicméně pro odběr krve pacientem je možno použít skleněné kapiláry kalibrované na daný objem (kapitola 2.1.2), případně odběrová VAMS (kapitola 2.1.4), nebo lze aplikovat případnou korekci krevního objemu stanovením obsahu draslíku nebo sodíku v DBS vzorcích [20, 21].

Metoda, která umožňuje kvantitativní stanovení draslíku a sodíku pro výpočet přesného objemu krve a zároveň umožňuje stanovení koncentrace aminokyselin v DBS eluátech v rámci jediné analýzy je prezentována v *příloze II.* Vybrané hlavní matriční složky v DBS vzorcích (např. sodík a draslík) jsou u standardní populace konstantní a byly použity pro stanovení objemu krve nanesené na DBS kartu. CE nabízí účinný nástroj pro rychlé stanovení cílových analytů současně s objemem DBS v rámci jedné analýzy. To může významně zjednodušit kvantitativní analýzy DBS, které obvykle vyžadují použití dalších off-line analytických metod, používání interního standardu nebo časově náročnou optimalizaci na očekávané hodnoty hematokritu. Navrhovaná metoda by se tak mohla stát základním pilířem pro stanovení neznámého objemu krve v DBS a pro kvantitativní analýzu relevantních analytů (aminokyseliny, léčiva, biologické markery, biomolekuly atd.) v rámci jediné analýzy.

4.2.2 Aplikace vyvinuta pro stanovení aminokyselin

Metoda, která umožňuje kvantitativní stanovení přesného objemu krve a koncentrace aminokyselin v DBS eluátech je prezentována v *příloze II.* Kvantitativní stanovení

aminokyselin je důležité pro diagnostiku vrozených metabolických poruch u novorozenců. Při této poruše je nutná včasná diagnóza již v novorozeneckém věku a je nutné vybrané aminokyseliny vyřadit z potravy a dodržovat přísnou dietu celý život. Díky univerzálnímu charakteru vodivostní detekce lze v jediné CE analýze současně stanovit anorganické, organické a biochemické analyty. Kapilární elektroforézu s kapacitně vázanou bezkontaktní vodivostní detekcí (CE-C^4D) lze tedy použít pro stanovení přesného objemu DBS kvantifikací hlavních anorganických kationtů (K^+, Na^+, Cl^-) a pro současné stanovení vybraných aminokyselin ve vzorku DBS. Do kapilární krve byly přidány různé koncentrace fenylalaninu (300, 600 a 1200 μM) a výsledné vzorky krve byly naneseny na vzorkovací DBS karty. Takto připravené DBS vzorky byly použity pro kvantitativní stanovení fenylalaninu v kapilární krvi a pro prokázání vhodnosti navržené metody pro stanovení abnormálních hladin fenylalaninu v lidském těle, které mohou souviset s fenylketonurií. Čtyři elektroferogramy demonstrující postupně se zvyšující hladiny fenylalaninu v kapilární krvi jsou znázorněny na obr. č. 15. Červený záznam ukazuje koncentraci fenylalaninu typickou pro zdravé jedince, černý záznam odpovídá zvýšené koncentraci fenylaninu při hyperfenylalaninemii, modrý záznam odpovídá koncentraci fenylalaninu pro mírnou fenylketonurii a zelený záznam odpovídá koncentraci fenylalaninu při závažné fenylketonurii.

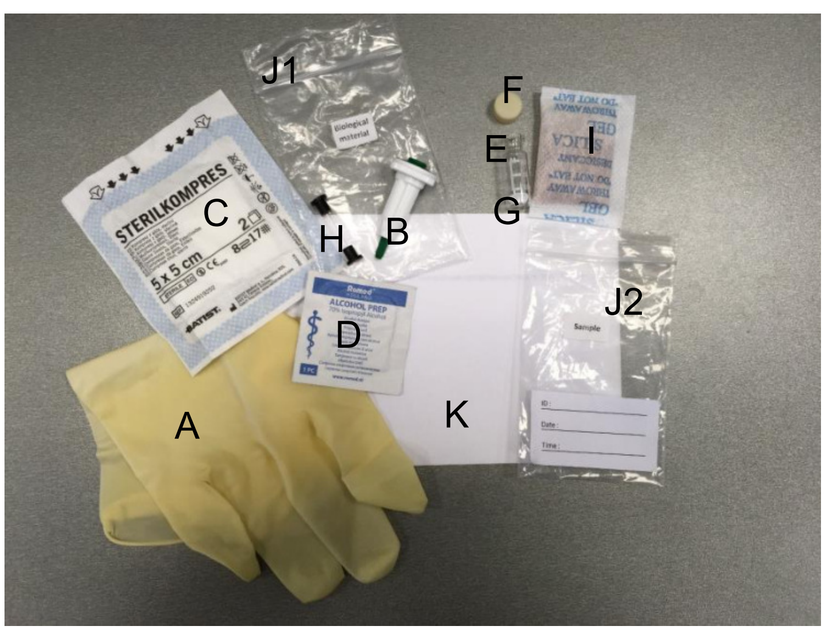


Obr. č. 15: CE-C^4D stanovení aminokyselin v DBS eluátu obsahujícím zvyšující se koncentrace fenylalaninu. Do kapilární krve bylo přidáno 0, 300, 600 a 1200 μM fenylalaninu.

4.2.3 Samoodběr s automatizovaným zpracováním DBS pro klinickou analýzu

Koncept navržený v *příloze IV* byl zaměřen na zjednodušení odběru krve a následného zpracování DBS, aby poskytl nový a uživatelsky příjemný analytický nástroj pro analýzu krve. V navrhovaném analytickém konceptu byl řešen aspekt vlastního odběru kapilární krve ve formě DBS následovaný plně automatizovanou analýzou s minimálními požadavky na analytickou laboratoř. Pro vlastní odběr mikrolitrového objemu kapilární krve z bříška prstu byla použita jednorázová souprava. Odebrané vzorky krve byly odeslány do laboratoře pro plně automatizované zpracování a analýzu DBS, které byly provedeny komerční CE přístrojem. Kvantitativní výsledky byly získány do 20 minut od dodání vzorku DBS do laboratoře.

Odběrová souprava obsahuje jednorázové zařízení pro samoodběr DBS a je určena pro jednoduchý odběr přesně definovaného objemu kapilární krve. Souprava je vhodná i pro osoby bez školení v odběru krve. Odběr krve a tvorbu DBS tak lze provádět doma ve známém prostředí a s podstatně vyšším komfortem ve srovnání s žilním odběrem v lékařských zařízeních. Dalšími výhodami domácího odběru DBS vzorků jsou méně invazivní odběr krve a kratší časová náročnost. Navíc náklady na běžné komponenty soupravy jsou minimální. Vzorkovací souprava je navržena tak, aby byla co nejuniverzálnější pro následné zpracování (viz obr. č. 16).

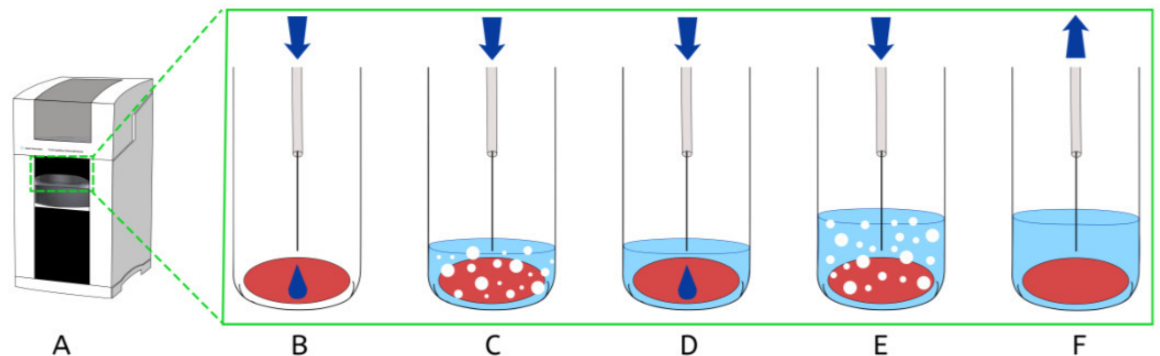


Obr. č. 16: Fotografie vybalené vzorkovací samoodběrové soupravy pro DBS. A – sterilní latexové rukavice; B – jednorázová lanceta; C – suchá gáza; D – dezinfekční gáza impregnovaná isopropyl alkoholem; E – skleněná vialka s uzávěrem (F); G – vyražený disk z DBS karty; H – odběrová kapilára se zátkami; I – sáček s vysoušedlem; J – uzavíratelné sáčky na zip s identifikačními štítky a návodem k použití, vše zabalené v obálce – K.

Následně byla práce v *příloze IV* zaměřena na vývoj plně automatizovaného procesu pro DBS eluci a CE-UV analýzu výsledného eluátu. Po vyjmutí vialky s DBS ze sáčku byla vialka uzavřena a vložena do karuselu CE autosampleru. Následné procesy jako dávkování eluentů do vialky a homogenizace vzorku (probublávání vzduchem z prázdné vialky) byly prováděny interním tlakovacím systémem CE přímo přes separační kapiláru. Jednotlivé kroky jsou podrobně popsány v tab. č. 4. Schéma tohoto automatizovaného zpracování DBS pomocí CE je znázorněno na obr. č. 17.

Tab. č. 4: CE sekvence pro plně automatizovanou DBS eluci a CE-UV analýzu. Všechny postupy byly autonomně prováděny CE přístrojem.

Akce	**Doba trvání**	**Tlak/Napětí**
1. DBS eluce		
plnění vialky 80 µl ACN	180 s	950 mbar
homogenizace eluátu vzduchem	60 s	950 mbar
plnění vialky 20 µl deionizované vody	140 s	950 mbar
homogenizace eluátu vzduchem	300 s	950 mbar
2. Kondicionování separační kapiláry		
promývání 0,1 M NaOH	60 s	950 mbar
promývání DI vodou	90 s	950 mbar
promývání roztokem BGE	120 s	950 mbar
3. CE-UV analýza		
dávkování DBS eluátu	12 s	55 mbar
CE separace a detekce	240 s	+25 kV

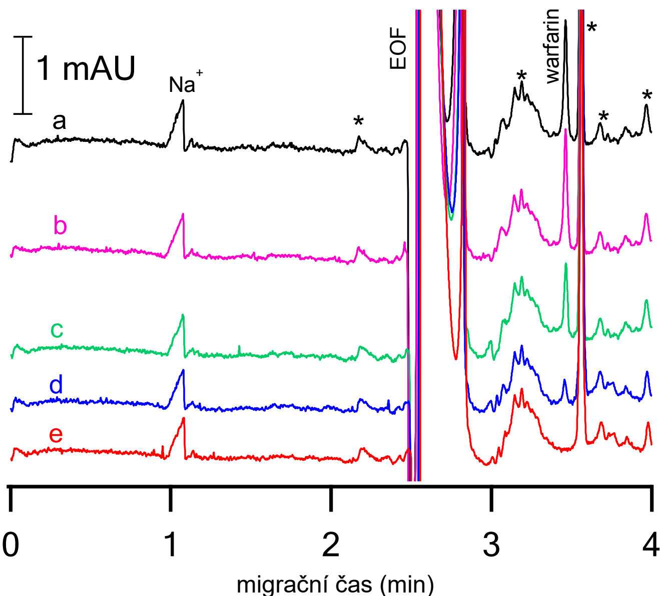


Obr. č. 17: Schéma automatizovaného zpracování DBS pomocí CE. A – umístění vialky s DBS do CE karuselu, B – plnění vialky 80 µl ACN, C – homogenizace vzduchem, D – plnění vialky 20 µl DI vody, E – homogenizace vzduchem, F – dávkování eluátu do CE.

4.2.4 Aplikace vyvinuté automatizované metody pro stanovení warfarinu

Warfarin je nejrozšířenější perorální antikoagulant používaný ve velkém počtu dlouhodobých klinických testů, které vyžadují přesnou kontrolu srážlivosti krve (trombotické a vaskulární patologie). Farmakologické účinky warfarinu se rutinně stanovují pomocí INR [94]. Hlavními průvodními nepříjemnostmi při stanovení INR jsou invazivní odběry žilní krve a pravidelné návštěvy klinických laboratoří. Alternativou, která výrazně zvyšuje komfort pacienta a zjednodušuje odběr vzorků, je stanovení warfarinu v kapilární krvi odebrané ve formě DBS. Stanovení warfarinu v DBS může nabídnout doplňkové informace k testu INR. Odběr DBS vzorků může pacient provádět doma a může představovat první krok k pohodlnějšímu terapeutickému monitorování warfarinu a zlepšení kvality pacientova života.

V *příloze IV* je představen nový analytický koncept pro analýzu warfarinu v DBS. Při léčbě warfarinem je důležité dodržovat přísná dietologická opatření, aby nedocházelo ke kolísání aktivity účinné látky. Navrhovaný koncept byl demonstrován domácím vzorkováním DBS kapilární krve obsahující warfarin, odesláním vzorků do naší laboratoře a provedením automatizované eluce a stanovení warfarinu v DBS pomocí CE. Jeden z dobrovolníků měl diagnostikovanou nadměrnou srážlivost krve, byl léčen warfarinem a sledován ve specializovaném klinickém centru. Léčba warfarinem trvala 6 měsíců a lék byl po tomto období změněn. Byli jsme tedy schopni získat a analyzovat skutečné DBS vzorky obsahující warfarin. Výsledné elektroferogramy DBS vzorkované pacientem v různých fázích léčby jsou znázorněny na obr. č. 18.

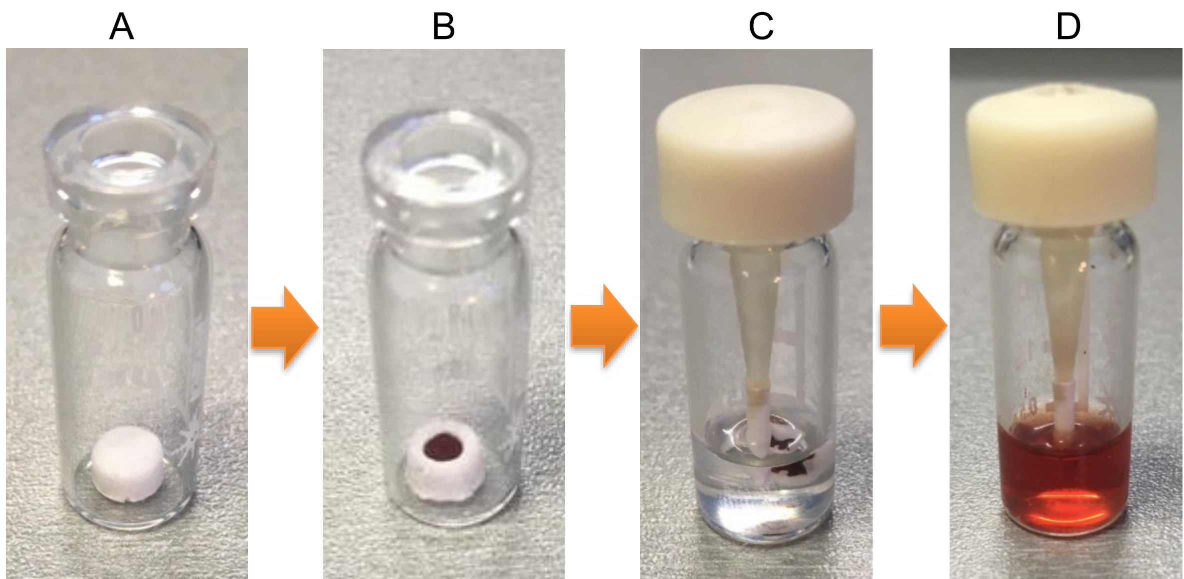


*Obr. č. 18: Automatizované CE-UV analýzy DBS po samoodběru dobrovolníkem léčeným warfarinem. Vzorky DBS byly odebrány 1 měsíc před (a), 1 den před (b), 3 dny po (c), 7 dní po (d) a 1 měsíc po (e) ukončení léčby warfarinem. * – neznámé matriční komponenty.*

4.2.5 Aplikace nových rozpustných odběrových materiálů

Vývoj alternativních odběrových materiálů je v popředí aktuálního výzkumu. Jednou z alternativ k celulózovým kartám je nedávno vyvinutý odběrový materiál VAMS, který absorbuje fixní objem krve, což umožňuje eliminaci vlivu hematokritu. VAMS mají odběrovou polymerní špičku vyrobenou z nerozpustného materiálu, která může mít vliv na extrakční výtěžnosti sledovaných analytů [95, 96]. Dalším alternativním odběrovým materiálem jsou rozpustné vzorkovací sorbenty na bázi alginátu, chitosanu a karboxymetylcelulózy [18]. Aplikace rozpustných sorbentů umožňuje odběr, transport i skladování kapilární krve obdobným způsobem jako u běžných odběrových DBS karet, navíc se složky krve mohou díky rozpustnosti sorbentů snadněji uvolňovat do DBS eluátu. Ve srovnání s celulózovými kartami však výroba alginátových a chitosanových vzorkovacích sorbentů vyžaduje poměrně sofistikované procesy, je časově náročná a používá značné množství chemických činidel (pěnidla, gelující ionty, modifikátory pH a změkčovadla). Nevýhodou je také vysoká cena chitosanu a nižší rozpustnost chitosanu a komerčních karboxymetylcelulózových materiálů [18]. Navíc rozpuštěné vzorkovací sorbenty DBS nemusí být přímo kompatibilní s následnou analýzou, proto je nutná úprava eluátů, která se obvykle provádí ručně a off-line [18].

V *příloze VI* byly řízenou lyofilizací připraveny různé polymerní pěny, které byly použity pro odběr a analýzu DBS. Polymerní pěny jsou levné, snadno se připravují, umožňují rychlé sušení odebraného malého objemu kapilární krve při okolní teplotě, úplnou rozpustnost ve vodných roztocích a přímou analýzu DBS spojením HF-LPME a CE. Sorpční polymerní pěna s přesnými rozměry byla umístěna do CE vialky a DBS byla vytvořena přímo v CE vialce, čímž se eliminovala další ruční manipulace s biologickým materiálem. Proces eluce DBS zahrnoval úplné rozpuštění polymerní pěny i DBS a zajistil zlepšenou dostupnost všech složek krve v DBS eluátech. V důsledku toho bylo dosaženo téměř úplné extrakce cílových analytů, která byla prokázána HF-LPME/CE stanovením acidických léčiv v DBS odebraných na vyrobené polymerní pěny. Složení polymerních pěn bylo optimalizováno pro odběr a analýzu DBS. Pěny byly připraveny z polyvinylpyrollidonu (PVP) nebo směsi karboxymetylcelulózy (CMC07) a oxidované-6-karboxycelulózy (OC) jednoduchým způsobem založeným na homogenizaci vodné polymerní disperze, pipetování daného objemu disperze do 96jamkové destičky a postupném zmrazení/lyofilizaci disperze. Výsledné polymerní pěny byly porézní disky jednotných rozměrů (cca 6 x 3 mm) (obr. č. 19 A) s plnou rozpustností ve standardních vodných roztocích. Na rozpustné polymerní pěny bylo pipetováno 10 µl kapilární krve (obr. č. 19 B). Následně byl vzorek krve sušen ve standardní CE vialce. Po usušení následovalo přečištění, eluce DBS pomocí HF-LPME (obr. č. 19 C-D) a analýza pomocí CE.



Obr. č. 19: Fotografie zobrazující tvorbu DBS na 6% PVP pěně s následnou DBS elucí a extrakcí. A – vložení 6% PVP pěny do CE vialky; B – DBS z kapilární krve vytvořena v polymerní pěně;

C – naplnění CE vialky 550 µl 25mM HCl a jednorázovým HF-LPME zařízením; D – průhledný DBS eluát po rozpuštění pěny a HF-LPME DBS eluátu.

V důsledku plné rozpustnosti vzorkovacích pěn bylo dosaženo vysoké extrakční výtěžnosti (ER) cílových analytů, což bylo prokázáno stanovením modelových acidických léčiv v DBS pomocí HF-LPME/CE. Do kapilární krve byla přidána léčiva a obohacená krev byla vzorkována na 6% PVP, 1% CMC07/2% OC a na další komerční odběrové sorbenty (Whatman, VAMS, Ahlstrom).

Pro DBS nanesené na 6% PVP či 1% CMC/2% OC byly hodnoty ER pro stanovení naproxenu a diklofenaku srovnatelné s ER pro DBS nanesené na kartách WhatmanTM 903. Hodnoty ER však byly 1,4krát, 1,8krát a 1,9krát vyšší pro ibuprofen, ketoprofen a warfarin v DBS nanesených na 6% PVP. Hodnoty ER pro DBS nanesené na 6% PVP byly ještě výrazně vyšší (až 2,8krát a 10,6krát) ve srovnání s DBS nanesenými na nerozpustné vzorkovací sorbenty VAMS a Ahlstrom (GenCollectTM 2.0).

Odběr krve na rozpustné polymerní pěny nabízel rychlejší dobu předúpravy a vyšší extrakční výtěžnosti ve srovnání se standardními odběrovými DBS sorbenty. Kromě toho rozpustné polymerní pěny vykazovaly nepatrné změny v koncentracích analytu pro DBS připravené z krevních vzorků s různými hodnotami hematokritu. Léčiva vzorkovaná na rozpustných pěnách a skladovaná při laboratorní teplotě byla stabilní po dobu čtyř týdnů. Nové PVP a CMC07/OC pěny proto představují atraktivní alternativu, ke standardním odběrovým DBS sorbentům, a mohou v budoucnu tvořit novou skupinu odběrových DBS sorbentů.

5 ZÁVĚR

Předložená dizertační práce se zabývá vývojem metod pro stanovení vybraných analytů v DBS, ale záběr práce má daleko širší rozsah. Kromě možností kvantifikace DBS se práce zabývá vývojem extrakčních metod a postupů pro přímé dávkování DBS eluátu do separační kapiláry. Další část je zaměřena na samoodběr a vzorkování kapilární krve za vzniku DBS a následné automatizované zpracování vzorku pro CE analýzu. V neposlední řadě je práce věnována vývoji nových rozpustných odběrových materiálů za účelem zvýšení extrakční výtěžnosti analytů z DBS a eliminace negativních vlivů při odběru vzorků krve (např. vliv hematokritu). Výsledky jsou prezentovány formou diskuze šesti vědeckých článků, které jsou připojeny jako přílohy v kapitole 9.

- Je prezentován komplexní výzkum, který je první systematickou experimentální studií zabývající se vlivem tloušťky membrány na extrakční výtěžnost při extrakcích přes SLM, které jsou přímo spojeny s CE. Přenosy modelových bazických léčiv přes porézní PP membránu jsou významně ovlivněny tloušťkou membrány a nejúčinnější přenos je získán pro nejtenčí (25 μm) membránu.
- Je představen nový koncept pro stanovení objemů kapilární krve v DBS vzorcích. Koncept je založen na principu CE-C^4D stanovení hlavních anorganických iontů (K^+, Na^+ a Cl^-). Koncentrace stanovených iontů lze použít pro výpočty přesného objemu odebrané krve v DBS. Zároveň je možné v rámci stejné analýzy provést kvantitativní stanovení konkrétních analytů. Vyvinutý koncept je vhodný například pro kvantifikaci aminokyselin ve vzorcích DBS s neznámými objemy, který je zvláště zajímavý při screeningu vrozených metabolických poruch.
- Je popsáno nové uspořádání, které umožňuje přímé spojení HF-LPME s CE pro účinné přečištění a prekoncentraci cílových analytů z komplexních biologických vzorků a jejich přímou analýzu. Navržené uspořádání je použito pro přímé stanovení vybraných modelových bazických léčiv v lidské moči a ve vzorcích DBS. Tato metoda je zvláště výhodná při analýze DBS, protože DBS eluce a HF-LPME eluentu jsou provedeny současně během jednoho kroku, po kterém následuje přímá CE-UV analýza. Doba zpracování DBS je tak snížena na minimum a ruční zpracování DBS je omezeno pouze na počáteční vyražení DBS z odběrové karty. Navrhovaný koncept HF-LPME/CE

je dále aplikován pro stanovení terapeutických koncentrací modelových acidických léčiv v DBS a může být obzvláště atraktivní pro farmakokinetické studie a biomedicínské aplikace. Kromě toho je navržená instrumentace vhodná také pro přímou analýzu dalších vysoce komplexních vzorků se stopovými koncentracemi cílových analytů.

- Je představen nový koncept samoodběru krve ve formě DBS, transport DBS do laboratoře poštou, plně automatizované zpracování a analýza DBS. Navržený koncept představuje vhodný nástroj pro klinické analýzy a může významně ovlivnit úroveň lékařské péče zainteresovaných subjektů. Eliminuje potřebu častých a pravidelných návštěv lékařských center a bolestivý odběr žilní krve obvykle nutný pro standardní klinické testy. Výsledky je navíc možné sdělovat subjektům telefonicky. Dalšími přednostmi jsou méně invazivní odběr kapilární krve pomocí jednorázové samoodběrové sady a pohodlí odběru krve ve známém prostředí doma. Přepravu DBS do laboratoře mohou provádět poštovní nebo balíkové doručovací společnosti a DBS je automaticky analyzována v laboratoři se zanedbatelným zásahem personálu. Eluce DBS a následná přímá analýza eluátu se provádí pomocí komerčně dostupného CE přístroje a kvantitativní výsledek je získán téměř okamžitě po dodání DBS do laboratoře.

- Jsou představeny polymerní pěny z PVP nebo směsi CMC07 a OC. Jejich složení je optimalizováno pro odběr a analýzu DBS. Pěny jsou připraveny jednoduchým postupem, který zahrnuje homogenizaci vodné polymerní suspenze, pipetování daného objemu suspenze do 96jamkové destičky a lyofilizaci suspenze. Výsledné polymerní pěny jsou porézní disky s jednotnými rozměry (cca 6 × 3 mm) a plně rozpustné ve standardních vodných roztocích. Rozpustné polymerní pěny umožňují odběr nepatrných objemů kapilární krve a rychlé sušení krve ve standardní CE vialce, po kterém následuje úprava DBS (eluce a HF-LPME) přímo ve vialce a přímá CE analýza. Odběr krve na rozpustné polymerní pěny nabízí kratší dobu přípravy a zlepšené extrakční výtěžnosti analytů ve srovnání se standardními odběrovými DBS sorbenty.

6 BUDOUCÍ PERSPEKTIVA

Výsledky a zkušenosti, které jsme získali v experimentech prezentovaných v této práci, přinesly nové otázky a cíle, které bychom v budoucnu rádi vysvětlili a kterých bychom chtěli v budoucnu dosáhnout.

Zde je jejich krátký výčet:

- aplikovat vyvinuté metody na další suché vzorky se složitou matricí (např. moč, sliny, hlen, mozkomíšní mok, potravinové výrobky, vzorky životního prostředí), a analýzu dalších analytů (např. polutanty, antibiotika, vitamíny, minerály, biomarkery),
- vyvinout automatizovanou metodu pro přímé spojení extrakcí přes SLM s CE, která by umožnila přímé dávkování a analýzu analytů z DBS,
- vyvinout přímé spojení HF-LPME s komerční CE, která by zahrnovala celý proces manipulace s kapalinami potřebný pro HF-LPME (impregnace HF organickým rozpouštědlem, plnění HF akceptorovým roztokem, dávkování akceptoru z HF),
- automatizovat zpracování DBS vzorků pomocí přístroje pro sekvenční analýzu přímo spojeného ke komerční CE,
- vyvinout postupy pro zvýšení citlivosti a selektivity automatizovaných CE analýz, např. pomocí zakoncentrování analytů v kapiláře, případně použití ESI-MS detekce,
- využít vyvinuté analytické metody na vzorky odebrané pomocí komerčně dostupných odběrových souprav nekvalifikovanými klinickými subjekty.

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8 SEZNAM PŘÍLOH

Příloha I:

RYŠAVÁ, L.; DVOŘÁK, M.; KUBÁŇ, P. The effect of membrane thickness on supported liquid membrane extractions in-line coupled to capillary electrophoresis for analyses of complex samples. *Journal of Chromatography A*, 2019, roč. **1596**, s. 226-232. ISSN: 0021-9673. DOI: 10.1016/j.chroma.2019.02.067.

Příloha II:

DVOŘÁK, M.; RYŠAVÁ, L.; KUBÁŇ, P. Capillary electrophoresis with capacitively coupled contactless conductivity detection for quantitative analysis of dried blood spots with unknown blood volume. *Analytical Chemistry*, 2020, roč. **92**, č. 1, s. 1557-1564. ISSN: 0003-2700. DOI: 10.1021/acs.analchem.9b04845.

Příloha III:

MIKOVÁ, B.; DVOŘÁK, M.; RYŠAVÁ, L.; KUBÁŇ, P. Hollow fiber liquid-phase microextraction at-line coupled to capillary electrophoresis for direct analysis of human body fluids. *Analytical Chemistry*, 2020, roč. **92**, č. 10, s. 7171-7178. ISSN: 0003-2700. DOI: 10.1021/acs.analchem.0c00697.

Příloha IV:

RYŠAVÁ, L.; DVOŘÁK, M.; KUBÁŇ, P. Dried blood spot self-sampling with automated capillary electrophoresis processing for clinical analysis. *Angewandte Chemie International edition*, 2021, roč. **60**, č. 11, s. 6068-6075. ISSN: 1433-7851. DOI: 10.1002/anie.202012997.

Příloha V:

MIKOVÁ, B.; DVOŘÁK, M.; RYŠAVÁ, L.; GEBAUER, P.; MALÁ, Z.; KUBÁŇ, P. At-line coupling of hollow fiber liquid-phase microextraction to capillary electrophoresis for trace determination of acidic drugs in complex samples. *Talanta*, 2022, roč. **238**, č. 2, 123068. ISSN: 0039-9140. DOI:10.1016/j.talanta.2021.123068.

Příloha VI:

RYŠAVÁ, L.; DORAZILOVÁ, J.; DVOŘÁK, M.; SEDLÁČEK P.; VOJTOVÁ L.; KUBÁŇ, P. Fully soluble polymeric foams for dried blood spot collection and direct analysis by capillary electrophoresis. *Analytica Chimica Acta*, 2023, roč. **1241**, 340793. ISSN 0003-2670. DOI: 10.1016/j.aca.2023.340793

8.1 Podíl na publikovaných článcích

Příloha I:

Lenka Ryšavá vypracovala koncepci experimentální práce, v rámci experimentální práce se věnovala přípravě a analýze vzorků pomocí CE. Spolupodílela se také na úpravě vzorků pomocí extrakce přes SLM. V rámci přípravy publikace připravila většinu kapitol, zajistila i grafickou interpretaci výsledků. Podle sytému Apollo jí v autorském kolektivu přísluší autorský podíl 80 %.

Příloha II:

Lenka Ryšavá se podílela na experimentální strategii, v rámci experimentální práce se věnovala přípravě a analýze vzorků pomocí CE pro stanovení endogenních aminokyselin z DBS. Spolupodílela se také na úpravě vzorků. V rámci přípravy publikace se podílela na části popisující analýzu aminokyselin z DBS a také na grafické podobě obrázků a abstraktu. Podle sytému Apollo jí v autorském kolektivu přísluší autorský podíl 45 %.

Příloha III:

Lenka Ryšavá se spolupodílela na experimentální práci týkající se grafického 3D návrhu a tisku extrakčních jednotek, zároveň konzultovala experimentální výsledky. Dále se zapojila do vyhodnocení a zpracování dat a také se autorsky spolupodílela na přípravě publikace především grafického abstraktu. Podle systému Apollo jí v autorském kolektivu přísluší autorský podíl 30 %.

Příloha IV:

Lenka Ryšavá navrhla a vypracovala návrh koncepce celé experimentální práce. V rámci experimentální práce se věnovala odběru vzorků, jejich následné analýze pomocí vyvinuté metody. V rámci přípravy publikace byla koordinátorkou, zajistila návaznost jednotlivých

kapitol, zajistila i grafickou stránku publikace (od grafického abstraktu až po jednotlivé obrázky), interpretace a diskusi výsledků. Podle systému Apollo jí v autorském kolektivu přísluší autorský podíl 60 %.

Příloha V:

Lenka Ryšavá se podílela na návrhu experimentálních cílů a přípravě krevních vzorků. Na publikaci se podílela na grafickém návrhu, a také na interpretaci výsledků. Zajišťovala 3D tisk držáku dutého vlákna pro vyvinutou HF-LPME metodu. Podle sytému Apollo jí v autorském kolektivu přísluší autorský podíl 20 %

Příloha VI:

Lenka Ryšavá byla zodpovědná za návrh experimentů a experimentálních strategií. Věnovala se především vývoji HF-LPME metody pro analýzu krevních vzorků na nově vyvinutých polymerních pěnách. Dále se spolupodílela na vývoji polymerních pěn a měření jejich struktury na SEM. Podle sytému Apollo jí v autorském kolektivu přísluší autorský podíl 35 %.

9 PŘÍLOHY

9.1 Příloha I

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The effect of membrane thickness on supported liquid membrane extractions in-line coupled to capillary electrophoresis for analyses of complex samples

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ABSTRACT

The effect of membrane thickness on extraction performance was systematically examined in extractions through supported liquid membranes (SLM), which were in-line coupled to capillary electrophoresis (CE). Three porous polypropylene membranes with different thickness (25, 100 and 170 μm) were used as supports for SLM extractions of model basic drugs (nortriptyline, papaverine, haloperidol and loperamide) from complex samples. The analytes were transferred through the SLMs by a pH gradient and were in-line injected, separated and quantified using a commercial CE instrument with ultraviolet (UV) detection. Transfers of the model drugs through SLM decreased with the increased membrane thickness (in the order: 25 > 100 > 170 μm) and highest transfers were achieved for the thinnest membrane. Interferences from complex sample matrices were efficiently eliminated, moreover, impregnation of the 25 μm membrane required significantly reduced volume of organic solvent. Mechanical stability of the impregnated 25 μm membrane was excellent during in-line injections, which necessitated direct contact of CE separation capillary with the SLM. Repeatability of the hyphenated SLM-CE-UV method (using the 25 μm membrane) was lower than 11% (RSD values of peak areas) and calibration curves were strictly linear in 0.5–30 μg/mL concentration range (coefficients of determination ≥ 0.997). Transfers of the basic drugs from donor solutions (standard and undiluted human urine/plasma) through the SLMs ranged from 45 to 231% and limits of detection were between 0.02 and 0.15 μg/mL.

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1. Introduction

Extractions through membranes formed by solid supports impregnated with carrier solutions were described already in 1970s [1,2]. These membranes were made of a layer of a glass fiber paper placed between two dialysis papers, the membranes were rather thick and the extractions took usually several hours to complete. Application of supported liquid membrane (SLM) for extraction purposes was first described in late 1970s [3,4] and SLMs were initially used in chemical industry [5,6]. Suitability of SLMs in sample pretreatment was later explored by Audunsson using thin sheet SLMs, which separated donor and acceptor solution streams in a flow system [7,8].

The original concept of extraction through SLM was generally accepted for sample treatment and SLMs are nowadays made of inert porous polymeric materials (usually polypropylene (PP) or polytetrafluorethylene (PTFE) foil or hollow fiber), which are impregnated with water immiscible solvents. Principles of analyte transfer through SLM were comprehensively described in former publications [5,7,8], moreover, numerous review articles can be found in the literature reporting on fundamental aspects and on applications of SLM extractions in various analytical fields [9–15].

SLM extractions rely on diffusive transfer of analytes of interest from one aqueous solution (donor solution, which is located on one side of the SLM) into the SLM and then from the SLM into another aqueous solution (acceptor solution, which is located on the opposite side of the SLM). The transfer of analytes into and from the SLM is achieved by selection of appropriate liquid membrane and by forming a pH gradient on both sides of the SLM. In the pH gradient, analytes are neutral in the donor solution, which promotes their distribution into the SLM. They are transferred as neutral species through the liquid membrane in the polymeric support and they reach the phase interface between SLM and acceptor solution.

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Acceptor solution pH is adjusted to a value, which promotes their complete ionization, and the analytes are transferred to the acceptor solution from the phase interface. Their back-extraction from acceptor into SLM is avoided since ionic analytes tend to remain in aqueous solutions. Composition of the liquid membrane and pH of the two aqueous phases play an important role in cross-SLM transfer of analytes as well as of matrix components and proper selection of these variables is essential for efficient elimination of matrix species (salts, proteins, lipids, etc.). Moreover, as the volume ratios of donor to acceptor solution are usually ranging from tens to hundreds and extraction efficiencies reach up to 90%, substantial analyte enrichment can be achieved in SLM extractions [9,10].

SLM extractions are mostly carried out off-line in purpose-made microextraction devices and resulting acceptor solutions are transferred for analyses to a suitable analytical instrumentation. This might be high performance liquid chromatography (HPLC), mass spectrometry (MS) and capillary electrophoresis (CE) for aqueous acceptors from three-phase SLM extractions or gas chromatography (GC) for organic acceptors after two-phase SLM extractions [10]. Less commonly, SLM extractions can be coupled to analytical instrumentation and injections of pretreated samples can be performed on-line with no need for manual transfer of the resulting acceptors [16]. SLM extractions were on-line coupled to HPLC, GC and other instruments [16–18], moreover, direct coupling to CE represents an attractive alternative since injection volumes are significantly reduced in CE and are well compatible with microliter volumes of acceptor solutions resulting from SLM extractions [19]. History, developments and principles of direct coupling of SLM extractions to CE were summarized in a recent tutorial [20].

In-line coupling of SLM extraction to CE was also achieved by direct contact of the separation capillary with the SLM surface and by injecting the analytes transferred through the SLM from the diffusive layer at the phase interface between SLM and acceptor solution [21]. Operational solutions in this microextraction system are stagnant (with no agitation) and transfer rates of analytes should be reciprocally proportional to the phase interface thickness according to definition of membrane permeability coefficient in [22]. Application of supporting materials with different thicknesses might thus have direct bearing on specific extraction parameters (e.g. time and efficiency), nevertheless, effects of supporting material thickness on transfer characteristics have not been investigated in SLM extractions coupled to CE. Surprisingly, effects of supporting material thickness on extraction performance have neither been investigated for planar sheet SLM extractions nor for HF-LPMEs. In the current practice, extractions through SLMs use commercially available supports with thickness of ca. 100 μm for planar sheet membranes and 200–300 μm for tubular HFs (wall thickness). Rarely, thinner (25 μm [23]) or thicker (450 μm [24]) supports were applied in SLM extractions but with no rationalization for their use.

In this contribution, three different planar membranes were investigated to comprehensively examine the effect of the supporting material thickness on transfer efficiency of SLM extractions in-line coupled to CE. It was demonstrated that supporting material thickness (and thus SLM thickness) plays an important role in the cross-SLM transfers of model analytes (basic drugs), which were faster and higher for thinner supports. Consumption of liquid membrane decreased considerably with support thickness and required 1 μL organic solvent only for impregnation of 25 μm thick PP membrane discs. Thin membranes (25 μm) were characterized by the same mechanical stability as thicker membranes (100 or 170 μm) during direct contact with CE separation capillary and efficient elimination of matrix components was achieved for extractions of undiluted human urine and plasma samples independently of the membrane thickness.

2. Materials and methods

2.1. Reagents, standard solutions and body fluids

All chemicals were of reagent grade and deionized (DI) water with resistivity higher than 18 MΩ cm was used throughout. Stock solutions of basic drugs (1 mg/mL, Sigma, Steinheim, Germany) were prepared from pure chemical (haloperidol) and from hydrochloride salts (nortriptyline, loperamide and papaverine) in methanol (Lach-Ner, Neratovice, Czech Republic) and were stored at −20 °C. Standard solutions of basic drugs were prepared by dissolving appropriate volumes of the stock solutions in DI water. Optimum BGE solution for CE-UV–vis determination of basic drugs consisted of 15 mM sodium dihydrogen phosphate with 15 mM phosphoric acid (pH 2.23) and was prepared daily. Organic solvent for impregnation of PP membranes was 1-ethyl-2-nitrobenzene (ENB, ≥ 98%, Fluka, Buchs, Switzerland). Operational solutions of HCl were prepared from concentrated HCl (37%, Lach-Ner) and solutions of NaOH were prepared from NaOH (Lach-Ner).

Physiological saline solution of 150 mM was prepared from NaCl (Pliva-Lachema, Brno, Czech Republic). Human urine samples were obtained from volunteers at the Institute of Analytical Chemistry after 24-hour collection and were kept at 4 °C for one day. Human plasma samples were purchased as lyophilized powders from Sigma, were prepared according to supplier's instructions (dissolved in 1 mL of DI water) and stored at −20 °C.

2.2. Capillary electrophoresis

A 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) was operated at a potential of + 25 kV applied at the injection side of the separation capillary for all runs. Detection of basic drugs was performed using the in-built UV–vis diode array detector at 200 nm. Separation capillary used was a fused silica capillary (75 μm ID/375 μm OD, L_{tot} = 45 cm and L_{eff} = 36.8 cm) supplied by Polymicro Technologies (Phoenix, AZ, USA). Prior to the first use, the bare capillary was flushed for 15 min each with 100 mM NaOH, DI water and BGE solution. At the beginning of each working day, the capillary was flushed for 2 min each with 50 mM NaOH, DI water and BGE solution. Between two consecutive CE analyses, the capillary was flushed with 50 mM NaOH (30 s), DI water (30 s) and BGE solution (60 s). At the end of each working day, the capillary was flushed for 2 min each with 50 mM NaOH, DI water and air. All flushing procedures were performed at a pressure of 950 mbar. Capillary temperature was maintained at 25 °C and hydrodynamic injections were performed at 50 mbar for 5 s. The CE instrument was controlled, and analytical signals were acquired, by ChemStation CE software (Agilent Technologies).

The high voltage electrode at the injection side was 5 mm shorter (G7100-60033, Agilent Technologies) than the standard electrode. The short electrode eliminated possible contamination of the low volumes of acceptor solutions due to their contact with electrode as was reported earlier [25] and ensured direct contact of the separation capillary with SLMs. The separation capillary protruded by approx. 8 mm from the short electrode.

2.3. Micro-extraction device and its function

Tailor-made microextraction devices were prepared according to the previously reported procedure [25] and were compatible with Agilent 7100 CE instrument. Each device consisted of a donor and an acceptor unit, which were separated by a flat sheet PP membrane impregnated with an organic solvent (SLM). Donor and acceptor units were scalpel-cut from 200 μL PP micropipette tips (F.L. Medical, Torreglia, Italy, Part No. 28063) to the length of 11 and 10 mm, respectively. For the 25 μm thick PP membranes, acceptor

Table 1
Parameters of PP membranes.

Membrane type	Celgard® 2500	Accurel PP 1E R/P	Accurel PP 2E-HF R/P
Thickness	25 µm	100 µm	170 µm
Porosity	55 %	n.a.	n.a.
Pore size	0.064 µm	0.1 µm	0.2 µm

n.a. – not available.

units were shortened to 7 mm (by cutting off 3 mm at the narrower end). Flat sheet PP membranes (discs with 11 mm diameter) were cut from Celgard® 2500 (Celgard LLC, Charlotte, NC, USA) and from Accurel PP 1E R/P and Accurel PP 2E-HF R/P (Membrana, Wuppertal, Germany) using a cork borer. Characteristic parameters of the three PP membranes are summarized in Table 1 and electron scanning microscope (ESM) images of the membranes are depicted in Figure S1 in Supplementary data.

The 11 mm discs of 25, 100 and 170 µm thick PP membranes were impregnated with 1, 5 and 7 µL of ENB, respectively. Immediately when the solvent soaked into the membrane pores, the disc was placed on top of a donor unit and pressed against bottom of an acceptor unit. Twenty µL of donor solution was pipetted into the donor unit followed by pipetting 20 µL of acceptor solution into the acceptor unit and extraction time was measured from this moment. Analytes present in the donor solution were diffusively transferred through the SLM based on their distribution coefficients related to the organic solvent impregnated in the membrane pores. At diffusive conditions, a decreasing concentration gradient of the transferred analytes is formed in the direction from the SLM surface and rather long extraction times are necessary to obtain equilibrium in the entire acceptor solution. In order to shorten the pretreatment time, the injection end of the separation capillary must be positioned as close to the SLM as possible. Thus, the extracted analytes were injected directly from the SLM surface in the acceptor unit of the device. Dimensions of the microextraction device were selected to fit the internal diameter of the Agilent PP sample vials (Part No. 5182-0567) and the device was placed in a stainless-steel compression spring with the following dimensions: wire thickness, 200 µm; OD, 5.8 mm; length, 7 mm; number of coils 7, (Pružiny Čermák, Brno, Czech Republic). The sample vial with the spring and the extraction device was closed with a polyurethane snap cap (Agilent Technologies, Part No. 5181-1512). The soft compression spring released the pressure during the direct contact of the separation capillary with the SLM surface and eliminated possible perforation of the SLM, see Figure S2 in Supplementary data. Microextraction devices were disposable and a new device was prepared for each extraction.

Transfer of analytes through the SLM was calculated based on the following equation:

$$Transfer\,(\%) = \frac{C_{a,final}}{C_{d,initial}} \times 100 \quad (1)$$

where $C_{a,final}$ is the final concentration of an analyte at the membrane facing acceptor solution and $C_{d,initial}$ is the initial concentration of the analyte in donor solution. Since donor and acceptor solution volumes were identical (20 µL), no volume corrections were carried out.

3. Results and discussion

3.1. Extractions through SLMs with different thicknesses

Four basic drugs were selected as model analytes. Chemical structures, pK_a values and log P values of nortriptyline, papaverine, haloperidol and loperamide are shown in Figure S3 in Supplementary data. BGE solution consisting of 15 mM NaH_2PO_4 and 15 mM H_3PO_4 at pH 2.23 [26] was used for CE-UV determination of the four basic drugs transferred through the different SLMs. PP membranes with three different thicknesses (25, 100 and 170 µm) were examined as supporting material for SLM extraction in-line coupled to CE. Initial experimental conditions involved extractions of a standard donor solution containing 10 µg/mL of the basic drugs prepared in 150 mM NaCl solution, which corresponds to NaCl concentration in physiological solution. Acceptor solution was DI water. SLMs were prepared by impregnating the membranes with appropriate volumes of ENB (exact ENB volumes can be found in Chapter 2.3), and the influence of membrane thicknesses on the cross-SLM transport was investigated for extraction time of 19 min. This time was selected as a good compromise for duration of SLM extraction since 19 min was sufficient to ensure equilibrium state at the SLM for 100 µm thick PP membranes previously [27]. Corresponding results are depicted in Fig. 1 and clearly demonstrate that highest analyte transfers were achieved for the 25 µm thick PP membrane.

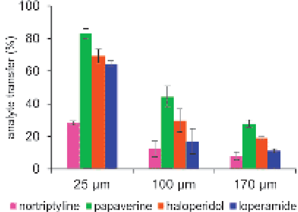

Fig. 1. Effect of membrane thickness on extraction of basic drugs. Extraction conditions: donor, 150 mM NaCl and 10 µg/mL of the four basic drugs (20 µL); acceptor, DI water (20 µL); SLM, ENB (1, 5 and 7 µL for 25, 100 and 170 µm membrane, respectively); extraction time, 19 min. CE conditions: BGE solution, 15 mM NaH_2PO_4 and 15 mM H_3PO_4 at pH 2.23; voltage, +25 kV; injection, 50 mbar for 5 s, detection wavelength, 200 nm.

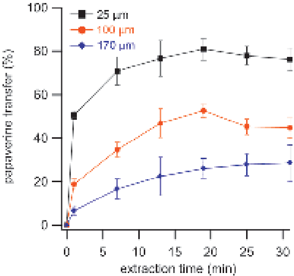

Fig. 2. Effect of membrane thickness on extraction time of papaverine. Extraction and CE conditions as for Fig. 1.

In order to confirm the above observations, time-dependent analyte transfers were determined for the three PP membranes. First CE-UV analysis was performed at 1 min extraction time and 5 additional analyses were carried out from the same SLM microextraction device in 6 min intervals. The total extraction time was 31 min and corresponding results for papaverine transfer through the three membranes are depicted in Fig. 2. At the selected extraction conditions, papaverine exhibits highest transfer through the ENB-impregnated SLMs and was thus selected for graphical presentation; similar time-dependent profiles were observed for extractions of the other three analytes and the three SLMs. Fig. 2 demonstrates rapid transfer of papaverine through the 25 µm thick membrane. More than 50% of the drug was transferred to the acceptor side of the SLM within 1 min and a plateau

(71–81% of transferred papaverine) was formed for subsequent extraction times 7–31 min. Maximum transfers of papaverine through 100 μm and 170 μm membranes were lower (45–51 % and 26–27 %, respectively) and were achieved at 13–31 min and at 25–31 min, respectively. The major differences in analyte transfers were observed in the early stage of the microextraction process demonstrating 10-fold and 5-fold improvement at 1 and 7 min extraction time, respectively, for 25 vs. 170 μm membranes. We assume that these differences were associated with significantly shorter diffusion path for 25 μm membrane and thus an accelerated transfer of analytes in the early stage of the extraction process as was theoretically elucidated by Pedersen-Bjergaard and coworkers [22]. In subsequent Chapters, all experiments were always carried out with the three membranes to investigate the effect of membrane thickness on extraction performance at semi-optimized and optimized conditions. Graphical outputs of the experiments were presented for the 25 μm thick membrane, which exhibited best extraction performance.

3.2. Optimization of the donor and the acceptor solution pH

The initial optimization of donor and acceptor solution composition was carried out with extractions of donor solutions containing 150 mM NaCl and 10 μg/mL of the four basic drugs at neutral or alkaline conditions (addition of 10 mM NaOH) and for two different acceptor solutions (DI water and 10 mM HCl). Electropherograms depicting analyte peaks after extractions at different donor and acceptor conditions are shown in Figure S4 in Supplementary data. Trace a shows extraction and in-line injection of a blank donor solution, which contained 150 mM NaCl but no drugs, and shows no analytical signals at migration times of the four analytes. The four additional traces (b – e) show electropherograms for extractions and in-line injections of the four basic drugs at different donor and acceptor conditions. Extractions of the drugs into DI water favoured transfers of papaverine, see traces b and d, whereas considerable increase in transfer of nortriptyline and haloperidol was observed for 10 mM HCl acceptors. An increase in transfer efficiency of all analytes was observed for extractions from alkaline donor solutions (traces d and e) compared to extraction from DI water (traces b and c). Highest transfers for nortriptyline, haloperidol and loperamide were achieved for extractions from alkaline donor solutions into acidic acceptor solutions and additional investigations including fine-tuning of NaOH and HCl concentrations were carried out. On the other hand, extractions of papaverine were not consistent with the above observations and highest transfer of papaverine was obtained from saline donor solution (without pH adjustment) into DI water as acceptor. We assume that the different papaverine behaviour was associated with its lowest pK_a (6.0) and log P (3.08) values (see Figure S3 in Supplementary data). Thus, if papaverine was the target analyte, different extraction conditions should be used preferentially.

3.2.1. Acceptor solution pH

Further optimizations were carried out with donor solutions consisting of different matrices (150 mM NaCl, undiluted human urine and undiluted human plasma) and only the results for body fluid samples are presented graphically. Donor solutions were adjusted by addition of 1 M NaOH to achieve final concentration of 10 mM NaOH and effect of acceptor solution composition (0–25 mM HCl) on transfers of the four drugs (spiked at 10 μg/mL) was investigated. Fig. 3 shows transfers of the four analytes from urine (panel A) and plasma (panel B) samples and confirms the previously observed improvement of transfers achieved at acidic acceptor conditions. The transfers improved most significantly in the 0–10 mM HCl concentration range and a marginal improvement was achieved by further acidification of acceptor solution to

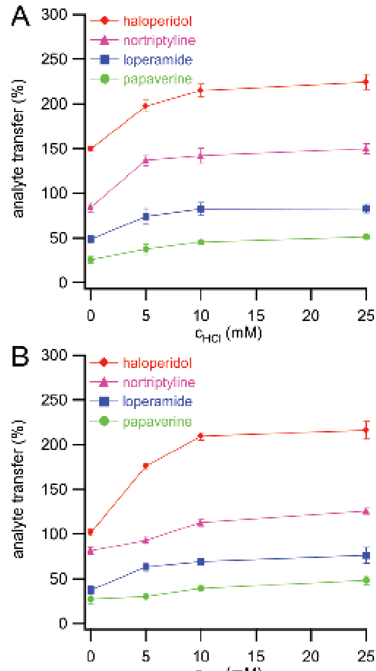

Fig. 3. Effect of HCl concentration in acceptor solution on SLM extractions. Extraction conditions: (A) donor, human urine spiked with 10 μg/mL of four basic drugs, pH adjusted by addition of 10 mM NaOH; (B) donor, human plasma spiked with 10 μg/mL of four basic drugs, pH adjusted by addition of 10 mM NaOH; SLM, 25 μm PP membrane impregnated with 1 μL ENB; extraction time, 19 min. CE conditions as for Fig. 1.

25 mM HCl. As the CE system was more susceptible to the low pH and high conductivity of the injected 25 mM HCl acceptor (broader peaks due to reduced stacking of the injected zone), 10 mM HCl was finally selected as the most convenient acceptor solution.

3.2.2. Donor solution pH

The same three matrices spiked with the four drugs (10 μg/mL) were extracted into 10 mM HCl acceptor solution and composition of the donor solutions was adjusted by addition of different volumes of 1 M NaOH solution to achieve final concentrations of 0–25 mM NaOH. Transfers of analytes as a function of donor NaOH concentration are shown in Fig. 4. A significant increase in transfer of haloperidol was observed for all alkaline donor solutions. Transfer of nortriptyline and loperamide increased for 5 and 10 mM NaOH and a reduction was obtained for 25 mM NaOH. Papaverine transfer was lowered by alkalization of donor solutions and as discussed before, best performance was achieved for extractions from body fluids with no pH adjustments. Based on these measurements, 10 mM NaOH solution was selected as the optimum donor solution composition.

3.3. Extractions of body fluids through membranes with different thicknesses

The optimum extraction conditions (10 mM NaOH as donor phase and 10 mM HCl as acceptor phase) were used for extractions of the four basic drugs from undiluted urine and plasma

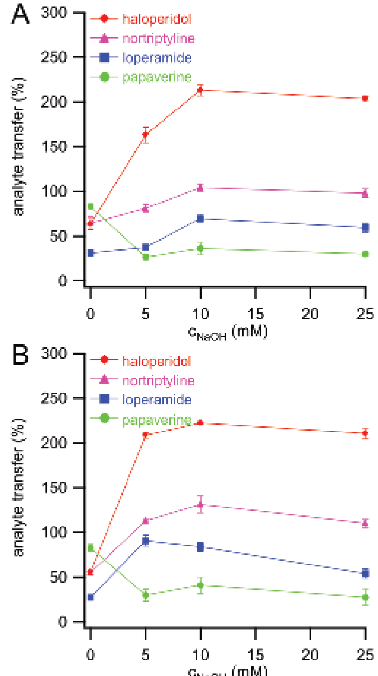

Fig. 4. Effect of NaOH concentration in donor solution on SLM extractions. Extraction conditions: (A) donor, human urine spiked with 10 μg/mL of four basic drugs, (B) donor, human plasma spiked with 10 μg/mL of four basic drugs; acceptor 10 mM HCl; SLM, 25 μm PP membrane impregnated with 1 μL ENB; extraction time, 19 min. CE conditions as for Fig. 1.

samples through the three PP membranes (25, 100 and 170 μm thick). Extraction time was selected as 19 min. Graphical presentation of analyte transfers through the three membranes extracted from plasma sample is depicted in Figure S5 in Supplementary data and clearly demonstrates advantages of using the thinnest PP membrane over the two thicker ones. About two-fold and four-fold better transfers were obtained for extractions through 25 μm membranes in comparison to 100 and 170 μm membranes, respectively. The same differences in transfer efficiency (about two- and four-fold) were obtained for extractions from urine samples, too.

3.4. Extraction time

Transfers of basic drugs to the phase interface were measured for extraction times 1–31 min in 6 min intervals. Fig. 5 illustrates gradually increasing transfers of all analytes from donor solution of human plasma to the phase interface in the first 13 min followed by a plateau and a decrease in the transfers of the analytes for 13–19 min and 25–31 min, respectively. Highest transfers of analytes were obtained for extraction times between 13 and 19 min. Repeatability of analyte transfers for different extraction times was considered and relative standard deviation (RSD) values up to 20% were observed for 1–13 min extractions, which improved considerably (RSD ≤ 9.9%) for longer extraction times (19–31 min). The improved RSD values are consistent with better equilibration of drug concentrations at the phase interface for longer extraction times. Since extraction time is typically the crucial parameter, which determines duration of the entire analytical protocol, and high repeatability is essential for good analytical performance, 19 min extraction time was selected for all subsequent experiments. If higher sample throughput was required, shorter extraction times (e.g. 7 and 13 min) might be applicable, however, at the expense of slightly reduced analyte transfers and extraction process repeatability.

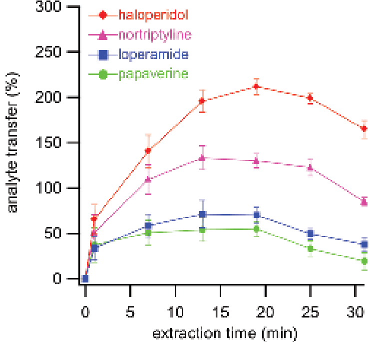

Fig. 5. Effect of extraction time on SLM extractions of basic drugs. Extraction conditions: donor, human plasma spiked with 10 μg/mL of the four basic drugs in 10 mM NaOH; acceptor, 10 mM HCl; SLM, 25 μm PP membrane impregnated with 1 μL ENB; extraction time, 19 min. CE conditions as for Fig. 1.

3.5. Analytical performance

Analytical performance of in-line coupling of SLM extraction to CE analysis for determination of basic drugs was examined at the following conditions. SLMs were formed by impregnating 25 μm thick PP membranes with 1 μL of ENB. Acceptor solutions were 10 mM HCl and donor solutions contained various concentrations of basic drugs and were adjusted by addition of 1 M NaOH to achieve final concentration of 10 mM NaOH. Extractions were carried out for 19 min and extracted analytes were injected into CE directly from the SLM surface in the acceptor unit of the microextraction device. For analyses of standard solutions, the basic drugs were always added to saline solution containing 150 mM NaCl. For analyses of real samples, drug-free human plasma and urine samples were used undiluted and were first extracted as blank samples without spiking with the drugs. No analytical signals were detected at migration times of the four analytes demonstrating that the drugs were not present in human plasma and urine samples or were at concentrations below the limit of detection (LOD) of the method. In analyses of body fluids after drug ingestion, interferences from metabolic products of the parent drugs might be observed. In such case, application of MS detector would be the proper approach to differentiate between the metabolites and the parent drugs.

The drug-free plasma and urine were spiked with the four basic drugs and repeatability of the analytical technique was evaluated for extractions of donor solutions containing 10 μg/mL of the four basic drugs from five individual microextraction devices. Repeatability, expressed as RSD values of peak areas for five analyses of the same sample (urine and plasma), was within the 4.0–9.9% range and is summarized in Table 2. Repeatability was also measured for analyses of five unique urine and three unique plasma samples and RSD values were lower than 11% demonstrating insignificant effect of sample matrix on the extraction and analytical process. The RSD values are summarized in Table 2 and comply with requirements on repeatability in bioanalytical method validation [28]. Table 2 also summarizes coefficients of determination for linear-

Table 2
Analytical parameters for the determination of basic drugs by in-line coupling of SLM extraction to CE. CE conditions as for Fig. 1. Extraction conditions as for Fig. 6. Calibration range = 0.5–30 µg/mL.

	Nortriptyline	Papaverine	Haloperidol	Loperamide
	Standard			
Transfer (%) 10 µg/mL	159.3	52.8	231.1	89.8
RSD (%) 10 µg/mL	5.1	3.5	4.3	7.3
r^2	0.999	0.998	0.997	0.998
LOD (µg/mL)	0.03	0.13	0.02	0.07
LOQ (µg/mL)	0.10	0.42	0.08	0.23
	Human urine			
Transfer (%) 10 µg/mL	145.8	52.3	212.8	76.8
RSD (%) 10 µg/mL[a]	7.3	6.7	5.1	7.6
RSD (%) 10 µg/mL[b]	9.7	7.1	7.0	9.9
r^2	0.999	0.998	0.997	0.998
LOD (µg/mL)	0.04	0.13	0.02	0.08
LOQ (µg/mL)	0.14	0.42	0.08	0.28
	Human plasma			
Transfer (%) 10 µg/mL	105.2	45.1	210.5	65.7
RSD (%) 10 µg/mL[a]	4.0	6.9	6.8	9.9
RSD (%) 10 µg/mL[c]	10.8	8.9	7.4	10.9
r^2	0.997	0.997	0.998	0.999
LOD (µg/mL)	0.05	0.15	0.03	0.09
LOQ (µg/mL)	0.16	0.50	0.09	0.31

[a] one urine and one plasma sample analysed 5-times ($n = 5$) after spiking with 10 µg/mL of drugs.
[b] five unique urine samples analysed 2-times ($n = 10$) after spiking with 10 µg/mL of drugs.
[c] three unique plasma samples analysed 2-times ($n = 6$) after spiking with 10 µg/mL of drugs.

ity measurements in 0.5–30 µg/mL concentration range ($r^2 = 0.997 - 0.999$), analyte transfers calculated for 10 µg/mL concentrations of analytes (45–231%) and LODs (0.02 – 0.15 µg/mL)/LOQs (0.08 – 0.5 µg/mL) of the SLM-CE-UV method. LODs and LOQs were defined as the lowest concentration of analyte giving analytical signal three and ten times higher than baseline noise (S/N = 3 and S/N = 10), respectively, and are suitable for most clinical applications [29].

Resulting electropherograms demonstrating in-line coupling of SLM extraction to CE-UV analysis of blank human urine and human urine spiked with the four basic drugs at 0.5 and 10 µg/mL concentrations are depicted in Fig. 6A and SLM-CE-UV analyses of blank human plasma and human plasma spiked with the basic drugs at 0.5 and 10 µg/mL concentrations are depicted in Fig. 6B.

4. Conclusions

A comprehensive investigation is presented, which is the first systematic experimental study of the effects of membrane thickness on extraction performance of SLM extractions in-line coupled to CE. Transfers of model basic drugs through porous PP membranes with different thicknesses were significantly influenced by the membrane thickness and most efficient transfers were obtained for the thinnest (25 µm) membrane. In addition, equilibrium of the extraction process was achieved faster and consumption of organic solvents for membrane impregnation was reduced for the 25 µm PP membrane. Only 1 µL of organic solvent was necessary per extraction, which was 5-fold and 7-fold lower than for 100 and 170 µm PP membranes. Mechanical stability of the SLM was not affected by membrane thickness and multiple injections from SLM surface, which required direct contact of the separation capillary with the membrane, were possible for the 25 µm thick membrane. Matrix components were efficiently eliminated from undiluted human body fluids (urine and plasma) demonstrating usefulness of thin SLMs in direct CE analyses of complex biological samples. This fundamental study experimentally verified that membrane thickness plays a key role in cross-SLM transfers of model analytes and confirmed the formerly elucidated principles of extraction through SLM. Thin membranes represent an interesting supporting material due to their more efficient cross-membrane transfer/reduced environmental impact and findings reported in this manuscript might be applied more generally to SLM extraction and HF-LPME techniques.

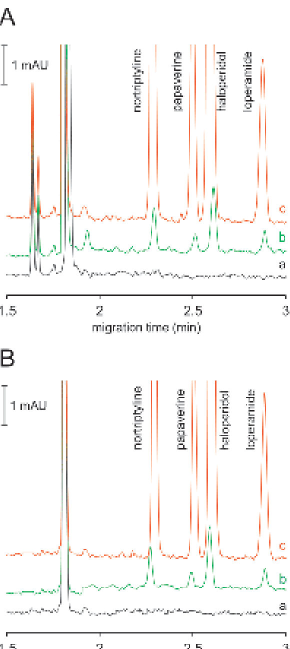

Fig. 6. In-line coupling of SLM extraction to CE for analysis of basic drugs in human urine (A) and human plasma (B). Extraction conditions as for Fig. 5 except donor, (a) blank urine/plasma in 10 mM NaOH, (b) urine/plasma spiked with 0.5 µg/mL of the four drugs in 10 mM NaOH, (c) urine/plasma spiked with 10 µg/mL of the four basic drugs in 10 mM NaOH; acceptor, 10 mM HCl. CE conditions as for Fig. 1.

Acknowledgments

Financial support from the Czech Academy of Sciences (Institute Research Funding RVO:68081715) and the Grant Agency of the Czech Republic (Grant No. 16-09135S) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.02.067.

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Supplementary data

The effect of membrane thickness on supported liquid membrane extractions in-line coupled to capillary electrophoresis for analyses of complex samples

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Figure S1. ESM images of three PP membranes with different thickness.

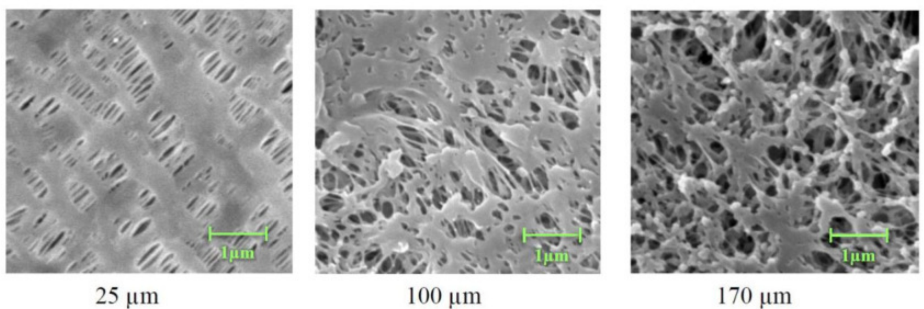


Figure S2. Schematic drawing of the process for in-line coupling of microextraction across SLM to a commercial CE instrument. (A) Sample vial before sample injection, (B) sample vial during injection of the pretreated sample.

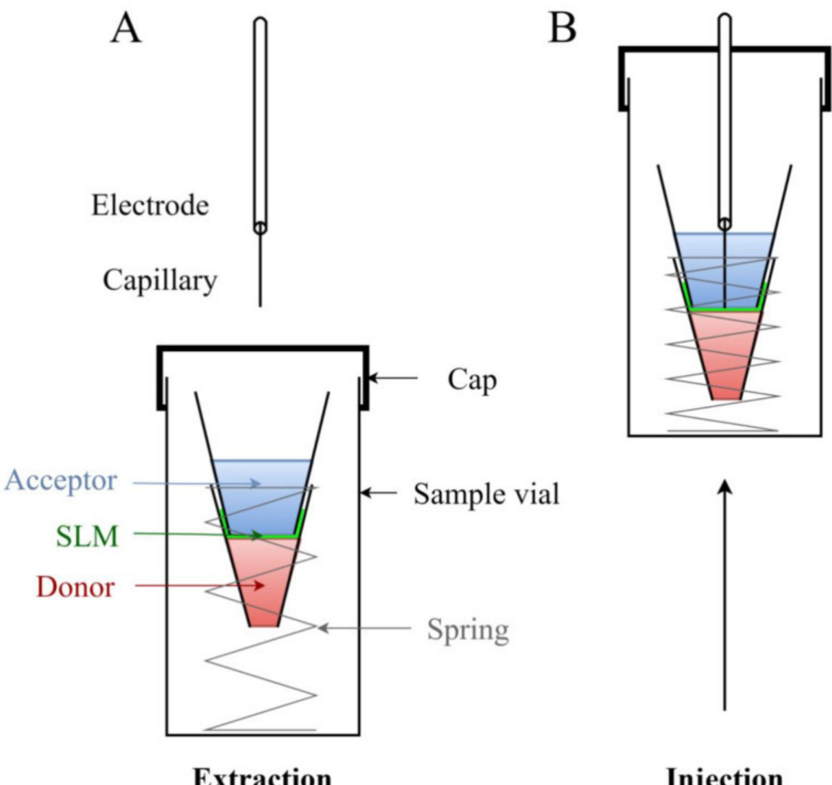


Figure S3. Chemical structures, pK_a values and log P values of the four basic drugs; (www.drugbank.ca, 16th December 2018).

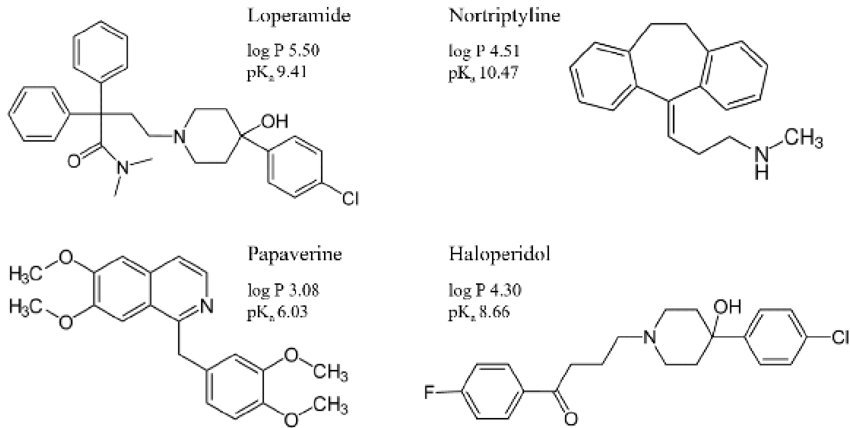
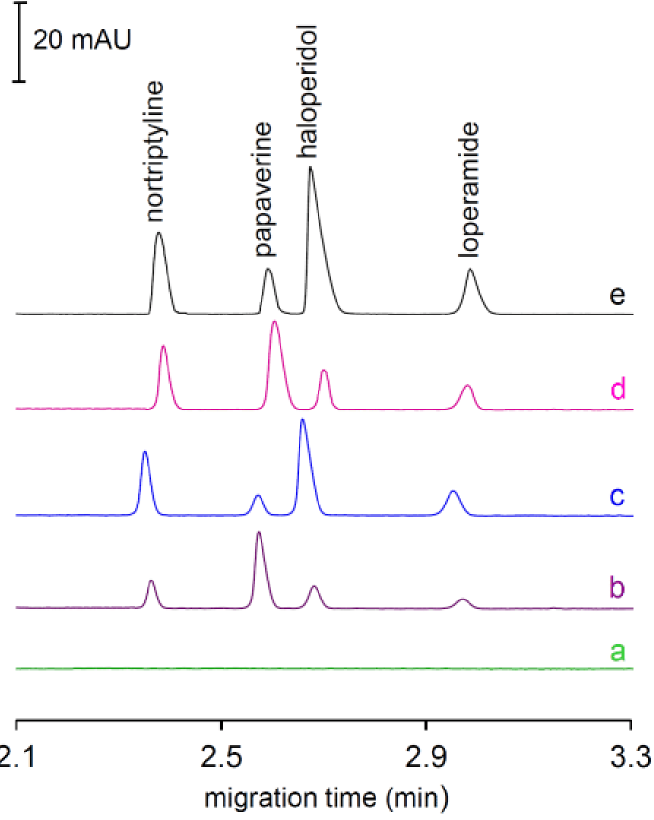
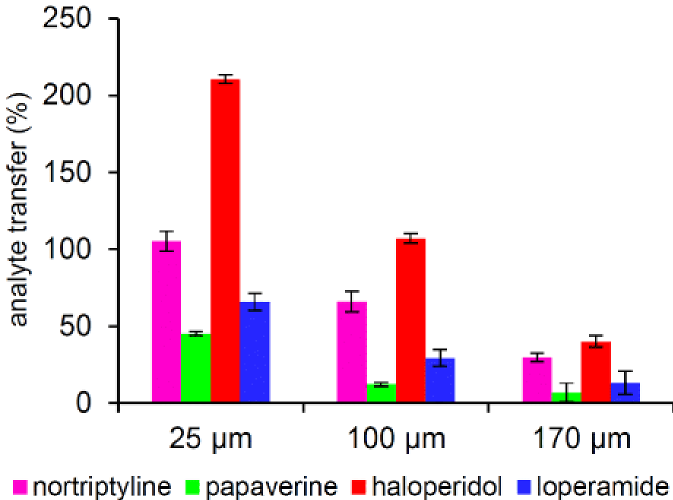


Figure S4. Transfer of basic drugs measured by in-line coupling of SLM extraction to CE at various conditions of donor and acceptor solutions. Extraction conditions: (a) donor, 150 mM NaCl; acceptor, DI water; (b) donor, 150 mM NaCl spiked with 10 μg/mL of four basic drugs; acceptor, DI water; (c) donor, 150 mM NaCl spiked with 10 μg/mL of four basic drugs in 10 mM NaOH; acceptor, DI water; (d) donor, 150 mM NaCl spiked with 10 μg/mL of four basic drugs; acceptor, 10 mM HCl; (e) donor, 150 mM NaCl spiked with 10 μg/mL of four basic drugs in 10 mM NaOH; acceptor, 10 mM HCl. CE conditions: BGE solution, 15 mM NaH_2PO_4 and 15 mM H_3PO_4 at pH 2.23; voltage, + 25 kV; injection, 50 mbar for 5 s, detection wavelength, 200 nm.



S4

Figure S5. Effect of membrane thickness on SLM extraction of human plasma. Extraction conditions: donor, human plasma spiked with 10 µg/mL of the four basic drugs in 10 mM NaOH; acceptor, 10 mM HCl; SLM, ENB (1, 5 and 7 µL for 25, 100 and 170 µm membranes, respectively); extraction time, 19 min. CE conditions as for Figure S4.



9.2 Příloha II

Article

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Capillary Electrophoresis with Capacitively Coupled Contactless Conductivity Detection for Quantitative Analysis of Dried Blood Spots with Unknown Blood Volume

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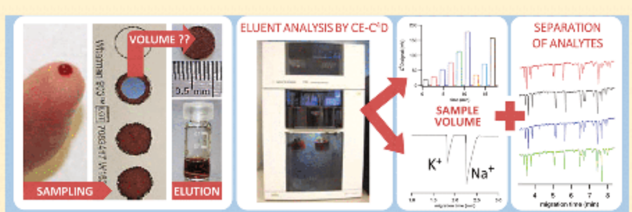
Supporting Information



ABSTRACT: Blood volume in dried blood spot (DBS) analysis is assumed to be constant for DBS punches with a fixed area. However, blood volume in the punch is dependent on several factors associated with the blood composition and is preferentially normalized by off-line analysis for quantitative purposes. Instead of using external instrumentation, we present an all-in-one approach for the simultaneous determination of exact blood volume in the DBS punch and the quantitation of target analytes. A DBS is eluted with 500 μL of elution solvent in a sample vial, and the eluate is directly subjected to an automated analysis by capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C^{4}D). The capillary blood volume in the eluate is calculated from the concentrations of the inorganic blood constituents (K^+, Na^+, or Cl^-) determined by CE-C^{4}D, which are linearly proportional to the blood volume originally sampled onto the DBS card. Alternatively, conductivity of the DBS eluate can be used for the blood volume determination by using C^{4}D in a nonseparation flow-through mode. The methods are suitable for the determination of blood volume in unknown DBS samples by punching out the entire DBS or by subpunching a small section of a large DBS with variations of the true vs the determined volume ≤5.5%. Practical suitability was demonstrated by the simultaneous CE-C^{4}D determination of K^+ and Na^+ (for DBS volume calculation) and amino acids (target analytes) in unknown DBS samples. Quantitative analysis of selected amino acids (related to inborn metabolic disorders) in the unknown DBS was compared with a standard analytical procedure using wet-blood chemistry, and an excellent fit was obtained. The use of CE-C^{4}D represents an important milestone in quantitative DBS analysis since the detection technique is universal, and the separation technique enables the determination of cations and/or anions and the use of multiple detectors, which further enhance selectivity/sensitivity of the analysis and the range of detectable analytes.

Human blood is predominantly sampled in a "wet" format, and the analysis of venous blood (serum or plasma) is the golden standard in clinical applications.[1] An alternative technique for blood sampling is the collection of capillary blood in form of dried blood spots (DBSs).[2] Since the introduction in 1963,[3] DBS sampling has been widely adopted for the determination of metabolic disorders in newborns[4−6] and has also gained significant interest in pharmacokinetic, toxicokinetic, and therapeutic drug monitoring studies.[7,8]

In DBS sampling, a microliter volume of capillary blood is collected on a paper-based sampling card from a heel prick or a finger prick. DBS sampling is thus suitable for all individuals including infants and elderly people, for whom venipuncture might be too invasive.[1] The spot is then dried for 3 h at ambient air to form the DBS, the card is packed into a plastic bag with a desiccant, and the DBS can be stored at ambient conditions for further use. Due to the sampling simplicity, DBSs can also be self-collected at home, which significantly increases the willingness of patients to participate in clinical studies.[7,8] The sampling cards with the DBSs are considered non-biohazardous material, and their transport to analytical laboratories can be accomplished by regular mail and parcel delivery companies.[1,6] Most analytes in DBSs are stable at ambient temperature for days to weeks, and thus, DBSs enable simple sampling and transport of blood samples from remote

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areas or areas with limited resources, where plasma/serum sampling and transport would not be practically/economically feasible. Further features of DBS sampling are general acceptance of DBS samples by legislative authorities, better stability of analytes in dry compared to wet blood samples, significant reduction of overall costs, and compatibility of DBS eluates with standard analytical techniques.[1,5−7]

Besides the benefits evidenced in the former paragraph, DBS sampling also faces several challenges. The small blood volume, which is one of the DBS benefits, limits the possibility of performing replicate analyses and simultaneously requires highly sensitive but expensive analytical methods, such as GC/MS/MS and LC/MS/MS.[1] The DBS is usually processed by punching a predefined part of the DBS, eluting, extracting, centrifuging, evaporating the extract to dryness, and reconstituting it with a suitable solvent, which are time and labor consuming. Automation of DBS elution/analysis is still in its infancy, and although systems for hyphenation of DBS elution to HPLC[9,10] and for direct injection from DBS into MS[11] were presented, they are rather complex and costly. DBS sampling is not suitable for air-sensitive and volatile analytes, moreover, analyte concentrations in capillary blood might differ from those in venous blood, and the correlation between these two matrices must be established before a clinical assay.

Perhaps the most important parameter influencing DBS sampling and subsequent quantitative analysis is the hematocrit level (volume percentage of blood cells) in blood. Hematocrit levels vary between 37% and 51% for healthy adults,[12] however, they can differ more significantly in specific populations, e.g., neonates, children, and people living at high altitudes.[8] The hematocrit level has a direct bearing on the DBS size and, thus, on the amount of blood in the subpunched part of the DBS. Moreover, different hematocrit levels induce different analyte distribution on the DBS card due to the DBS nonhomogeneity and the so-called volcano effect, which can have further adverse effects on the quantitative analysis.[7,13]

Various approaches were described for the correction or elimination of the above-reported adverse effects on the quantitative DBS analysis. These included, for example, punching the whole DBS instead of a DBS subpunch[7] and the use of perforated or precut DBS cards.[14,15] Nevertheless, these two approaches are burdened by sampling of an exact blood volume to the DBS card and are not compatible with one of the major DBS advantages, i.e., sampling at home by a nonskilled person with no need for a precise volumetric device. Correction of the blood volume by the determination of potassium or sodium content in the DBS subpunch was suggested, nevertheless, this approach requires additional instrumentation/analyses.[16,17] Standardization of the hematocrit in calibration standards close to the expected hematocrit levels of the blood samples is possible but is time-consuming/laborious.[7] The differences in sampled blood volumes might be also eliminated by the recently developed volumetric absorptive microsampling (VAMS) devices, which absorb a fixed blood volume,[18] or corrected by conductivity measurements of the final DBS eluate before analysis.[19] Unfortunately, the use of VAMS and conductivity measurements is limited by a rather high cost of the VAMS devices[20] and by the use of a lab-made ring disc electrode for conductivity measurements,[19] respectively.

Capillary electrophoresis (CE) is the analytical method of choice for rapid analyses of minute sample volumes[21,22] and is thus perfectly suited to the analysis of DBSs. Moreover, CE enables simultaneous determination of multiple sample constituents with different physicochemical properties when combined with a universal detector, such as with a capacitively coupled contactless conductivity detector (C^4D).[23] These characteristics are considered useful for quantitative analyses of particular analytes in DBS samples and for correction of varying blood volumes associated with DBS sampling. A novel analytical approach is presented in this contribution, which is based on the use of a commercial CE-C^4D instrument, it enables the determination of the exact blood volume in unknown DBS samples by quantifying major inorganic ions in the DBS eluate or by measuring the eluate conductivity and is fully automated. In the optimized procedure, K^+, Na^+, and cationic analytes (amino acids related to inborn metabolic disorders) are simultaneously determined by CE-C^4D in the DBS eluate. Concentrations of K^+ and Na^+ are used for the calculation of the exact blood volume in the unknown DBS sample, and the volume is subsequently used for the determination of the original amino acid concentrations. The proposed technique can be further extended to the anionic analytes by using Cl^- for the DBS volume calculations. Moreover, the nondestructive character of C^4D favors this technique also for simultaneous coupling with other commercially available CE detectors (UV−vis, MS, and LIF),[24,25] which might further enhance analytical selectivity/sensitivity and extend the range of detectable analytes.

■ EXPERIMENTAL SECTION

Reagents, Standard Solutions, and DBS Samples. All chemicals were of analytical reagent grade, and deionized (DI) water had a resistivity higher than 18 MΩ·cm. Stock solutions of sodium, chloride, and potassium (1 M) were prepared from NaCl and KCl, respectively (Pliva-Lachema, Brno, Czech Republic) in DI water. Details on the preparation of stock solutions of amino acids are given in the Supporting Information.

All stock solutions were stored in a refrigerator at 4 °C. Standard solutions of inorganic ions and amino acids were prepared by dissolving appropriate volumes of the stock solutions in DI water, DI water/methanol (50/50, v/v), or pure methanol (Lach-Ner, Neratovice, Czech Republic). A 100 mM NaOH solution for separation capillary conditioning was prepared from NaOH pellets (Lach-Ner) in DI water. DBS samples were obtained by spotting the capillary blood from a finger prick onto a Whatman 903 Protein Saver sampling card (GE Healthcare Ltd., Cardiff, UK). The DBS samples were collected from volunteers at the Institute of Analytical Chemistry who signed written informed consent.

Capillary Electrophoresis. CE analyses were performed with a 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with an Admet C^4D (Admet, Prague, Czech Republic). The optimum background electrolyte (BGE) solution consisted of 1.6 M acetic acid with 0.1% (v/v) Tween 20 and 1 mM 18-crown-6 at pH 2.1. The BGE solution was prepared daily from a 5 M stock solution of acetic acid (≥99.9%, Fluka, Buchs, Switzerland), 1% (v/v) stock solution of Tween 20 (Sigma), and 10 mM stock solution of 18-crown-6 (Sigma). Inorganic cations and amino acids were separated at a potential of +30 kV applied at the injection side of the separation capillary. For the analyses of inorganic anions, the polarity of the electric potential was reversed to −30 kV. C^4D was operated at 1.84 MHz and 50 V_{pp} through a fused silica (FS) separation capillary (25 μm i.d./375 μm o.d., L_{tot} = 50 cm

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and L_{eff} = 37 cm) supplied by Polymicro Technologies (Phoenix, AZ, USA). Prior to the first use, the bare capillary was preconditioned by flushing with 100 mM NaOH (15 min), DI water (5 min), and BGE solution (5 min). Between two consecutive CE analyses, the capillary was flushed with 100 mM NaOH (2.5 min), DI water (1.5 min), and BGE solution (5 min). At the end of each working day, the capillary was flushed with 100 mM NaOH (5 min), DI water (5 min), and air (5 min). All flushing procedures were performed at the pressure of 950 mbar. Capillary temperature was maintained at 25 °C, and hydrodynamic injections were performed at 100 mbar for 30 s. The CE instrument was controlled, and the analytical signals were acquired by ChemStation CE software (Agilent Technologies).

The high voltage electrode (P/N G7100-60033, Agilent Technologies) at the injection side was 5 mm shorter than the standard electrode at the capillary outlet. The short electrode eliminated the possible contamination of the DBS eluate by liquid residues located between the tubular electrode and the separation capillary and simultaneously also the possible contamination of the electrode by the DBS eluate. The separation capillary protruded by approximately 8 mm from the short electrode to ensure a proper injection from the Agilent 7100 CE glass snap-cap vial (2 mL; P/N 5182-9697) containing 500 μL of the DBS eluate. The injection end of the separation capillary was immersed into a glass vial with DI water prior to the injection of the DBS eluate in order to avoid the contamination of the DBS eluate by the BGE solution.

Conductivity Measurements. Conductivity measurements were performed using the same CE-C^4D instrument (Agilent Technologies/Admet). A short segment of a FS capillary (25 μm i.d./375 μm o.d., L_{tot} = 30 cm and L_{eff} = 17 cm) from Polymicro Technologies was used for conductivity measurements, and the C^4D/capillary temperature was maintained at 25 °C. The bare capillary was preconditioned by flushing with 100 mM NaOH (5 min) and DI water (2 min). For C^4D measurements, a standard solution or a DBS eluate was flushed through the capillary for 0.75 min, flushing was stopped for another 0.25 min, during which the conductivity was measured, and finally, the capillary was flushed with DI water for 1 min. Between the C^4D measurements of two DBS eluates, a 1 min flush with 100 mM NaOH was included before the DI water flush; the flushing pressure was 950 mbar in all cases. The CE electrodes and C^4D data acquisition were the same as in the previous paragraph.

DBS Sampling, Spiking, Storage, and Elution. DBS samples were obtained by pipetting various volumes of capillary blood from a finger prick onto the Whatman 903 Protein Saver sampling card. Before pricking, the fingertip was wiped off with a lint-free cotton pad soaked with ethanol (Lach-Ner). Ethanol at the fingertip was evaporated to avoid blood precipitation, and the fingertip was pricked using an automated skin puncture device (ACCU-CHEK FastClix, Roche, Mannheim, Germany) with disposable lancets, which were discarded after use. The first drop was wiped off with a dry cotton pad, and exact volume was withdrawn from the subsequent drop using a micropipette with an adjustable volume (2−20 or 5−50 μL, ProLine Plus, Biohit, Helsinki, Finland) into a graduated polypropylene micropipette tip (Sorenson Bioscience Inc., Salt Lake City, UT, USA; One Touch 1−20 or 1−330 μL). The blood from the tip was quantitatively dispensed onto the sampling card (into the center of the sampling circle) and was air-dried in a holder for 3 h to form the DBS. The DBS with an "unknown" volume was formed by a lab-assistant by pipetting a certain volume of capillary blood, which was not known to the analyst, onto the DBS sampling card and dried as described above. For a DBS with really unknown volume, capillary blood was dropped directly from the finger prick into the center of the DBS sampling circle (the whole circle was filled with blood) and dried as described above. For spiking and standard addition calibration, 99 μL of capillary blood was mixed with 1 μL of amino acids standard solution in a 250 μL PCR tube, the spiked blood was pipetted by the ProLine Plus micropipette to the sampling card, and the resulting DBSs were dried as described above. Each DBS card was stored in a closed plastic bag with a desiccant in a refrigerator at 4 °C for up to three months.

The elution of capillary blood from the DBS for conductivity and CE measurements was performed according to the following procedure and is depicted in Figure S1. (i) The entire DBS was cut from the sampling card using a 10 mm cork-borer. For the analysis of a DBS with an unknown volume, only a part of the DBS was cut with a smaller (6, 7, or 8 mm; see Results and Discussion) cork-borer. (ii) The resulting disc of the Whatman 903 card with the DBS was placed at the bottom of a clean Agilent glass snap-cap vial (2 mL; P/N 5182-9697) using a tweezer. The diameter of the DBS disc was lower than the i.d. of the glass vial. (iii) The DBS was eluted directly in the glass vial with a specific volume of DI water, methanol/DI water, or methanol. For elution with DI water, 500 μL of DI water was pipetted into the vial, and the vial was closed and agitated at 1000 rpm for 10 min. For elution with methanol/DI water, 250 μL of methanol was pipetted into the vial, and the vial was closed and agitated at 1000 rpm for 5 min. Subsequently, 250 μL of DI water was added to the vial, and the vial was closed and agitated at 1000 rpm for another 5 min. For elution with methanol, 500 μL of methanol was pipetted into the vial, and the vial was closed and agitated at 1000 rpm for 10 min. The vials were agitated using a Vibramax 110 (Heidolph Instruments GmbH, Schwabach, Germany) agitator. In order to minimize manipulation with the DBS sample and to avoid contamination of the DBS eluate, the Whatman 903 disc was not removed from the vial after the DBS elution and the CE injection was performed directly from the free solution above the disc. DBS eluates were used only on the day of elution and were stored at 4 °C.

■ RESULTS AND DISCUSSION

Conductivity Measurements. The analytical response of the C^4D was examined with a set of standard NaCl solutions and showed a linear relationship in the intended conductivity range (see procedures and Figures S2 and S3 for the resulting conductivity plot and the calibration curve). The stability of the DBSs for conductivity measurements and the elution time required for the complete transfer of the DBS to the elution solvent were examined with real 10 μL DBSs. No statistically significant variations were observed for up to one month old DBSs, and complete elution was achieved by agitating DBSs at 1000 rpm for 10 min (see Experimental Section Figure S4 and corresponding text in the Supporting Information).

The response of the C^4D to the conductivities of real DBS samples was examined with sets of DBSs prepared by pipetting 7, 8, 10, 12, 15, and 20 μL of capillary blood onto the DBS

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sampling cards, drying them, and eluting them with 500 μL of DI water. One individual sampled three DBS sets on 1 day (at 8:00 a.m., 12:00 p.m., and 4:00 p.m.), and all sets were processed the next day. The same individual sampled one DBS set on three consecutive days, and the sets were processed 1 day after the last set was collected. In addition, five individuals sampled one DBS set each, and the sets were processed the next day; hematocrit levels were within the range specified for healthy adults in ref 12. Note that the DBS samples were prepared by each individual, and thus, the observed variations in conductivity did not only account for the differences in blood composition at different day-times and days and among different individuals but also for the sampling procedure.

Linear relationships were obtained for all DBS sets (coefficients of determination (r^2) ≥ 0.9926), demonstrating that the automated C^4D measurement represents an attractive method for the determination of blood volume in unknown DBS samples by conductivity measurements of the DBS eluates. The results are summarized in Table S1 and show no statistically significant differences (at the 0.05 significance level) in the calibration curve parameters. Relative standard deviation (RSD) values for the slope and the intercept were 1.2%, 0.9%, and 2.4% (slope) and 0.7%, 1.3%, and 3.4% (intercept) for interday, intraday, and individual-to-individual DBS sets, respectively.

Elution with Different Solvents. The elution of DBS is usually carried out with DI water, organic solvents, or aqueous mixtures of organic solvents. The elution with organic or partially organic solvents might be especially useful when the target analytes are hydrophobic compounds and when the matrix effects are to be suppressed. The DBS sets (7−20 μL) were eluted with three different elution solvents (DI water, 50% (v/v) methanol/DI water, and 100% methanol), corresponding conductivities were measured with the C^4D, and the resulting calibration curves are depicted in Figure 1. All

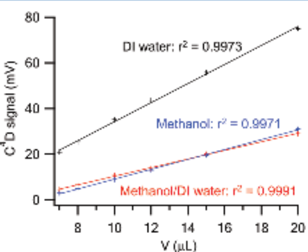

Figure 1. Calibration curves with coefficients of determination for conductivity measurements of DBSs with different blood volumes eluted with the three different elution solvents (500 μL).

three curves showed perfect linearity (r^2 ≥ 0.9971) while being characterized by different slope and intercept values. This is, however, not surprising since DI water, methanol/DI water mixtures, and methanol have different conductivities and conductivities of inorganic ions (which are eluted from the DBSs and contribute most significantly to the total blood conductivity) in the three solvents change with methanol content.

Moreover, DBS processing with organic solvents eliminates the elution of some blood constituents from the DBS, which might otherwise partly contribute to the eluate conductivity as well. This has been evidenced by a complete discoloration of the DBS disc eluted with DI water, demonstrating that all blood constituents were transferred to the aqueous solution resulting in a red-brownish color of the eluate. On the contrary, the DBS discs remained visually almost unchanged after elution with methanol/DI water and methanol, and the eluates were colorless. The red blood cells, proteins, and other macromolecular compounds were preserved in the discs during the elution with methanol (or methanol/DI water), and only low molecular weight compounds were eluted into the free solution.

Conductivity Measurement of Blood Volume in DBS Samples. The suitability of the proposed C^4D approach was examined by the determination of the exact blood volume in "unknown" DBS samples. Spots with three different "unknown" volumes (from the low, mid, and far end of the calibration range) were formed on the card, and their exact volumes were determined by measuring the conductivities of their eluates and by subsequent calculations using the derived calibration equations. The results are summarized in Table 1 and reveal that the calculated volumes were in perfect accordance with the truly pipetted blood volumes and did not differ by more than 3.7%. The "unknown" DBS volumes were also determined for the two remaining elution solvents (methanol, methanol/DI water), and comparable agreements (≤3.0% difference) were achieved.

CE-C^4D for the Determination of Inorganic Ions in DBS Samples. CE-C^4D is a universal analytical method, which can be used for the determination of anions and cations with various physicochemical properties.[26] In the actual experiments, a BGE solution that enables the determination of inorganic cations, anions, and selected target analytes is desirable, and according to our former experience,[23] acetic acid-based BGE solutions were examined. The optimization procedure is comprehensively described in the last section of the manuscript and in the Supporting Information.

The optimized BGE solution consisted of 1.6 M acetic acid (pH 2.1) with the addition of 1 mM 18-crown-6 and 0.1% (v/v) Tween 20 and provided the simultaneous CE-C^4D determination of inorganic cations and amino acids. 18-Crown-6 was added to the BGE to aid K^+ and NH_4^+ separation and Tween 20 to avoid excessive attachment of macromolecular blood constituents to the capillary walls. The BGE was also suitable for the determination of inorganic anions by simply switching the HV polarity and enabled comprehensive determination of inorganic ion content in blood by two separate CE runs (Figure 2).

A detailed characterization of the CE-C^4D system for the determination of inorganic cations and anions was carried out with standard solutions and real DBS eluates. The CE-C^4D performance worsened significantly for the DBS eluates, and an extended procedure (including consecutive flushing with 100 mM NaOH, DI water, and BGE solution), which is summarized in the Supporting Information, was required to ensure stable CE-C^4D performance.

Elution with Different Solvents. Linearity of the CE-C^4D method for the determination of inorganic cations and anions in DBSs with different blood volumes was also examined. Standard solutions containing specific concentrations of K^+, Na^+, and Cl^- typical for capillary blood were used, and the results are summarized in Figure S5. Excellent linearity was achieved for all three elution solvents (DI water, methanol/DI water, and methanol). Linearity of the CE-C^4D

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Table 1. Comparison of "Unknown" and Calculated DBS Volumes Determined by Total Conductivity Measurements[a]

	A						B						C					
pipetted "unknown" volume (μL)	8.5		11.5		18.5		8.5		11.5		18.5		8.5		11.5		18.5	
calculated volume (μL)	8.53	0.4%[b]	11.16	3.0%[b]	18.16	1.8%[b]	8.55	0.6%[b]	11.23	2.3%[b]	18.12	2.0%[b]	8.30	2.4%[b]	11.14	3.1%[b]	17.93	3.1%[b]
universal calibration (μL)	8.46	0.5%[b]	11.08	3.7%	18.05	2.4%[b]	8.51	0.1%[b]	11.19	2.7%[b]	18.06	2.4%[b]	8.40	1.1%[b]	11.27	2.0%[b]	18.10	2.2%[b]
RSD (%), total conductivity	2.3		3.7		3.0		3.4		2.5		2.3		2.7		3.2		1.1	

[a](A) The same individual sampled three DBS sets at different times on the same day. (B) The same individual sampled one DBS set on three consecutive days. (C) Five individuals sampled one DBS set on the same day. DBSs were eluted with 500 μL of DI water. Universal calibration used all calibration data for the calculation of the "unknown" volumes. [b]% difference of the "unknown" and the calculated volume.

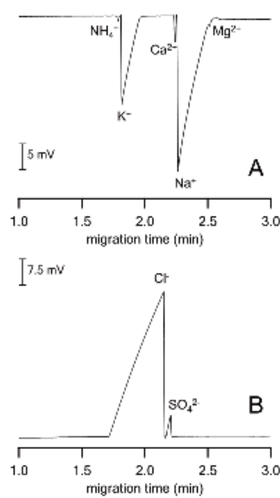

Figure 2. CE-C^{4}D determination of inorganic cations (A) and anions (B) in DBS eluate (20 μL of DBS eluted with 500 μL of DI water). BGE solution: 1.6 M acetic acid, 1 mM 18-crown-6, 0.1% (v/v) Tween 20, pH 2.1; separation voltage: +30 kV (A) and −30 kV (B); injection: 100 mbar × 30 s; C^{4}D: 1.84 MHz at 50 V_{pp}.

of the real DBS samples was subsequently examined with several sets of DBSs prepared by pipetting 7, 10, 13, 16, 19, and 22 μL of capillary blood onto the DBS sampling cards, drying them, and eluting them with methanol/DI water. The DBS calibration sets were spotted onto the sampling cards in a similar way as reported in the section Conductivity Measurements (i.e., three DBS sets were sampled by one individual at different times of 1 day, the same individual sampled one DBS set on three consecutive days, and five DBS sets were prepared by five different individuals on the same day). The overall results are summarized in Table S2 and confirmed the linear relationship between the DBS volume and peak area of the three major inorganic ions in the DBS eluates. Moreover, the variations of the calibration equations for the DBS sets sampled on 1 day, sampled on three consecutive days, and sampled by five different individuals were marginal with RSD values lower than 2.6%, 4.5%, and 5.9%, respectively.

Subsequently, three sets of DBSs with volumes between 7 and 22 μL were prepared and eluted with the three different elution solvents, and calibration curves were plotted for the three inorganic ions after quantitative CE-C^{4}D determination. Figure 3 depicts linear relationships for the three eluting solvents ($r^2 \geq 0.9949$) with slightly different slope values. This indicates that the content of the inorganic ions eluted from the DBS might be different for different solvents and the same elution solvent must be used for quantitative measurements of the calibration and the unknown DBS samples. A comprehensive discussion on the suitability of the three elution solvents on DBS processing is given in the Supporting Information and in Table S3, and the procedure combining the elution with methanol/DI water was adopted for quantitative measurements of "unknown" DBS samples in the subsequent experiments.

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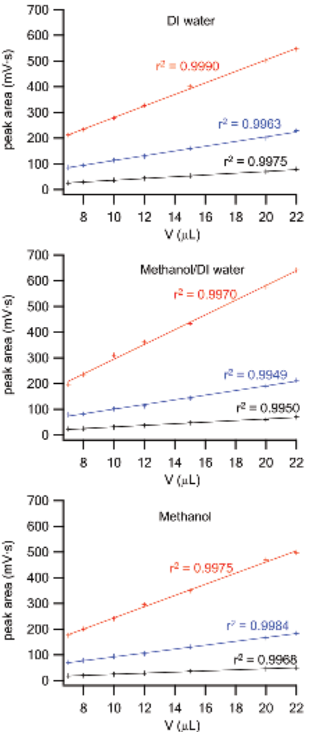

Figure 3. Calibration curves with coefficients of determination for peak area measurements of inorganic ions in DBS eluates prepared in three different elution solvents. Red trace, Cl^-; blue trace, Na ; black trace, K^+.

CE-C^{4}D Determination of Blood Volume in DBS Samples. The "unknown" DBSs were formed as reported in the section Conductivity Measurements. Two different blood volumes (from the far end of the calibration range) were pipetted onto the card, and the exact volumes were determined by quantitative CE-C^{4}D determination of the three inorganic ions in the eluates and by subsequent calculations using the derived calibration equations. The results are summarized in Table 2 and reveal that the calculated volumes were in perfect accordance with the real volumes of the "unknown" DBS samples and did not differ by more than 5.5%.

Finally, five unknown DBSs were formed by filling the whole sampling circle with capillary blood, and the central part of the DBS was subsequently punched out using a 6, 7, or 8 mm cork borer. The three sizes were selected in order to cover the blood volumes within the calibration range, and the particular DBS subpunches were eluted with methanol/DI water. Blood volumes in the eluates were determined using the formerly derived calibration equations and are summarized in Table S4, which also includes blood volume calculations based on the eluate conductivity for comparison. The differences in the volumes determined by the calibration curves of the three ions are statistically not significant (RSD $\leq$ 3.7%) and confirm the applicability of the proposed approach for the determination of blood volume in unknown DBSs.

Determination of Endogenous Amino Acids in DBS Samples. Thanks to the universal character of the conductivity detection, inorganic, organic, and biochemical species can be determined simultaneously in one CE run. The CE-C^{4}D can thus be used for the determination of the exact DBS volume by quantifying major inorganic cations and for the simultaneous determination of selected endogenous amino acids in the unknown DBS sample.

DBS samples were prepared by spotting 20 µL of capillary blood onto the DBS sampling card, drying the spots for 3 h, and eluting the spots with methanol/DI water. The eluates were subjected to the CE-C^{4}D determination in the cationic mode, and acetic acid content in the BGE solution (containing 1 mM 18-crown-6 and 0.1% (v/v) Tween 20) was varied between 1.2 and 1.7 M. The best separation of endogenous amino acids was achieved at 1.6 M acetic acid (see Figure S6), and a representative electropherogram demonstrating the simultaneous determination of inorganic cations and amino acids is depicted in Figure S7. In addition, zoomed-in regions of electropherograms recorded for the CE-C^{4}D analyses of amino acids in DBS eluates from five different individuals are depicted in Figure 4. Slight differences were observed for the content of some amino acids, which are consistent with the

Table 2. Comparison of "Unknown" And Calculated Dbs Volumes from the Peak Areas of the Three Inorganic Ions Determined by CE-C^{4}D[a]

	A				B				C			
pipetted "unknown" volume (µL)	19.5		20.5		19.5		20.5		19.5		20.5	
calculated volume, K^+ (µL)	19.28	1.2%[b]	20.38	0.6%[b]	18.98	2.7%[b]	19.82	3.3%[b]	19.25	1.3%[b]	20.05	2.2%[b]
universal calibration, K^+ (µL)	18.42	5.5%[b]	19.48	5.0%[b]	20.21	3.7%[b]	21.11	3.0%[b]	18.86	3.3%[b]	19.64	4.2%[b]
RSD (%), peak area, K^+	2.1		3.2		3.7		0.9		6.1		6.2	
calculated volume, Na^+ (µL)	19.63	0.7%[b]	20.14	1.8%[b]	19.59	0.5%[b]	20.13	1.8%[b]	19.61	0.6%[b]	19.94	2.7%[b]
universal calibration, Na^+ (µL)	20.41	4.7%[b]	20.94	2.2%[b]	19.08	2.2%[b]	19.59	4.4%[b]	19.35	0.8%[b]	19.67	4.1%[b]
RSD (%), peak area, Na^+	3.3		0.7		5.2		7.0		6.9		7.6	
calculated volume, Cl^- (µL)	20.15	3.3%[b]	20.75	1.2%[b]	19.50	0.0%[b]	20.13	1.8%[b]	19.57	0.3%[b]	20.35	0.7%[b]
universal calibration, Cl^- (µL)	20.24	3.8%[b]	20.84	1.6%[b]	19.07	2.2%[b]	19.69	4.0%[b]	19.91	2.1%[b]	20.71	1.0%[b]
RSD (%), peak area, Cl^-	4.1		2.3		2.3		5.3		3.5		1.4	

[a](A) The same individual sampled three DBS sets at different times on the same day. (B) The same individual sampled one DBS set on three consecutive days. (C) Five individuals sampled one DBS set on the same day. DBSs were eluted with 500 µL of methanol/DI water. Universal calibration used all calibration data for the calculation of the "unknown" volumes. [b]% difference of the "unknown" and the calculated volume.

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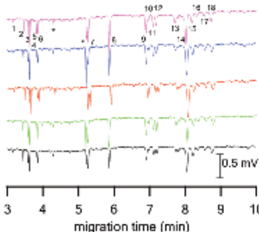

Figure 4. Detail of the CE-C^{4}D determination of amino acids in DBS eluate (20 μL of DBS eluted with methanol/DI water 50/50 (v/v)) of five individuals. CE-C^{4}D conditions as for Figure 2. Peak description: 1, Chol; 2, Crea; 3, Orn; 4, Lys; 5, Arg; 6, His; 7, Gly; 8, Ala; 9, Val; 10, Ile; 11, Leu; 12, Ser; 13, Thr; 14, Gln; 15, Glu; 16, Phe; 17, Tyr; 18, Pro; *, unknown.

rather narrow ranges of amino acid concentrations in the blood of healthy individuals.[27] Validation parameters for the CE-C^{4}D analysis of amino acids eluted from DBSs for five independent samples (from the same individual and from different individuals) are reported in Table S5.

Capillary blood was spiked with different concentrations of Phe (300, 600, and 1200 μM; see the Experimental Section for the spiking procedure), and the resulting blood samples were spotted onto the DBS sampling cards along with unspiked capillary blood. The set of the DBS samples was employed for the quantitative determination of Phe in capillary blood (using a standard addition method) and to demonstrate the suitability of the proposed method for the determination of abnormal levels of Phe in the human body, which might be related to PKU. Four electropherograms demonstrating the gradually increasing Phe levels in capillary blood are depicted in Figure 5. The red trace shows a Phe concentration typical for healthy individuals; the black trace corresponds to a trigger Phe concentration in PKU, and the blue/green traces correspond to Phe concentrations typical for a serious PKU. Details about LODs and linearities achieved for Phe (and Tyr, also related to PKU) are summarized in Table S5.

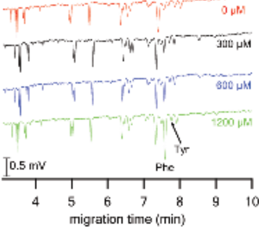

Figure 5. CE-C^{4}D determination of inorganic cations (not shown) and amino acids (detailed view) in DBS eluate (20 μL of DBS eluted with 500 μL of methanol/DI water 50/50 (v/v)) containing increasing concentrations of Phe. CE-C^{4}D conditions as for Figure 2. Capillary blood was spiked with 0, 300, 600, and 1200 μM Phe.

DBSs with Unknown Blood Volume for Amino Acid Analysis. To demonstrate the applicability of the CE-C^{4}D method for the quantitative determination of amino acids in DBSs with unknown volume, capillary blood was spotted from a finger prick onto a DBS sampling card to fill the whole sampling circle and the central 8 mm part of the DBS was punched out. The DBS subpunch was then eluted according to the previously described procedure, and the eluate was subjected to CE-C^{4}D analysis. The volume of the capillary blood in the subpunch was calculated from the peak areas of K^+ and Na^+ and corresponded to 19.4 ± 0.8 μL, which was subsequently used for exact quantitation of Phe in the DBS. On the basis of the previously employed standard addition method, Phe concentration was calculated to be 61.3 ± 2.6 μM. The same capillary blood (not sampled to the DBS card) was processed according to a procedure described in ref 27 using precipitation with an excess of methanol and centrifugation. The supernatant was only diluted with DI water/methanol to achieve the same final dilution (~25.8-fold) and the same methanol content (50%, v/v) as was used for the elution of the DBS subpunch. The resulting supernatant was directly analyzed by CE-C^{4}D, and Phe concentration (64.5 ± 2.8 μM) was determined from three consecutive injections.

■ CONCLUSIONS

In this contribution, a novel approach for the determination of capillary blood volumes in DBS samples is presented. The method relies on conductivity measurements in contactless mode and enables quantitative determination of exact blood volumes in DBS eluates. Conductivity of the DBS eluates is linearly proportional to the capillary blood volume originally spotted onto the DBS sampling card. Moreover, linear relationships are also obtained for the peak areas of major inorganic ions (K^+, Na^+, and Cl^-) determined by CE-C^{4}D vs the blood volume in the DBS eluates, and the derived calibration curves can be used for the calculations of the exact blood volumes in unknown DBSs. Since CE-C^{4}D is a universal analytical method and enables the determination of ions with different physicochemical properties, it can be used for the simultaneous determination of inorganic ions and various organic and biochemical compounds. Thus, one CE-C^{4}D analysis can be used for the determination of the exact blood volume in the DBS eluate (by quantifying K^+/Na^+ or Cl^- in the cationic or anionic CE modes, respectively) and for the quantitative determination of particular analytes. The developed approach is demonstrated to be suitable for automated CE-C^{4}D quantification of amino acids in DBS samples with unknown volumes, which might be particularly interesting in the screening of inborn metabolic disorders. Moreover, since C^{4}D is a nondestructive detection technique, CE-C^{4}D can be in-line coupled with other CE detection modes (e.g., MS), which might further enhance selectivity/sensitivity of the quantitative DBS analyses and considerably extend the range of detectable analytes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b04845.

(i) DBS elution scheme, (ii) continuous C^{4}D monitoring of eluate conductivity, (iii) effect of agitation time/speed on DBS elution, (iv) DBS stability, (v) CE stability, (vi) linear regression parameters for conductivity measurements, (vii) linearity measurements for peak areas of inorganic ions vs DBS volumes, (viii) DBS elution with various solvents, (ix) effect of BGE

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composition on CE-C^{4}D determination of amino acids, and (x) effect of elution solvent composition on the elution of amino acids from DBSs (PDF)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Capillary Electrophoresis with Capacitively Coupled Contactless Conductivity Detection for Quantitative Analysis of Dried Blood Spots with Unknown Blood Volume

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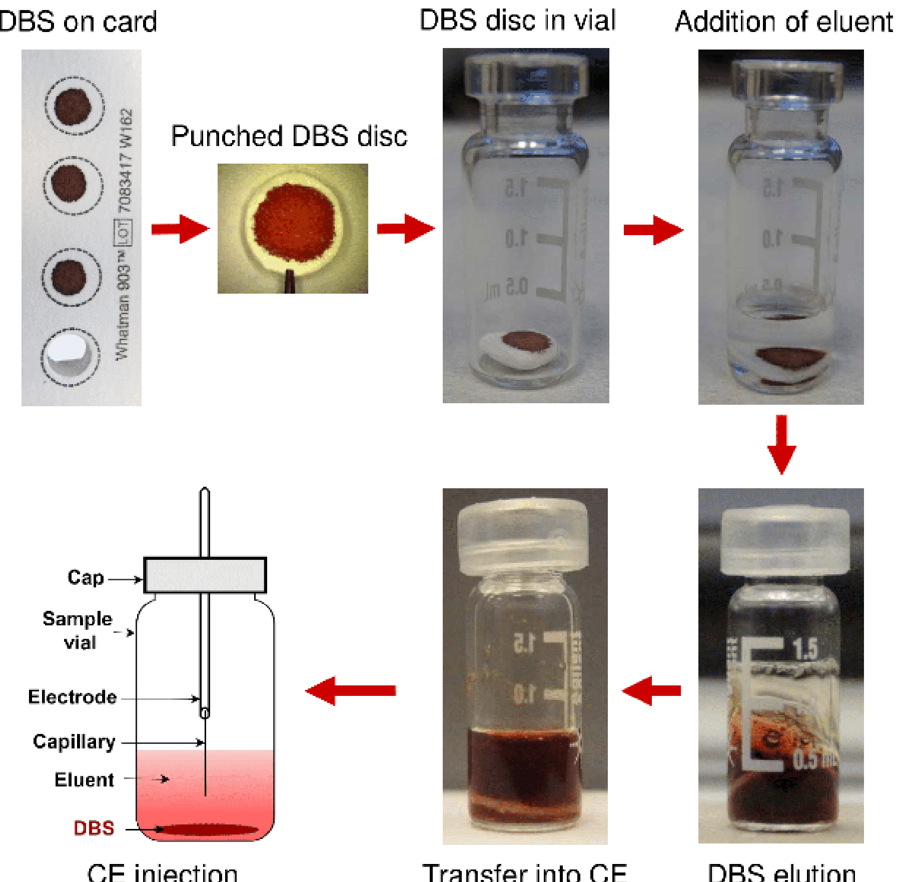
Figure S1. Visual description of the entire DBS elution process and injection into CE-C^{4}D.



Preparation of amino acid stock solutions. Stock solutions of the following amino acids were prepared in DI water from pure chemicals (L-forms, Pliva-Lachema and Sigma, Steinheim, Germany): creatinine (Crea), lysine (Lys), arginine (Arg), histidine (His), glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), serine (Ser), threonine (Thr), asparagine (Asn), methionine (Met), citrulline (Cit), tyrosine (Tyr), tryptophan (Trp), phenylalanine (Phe), proline (Pro), ornithine (Orn), glutamic acid (Glu), glutamine (Gln), cysteine (Cys) and aspartic

acid (Asp); concentrations were 10 mM, except for Tyr and Trp (1 mM) and Phe (25 mM). Stock solution of choline (Chol, 10 mM) was prepared in DI water from choline chloride.

Conductivity measurements. Linear response of the C^4D in the intended conductivity range was examined with a set of standard solutions of NaCl prepared in DI water. NaCl concentrations for the calibration protocol were based on the NaCl concentration in a physiological solution (~ 150 mM) and took into account the final dilution during DBS elution with 500 μL of the elution solvent. The artificial "eluates" (corresponding to 7, 8, 10, 12, 15 and 20 μL of blood) were prepared by mixing stock solution of NaCl with DI water and had final concentrations of 2.1, 2.4, 3.0, 3.6, 4.5 and 6 mM NaCl, respectively. The NaCl solutions were flushed through the C^4D according to the procedure described in the **Experimental Section** and conductivity signals were obtained by subtracting the conductivity signal of DI water from the conductivity signal of each particular NaCl solution. A record of the whole calibration protocol is depicted in Figure S2 and the resulting calibration curve is shown in Figure S3. The curve was linear in the whole calibration range and coefficient of determination was 0.9988. These experiments confirmed that response of the C^4D is linear in the selected conductivity range and that the CE instrument can be used for automated C^4D measurements of DBS eluates.

DBS processing involves elution of the capillary blood from the DBS by a suitable solvent. During the elution, various blood constituents are released into the solvent, which in the case of aqueous solutions include, among others, salts, red blood cells, proteins, fats and various macromolecular compounds. Proteins, fats and macromolecules might adhere to the inner capillary wall during C^4D measurements and change the conductivity reading of DBS eluates. A simple flushing procedure was thus applied to avoid capillary contamination by these blood constituents and an additional flush with 100 mM NaOH for 1 min was included after C^4D

measurement of a real DBS eluate. Constant conductivity readings for DI water were obtained after the flushing procedure and confirmed no attachment of blood constituents to the capillary wall.

Figure S2. Conductivity measurements of standard NaCl solutions corresponding to capillary blood volumes. The number above each step reports NaCl concentration (in mM) and U denotes an "unknown" NaCl sample.

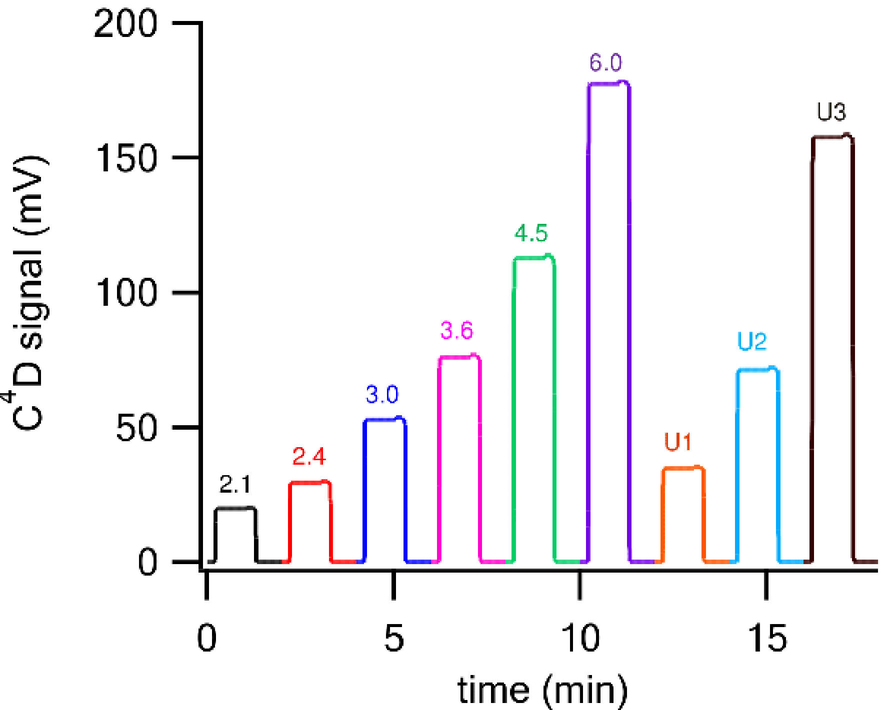
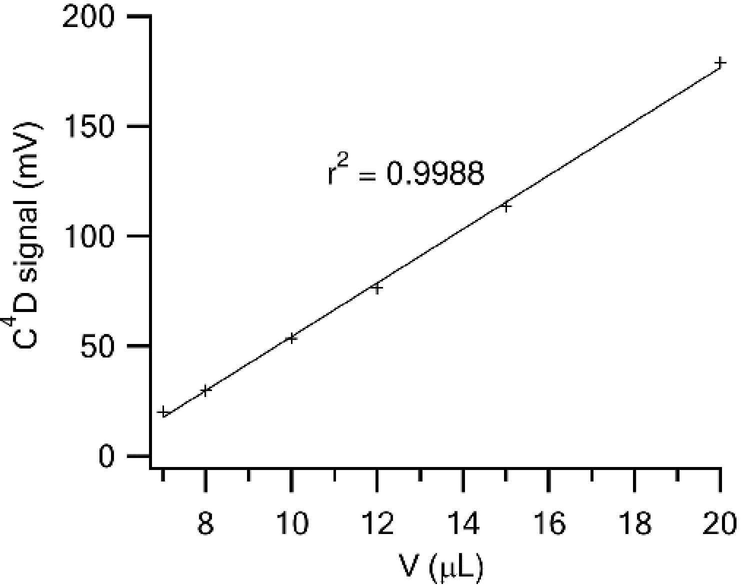


Figure S3. Calibration curve with the coefficient of determination for the conductivity measurements of standard NaCl solutions performed in Figure S2.



Processing time for a complete elution of the blood constituents from the DBS was examined by preparing a set of identical DBSs (10 μL of blood) and eluting them at different agitation speed (150 – 1500 rpm) and time (0 – 23 min) by 500 μL of DI water. Conductivity of the resulting eluates was determined by C^4D according to the aforementioned procedure. Corresponding results are depicted in **Figure S4** and demonstrate an incomplete elution of the DBS constituents for low agitation speeds (150 and 500 rpm) and a complete elution at high agitation speeds (1000 and 1500 rpm). Stable conductivity readings were achieved after elution for 8 min at 1000 and 1500 rpm. DBS elution was thus performed at 1000 rpm for 10 min in all subsequent experiments.

Figure S4. Effect of agitation rate and time on elution of real DBS samples. DBS spots (10 μL of capillary blood), DI water (500 μL) was used as the elution solvent.

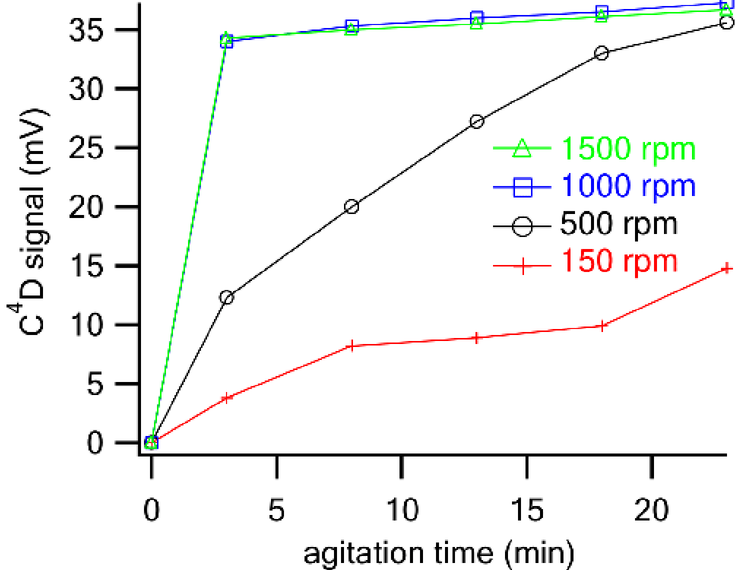


Table S1. Parameters of the conductivity measurements. (A) The same individual sampled three DBS sets on different times of one day. (B) The same individual sampled one DBS set on three consecutive days. (C) Five individuals sampled one DBS set on the same day.

	A		B		C	
	Slope	Intercept	Slope	Intercept	Slope	Intercept
	0.9359	-5.2868	0.9379	-5.2788	0.9460	-5.2052
RSD (%)	1.2%	0.7%	0.9%	1.3%	2.4%	3.4%
	r^2		r^2		r^2	
	0.9963		0.9926		0.9926	
RSD (%)	0.1%		0.2%		0.4%	

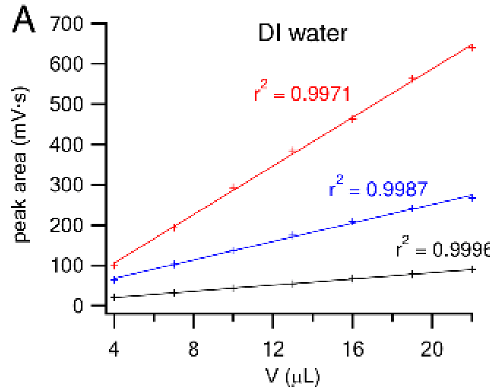
Stability of the DBSs for the conductivity measurements was examined by preparing a set of identical 10 μL DBSs and by eluting them immediately, one week and one month after drying. 500 μL of DI water was used as the elution solvent (see **Experimental Section**). Five DBSs were used for each group of experiments and the DBSs for the two later groups were stored refrigerated at 4 °C. Conductivity measurements of the eluates were statistically not different at the 0.05 significance level and, compared to the freshly eluted DBSs, the conductivities differed by 3.3% and 2.9% for the one week and the one month old DBS samples, respectively.

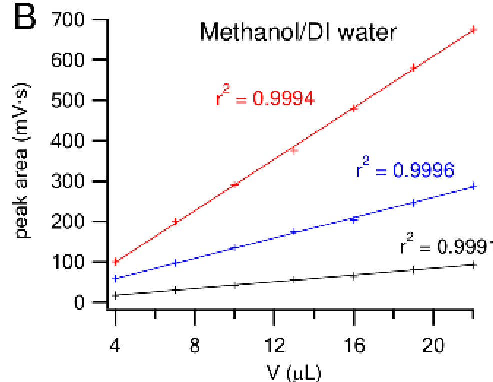
The suitability of the optimized BGE solution for CE-C^{4}D analyses of inorganic cations and anions was examined by standard solutions containing concentrations of K^+, Na^+ and Cl^- typically found in capillary blood and in real DBS eluates. Excellent analytical performance was obtained in BGE solution consisting of 1.6 M acetic acid, 0.1% (v/v) Tween 20 and 1 mM 18-crown-6 (pH 2.1). The capillary was flushed with the BGE solution only between two consecutive CE runs and RSD values for migration times (t_m) and peak areas (PA) were better than 0.1% and 2.8%, respectively. The same analytical procedure was tested with aqueous eluates of DBSs, which significantly worsened analytical parameters of the method due to the presence of the macromolecular blood components, and the RSD values increased up to 8% (t_m) and 50% (PA). The capillary flush with the BGE solution between two CE analyses of the real DBS eluates was not sufficient to renew the separation capillary inner surface and a thorough NaOH flush (100 mM, 2.5 min) was required. Using the flushing procedure with NaOH, DI water and BGE solution, the RSD values (for K^+, Na^+ and Cl^-) dropped back to their typical values and did not exceed 0.5% (t_m) and 5% (PA) even for the highest DBS volume. The same flushing procedure was also included for CE-C^{4}D analyses of DBSs eluted with methanol/DI water and methanol. Although the transfer of the interfering blood constituents to these elution solvents was eliminated due to the presence of methanol, the above optimized

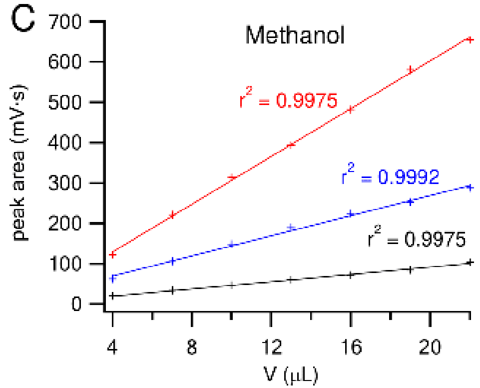
flushing procedure was applied in order to maintain constant capillary conditions and thus stable CE-C^4D performance.

Linearity of the optimized CE-C^4D method for the determination of inorganic cations and anions in DBSs with different blood volumes was examined with standard solutions containing specific concentrations of K^+, Na^+ and Cl^- in DI water, respectively. These concentrations were based on the typical content of the ions in capillary blood, took into account dilution of the capillary blood during DBS elution (500 µL of elution solvent) and ranged from 0.4 to 2.2 mM, 0.8 to 4.4 mM and 0.88 to 4.84 mM (corresponding to 4 – 22 µL of blood) for K^+, Na^+ and Cl^-, respectively. The calibration curves (peak areas vs. "blood volume") were strictly linear in the whole calibration range and coefficients of determination were 0.9996, 0.9987 and 0.9971 for K^+, Na^+ and Cl^-, respectively. Corresponding calibration curves are depicted in **Figure S5A.** The same standard solutions of the inorganic ions were prepared in 50% (v/v) methanol/DI water and 100% methanol and were analysed by the CE-C^4D method. The calibration curves are depicted in **Figure S5B** and **S5C** along with corresponding coefficients of determination. Quantitative results of the calibration curves determined for the ions prepared in the three different matrices were statistically not different at the 0.05 significance level and confirmed that linear response and comparable analytical signals are achieved independently of the sample medium.

Figure S5. Calibration curves with coefficients of determination for peak area measurements of inorganic ions in standard solutions prepared in three different elution solvents. K^+, Na^+ and Cl^- concentrations correspond to their concentrations in blood volume (4 – 22 µL) diluted with 500 µL of the eluent. Red trace – Cl^-, blue trace – Na^+, and black trace – K^+.







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Stability of the DBSs for the inorganic ion determination by CE-C^4D was examined by preparing a set of identical 20 μL DBSs and by eluting them immediately, one week and one month after drying. The DBSs were eluted with methanol/DI water (see **Experimental Section**), which eliminated the co-elution of the proteinaceous matrix and ensured stable CE-C^4D performance. Five DBSs were used for each group of experiments and the DBSs for the two later groups were stored refrigerated at 4 °C. Quantitative analysis of K^+, Na^+ and Cl^- revealed no statistically significant differences (at the 0.05 significance level) in content of the three inorganic ions in the eluates and the results for the one week and for the one month old DBS samples differed by less than 2.1% and 2.7%, respectively, in comparison to the results for the freshly eluted DBSs.

Table S2. Parameters of the linearity measurements of peak areas for the three inorganic ions determined by CE-C^4D. (A) The same individual sampled three DBS sets on different times of one day. (B) The same individual sampled one DBS set on three consecutive days. (C) Five individuals sampled one DBS set on the same day. DBSs were eluted with 500 μL methanol/DI water.

	A			B			C		
	K^+	Na^+	Cl^-	K^+	Na^+	Cl^-	K^+	Na^+	Cl^-
slope	3.089	11.162	28.957	3.442	10.449	28.192	3.166	10.588	29.335
RSD (%), slope	1.7%	1.5%	2.6%	1.2%	4.5%	2.8%	5.9%	4.7%	0.1%
r^2	0.9977	0.9960	0.9997	0.9971	0.9988	0.9997	0.9963	0.9971	0.9996
RSD (%), r^2	0.06%	0.03%	0.01%	0.15%	0.04%	0.02%	0.12%	0.11%	0.01%

Processing of DBS with various elution solvents revealed that proteins and other macromolecular blood constituents were eluted from the DBSs by DI water, but had no negative effect on the quantitative CE-C^4D determination of the three inorganic ions. The inorganic ions migrate faster than the matrix ions and the capillary was flushed with NaOH/BGE solution after

each separation, which ensured stable EOF and migration times of the fast analyte ions (RSD values of $t_m \leq 0.5\%$). Note, however, that the presence of the matrix components in the eluate might have an adverse effect on the determination of other ions (i.e., drugs, amino acids, etc.) due to the matrix/analyte comigration and enhanced shifts of migration times for slower analytes. For these reasons, DI water was not used for DBS elution in the subsequent section. The presence of matrix components was not evidenced in the DBS eluates eluted with methanol/DI water and methanol.

Table S3. Determination of calibration curve parameters for the inorganic ions in standard solutions and DBS samples eluted with different elution solvents.

	DI water		Methanol/DI water		Methanol	
Ion	Slope	r^2	Slope	r^2	Slope	r^2
			Standard solutions			
Na^+	12.854	0.9971	13.103	0.9994	13.705	0.9975
K^+	4.160	0.9987	4.183	0.9996	4.586	0.9992
Cl^-	29.189	0.9996	30.107	0.9991	29.689	0.9975
			DBS samples			
Na^+	10.568	0.9963	9.674	0.9949	8.594	0.9984
K^+	3.553	0.9975	3.110	0.9950	2.323	0.9968
Cl^-	26.030	0.9990	29.212	0.9970	23.428	0.9975

Table S4. Blood volume determined in DBS sub-punches of 6, 7 and 8 mm size. DBSs were eluted with 500 µL methanol/DI water.

Sub-punch diameter (mm)	**6**	**7**	**8**
Calculated volume, K^+ (µL)	8.05	12.81	19.82
Calculated volume, Na^+ (µL)	8.59	13.66	21.94
Calculated volume, Cl^- (µL)	8.18	13.20	21.07
Calculated volume, Conductivity (µL)	8.37	12.89	21.30
RSD (%)	2.5%	2.5%	3.7%

Optimization of BGE solution composition was aimed at a complete separation of selected amino acids related to inborn metabolic disorders (namely Phe and Tyr for the determination of PKU, but might be also applicable to Val, Leu, Ile for the determination of MSUD). The effect of the acetic acid concentration on the CE separation of the endogenous DBS amino acids in the 1.4 – 1.7 M range is depicted in **Figure S6** and a representative electropherogram demonstrating the simultaneous determination of inorganic cations and amino acids is depicted in **Figure S7**. Selected validation data of the CE-C^4D method are presented in **Table S5**. Limit of detection (LOD) was defined as 3 × S/N and coefficients of determination (r^2) were reported for 1.5 – 48 µM concentration range in DBS eluent.

Figure S6. Optimization of acetic acid concentration in the BGE solution for the CE-C[4]D determination of amino acids. BGE solution: 1.4 – 1.7 M acetic acid, 1 mM 18-crown-6, 0.1% (v/v) Tween 20, pH 2.1; separation voltage: + 30 kV; injection: 100 mbar × 30 s; 25/360 μm i.d./o.d. fused silica capillary, detection: 1.84 MHz at 50 V_{pp}.

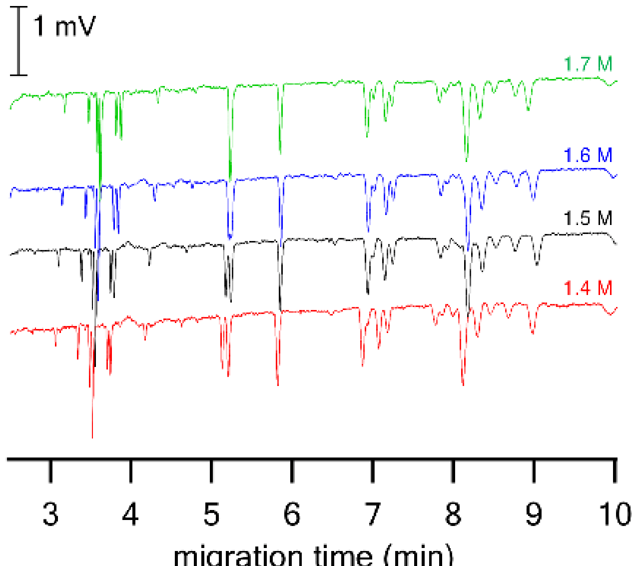


Figure S7. CE-C^{4}D determination of inorganic cations and amino acids in DBS eluate (20 µL DBS eluted with 500 µL methanol/DI water 50/50 (v/v)). CE-C^{4}D conditions as for Figure 2.

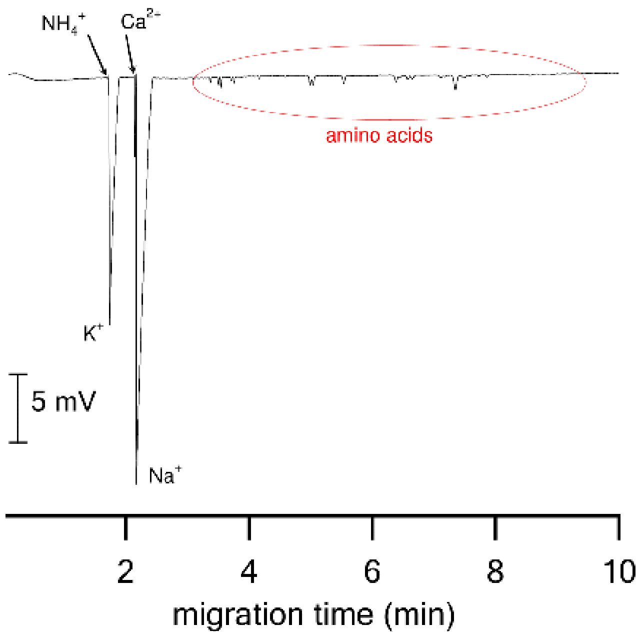
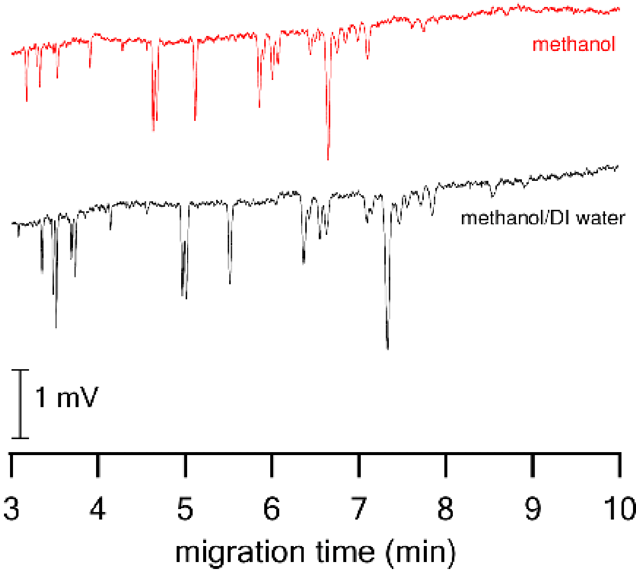


Table S5. Validation parameters for five subsequent CE analyses of five DBSs formed by one individual and five different individuals.

	Same individual		Different individuals			
	%RSD t_m	%RSD PA	%RSD t_m	%RSD PA	LOD (µM)	r^2
K^+	0.8%	2.2%	1.5%	3.4%		
Na^+	0.6%	3.0%	1.4%	3.3%		
Phe	1.0%	3.7%	1.9%	1.7%	0.46	0.9971
Tyr	1.0%	3.2%	1.9%	3.1%	0.59	0.9969

Elution of the amino acids from the DBS samples was examined with methanol and methanol/DI water. Electropherograms of the CE-C^{4}D analyses of the DBS eluates obtained by elution with methanol/DI water and pure methanol are depicted in **Figure S8** and demonstrate rather similar profiles of the amino acids. Note, however, that a considerable difference is visible for the early migrating amino acids (migration times 3 – 4 min) in the two traces and demonstrates different elution properties of the fully organic and the partly aqueous elution solvent. This can be explained by the general characteristics of the rapid amino acids (Crea, Lys, Arg, His), which are all hydrophilic and are thus preferentially eluted into the partly aqueous solvent.

Figure S8. Detail of the CE-C^{4}D determination of amino acids in DBS eluate (20 µL DBS) eluted with 500 µL of pure methanol and methanol/DI water 50/50 (v/v). The shift in migration times of amino acids is due to injection of different content of methanol into separation capillary for the two solvents.



9.3 Příloha III

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Hollow Fiber Liquid-Phase Microextraction At-Line Coupled to Capillary Electrophoresis for Direct Analysis of Human Body Fluids

Blanka Miková, Miloš Dvořák, Lenka Ryšavá, and Pavel Kubáň*

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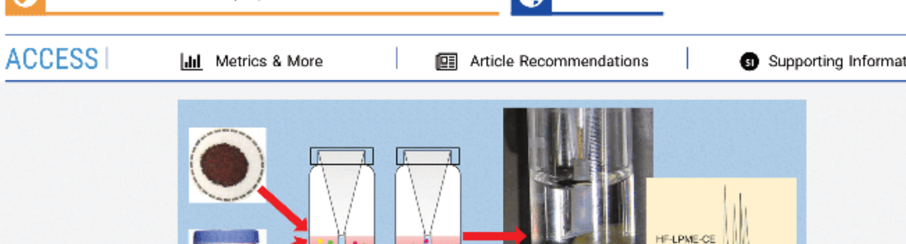

ABSTRACT: A simple and cheap all-in-one concept for at-line coupling of hollow fiber liquid-phase microextraction (HF-LPME) to commercial capillary electrophoresis (CE) is demonstrated, which enables the direct analysis of complex samples. A disposable microextraction device compatible with injection systems of Agilent CE instruments is proposed, which consists of a short segment of a porous HF attached to a tapered polypropylene holder. The holder maintains a constant position of the HF in a CE vial during extraction and simultaneously guides the injection end of a separation capillary into the HF lumen for automated CE injection and analysis. In a typical analytical procedure, the HF is impregnated with a water-immiscible solvent, its lumen is filled with 5 μL of an aqueous acceptor solution, and the microextraction device is placed in a 2 mL glass CE vial containing 550 μL of a donor solution. The vial is agitated at 750 rpm for 10 min, and the resulting acceptor solution is injected directly from the HF lumen into the commercial CE. No additional manual handling is required, except for the transfer of the CE vial to the CE autosampler. Multiple complex samples can be simultaneously pretreated in a multiple-well plate format, thus significantly reducing the total analysis time. Suitability of the analytical method is demonstrated by the direct determination of model basic drugs (nortriptyline, haloperidol, loperamide, and papaverine) in physiological solutions, urine, and dried blood spot (DBS) samples. Repeatability of the method is better than 12.8% (%RSD), extraction recoveries range between 34 and 76%, and enrichment factors are 37−84. The method is linear in a range of 2 orders of magnitude ($R^2 \geq 0.9977$) with limits of detection of 0.7−1.55 μg/L. The method has a high potential for the direct analysis of DBS samples since DBS elution and HF-LPME are performed simultaneously during the 10 min agitation. The manual DBS handling is thus reduced to inserting the DBS punch into the CE vial only. Moreover, the universal character of the HF-LPME might extend the applicability of the method to a wide range of analytes/matrices, and combination with other commercial detectors might improve the selectivity/sensitivity of the CE analysis.

■ INTRODUCTION

For more than three decades, substantial interest has been devoted to the development of microextraction techniques[1−6] and particularly to liquid-phase microextraction (LPME) through a supported liquid membrane (SLM).[1,5,7−9] In LPME through SLM, target analytes are transferred from a milliliter volume of an aqueous sample into an SLM and subsequently into a microliter volume of an acceptor solution on the opposite side of the SLM. The SLM is usually formed as a thin layer of a water-immiscible solvent anchored in the supporting material and acts as a selective barrier for the transfer of target analytes. Sample matrix components (salt ions, proteins, and other interfering species) are efficiently retained by the SLM, resulting in an excellent sample cleanup. Moreover, because the acceptor volume is usually lower than the sample volume, the enrichment of target analytes in acceptor solution is achieved, thus making the extraction process suitable for trace analyses.

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LPME is mostly performed off-line, i.e., the sample treatment is performed in a separate procedure and the resulting acceptor is manually transferred to the analytical system for injection and analysis. The off-line approach might be suitable for most standard procedures, nevertheless, some applications might benefit from the coupling of LPME to an analytical system, and various set-ups for their direct coupling have been described.[10,11] The volume of the acceptor solution after LPME is usually in the microliter range and is thus perfectly compatible with injection volumes in liquid and gas chromatography and in nonseparation analytical techniques. The direct coupling of LPME to such analytical instruments can, therefore, be achieved by transferring the entire acceptor solution into their injection systems using a syringe or a peristaltic pump.[12−14]

Capillary electrophoresis (CE), on the contrary, uses significantly lower injection volumes, and the transfer of the entire acceptor solution to CE is definitely more challenging. Nevertheless, CE has shown a high potential for clinical analysis recently[15−17] and is thus very attractive for the analysis of minute complex samples. Compatibility between the LPME acceptor volumes and the CE injection system was achieved by reducing the extraction unit size,[18] on-capillary treatment of the microliter sample volume,[19] or injection of a fragment of the acceptor solution only.[20] Lab-made CE instruments were exclusively used for the direct coupling of CE to LPME, which enabled the simple control of the transfer between the extraction system and the separation capillary. Nevertheless, the use of the lab-made CEs required manual manipulations with the capillary, and automation of the LPME/CE procedure was not possible. Later on, the direct coupling of LPME to commercial CE was achieved by using purpose-made microextraction devices compatible with the sample vials in CE autosamplers. The tailored devices allowed for the automation of the extraction/separation process, however, at the expense of repeated use of the devices and thus possible sample carry-over issues[21,22] or limited preconcentration ability.[20,23]

Even more convenient compatibility of the acceptor solution and the CE injection volumes can be achieved by the direct coupling of a single drop microextraction (SDME) to CE.[24,25] In SDME−CE, a small drop of an aqueous acceptor solution is covered with a thin layer of a water-immiscible solvent at the tip of the separation capillary, which is immersed directly into a sample solution or into its headspace. All operations, including filling the capillary with various solutions, formation of the solvent layer on the acceptor drop, and back-injection of the acceptor solution after SDME, are performed with a commercial CE and can be automated. On the other hand, the drop at the capillary tip is not very stable, the technique is suitable for a limited range of analytes only, and analysis of highly complex samples, such as biological samples or body fluids, is not readily possible.[25−27]

In this contribution, we present an environmentally friendly and cost-effective setup for at-line coupling of hollow fiber liquid-phase microextraction (HF-LPME) to CE for the direct analysis of complex biological samples. The HF-LPME procedure ensures efficient sample cleanup and preconcentration of model analytes from urine and dried blood spot (DBS) samples into a microliter volume of an acceptor solution filled in the lumen of the HF. The HF is impregnated with a negligible volume of a water-immiscible solvent to form the SLM, which enables a selective transfer of model analytes from the sample to the acceptor solution and retains interfering matrix components in the sample. The HF-LPME process is performed in a CE sample vial fitted with a tailor-made disposable microextraction device, which is suitable for at-line CE injection of the acceptor solution directly from the HF lumen. Thus, the CE injection requires no operator's intervention and can be fully automated for batch-wise measurements. The developed setup is suitable for various complex samples, but it might be particularly attractive for DBS analysis since DBS elution and HF-LPME of target analytes are performed simultaneously during one common agitation cycle. Moreover, the DBS elution/HF-LPME process is performed in a multiple-well plate format and pretreatment of up to 50 samples at a time is possible, which significantly reduces the sample processing/analysis time.

EXPERIMENTAL SECTION

Reagents, Standard Solutions, and Body Fluids. Analytical reagent grade chemicals and deionized water with resistivity higher than 18 MΩ·cm were used for the experiments. Stock solutions of nortriptyline hydrochloride, loperamide hydrochloride, papaverine hydrochloride, and haloperidol (Sigma, Steinheim, Germany) were prepared at a concentration of 1.000 mg/L of the drug in pure methanol (Lach-Ner, Neratovice, Czech Republic) and were stored in a deep-freezer at −20 °C. Standard solutions and donor solutions were prepared by dissolving appropriate volumes of the above-reported stock solutions in deionized water and in various HCl or NaOH solutions. Organic solvents for SLM impregnation were 1-ethyl-2-nitrobenzene (ENB, ≥ 98%, Fluka, Buchs, Switzerland), 4-nitrocumene (4-NC, ≥ 98%, Tokyo Chemical Industry, Tokyo, Japan), 2-nitrophenyl octyl ether (NPOE, ≥ 99%, Fluka), and dihexyl ether (DHE, 97%, Sigma-Aldrich, Steinheim, Germany). Operational solutions for extractions were prepared by dissolving HCl (37%, Lach-Ner) or NaOH (pellets, Lach-Ner) in deionized water. Stock solutions of 500 mM acetic acid and 500 mM sodium acetate were prepared from the corresponding chemicals (Fluka and Sigma). A background electrolyte (BGE) solution for the CE-UV determination of the basic drugs was prepared from these two stock solutions and was mixed with acetonitrile (Lach-Ner) and deionized water.

A physiological solution was prepared by dissolving NaCl (Pliva-Lachema, Brno, Czech Republic) in deionized water and had a concentration of 150 mM (∼0.9% w/v). Body fluids were collected from volunteers at the Institute of Analytical Chemistry. All volunteers signed a written informed consent. Human urine samples were obtained by 24 h collection and were kept in a refrigerator at 4 °C for 1 day and then in a deep-freezer at −20 °C for one month. DBS samples were formed by spotting 22 μL of capillary blood from a finger prick onto a Whatman 903 Protein Saver (GE Healthcare Ltd., Cardiff, UK) sampling card and by drying the spots at laboratory temperature for 3 h. For DBS samples spiked with the drugs, 22 μL of capillary blood was mixed with 1 μL of a standard drug solution in a PCR microvial (250 μL) and 22 μL of the mixture was transferred to the sampling card. The sampling cards with the DBSs were stored in zip-lock plastic bags with a desiccant (in a refrigerator at 4 °C) for three months. The DBS sample for extraction and CE analysis was prepared by punching the whole DBS from the card, inserting it into a glass snap-cap CE vial (2 mL; Agilent Technologies, Waldbronn, Germany, P/N 5182-9697) with tweezers, and pipetting 550 μL of an elution solution to the vial. The DBS elution was

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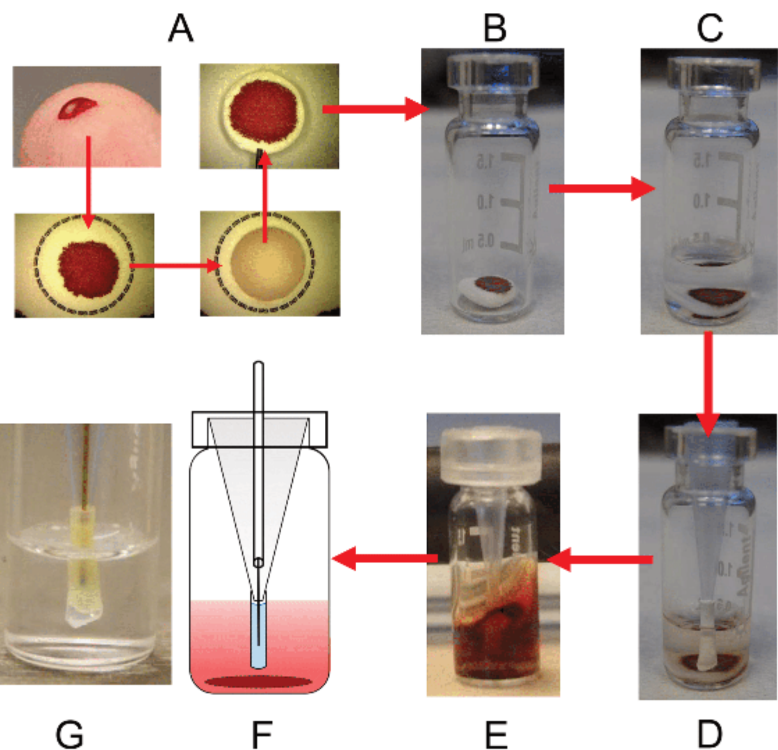

Figure 1. Schematic drawing of the DBS formation/elution process coupled with HF-LPME and at-line injection into CE.

carried out simultaneously with the HF-LPME and is described in the section Microextraction Devices.

Capillary Electrophoresis. Separation, detection, and quantification of the basic drugs were carried out using a 7100 CE instrument (Agilent Technologies) equipped with an in-built UV–Vis diode array detector. The detection wavelength was set at 200 nm. The BGE solution was optimized earlier,[28] consisting of 30 mM sodium acetate, 30 mM acetic acid, and 30% (v/v) acetonitrile with apparent pH 5.2, and was prepared daily. The basic drugs were separated at a potential of +15 kV, applied at the injection side of a fused silica separation capillary (75 μm i.d./375 μm o.d., L_{tot} = 45 cm and L_{eff} = 36.8 cm), which was supplied by Polymicro Technologies (Phoenix, AZ). Details on capillary equilibration can be found in the Supporting Information. Standard and acceptor solutions were injected hydrodynamically at 50 mbar for 5 s. Equilibration, injection, separation, and data acquisition were controlled by the ChemStation CE software (Agilent Technologies).

The short tubular electrode (Agilent Technologies, P/N G7100-60033) at the injection side was 5 mm shorter than the standard tubular electrode (Agilent Technologies, P/N G7100-60007), which was mounted at the CE outlet side. The separation capillary protruded by 8 mm from the short electrode. The short electrode was a necessary prerequisite for a proper injection procedure since it did not contact the HF holder, it eliminated possible contamination of the acceptor solution and the protruding injection end of the separation capillary was immersed directly in the acceptor solution. A detailed description and a schematic drawing of the injection process are reported in the section Microextraction Devices and in Figure 1, respectively.

Microextraction Devices. Each microextraction device was tailor-made from a 1000 μL polypropylene (PP) micropipette tip (FL Medical, Torreglia, Italy, Part No. 28053) and a porous PP HF (Accurel PP 300/1200, Membrana, Wuppertal, Germany). The tip was cut with a razor blade to a length of 22 mm (measured from the narrow end). The upper part of this 22 mm long segment had an o.d. slightly larger than 6 mm and was stopped by the vial orifice when inserted into the glass CE vial (see Figure 1D,F). The tapered tip segment acted as a holder for the porous HF and as a guide for the tubular electrode and the separation capillary. The HF (tubular membrane with a wall thickness of 300 μm, an i.d. of 1200 μm, and a pore size of 0.2 μm) was cut into 9 mm long extraction units, which were closed at the bottom by mechanical pressure (squeezing the bottom 1 mm by tweezers) and were sealed by heating the squeezed part for 2 s at 90 °C. The HF extraction unit was stretched over the narrow end of the holder to form the whole microextraction device. The devices were preassembled and stored in a plastic box, were

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disposable (estimated costs are 0.01 €/device), and were used for one extraction only.

Before HF-LPME, the HF was impregnated with a suitable organic solvent for 10 s and excess of the solvent was wiped off by a lint-free tissue. Immediately after the impregnation, 5 μL of an acceptor solution was transferred to the HF lumen by a Hamilton syringe (10 μL, Hamilton, Bonaduz, Switzerland, flat tip) and the whole microextraction device was placed into the glass CE vial with a donor solution. A photograph and a schematic drawing of the microextraction device in the CE vial are shown in Figure 1D,F, respectively.

The HF-LPME was carried out in a multiple-well plate format by placing the vial with the microextraction device and the donor solution into a vial holder (Agilent Technologies, P/N 9301-0722), which was fixed in a Vibramax 100 (Heidolph Instruments GmbH, Schwabach, Germany) agitator (Figure S1 in the Supporting Information). The holder can accommodate multiple vials, and up to 50 samples can be pretreated at the same time. For the HF-LPME of a DBS sample, a 10 mm disc (containing the whole DBS) was punched out from the DBS sampling card (Figure 1A) and was placed on the bottom of the vial (Figure 1B) before 550 μL of an elution solution was added (Figure 1C). Elution of the capillary blood from the DBS and HF-LPME took place simultaneously during the vial agitation (Figure 1E). When the extraction was completed, the vial with the extracted sample required no handling other than transfer to the autosampler carousel of the CE instrument. The acceptor solution was injected directly from the HF lumen after immersing the injection end of the separation capillary into the acceptor solution (Figure 1F,G). The eluate and the DBS disc were not removed from the vial before the CE injection. HF-LPMEs of standard solutions and urine samples differed only by pipetting of 550 μL of the donor solution instead of the elution solvent into the vial. The subsequent steps (Figure 1D–G) were same as for the DBS samples.

Calculations. Extraction recoveries and enrichment factors of the HF-LPMEs were calculated according to ref 8 and are summarized in the Supporting Information.

■ RESULTS AND DISCUSSION

Capillary Electrophoresis Optimization for At-line Injection from the HF. All experiments were carried out with the tailor-made microextraction devices by filling a non-impregnated HF with 5 μL of a standard solution of the four basic drugs (10 mg/L), inserting the device in the sample vial, and using no donor solution. A constant height of the HF inside the CE vial was ensured by the holder made of the micropipette tip, which also guided the separation capillary into the HF lumen. Two tubular electrodes (standard and short length) were examined for injections from the HF lumen. During injections with the standard length electrode, the electrode contacted the funnel-like shape of the holder and pushed the holder into the vial. Since this can lead to damage of the vial or the electrode, CE injection with the short electrode was preferred. The short electrode moved freely into and out of the holder and ensured a correct position of the separation capillary with regard to the HF lumen position (see Figure 1F,G).

The length of the separation capillary protruding from the short tubular electrode was optimized between 5 and 10 mm (with 1 mm increments), and two extreme situations were observed. Air was injected into the separation capillary when the capillary protruded by 5 and 6 mm, because the injection end was too short. For the separation capillary protruding by 10 mm, the injection end contacted the sealed bottom of the lumen and penetrated the HF or detached the HF from the holder. The standard solution was properly injected from the HF lumen for the separation capillary protruding by 7–9 mm, and 8 mm was chosen as optimum for the subsequent CE experiments. To examine the repeatability of the CE injection from the tailor-made microextraction devices, five subsequent injections were carried out from one device and one injection/device was carried out from five unique devices. The relative standard deviation (RSD) values were 2.4–4.7 and 2.1–4.9% for the first and the second set of injections, respectively, and did not significantly differ from those for standard CE injections performed from the glass CE vial filled with 800 μL of the same standard solution (1.9–4.5%).

Parameters of the HF-LPME At-line Coupled to CE. All variables affecting the HF-LPME performance were examined at the following initial conditions. A donor solution containing 1 mg/L of the four drugs was prepared in alkaline media (10 mM NaOH) to promote their transfer into the SLM. NaCl (150 mM) was added to the donor solution to simulate typical NaCl concentrations in body fluids and physiological solutions. The acceptor solution was 10 mM HCl to promote the drugs' release from the SLM. HF-LPMEs were carried out by agitating CE vials at 500 rpm for 20 min.

Organic Solvent. The composition of the organic solvent is the most important parameter in LPME, and four individual solvents, previously used for microextractions of basic drugs, were tested. Highest extraction recoveries (~50%) of nortriptyline, haloperidol, and loperamide were achieved for HF-LPMEs through DHE; nevertheless, papaverine behaved differently and was most efficiently extracted through ENB. Based on the above observations, a mixed solvent of DHE and ENB (1:1 (v/v)) was used for the SLM impregnation, which improved the extraction efficiencies of all drugs and reached the extraction recoveries between 50 and 70%. Improvement of extraction efficiencies of basic drugs through mixed solvent SLMs was also reported previously,[29] and the DHE/ENB mixture was used as the SLM in all subsequent experiments. Extraction recoveries for individual solvents and for the mixed DHE/ENB solvent are depicted in Figure 2.

Agitation. The transfer of analytes in HF-LPME is based on their availability at the HF-phase interface; thus, the efficiency of the cross-SLM transfer can be increased by agitation. The transfer of the basic drugs was low at fully stagnant conditions (0 rpm), and extraction recoveries did not exceed 3%. The agitation speed was increased to 150, 300, 450, 600, 750, 900, and 1050 rpm, and its effect is demonstrated in Figure S2 in the Supporting Information. A gradual increase in extraction recoveries was achieved between 150 and 750 rpm, and a plateau was formed for faster agitation speeds. Although the extraction recoveries were comparable for 750–1050 rpm, significantly higher RSD values were obtained for the fastest agitation speed. At 1050 rpm, a rather pronounced vortex was formed in the sample vial and the HF was not immersed properly in the donor solution, resulting in the compromised repeatability of the HF-LPME process. Agitation at 750 rpm was thus selected for further experiments.

Extraction Time. In diffusive extractions, time plays a significant role since the extraction process needs to reach the equilibrium state, which may take several minutes (for microliter volumes) up to several hours (for milliliter–liter volumes).[7,9] In the developed setup, volumes of sample and

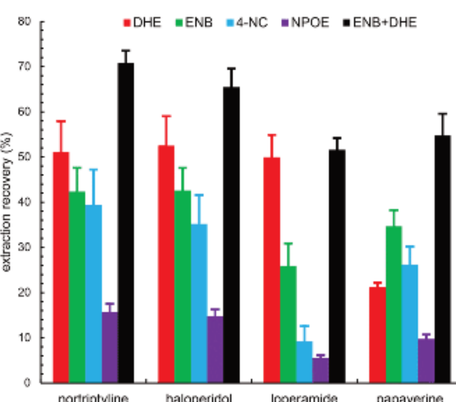

Figure 2. Effect of organic solvent composition on HF-LPME at-line coupled to CE for the determination of model basic drugs. HF-LPME conditions: acceptor, 5 μL of 10 mM HCl; donor, 550 μL of the four basic drugs (1 mg/L) in 10 mM NaOH including 150 mM NaCl; agitation speed, 500 rpm; extraction time, 20 min.

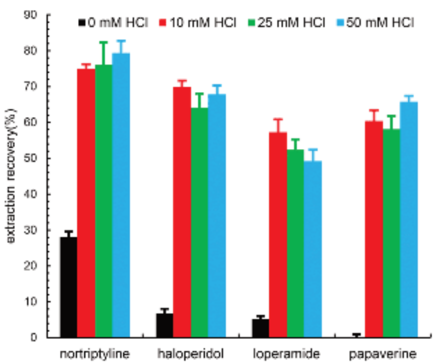

Figure 3. Effect of HCl in acceptor solution (5 μL) on HF-LPME at-line coupled to CE for the determination of model basic drugs. HF-LPME conditions: SLM, DHE/ENB (1:1, v/v); donor, 550 μL of the four basic drugs (1 mg/L) in 10 mM NaOH including 150 mM NaCl; agitation speed, 750 rpm; extraction time, 10 min.

acceptor solutions are in the microliter range and extraction time was examined between 0 and 20 min. The corresponding curves for the four analytes are depicted in Figure S3 in the Supporting Information and demonstrate a gradual increase in extraction efficiency for the first 10 min and leveling off during the next 10 min. Extraction efficiencies reached 60−80%, and a nearly exhaustive transfer from the sample to the acceptor solution was observed for nortriptyline and haloperidol. Repeatability of the extraction process was characterized by RSD values below 10% and did not change significantly for the 10−20 min extraction times; an extraction time of 10 min was selected for all subsequent measurements.

Acceptor and Donor Solutions. The transfer of target analytes in HF-LPME is based on a pH gradient, which is usually achieved by pH adjustments of the operational solutions. The analytes are in their neutral form in the donor solution, which facilities their transfer into the SLM, whereas they are ionized in the acceptor solution, which facilitates their release from the organic phase into the aqueous acceptor. For extractions of basic drugs, this is usually achieved by alkalization of the donor solution and acidification of the acceptor solution.

Optimization of the acceptor solution was performed with varying concentrations of HCl (0−50 mM) at a constant composition of the donor solution—NaOH (10 mM), NaCl (150 mM), and the four drugs (1 mg/L). The results are depicted in Figure 3 and demonstrate significantly improved extraction recoveries for acidic acceptor solutions. HF-LPME into deionized water resulted in no extraction of papaverine, extraction recoveries for haloperidol and loperamide were ≤10%, and for nortriptyline was ∼30%. This situation conforms with the fundamental principles of HF-LPME and can be explained by a higher degree of ionization of the drugs with higher pK_a values (6.03, 8.66, 9.41, and 10.47 for papaverine, haloperidol, loperamide, and nortriptyline, respectively) and thus their more efficient release from the SLM. No significant differences were observed for extractions into 10−50 mM HCl acceptors, and comparable extraction recoveries (60−80%) were achieved. Figure S4 in the Supporting Information demonstrates an additional feature of the increased HCl concentration in the acceptor solution on subsequent CE separations, i.e., deteriorated peak shapes for higher HCl concentrations. These are related to the higher conductivity of the injected zone in comparison to the BGE solution conductivity and are most pronounced for 50 mM HCl, which disables baseline separation of the target analytes. A 10 mM HCl acceptor solution was selected due to the excellent extraction performance and good compatibility with the BGE solution, resulting in sharp and well-defined analyte peaks.

For the donor solution with no pH adjustment (donor in deionized water at pH ∼ 6), the transfer of nortriptyline, haloperidol, and loperamide was not efficient (extraction recoveries <3%) and papaverine was transferred with a recovery ∼30%. At pH ∼6, the first three drugs are almost completely ionized, whereas papaverine is ionized from about 50% only. By alkalization of the donor solution, ionization of all analytes was suppressed to the minimum and their efficient transfer from the donor to the SLM was achieved. The observations are evidenced in Figure 4, and alkalization of the donor solution by 10 mM NaOH was further applied. Note, however, that alkalization of some biological samples might not be efficient with the 10 mM NaOH and thus a higher concentration of NaOH might be necessary for extractions of samples with a higher buffering capacity, such as urine and blood. Figure 4 also confirms that no deterioration of extraction recoveries was observed for NaOH concentrations up to 100 mM.

Validation of the HF-LPME At-line Coupled to CE. The donor solution for calibration purposes consisted of 10 mM NaOH, 150 mM NaCl, and the four drugs in the concentration range 2−300 μg/L (6-point calibration). For validation purposes, concentrations of the drugs in the donor solution were 10 and 100 μg/L. The acceptor solution was 10 mM HCl, and HF-LPMEs were carried out at 750 rpm for 10 min. Extraction recoveries ranged from 62 to 76%, which corresponded to enrichment factors of 68−84. Repeatability of the analytical process, including the HF-LPME and CE

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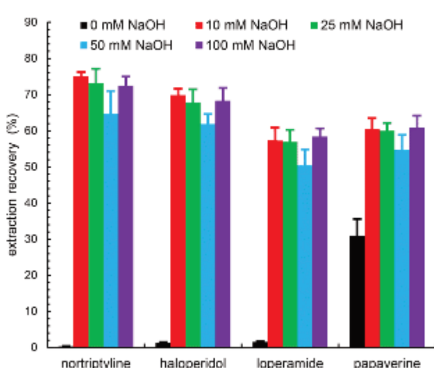

Figure 4. Effect of NaOH in donor solution on HF-LPME at-line coupled to CE for the determination of model basic drugs. HF-LPME conditions: SLM, DHE/ENB (1:1, v/v); acceptor, 5 μL of 10 mM HCl; donor, 550 μL of the four basic drugs (1 mg/L) in 150 mM NaCl; agitation speed, 750 rpm; extraction time, 10 min.

analysis, was better than 8.1 and 9.3% (RSD) for the 100 and 10 μg/L concentration levels, respectively. Calibration curves were linear in the entire calibration range, with coefficients of determination better than 0.9990. Limits of detection (LODs) were defined as analytical signals three times higher than background noise and ranged from 0.7 to 0.95 μg/L; all validation data are shown in Table 1. HF-LPME/CE-UV analyses of a blank (10 mM NaOH, 150 mM NaCl) and three standard donor solutions (10 mM NaOH, 150 mM NaCl spiked at 5, 10, and 50 μg/L of the four drugs) are depicted in Figure S5 in the Supporting Information.

The HF-LPME can be performed in a multiple-well plate format, with a large number of samples being processed simultaneously. Thus, the sample treatment throughput increases significantly and multiple extracts have to be analyzed by CE after the HF-LPME. Since each CE analysis lasts approximately 9 min, CE analysis is the limiting step of the analytical work-flow and a major part of the pretreated samples will be analyzed with a certain delay after their HF-LPME processing. As the extraction process involves the transfer of analytes from the donor to the acceptor solution on the basis of the pH gradient and the established equilibrium, long-term stability of this equilibrium (i.e., of the concentration of target analytes in the resulting acceptor solutions) was examined. Thirty unique microextraction devices were placed in 30 glass vials filled with the above-specified donor solution (at 50 μg/L of the four drugs). The set of the 30 samples was extracted during one 10 min HF-LPME run, and subsequently, all vials were transferred to the CE autosampler carousel. An unattended sequence was programmed and run, which included analysis of the 30 pretreated samples and 6 calibration standards with subsequent quantitation. The quantitative data are reported in Table S1 in the Supporting Information and confirm the equilibrium stability. Once the drugs were transferred through the SLM to the acidic acceptor solution, they were fully ionized, did not interact with the SLM, and did not diffuse back to the SLM/donor solution. Repeatability of peak areas was better than 11% (RSD) and proved that the multiple-well plate HF-LPME with subsequent automated CE analysis is suitable for the direct determination of drugs pretreated from multiple complex samples.

HF-LPME/CE-UV Analysis of Human Body Fluids. Urine and DBS samples were used unspiked and spiked with the four basic drugs. Therapeutic concentrations of the drugs range from 1 to 2000 μg/L in blood samples; no data are available for urine.[30] Urine has a high buffering capacity, contains high concentrations of inorganic salts, urea, and creatinine, and the addition of 10 mM NaOH was not sufficient for alkalization of urine samples. Thus, 495 μL of urine was mixed with 55 μL of 1 M NaOH solution and transferred to the glass vial for the HF-LPME. The resulting 100 mM NaOH increased the pH of all urine samples to ≥12.3 (independently of their composition) and the ionization of the basic drugs was efficiently suppressed. Importantly, it has been demonstrated earlier in the Acceptor and Donor Solutions section that donor NaOH concentrations up to 100 mM have no negative effect on the HF-LPME performance. Five drug-free urine samples were analyzed as blanks, and no comigration of the matrix components with the target analytes was observed. Figure S6 in the Supporting Information depicts a representative electropherogram of an HF-LPME/CE-UV analysis of blank urine (bottom trace) and shows several peaks related to the matrix (labeled with a cross) and to the extraction system (labeled with an asterisk). They are all baseline separated from the peaks of the target analytes, as can be seen from the three other electropherograms, which depict HF-LPME/CE-UV analyses of the same urine spiked with 2, 5, and 50 μg/L of the four drugs.

Table 1. Analytical Parameters for HF-LPME Coupled to CE/UV–Vis Analysis of Complex Samples[a,b]

matrix	standard				urine				dried blood spot			
analyte	Nor	Hal	Lop	Pap	Nor	Hal	Lop	Pap	Nor	Hal	Lop	Pap
extraction recovery (%), 10 μg/L	76	72	64	67	67	70	47	52	36	60	34	52
extraction recovery (%), 100 μg/L	74	73	62	70	69	69	50	54	38	59	34	53
enrichment factor	81–84	79–80	68–70	74–77	74–76	76–77	52–55	57–59	40–42	65–66	37	57–58
RSD (%), 10 μg/L	4.7	6.9	9.3	8.9	10.2	10.2	11.5	9.9	11.6	12.6	12.8	12.4
RSD (%), 100 μg/L	4.1	3.9	8.1	3.6	8.3	9.8	8.8	7.5	10.0	9.1	9.8	7.6
R^2	0.9990	0.9998	0.9995	0.9999	0.9995	0.9997	0.9981	0.9996	0.9991	0.9995	0.9977	0.9996
LOD (μg/L)	0.7	0.7	0.95	0.75	0.8	0.9	1.1	0.85	1.2	0.9	1.55	0.9
LOQ (μg/L)	2.3	2.3	3.2	2.5	2.7	2.9	3.7	2.8	4.0	2.9	5.2	2.9

[a]Nor = nortriptyline, Hal = haloperidol, Lop = loperamide, and Pap = papaverine [b]HF-LPME conditions as for Figures S4 and S5 and Figure 5 and CE conditions as for Figure 5.

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DBS samples were prepared by pipetting 22 μL of unspiked and spiked capillary blood onto the sampling cards according to the procedures described in the Experimental Section. Standard DBS processing involves punching the spot, elution of the capillary blood with a suitable solution, sample pretreatment, and transfer to an analytical instrument. In the proposed HF-LPME procedure, the elution and the pretreatment can be performed simultaneously, since as soon as blood elutes from the DBS, blood components diffuse into the free elution solution and can partition into the SLM. The actual setup for HF-LPME of DBS thus facilitates a two-step procedure, and the agitation time required to achieve equilibrium for the transfer of the drugs to the acceptor solution might differ from that for liquid samples. The effect of elution/extraction time was examined for 0−20 min using DBSs spiked with 50 μg/L of the four drugs, and resulting curves are depicted in Figure S7 in the Supporting Information. Equilibrium was achieved after 10 min of elution/extraction, and the DBS processing time was consistent with the extraction time for the liquid samples (standard, urine). NaOH (10 mM) was used as the elution solvent, which ensured adequate alkalization (pH ≥ 12.2) of the resulting eluates. Capillary blood is diluted during the elution step, and thus alkalization by 10 mM NaOH is sufficient. In addition, the dry nature of the DBS constitutes no limitations for the HF-LPME. The DBS elution process gradually releases the analytes into the donor solution, which are immediately transferred through the SLM to the acceptor solution, and induces no measurable increase in the HF-LPME time.

A representative electropherogram of a DBS blank (formed by spotting drug-free capillary blood) is depicted in Figure 5 (bottom trace). CE-UV measurements revealed several peaks related to the extraction system (organic solvents used in SLM, labeled with an asterisk), which were baseline separated from the target analytes with no comigration. The other three electropherograms depict CE-UV analyses of DBSs formed by spotting the same drug-free capillary blood spiked with 2, 5, and 50 μg/L of the four drugs.

The validation of the HF-LPME/CE-UV method for the determination of the four basic drugs in urine and DBS samples is summarized in Table 1, and most parameters are comparable to those for standard solutions. Extraction recoveries for urine (47−70%) were comparable to those for standard solutions, whereas some decrease was observed for recoveries of nortriptyline and loperamide (36 and 34%, respectively) for extractions from DBSs. This can be attributed to the drug−protein binding since the drugs are spiked directly into capillary blood, and their availability for the HF-LPME might be affected by these interactions.[8] Enrichment factors ranged from 52 to 77 for urine and from 37 to 66 for DBS samples. Repeatability of the elution/extraction/analysis of the four drugs was better than 12.8% (RSD) and conformed with the requirements for the bioanalytical method validation (http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf, accessed on January 15th 2020). Linear calibration curves with coefficients of determination ≥0.9977 were achieved, and LODs were between 0.8 and 1.55 μg/L.

CONCLUSIONS

A new microextraction setup is described, which enables at-line coupling of HF-LPME to CE for efficient cleanup and preconcentration of target analytes from complex biological samples and for their direct analysis. The HF-LPME is performed with a disposable microextraction device, which consists of a tapered plastic holder with a porous HF, and is compatible with Agilent CE instruments for automated analysis. The holder maintains constant position of the HF in a CE vial during extraction and simultaneously guides the separation capillary into the HF lumen during CE injection. In a typical procedure, the HF is impregnated with a microliter volume of a water-immiscible solvent, its lumen is filled with 5 μL of an aqueous acceptor solution, and the microextraction device is placed in a CE vial with 550 μL of an aqueous complex sample. Subsequently, the vial is positioned into an agitator and up to 50 samples can be pretreated simultaneously using a multiple-well plate format. The resulting acceptor solutions are analyzed by direct injection from the HF lumen, with no additional manual handling except for loading the CE vials into the CE autosampler. The transfer of the analytes to the acceptor solution is driven by a pH gradient and by the selectivity of the SLM; thus, composition of operational solutions and SLM was comprehensively optimized. The analytes are efficiently preconcentrated in the HF lumen, whereas matrix components are eliminated from the transfer by the SLM. The established equilibrium is constant over a considerable period of time and enables automated batch-wise CE analyses of the pretreated samples with a throughput of seven samples per hour. The proposed setup was exemplified with the direct determination of a set of model basic drugs in human urine and DBS samples. The setup is particularly beneficial in the analysis of DBSs since DBS elution and HF-LPME are performed simultaneously during one agitation cycle, which is followed by the at-line CE-UV analysis. The

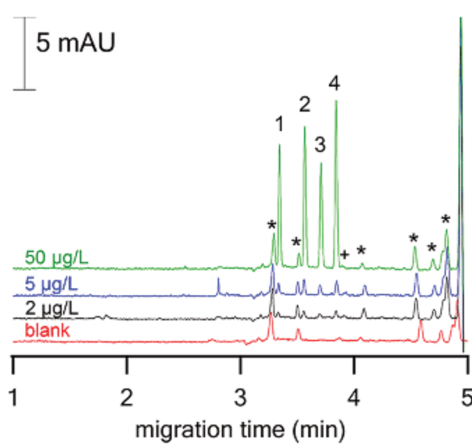

Figure 5. At-line coupling of HF-LPME to CE-UV for the direct determination of model basic drugs in DBS. HF-LPME conditions: SLM, DHE/ENB (1:1, v/v); acceptor, 5 μL of 10 mM HCl; donor, DBSs spiked with the four basic drugs and eluted with 550 μL of 10 mM NaOH; agitation speed, 750 rpm; extraction time, 10 min. CE conditions: BGE solution, 30 mM sodium acetate, 30 mM acetic acid, and 30% (v/v) acetonitrile at apparent pH 5.2; separation voltage, + 15 kV; injection, 50 mbar for 5 s; detection wavelength, 200 nm; 1 = nortriptyline, 2 = haloperidol, 3 = loperamide, 4= papaverine; + = unknown compounds from sample matrix, * = unknown compounds from SLM solvents.

DBS processing time is thus reduced to a minimum, and the manual DBS handling is minimized to the initial DBS punching. Moreover, the universal character of the HF-LPME and the CE might extend the applicability of the proposed setup to a wide range of analytes/matrices, and combination with mass spectrometric or laser-induced fluorescence detectors might further improve selectivity/sensitivity of the analytical method.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c00697.

Discussions of capillary equilibrations and calculations of extraction parameters, a photograph of a multiple-well plate extraction, effects of agitation speed and extraction time, CE electropherograms of basic drugs for different HCl concentrations, HF-LPME/CE-UV determination of basic drugs in standard solutions and urine samples, table of repeatability of the simultaneous multiple-well plate extraction process, and the effect of elution/extraction time on the extraction recovery of basic drugs from DBS samples (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Hollow fibre liquid-phase microextraction at-line coupled to capillary electrophoresis for direct analysis of human body fluids

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Capillary equilibration. Initial equilibration was performed by flushing the separation capillary with 100 mM NaOH (15 min), deionized water (5 min), and BGE solution (15 min). A daily equilibration included flushing the capillary with 100 mM NaOH (5 min), deionized water (5 min), and BGE solution (5 min). The capillary was flushed with 100 mM NaOH (1 min), deionized water (1 min), and BGE solution (1 min) between two consecutive CE analyses and with 100 mM NaOH, deionized water, and air (5 min each) at the end of a working day. The capillary was pressurized at 950 mbar during all flushing procedures and the capillary cartridge temperature was maintained at 25 °C.

Calculations. Extraction recoveries and enrichment factors of the HF-LPMEs were calculated according to equation (1) and (2), respectively:

$$Extraction\ recovery\ (\%) = \frac{n_{a,\ final}}{n_{d,\ initial}} \times 100 = \frac{V_a}{V_d} \times \frac{C_{a,\ final}}{C_{d,\ initial}} \times 100 \qquad (1)$$

$$Enrichment\ factor = \frac{C_{a,\ final}}{C_{d,\ initial}} \qquad (2)$$

where $n_{a,final}$ and $n_{d,initial}$ are the number of the analyte moles finally transferred into the acceptor solution and the number of the analyte moles initially present in the donor solution, respectively. V_a is the acceptor solution volume, V_d is the donor solution volume, $C_{a,final}$ is the final analyte concentration in the acceptor solution and $C_{d,initial}$ is the initial analyte concentration in the donor solution.

Figure S1. Photograph of an agitator with a holder for simultaneous extraction of up to 50 samples.

Figure S2. Effect of agitation speed on HF-LPME at-line coupled to CE for the determination of model basic drugs. HF-LPME conditions: SLM, DHE:ENB (1:1, v/v); acceptor, 5 μL of 10 mM HCl; donor, 550 μL of the four basic drugs (1 mg/L) in 10 mM NaOH including 150 mM NaCl; extraction time, 20 min.

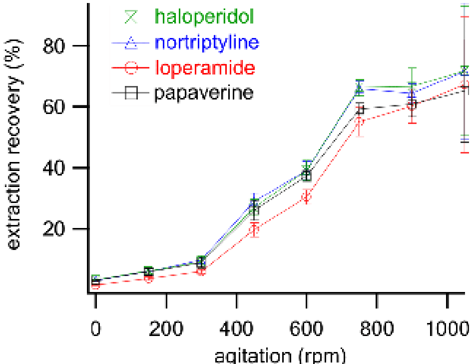


Figure S3. Effect of extraction time on HF-LPME at-line coupled to CE for the determination of model basic drugs. HF-LPME conditions: SLM, DHE:ENB (1:1, v/v); acceptor, 5 μL of 10 mM HCl; donor, 550 µL of the four basic drugs (1 mg/L) in 10 mM NaOH including 150 mM NaCl; agitation speed, 750 rpm.

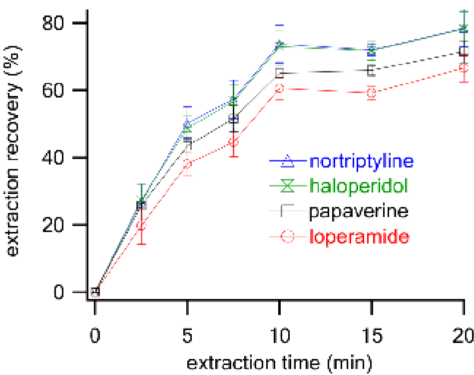


Figure S4. Effect of HCl concentration on CE determination and peak shapes of model basic drugs after HF-LPME of the four basic drugs at 1 mg/L. CE conditions: BGE solution, 30 mM sodium acetate, 30 mM acetic acid and 30% (v/v) acetonitrile at apparent pH 5.2; separation voltage, + 15 kV; injection, 50 mbar for 5 s; detection wavelength, 200 nm; 1 – nortriptyline, 2 – haloperidol, 3 – loperamide, 4 – papaverine.

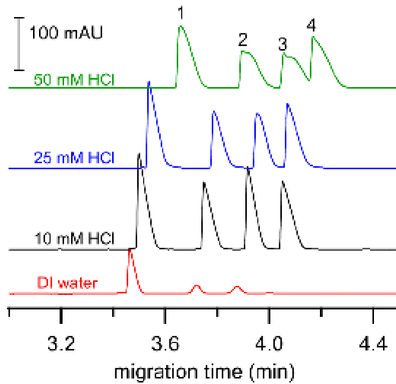


Figure S5. At-line coupling of HF-LPME to CE-UV for the direct determination of the four basic drugs in standard solutions. HF-LPME conditions: SLM, DHE:ENB (1:1, v/v); acceptor, 5 μL of 10 mM HCl; donor, 550 μL of the four basic drugs in 10 mM NaOH including 150 mM NaCl; agitation speed, 750 rpm; extraction time, 10 min. CE conditions as for Figure S4; * – unknown compounds from SLM solvents.

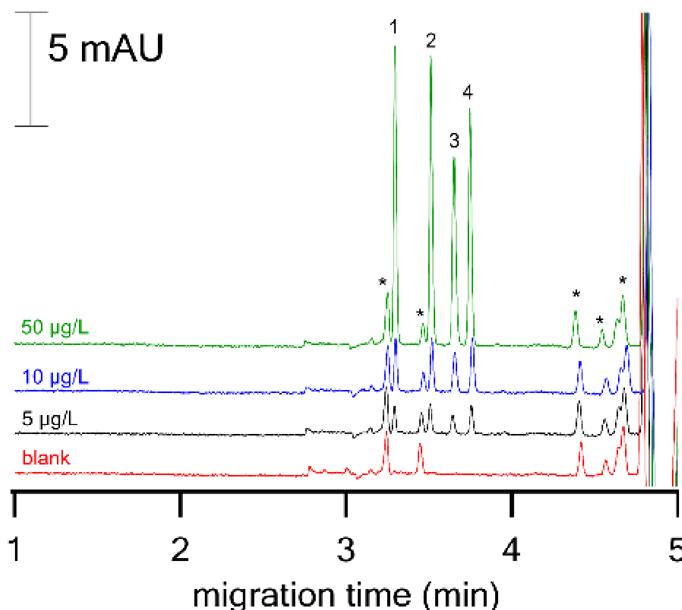


Table S1. Effect of the simultaneous multiple-well extraction process on the repeatability of the HF-LPME-CE/UV-Vis of the four basic drugs.

Analyte	RSD(%), n = 30
Nortriptyline	9.1
Haloperidol	11.0
Loperamide	8.7
Papaverine	7.4

Figure S6. At-line coupling of HF-LPME to CE-UV for the direct determination of model basic drugs in urine. HF-LPME conditions: SLM, DHE:ENB (1:1, v/v); acceptor, 5 μL of 10 mM HCl; donor, 550 μL of urine spiked with the four basic drugs and adjusted to 100 mM NaOH; agitation speed, 750 rpm; extraction time, 10 min. CE conditions as for Figure S4; + – unknown compounds from sample matrix, * – unknown compounds from SLM solvents.

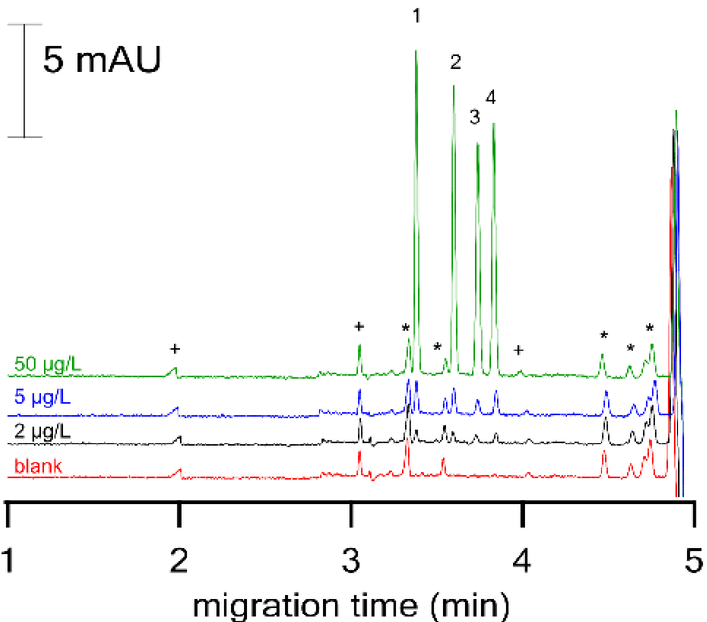
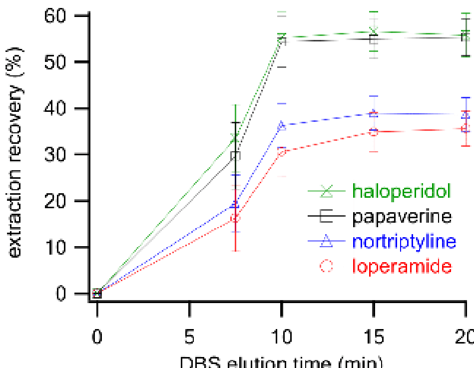


Figure S7. Effect of agitation time on simultaneous DBS elution/HF-LPME of the four basic drugs. HF-LPME conditions as for Figure S6 except for donor, capillary blood spiked with the four drugs at 50 μg/L, DBS eluted with 550 μL of 10 mM NaOH.



9.4 Příloha IV

Research Articles

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Dried Blood Spot Self-Sampling with Automated Capillary Electrophoresis Processing for Clinical Analysis

*Lenka Ryšavá, Miloš Dvořák, and Pavel Kubáň**

Abstract: *A simple and convenient concept of blood sampling followed by a fully automated analysis is presented. A disposable sampling kit is used for accurate self-sampling of capillary blood from a finger prick. A high-throughput blood sampling is thus enabled, which is essential in many clinical assays and considerably improves life quality and comfort of involved subjects. The collected blood samples are mailed to a laboratory for a fully automated dried blood spot (DBS) processing and analysis, which are performed with a commercial capillary electrophoresis instrument. Quantitative results are obtained within 20 min from the DBS delivery to the laboratory. The presented concept is exemplified by the determination of warfarin blood concentrations and demonstrates excellent analytical performance. Moreover, this concept is generally applicable to a wide range of endogenous and exogenous blood compounds and represents a novel and attractive analytical tool for personalized health monitoring.*

Introduction

Traditional clinical approaches are based on the analysis of body fluids, and blood is the preferred sample in most clinical assays.[1] To obtain blood samples, the presence of the investigated subjects/patients in clinical laboratories is required, which is time-consuming for both the subjects/patients and the laboratory staff, and usually involves painful venous phlebotomy. The above-mentioned shortcomings have led to recent developments in the field of patient-centric microsampling that can provide a wide range of benefits including less painful/disruptive blood sampling methods.[2–4] It involves self-sampling of a microliter volume of capillary blood, which is withdrawn from a finger prick and forms a dried blood spot (DBS).[3,4] Besides, it aims at enabling the collection of samples in a familiar and convenient environment (usually at home) and at treating every subject individually (personalized medicine). Blood self-sampling allows easier collection of clinical data by decreasing the impact on the subjects also in terms of reduced travel expenditure and time away from work and may increase the willingness for frequent and regular monitoring during therapies. It has been shown highly effective for the treatment of diabetes,[5] nevertheless, expanding the analytical target range is one of the most important challenges of the forthcoming decade(s).[6]

The standard DBS collection procedure uses paper-based sampling cards, which adsorb capillary blood, and the spots are dried up in ambient air for several hours.[7] The sampling cards with the DBSs present no biohazard and can be transported to analytical laboratories by any means, for example, mail, parcel delivery companies, etc.[8] More recently, novel approaches were proposed, which simplify the DBS collection and avoid the detrimental effects of DBS sampling on quantitative DBS analyses.[9–14] One of the proposed techniques is capillary blood collection by end-to-end glass capillaries, which is economical and allows the withdrawal of exact blood volumes from finger pricks with high accuracy and precision.[13] Moreover, by immediate transfer of the collected blood onto a pre-punched collection card for storage, the adverse hematocrit effects on the quantitative DBS analyses are eliminated.[11,12,15] The most essential advantage of using the glass capillaries is the fact that accurate/precise blood collection is achieved also by subjects with no training in blood collection.[13] The DBS self-sampling can be done at home and the withdrawal of a minute blood sample makes the experience more acceptable for subjects with severe anemia, infants, and children. The subjects have less anxiety and inconvenience and the blood samples can be collected at a higher sampling rate than with the standard venous phlebotomy.[10] The DBS self-sampling might thus open new horizons in personalized medicine, chronic disease monitoring, drug dosing optimization, and therapeutic drug monitoring (TDM).[8,10,16]

The collected DBS is usually processed by punching a predefined part of the DBS (or using the entire pre-punched DBS), which is followed by elution, extraction, centrifugation, evaporation, and reconstitution of the extract with a suitable solvent.[17] The complete process is time- and labor-consuming and its facilitation is at the forefront of current research. During the last decade, rather complex and costly systems have been presented, which aimed at the hyphenation of the DBS elution to the analysis by HPLC/MS or at the direct DBS analysis by MS and these techniques have successfully served for automated DBS analyses.[17–19] In comparison to HPLC and MS, capillary electrophoresis (CE) offers a cheaper, simpler, and more effective instrumental configuration, while it is still perfectly suitable for automated sample treatment and analysis.[20–22] Moreover, CE is predestined for handling/injecting minute sample volumes (typically obtained by DBS processing) and is distinguished by a high separation effi-

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ciency and the ability to simultaneously determine multiple sample constituents in a short time. All these characteristics are beneficial for clinical/bioanalytical assays and it is rather surprising that CE played an insignificant role in the DBS analyses in the past.[23]

In this contribution, a novel concept of capillary blood self-sampling followed by a fully automated DBS processing/analysis is presented. A low-cost, disposable DBS self-sampling kit is developed, which enables accurate and precise at-home collection of capillary blood from a finger prick onto a pre-punched DBS sampling disc accommodated in a standard snap-cap vial. Exactly 20 μL of blood is dispensed onto the disc using a glass capillary, the vial is placed into a zip-lock bag with a desiccant, dried up and sent to a laboratory. On arrival to the laboratory, the snap-cap vial with the DBS is loaded into a commercial CE instrument and the DBS is eluted by an automated sequence. As opposed to current automated DBS elution systems,[17] the laboratory staff does not physically contact the DBS material, which is protected in the vial. The resulting eluate is injected directly from the vial into the CE separation capillary for analysis. The DBS elution and analysis are performed fully autonomously by a single off-the-shelf analytical instrument and enable the direct pretreatment/analysis of the self-sampled DBS with an efficient elimination of matrix interferences and no operator's intervention.

The proposed concept was optimized by warfarin-spiked DBS samples and its suitability for clinical assays was exemplified by self-sampling of warfarin-containing DBSs, mailing the DBSs to the laboratory and automated DBS analysis by CE. Warfarin was selected as the most widespread oral anticoagulant used in a large number of long-term clinical assays that require regular control of blood coagulation, and due to the absence of a proper clinical method for the exact blood warfarin determination.[24–27] Such method might complement the current warfarin clinical monitoring (blood clotting expressed as international normalization ratio (INR)). Besides, although various subjects exhibit various INR values for the same warfarin blood concentration, the ratio between INR and warfarin blood concentration is rather stable for each subject[26] and the latter might be clinically relevant after the initial dosing setup. Moreover, with some subjects, measurements of blood warfarin levels are essential as they may eliminate uncertainty due to resistance to anticoagulants, interaction with pharmaceuticals, interaction with foods, and possible warfarin intoxication.[27]

During clinical assays, subjects are often obliged to daily visits to medical centers for intravenous blood phlebotomy at the start of the therapy followed by less frequent but regular visits/blood withdrawals in the later therapeutic stages. The suggested concept can thus significantly improve their life comfort due to the elimination of the painful phlebotomy and the absence of the travels/visits to medical centers and may open new horizons for assays requiring high-throughput sample collection/analysis. More importantly, the universal character of the DBS collection and CE analysis provides a general tool for a regular, patient-centric health status monitoring. Such tools are essential in determination of endogenous blood constituents, monitoring of chronic diseases, optimization of drug dosing, TDM, etc. and might propel a shift from the actual sick-care to a prevention-based healthcare system. Besides, the proposed workflow may also open new directions for on-site analysis without the need for the transport to the laboratory by coupling the DBS self-sampling with miniaturized and portable separation instruments.[28–30]

Results and Discussion

Kit for the DBS Self-Sampling

A single-use DBS sampling kit was designed for a convenient self-sampling of capillary blood and for compatibility with commercial analytical instruments. Details on the vendors of the kit sub-units are given in the Supporting Information (SI). The kit contained a sterile latex glove, a disposable lancet, a dry cotton pad, a disinfection pad soaked with isopropyl alcohol, a glass snap-cap vial (2 mL) with a polyurethane (PU) cap, a pre-punched DBS sampling disc (10 mm diameter), a glass capillary (20 μL, 30 mm long) with plugs, a bag with desiccant, two plastic zip-lock bags, sample identification label and "Instructions for use" in an envelope. Photographs of the DBS sampling kit are depicted in Figure S3 in the SI.

DBS Collection Using the Self-Sampling Kit, Delivery to the Laboratory, and Storage

The kit includes disposable equipment for DBS self-sampling and is intended for a simple withdrawal of an exactly defined volume of capillary blood. The kit is suitable also for individuals with no training in blood collection. Blood collection and DBS formation can, thus, be performed at home in a familiar environment and with a considerably higher comfort compared to venous phlebotomy in medical centers. Additional advantages of the at-home DBS self-sampling are less invasive blood withdrawal, reduced time requirements, increased sampling frequency, and higher willingness to undergo regular medical monitoring. Moreover, the cost of the off-the-shelf components is minimal and the price of the entire sampling kit is less than 1 €. Collected DBS samples are mailed directly to laboratory for analysis with no need for additional travelling expenditure and/or time away from work (see Figure 1).

To make the sampling kit as much universal as possible, the following aspects were considered. (i) A standard 2 mL snap-cap vial compatible with most commercial analytical instruments was selected. In the presented concept, CE was used as the analytical method of choice, but the DBS self-sampling procedure followed by a manual DBS elution can be used also with commercial HPLC, GC, MS and other analytical instruments. (ii) The DBS sampling disc was pre-punched from an approved Whatman™ 903 sampling card using a 10 mm puncher and was placed on the bottom of the vial. The size of the sampling disc was chosen to exactly fit the

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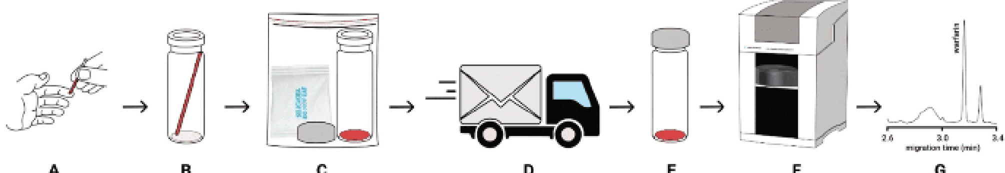

Figure 1. A scheme of the proposed analytical concept of a DBS self-sampling and an automated CE analysis. A) Capillary blood withdrawal, B) blood adsorption by DBS sampling disc, C) DBS packing, D) transport to laboratory, E) DBS loading into CE carousel, F) DBS elution/CE analysis, G) quantitative CE data.

internal diameter of the vial and not to move out of the position within the vial.

Capillary Blood Collection

Capillary blood and DBSs were sampled by volunteers from the Institute of Analytical Chemistry after signing a written informed consent. Before the DBS self-sampling, the content of the kit was removed from the bags. The latex glove was put on one hand and the isopropyl alcohol-soaked pad was used for disinfection of the index finger of the other hand. Alcohol on the fingertip was left to evaporate to avoid blood precipitation. The disinfected fingertip was pricked with the disposable lancet and the first drop of blood was wiped off with the dry cotton pad. The next drop was withdrawn into the glass capillary to collect the exactly defined volume of capillary blood. The glass capillary was subsequently placed into the snap-cap vial to contact the DBS sampling disc. There was no need for a precise centering of the capillary in the vial and the capillary entered the vial under an angle, which disabled blood contact with the vial wall. The entire blood volume was thus quantitatively adsorbed by the sampling disc and the capillary was disposed of into the "Biological material" zip-lock bag by turning the vial upside down. The procedures are depicted in Figure S4 and in Video S1 in the SI).

Capillary blood volume collected on the DBS sampling disc is a crucial parameter in quantitative DBS analysis. End-to-end glass capillary can withdraw an exact volume of blood and dispense it quantitatively onto the sampling disc without a special training.[13] A wide selection of the capillaries is available with different dimensions and volumes. The internal height of the snap-cap vial with the sampling disc is approx. 30 mm and thus capillaries with a height ≥ 30 mm were considered convenient. The maximum volume of capillary blood the pre-punched 10 mm sampling discs adsorbed was 26 ± 3 µL for three different hematocrit levels (low, medium, high[13]) and for this reason capillaries with 10, 20 and 30 µL volumes were investigated. 10 µL capillaries provided small spots on the sampling discs but the low blood volume might have a negative consequence on the analytical method sensitivity. 30 µL capillaries oversaturated the sampling discs and irreproducible volumes of blood remained in the capillaries. 20 µL capillaries formed well-defined spots on the sampling discs, blood was quantitatively adsorbed by the sampling material and 20 µL capillaries were thus selected for further experiments. The accuracy/precision of the 20 µL capillaries were comprehensively examined by the withdrawal of deionized (DI) water and capillary blood and the results are presented in chapter Precision and accuracy of the 20 µL glass capillaries in the SI.

Capillary Blood Drying

The standard procedure after capillary blood collection is drying the blood sample to form the DBS. In a typical DBS sampling, capillary blood is dried at ambient air for 2–3 h.[8] Capillary blood collected on the pre-punched disc accommodated in the snap-cap vial can exhibit different drying behavior due to the limited air circulation and the collected blood might dry up slowly or the drying process might be impaired. Two procedures for the in-vial drying of blood samples were, therefore, examined.

The first procedure involved drying the blood sample in an open snap-cap vial (placed vertically) at ambient air conditions. The drying time was monitored for 1–24 h by weighting the vial with the dry DBS sampling disc, the freshly prepared blood spot, and the blood spot during the drying procedure. DBSs were completely dried up after 12 h (see Figure S5, black trace, in the SI).

The second procedure involved a desiccant bag, which was placed together with the uncapped vial with a freshly spotted capillary blood in the "Sample" zip-lock bag. The desiccant and the vial were placed horizontally in the zip-lock bag and the bag was closed. The desiccant in the bag adsorbs the vapors during the blood drying up and enables the DBS formation. The drying time was examined for 1–24 h according to the above-reported weighting procedure. The DBSs were formed after 3 h drying time (see Figure S5, red trace, in the SI), which was 4-fold faster than without desiccant and was comparable to the standard DBS drying time.

Figure S6 in the SI depicts the effect of the drying time (with desiccant) on the CE analysis of DBS eluates (for details on elution conditions, see chapter Manual DBS elution). Elution and analysis of DBSs, which were not dried properly (0, 1, and 2 h), reveal lower elution efficiency of a model analyte and peak dispersion/shift due to the co-elution of matrix components. Proper DBS drying is thus essential; the addition of the desiccant to the zip-lock bag after the capillary blood self-sampling and drying the spot for

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3 h before shipment was applied to all blood samples (see also chapter DBS drying before shipment to the laboratory in the SI).

Delivery to the Laboratory and Storage

Immediately after the blood self-sampling, the snap-cap vial with the spot, the desiccant, and the PU cap were placed into the zip-lock bag with the identification label (sample ID, date/time of collection), which was filled in. The remaining components of the kit (blood-contaminated) were placed into the other zip-lock bag. The 3 h drying time was allowed to form the DBS. The two zip-lock bags were packed into an envelope and mailed to the laboratory for further processing/analysis and disposal of the biological material, respectively. The DBS samples were processed immediately after delivery or were stored at 4 °C until processing.

Optimization of the CE Instrument Set-up with Warfarin as a Model Analyte

All warfarin concentrations in the manuscript indicate warfarin concentrations in the original and undiluted capillary blood. For the optimization of the separation capillary alignment, DBSs were prepared from drug-free capillary blood spiked with 25 μg mL^{-1} of warfarin. The initial elution conditions (eluate volume: 500 μL; agitation speed: 1000 rpm; agitation time: 10 min) were adopted from our previous work[12] and eluate volumes of 500 and 100 μL were examined during the optimization procedure. The elution mixture consisted of acetonitrile (ACN) and DI water (4:1, v/v) and was based on the results in chapter Composition of the elution mixture in the SI. The CE-UV method was adopted from[31] and its optimization for warfarin analysis is described in the SI.

The interspace between the inner wall of the CE tubular electrode and the outer wall of the CE separation capillary can hold a few μLs of liquid (from flushing, etc.) and has been reported as a contamination source of samples with minute volumes.[12] Taking into account the low volume of DBS eluates and the necessity to eliminate their possible contamination by the contact with the CE electrode (and vice versa) a short electrode assembly was used at the CE injection side, which does not contact the eluates.

Also, due to the low liquid levels of the eluate (i.e. the height of the free eluate in the vial above the DBS disc), the length of the injection (inlet) end of the separation capillary had to be optimized. The optimum length of the injection end protruding from the tubular electrode depends on the eluate height and was 7 and 10 mm for the 500 and 100 μL eluate volumes, respectively. Longer injection end (11 mm) resulted in the direct contact with the DBS disc, reduced injection volume due to partial capillary clogging, possible contamination of the injection end by blood precipitates, and possible capillary rupture. Shortening the injection end affected the sampling process by injection of air, which was observed for injection end lengths <6 mm (500 μL eluate volume) and ≤ 9 mm (100 μL eluate volume), and resulted in the electric current failure during the subsequent CE analysis. The procedure for setting up exact capillary lengths is described in chapter Capillary electrophoresis and Figure S2 in the SI.

Manual DBS Elution

Details on the manual DBS elution are presented in the SI and describe optimization of the composition of the eluent mixture, elution time and elution volume. Best elution characteristics were achieved for the sequential DBS elution with ACN and DI water at a 4:1 v/v ratio (see Figure S7 and S8 in the SI). Elution times of 1 min (ACN) and 5 min (DI water) at an agitation speed of 1000 rpm were applied, which ensured exhaustive elution of warfarin (99–100 %) and excellent repeatability (RSD <4.7 %), see Figure 2. The optimized eluent volume was 100 μL (80 μL ACN and 20 μL DI water), which enabled minimum DBS dilution and reliable CE injection of the eluate for a sensitive warfarin determination (see Figure S9 in the SI). A graphical Scheme of the entire manual elution process is shown in Figure S10 in the SI.

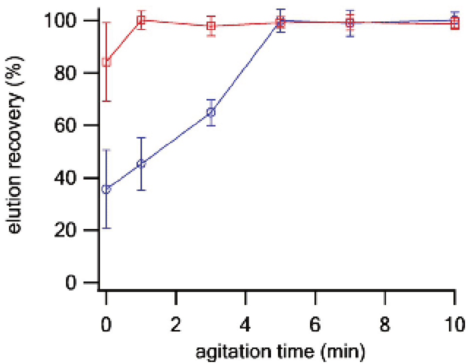

Figure 2. Effect of agitation time on the elution of warfarin from DBS samples. Red trace: effect of ACN agitation time (DI water agitation time 5 min), blue trace: effect of DI water agitation time (ACN agitation time 1 min). Elution was carried out with 400 μL of ACN followed by 100 μL of DI water at the agitation speed of 1000 rpm.

Analytical Performance

The analytical performance of the CE-UV method for the determination of warfarin in DBSs was examined at the above-optimized experimental conditions. Drug-free DBSs from healthy individuals ($n=5$) were eluted/analyzed and the analytical signals showed no peak(s) in the migration time of warfarin.

Warfarin was not present in the blood samples or was present at concentrations below the method LOD. Several matrix components were detected in the eluates, nevertheless, they migrated at times different from warfarin and did not interfere with its determination. Subsequently, the drug-free

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blood was spiked with warfarin and analytical parameters of the method were determined.

The repeatability of the analytical procedure was evaluated for blood spiked with 0.5, 1.0, and 3.0 $\mu g mL^{-1}$ of warfarin. Five DBSs were analyzed for each concentration and the repeatability, expressed as RSD values of warfarin peak areas, was better than 5.4%. It evidenced an insignificant effect of the sample matrix on warfarin elution from DBSs and excellent repeatability of the analytical procedure. The RSD values are summarized in Table 1 and comply with the requirements on repeatability in bioanalytical method validation.[32] Also summarized in Table 1 are coefficients of determination for linearity measurements in the 0.35–5 $\mu g mL^{-1}$ concentration range ($r^2 = 0.9999$) and LOD/LOQ (0.11/0.35 $\mu g mL^{-1}$) of the CE-UV method. LOD and LOQ were defined as the lowest concentration of warfarin giving analytical signal three and ten times higher than baseline noise (S/N = 3 and S/N = 10), respectively, and were well below its lower therapeutic concentration (1 $\mu g mL^{-1}$).[33]

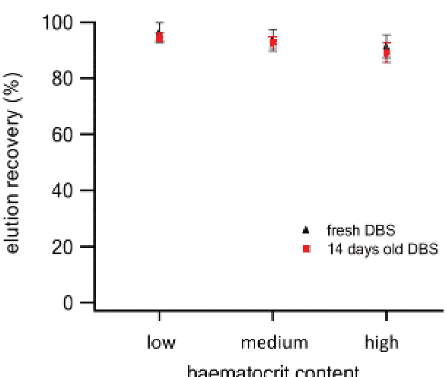

Figure 3. Effect of DBS hematocrit levels and DBS storage time on the elution of warfarin from DBS samples. Elution conditions as for Figure 2 except for volumes (ACN – 80 µL and DI water – 20 µL) and agitation times (1 min and 5 min).

Table 1: Analytical parameters for the determination of warfarin in DBS samples by manual DBS elution and CE-UV determination. Elution conditions as for Figure 3 and CE-UV conditions as for Figure 5, $n = 5$.

Parameter	
RSD (%), 0.5 $\mu g mL^{-1}$	5.4
RSD (%), 1.0 $\mu g mL^{-1}$	4.7
RSD (%), 3.0 $\mu g mL^{-1}$	5.2
r^2 (0.35–5.0 $\mu g mL^{-1}$)	0.9999
LOD ($\mu g mL^{-1}$)	0.11
LOQ ($\mu g mL^{-1}$)	0.35

Effect of Blood Hematocrit and DBS Ageing on Elution Characteristics

Various hematocrit levels and DBS ageing may have a direct impact on the elution of selected analytes from DBSs and the DBSs stability.[9] The following experiment was performed to examine the robustness of the proposed analytical procedure. Capillary blood samples with low, medium, and high hematocrit levels[13] ($n = 5$ for each level) were prepared and spiked with 1 $\mu g mL^{-1}$ of warfarin. The resulting DBSs were eluted and analyzed immediately after the drying period (3 h) and after 14 days (in the latter case, each vial with the DBS was stored in a zip-lock bag with the desiccant at ambient room conditions). The results are depicted in Figure 3 and demonstrate statistically insignificant differences (1–2%) for the analyses of the fresh vs. the aged DBSs.

Elution of warfarin from DBSs with different hematocrit levels resulted in gradually increasing warfarin concentrations for lower hematocrit levels and increased from 92 to 97% (fresh DBSs) and 90 to 96% (aged DBSs) for the high vs. the low hematocrit level. The difference values are within the bioanalytical method validation range[32] and DBS ageing had also no effect on the CE performance (see Figure S11 in the SI). The proposed concept can thus be used for warfarin determination in DBS samples independently of their hematocrit content and age.

Automated DBS Elution/CE Analysis

The proposed concept was aimed at the simplification of blood collection and subsequent blood processing to offer a novel and user-friendly analytical tool for blood analysis. Two major aspects were considered in the proposed analytical concept: (i) the self-sampling of capillary blood in the form of DBS, which was addressed earlier, and (ii) automation of the DBS processing/analysis with minimum requirements on the analytical laboratory staff.

To comply with both aspects, a fully automated process for the DBS elution and CE-UV analysis of the resulting eluate was elaborated. After removal from the zip-lock bag, the snap-cap vial with the DBS was closed with the PU cap and loaded into the CE autosampler carousel. All subsequent liquid handling (eluent transfers to the snap-cap vial) and sample convection (air bubbling from an empty vial) procedures were performed by the internal CE pressurizing system directly through the separation capillary. Short electrode assemblies were used on both sides of the CE system. DBS elution was performed at the CE outlet side and eluate injection at the CE inlet side, respectively. The capillary protruded by 12 and 10 mm from the electrodes at the CE outlet and inlet side, respectively. The procedure for setting up exact capillary lengths is described in chapter Capillary electrophoresis and Figure S2 in the SI.

Eluent types/volumes and convection times were adopted from the manual elution procedure. First, 80 µL of ACN was transferred to the vial and the eluent was bubbled with air for 1 min to eliminate DBS matrix components. Second, 20 µL of DI water was transferred to the vial and warfarin was eluted from the DBS by bubbling air through the eluent mixture for 5 min. Shorter times (2, 3 and 4 min) resulted in incomplete

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warfarin elution and longer times (7 and 10 min) confirmed the completion of warfarin elution. The completion of the elution process was additionally evidenced by running the fully automated DBS elution/analysis, which was followed by a subsequent off-line agitation of the vial at 1000 rpm for 5 min and CE analysis. No statistically significant difference in warfarin peak areas was observed for the consecutive CE analyses. After the DBS elution, the separation capillary was equilibrated by sequential NaOH, DI water, and BGE solution flush and the resulting DBS eluate was directly injected from the vial into the separation capillary for CE separation and warfarin quantification. Details of the sequence for the CE-automated DBS elution/analysis are summarized in Table 2 and a copy of the sequence in the ChemStation CE software is shown in the SI. A graphical Scheme of the entire analytical process and a time-lapse video of the automated DBS elution are presented in Figure 4 and Video S2 in the SI, respectively.

The total analytical time was 20 min and enabled a fully autonomous DBS elution and analysis with a sample throughput of 3 samples per hour. The analytical parameters of the automated method were determined for a set of drug-free DBS samples spiked with warfarin according to procedures reported in chapter Analytical performance. The data are summarized in Table 3 and are consistent with those achieved for the manual DBS elution and CE-UV analysis. The sample throughput of the fully automated DBS elution/analysis is approximately 2-fold lower than that of the manual DBS elution/analysis in 96-well plate format. Nevertheless, the automated method eliminates the multiple manual steps (see Figure S10 in the SI), can be performed fully autonomously in a 24 h/7 days regime and the total cost per sample is more than 7-fold lower (see Table S2 and corresponding text in the SI for the total cost-analysis). Besides, no external instrumentation is required and possible operator-induced errors are avoided, which further simplify and streamline the analytical workflow.

Table 2: CE sequence for a fully automated DBS elution and CE-UV determination of warfarin. All procedures were autonomously performed by the CE instrument. BGE solution and detection wavelength as for Figure 5.

Action	Duration	Pressure/Voltage
1. DBS elution[a]		
1a. Vial filling with 80 µL of ACN	180 s	950 mbar (95 000 Pa)
1b. Bubbling with air[b]	60 s	950 mbar (95 000 Pa)
1c. Vial filling with 20 µL of DI water	140 s	950 mbar (95 000 Pa)
1d. Bubbling with air[b]	300 s	950 mbar (95 000 Pa)
2. Separation capillary conditioning		
2a. 0.1 M NaOH flush	60 s	950 mbar (95 000 Pa)
2b. DI water flush	90 s	950 mbar (95 000 Pa)
2c. BGE solution flush	120 s	950 mbar (95 000 Pa)
3. CE-UV analysis		
3a. DBS eluate injection	12 s	55 mbar (5500 Pa)
3b. Warfarin separation	240 s	25 kV

[a] the snap-cap vial with the DBS was at the CE outlet to enable pressurizing at 950 mbar (95 000 Pa). [b] bubbling with air was achieved by pressurizing an empty snap-cap vial.

Table 3: Analytical parameters for the determination of warfarin in DBS samples by the fully automated DBS elution and CE-UV determination. Elution and CE conditions as for Table 2 and Figure 5, $n=5$.

Parameter	
RSD (%), 0.5 $\mu g\,mL^{-1}$	6.2
RSD (%), 1.0 $\mu g\,mL^{-1}$	5.1
RSD (%), 3.0 $\mu g\,mL^{-1}$	4.8
r^2 (0.35–5.0 $\mu g\,mL^{-1}$)	0.9998
LOD ($\mu g\,mL^{-1}$)	0.11
LOQ ($\mu g\,mL^{-1}$)	0.35

Application to Warfarin-Containing Blood Samples

Justification of the proposed concept was demonstrated by at-home DBS sampling of warfarin-containing capillary blood, mailing the samples to our laboratory, and running the automated DBS elution/CE determination of warfarin. One of the volunteers has been diagnosed with excessive blood clotting and was warfarin-treated and monitored in a specialized clinical center. The warfarin treatment took 6 months and the medication was changed after this period. Thus, we were able to obtain and analyze real warfarin-containing DBS samples.

The volunteer collected five DBS samples at different stages of the treatment and resulting CE-UV electropherograms of the DBS eluates are depicted in Figure 5. Quantitative analyses of warfarin in the DBS eluates (each eluate was analyzed three times) were based on an external calibration curve plotted for warfarin-spiked drug-free DBSs.

The first DBS was sampled one month before the end of warfarin treatment (Figure 5a) and evidenced the presence of warfarin ($2.41\pm0.15\ \mu g\,mL^{-1}$) within the therapeutic range.[33]

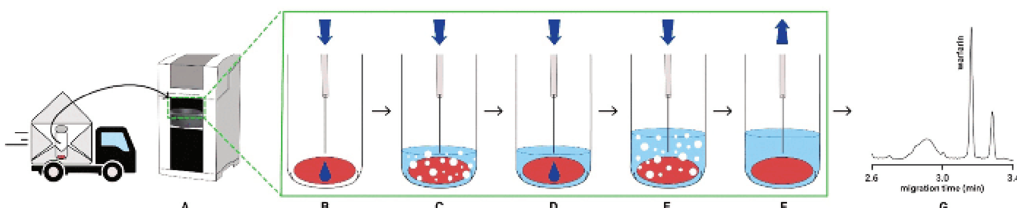

Figure 4. A scheme of the automated DBS processing and CE-UV analysis. A) DBS loading into CE carousel, B) vial filling with 80 µL of ACN, C) air bubbling, D) vial filling with 20 µL of DI water, E) air bubbling, F) CE injection, G) CE-UV separation.

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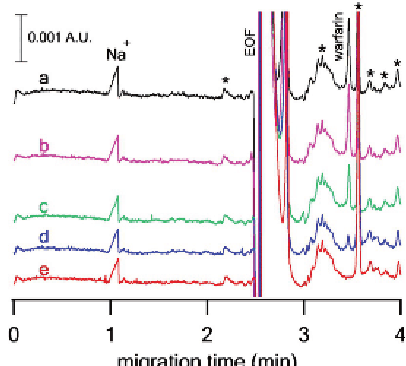

Figure 5. Automated CE-UV analyses of DBSs after self-sampling by a warfarin-treated volunteer. DBS samples were collected 1 month before (a) 1 day before (b), 3 days after (c), 7 days after (d), and 1 month after (e) warfarin discontinuation. Elution and CE-UV conditions as for Table 2; BGE solution, 30 mM acetic acid, 30 mM sodium acetate and 30% (v/v) ACN at pH 5.2; detection wavelength, 205 nm; *: unknown matrix compounds; EOF – electroosmotic flow.

The second DBS was sampled the day before warfarin discontinuation (Figure 5b) and the determined warfarin concentration ($2.46 \pm 0.11\ \mu g\,mL^{-1}$) confirmed the stable warfarin blood level during treatment. Two additional DBSs were sampled 3 days (Figure 5c) and 7 days (Figure 5d) after warfarin discontinuation with the determined warfarin concentration of 1.62 ± 0.08 and $0.45 \pm 0.03\ \mu g\,mL^{-1}$, respectively. The last DBS was sampled one month after the warfarin discontinuation and confirmed its complete elimination from the body as well as its baseline separation from all co-eluted DBS matrix components (Figure 5e) and justified the suitability of the proposed analytical concept. The concentrations determined in DBS samples after warfarin discontinuation (Figure 5c–e) correlate well with its mean half-life (36–42 h) in blood plasma.[24] The quantitative results were compared with the determination of warfarin in the DBS eluates using a UHPLC-MS/MS method with statistically non-significant differences. Details on the UHPLC-MS/MS method and the quantitative results comparison are presented in Experimental and in Table S3 in the SI, respectively.

Conclusion

A novel concept of blood self-sampling in the form of DBS, transport of the DBS to a laboratory by mail, and fully automated DBS processing/analysis is presented. The proposed concept reflects the actual analytical trends, constitutes a convenient tool for clinical analyses and may significantly improve the comfort and life quality of involved subjects. It eliminates the need for frequent and regular visits to medical centers and the painful phlebotomy usually associated with standard clinical methods. Moreover, the results can be communicated to the subjects by phone thus further reduce the travel expenditure to the minimum. Other merits are the less invasive capillary blood collection from a finger prick with a single-use DBS self-sampling kit and the comfort of blood sampling in a familiar environment at home. The DBS transport to the laboratory can be accomplished by mail or parcel delivery companies and the DBS is automatically analyzed in the laboratory with negligible staff intervention. The DBS elution and subsequent on-line analysis of the eluate are performed using a single off-the-shelf CE instrument and the quantitative result is obtained almost instantly after the DBS delivery to the laboratory.

The proposed analytical concept is exemplified by DBS sampling and analysis of warfarin and may open new horizons for clinical assays requiring high-throughput sample collection/analysis. It is simple, cheap, rapid, robust, and accurate and ensures exhaustive elution of the model analyte from DBSs and sufficient sensitivity for its determination at therapeutic levels. Moreover, the concept can be applied to a wide range of clinically relevant compounds, which possess similar characteristics as the model analyte and are thus amenable to the at-home DBS self-sampling with the subsequent automated CE analysis. For compounds with lower therapeutic levels, the actual concept can be extended by using on-capillary CE preconcentration techniques or selective/sensitive detectors,[34] which will enable adequate sensitivity for most TDM assays.[33]

The proposed concept, therefore, provides a general solution to personalized health monitoring (endogenous blood constituents monitoring, chronic disease monitoring, drug dosing optimization, TDM, etc.), and might propel a shift from the actual sick-care to a prevention-based healthcare system. It also offers an elegant analytical tool for critical (i.e. pandemic) situations, which require considerably reduced personal contacts between the subjects and the clinicians but maintain the need for frequent and regular clinical blood testing.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: analytical methods · blood self-sampling · dried blood spots · electrophoresis · warfarin

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Supporting Information

Dried Blood Spot Self-Sampling with Automated Capillary Electrophoresis Processing for Clinical Analysis

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Supporting Information for the manuscript

Dried blood spot self-sampling with automated capillary electrophoresis processing for clinical analysis

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EXPERIMENTAL SECTION

Reagents, standard solutions, and laboratory equipment

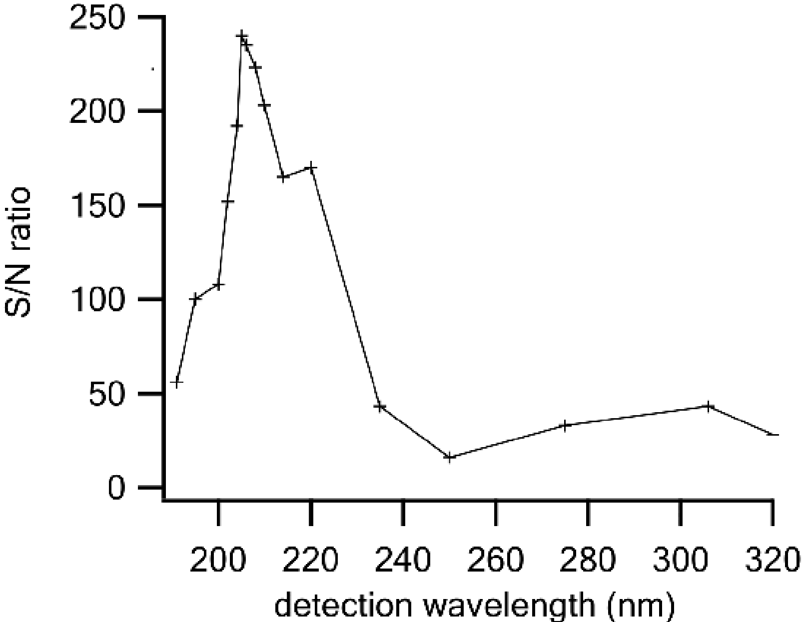
Analytical reagent grade chemicals were used for the experiments and deionized (DI) water with resistivity higher than 18 MΩ·cm was used throughout. The stock solution of warfarin (1 mg/mL) was prepared from an analytical standard (Sigma, Steinheim, Germany) by dilution with methanol (MeOH, Lach-Ner, Neratovice, Czech Republic) and was stored at – 20 °C. Standard solutions of warfarin were prepared daily by dissolving appropriate volumes of the stock solution in DI water. Background electrolyte (BGE) solution for capillary electrophoresis (CE) measurements was prepared daily from acetonitrile (ACN, Lach-Ner), DI water, and stock solutions of 1 M acetic acid (HAc, Fluka, Buchs, Switzerland) and 1 M sodium acetate (NaHAc, Sigma). The stock solutions were stored at a temperature of 4 °C in a refrigerator. LC/MS grade solvents for mobile phase preparation, ACN, DI water and formic acid, were purchased from Honeywell Burdick and Jackson (Seelze, Germany). Manual liquid handling procedures were performed with adjustable micropipettes (2 – 20 µL, 20 – 200 µL and 200 – 1000 µL, Proline Plus, Biohit, Helsinki, Finland). The agitation of snap-cap vials with DBS samples was done with a Vibramax 110 (Heidolph Instruments GmbH, Schwabach, Germany) agitator.

Capillary electrophoresis

CE analyses were facilitated using an Agilent 7100 CE (Agilent Technologies) instrument at a potential of + 25 kV applied at the injection side of a separation capillary. For manual DBS elution and CE analysis, a short (P/N G7100-60033, Agilent Technologies) and a standard (P/N G7100-60007) electrode were used at the injection and at the outlet side of the CE instrument, respectively. The short electrode was 5 mm shorter than the standard electrode. For CE-automated DBS elution and analysis, both electrodes were short. Detection was performed using an in-built UV-Vis diode array detector and warfarin was detected at 205 nm. Warfarin

was previously detected at 200 nm[1] and 306 nm[2] in CE, nevertheless, our measurements showed UV-absorbance spectrum with a maximum at 205 nm. Detection wavelength optimization was carried at 190 – 320 nm and the resulting signal-to-noise (S/N) ratios are illustrated in **Figure S1** and confirm our detection wavelength selection.

Figure S1. S/N ratios for warfarin CE-UV determination at various detection wavelengths. CE conditions: BGE solution, 30 mM HAc, 30 mM NaHAc and 30% ACN at pH 5.2; voltage, + 25 kV; injection, 55 mbar (5500 Pa) for 12 s; warfarin concentration 1 μg/mL.



Separation capillary was a bare fused silica capillary (75 μm i.d./375 μm o.d., L_{tot} = 45 cm and L_{eff} = 36.8 cm) supplied by Polymicro Technologies (Phoenix, AZ, USA) and protruded by 10 and 12 mm from short CE electrodes on the inlet and outlet side, respectively (see **Figure S2A**). Polyimide coating (2 mm) was burned off on both capillary ends. As it is difficult to

precisely measure the capillary lengths protruding from the electrodes inside the CE instrument, setting up the correct lengths of the capillary ends can be most conveniently done by measuring the lengths of the separation capillary exposed from the CE cartridge. For the inlet side, the total exposed length was 52.5 mm and for the outlet side, the total exposed length was 54.5 mm, see **Figure S2B**.

CE separation of warfarin, blood interferences, and other standard acidic drugs was examined in two different BGE solutions consisting of 30 mM acetate buffer with the addition of 30% MeOH or 30% ACN (v/v). The optimum BGE solution for CE-UV determination of warfarin consisted of 30 mM NaHAc, 30 mM HAc and 30% (v/v) ACN with an apparent pH 5.2.[3] In this BGE solution, warfarin was baseline resolved from all blood interferences as well as from ibuprofen, naproxen, ketoprofen, and diclofenac as the typical acidic drugs, which might potentially interfere with the warfarin determination. Peak shapes were also considerably better (sharp and symmetrical) resulting in a better separation efficiency in comparison to the MeOH-based BGE solution. Initial equilibration of a new capillary was carried out for 15 min each with 100 mM NaOH, DI water, and BGE solution. At the beginning of each working day, the capillary was flushed with 100 mM NaOH, DI water, and BGE solution for 2 min each. Between two consecutive CE analyses, the capillary was flushed with 100 mM NaOH (1 min), DI water (1.5 min), and BGE solution (2 min). At the end of each working day, the capillary was flushed with 100 mM NaOH, DI water, and air for 5 min each. All flushing procedures were performed at the pressure of 950 mbar (95000 Pa) and the capillary temperature was maintained at 25 °C. Hydrodynamic injections were performed at 55 mbar (5500 Pa) for 12 s; these conditions ensured best S/N ratios and number of theoretical plates for injections of warfarin standard solutions. The CE instrument was controlled, and analytical signals were acquired by ChemStation CE software (Agilent Technologies).

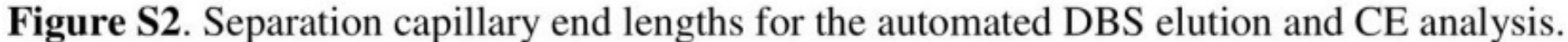
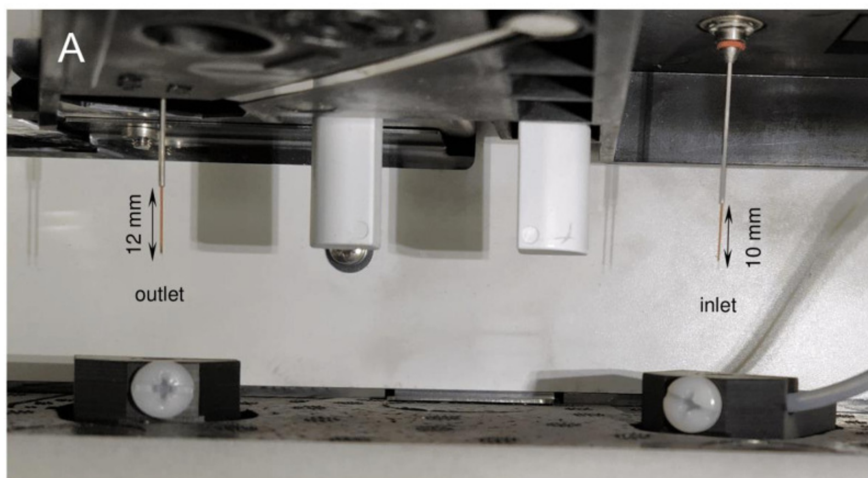
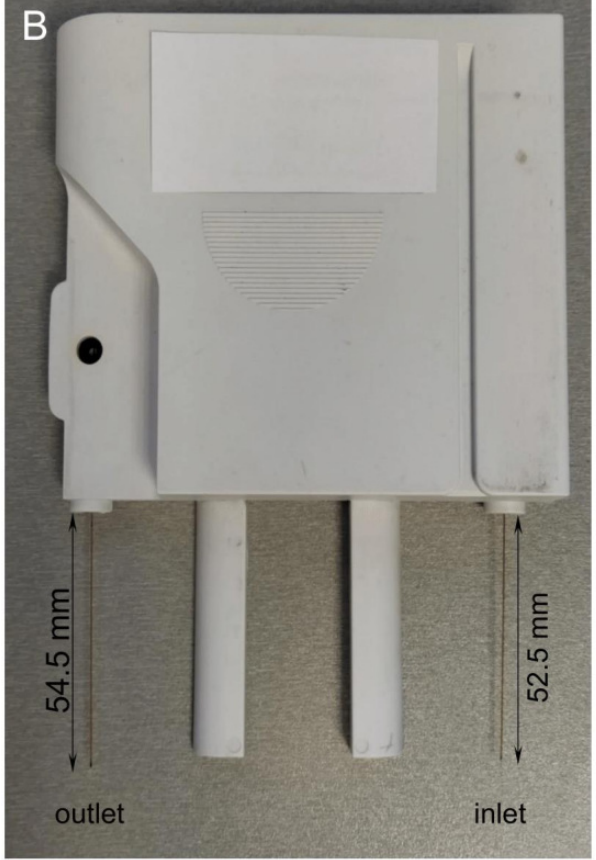


Figure S2. Separation capillary end lengths for the automated DBS elution and CE analysis.





Ultra high performance liquid chromatography – mass spectrometry/mass spectrometry (UHPLC-MS/MS)

Quantitative results of warfarin in real DBS samples were confirmed by UHPLC-MS/MS determination of warfarin in the resulting eluates. An Agilent 1290 Infinity II UHPLC System (Agilent Technologies, Palo Alto, CA, USA) coupled with electrospray ion source (ESI) and Agilent 6490 Triple Quadrupole mass spectrometry system was used for UHPLC-MS/MS analyses.

UHPLC separations were performed using a Zorbax C18 Eclipse plus column, 3.0 × 50 mm; 1.8 µm (Agilent Technologies, USA), heated at 40 °C. A mixture of 0.1% formic acid in DI water (A) and 0.1% formic acid in ACN (B) was used as the mobile phase. The flow rate was set at 0.4 mL/min and the injection volume was 2 µL. The gradient elution was carried out by increasing percentage of ACN as follows: a linear gradient from 20 to 80% B during 5.0 min followed by elution at 80% B for 1.5 min. The column was then re-equilibrated to initial conditions (20% B) for 1 min. The total run-time was 7.5 min. The retention time of warfarin was 3.22 min.

Quantitative analysis was carried out in a multiple reaction monitoring (MRM) mode using the transition m/z 309.1 → 163 in positive ion mode. Other MRM transitions were used for qualitative confirmation. The following MS parameters were fixed for all substances: gas temperature: 250 °C; gas flow: 8 L/min; nebulizer: 33 psi (227527 Pa); sheet gas temperature: 400 °C; sheet gas flow: 12 L/min; capillary voltage: 3000 V; nozzle voltage: 300 – 600 V.

A summary of precursor and product ions, collision energies and fragmentor voltages is presented in the following **Table S1**.

Table S1. Selected parameters of the MS/MS detection.

Precursor ion [*m/z*]	Product ion [*m/z*]	Collision energy [V]	Fragmentor [V]
309.1	163	12	91
309.1	251	24	91
309.1	43.1	36	91
309.1	147	91	91
307.1	161	20	125
307.1	250	24	25

Agilent MassHunter software (version 10.0) was used for data acquisition and quantification of the analysis results.

DBS collection

Capillary blood and DBSs were sampled by volunteers from the Institute of Analytical Chemistry after signing a written informed consent. The Institute of Analytical Chemistry has not an ethical committee. The research was approved by the director of the Institute of Analytical Chemistry and the leading scientists of the Departments at the Institute of Analytical Chemistry. The DBS sample collection was carried out according to the Declaration of Helsinki. Six volunteers (one of them being treated with warfarin) were given a set of DBS sampling kits (see **Figure S3**) and the Instructions for use (see **Figure S4**).

DBS self-sampling kit details

Two photographs of the DBS self-sampling kit are depicted in **Figure S3**. The set contained a sterile latex glove: A (Paul-Hartmann AG, Heidenheim, Germany), a disposable lancet: B (Haemolance Plus Normal Flow, HTL Strefa, Ozorków, Poland), a dry cotton pad: C (Batist Medical a.s., Červený Kostelec, Czech Republic), a disinfection pad soaked with isopropyl alcohol: D (Van Oostveen Medical B.V., Wilnis, The Netherlands), a glass snap-cap vial (2 mL): E with a polyurethane (PU) cap: F (Agilent Technologies, Waldbronn, Germany, P/N 5182-9697 and P/N 5181-1512), a DBS sampling disc: G (10 mm, inside the vial) pre-punched from Whatman™ 903 Protein Saver sampling card (GE Healthcare Ltd, Cardiff, UK), a glass capillary with plugs: H (20 μL, 30 mm, Keraglass a.s., Otvovice, Czech Republic), a bag with desiccant: I (SPX Flow Technology, Florida, USA), two plastic zip-lock bags: J (Ecofol s.r.o., Ústí nad Labem, Czech Republic) with identification labels, and Instructions for use (see **Figure S4**), all packed in an envelope: K.

Figure S3. Photographs of the packed and the exposed content of the DBS self-sampling kit.

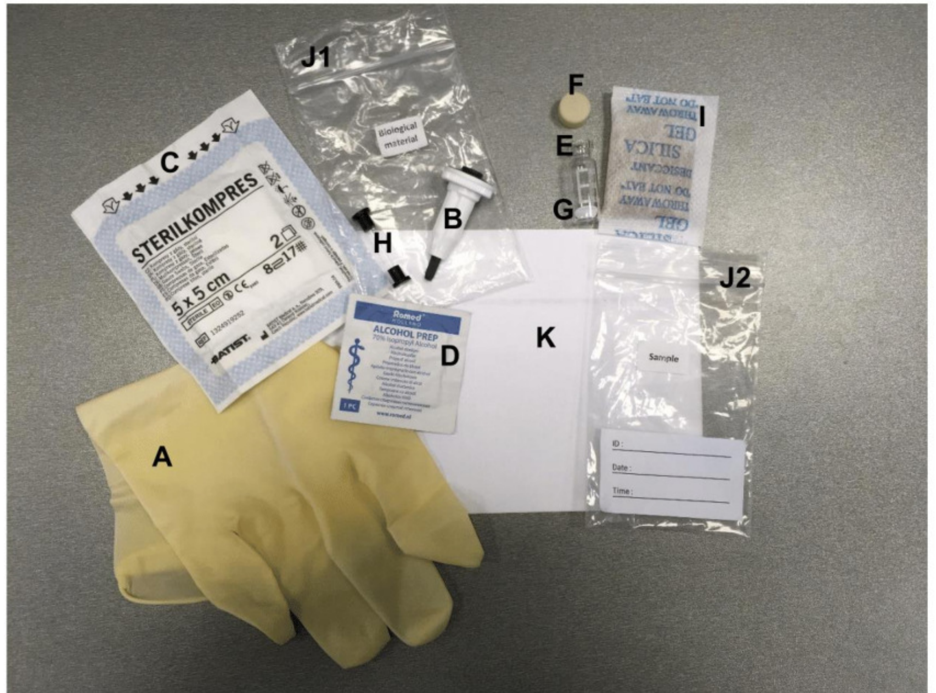


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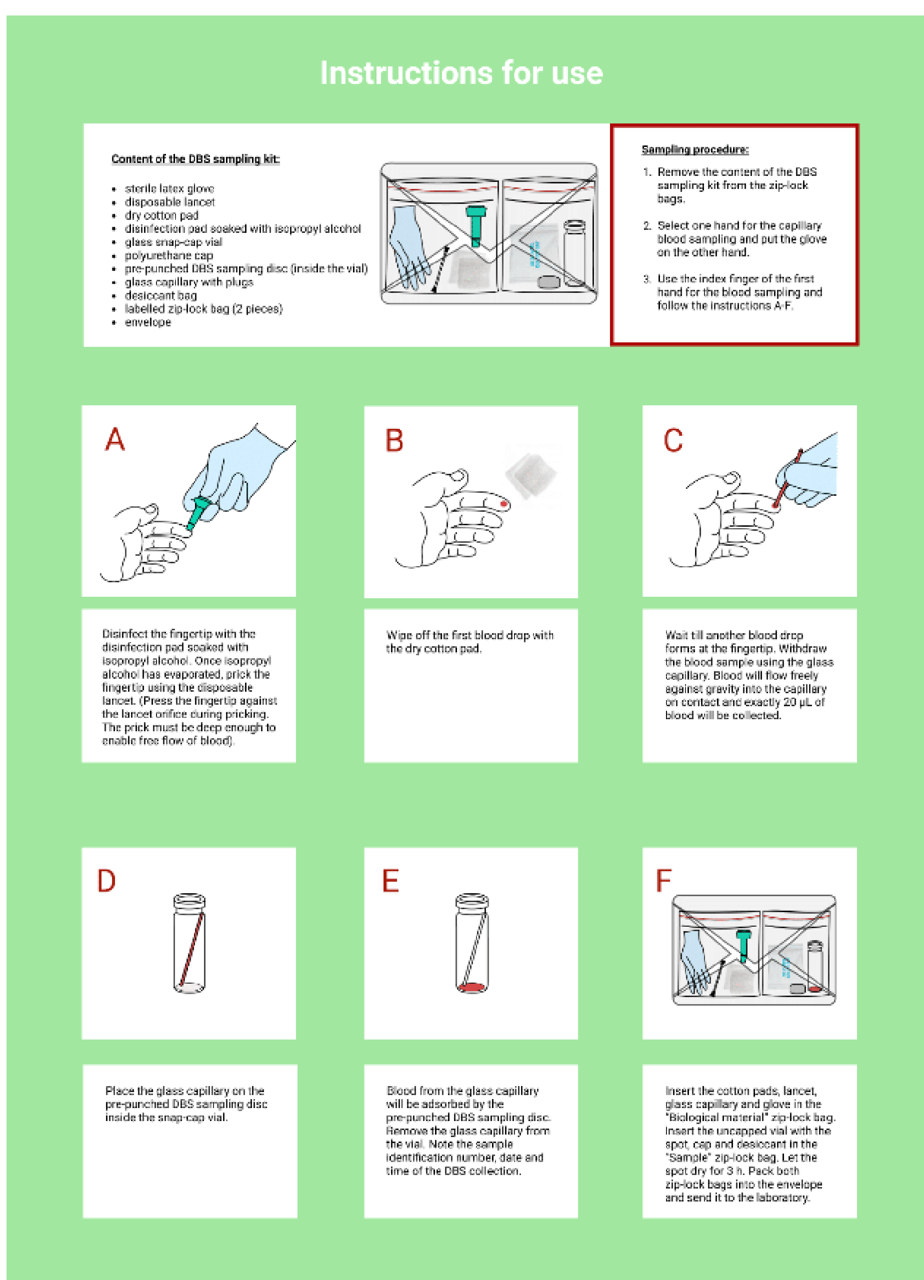
Figure S4. Instructions for use for the DBS sampling kit.



S10

The entire analytical workflow is schematically depicted in **Figure 1** and consists of the following steps, which are detailed in chapters *Kit for the DBS self-sampling* and *DBS collection using the self-sampling kit, delivery to the laboratory and storage* in the main manuscript. Capillary blood was withdrawn from a finger prick into a glass capillary (**Figure 1A**) and was quantitatively dispensed onto a pre-punched sampling disc (**Figure 1B** and **1C**). The DBS sample and all components of the sampling kit were inserted in labelled zip-lock bags and shipped to the laboratory (**Figure 1D**). In the laboratory, the snap-cap vial was closed (**Figure 1E**) and elution of the DBS sample was carried out manually or fully automatically by the CE system (**Figure 1F**). The resulting DBS eluates were quantitatively analysed by CE (**Figure 1G**). The collected DBS samples were stored in closed zip-lock bags with a desiccant at 4 °C for up to three months.

Drug-free and warfarin-spiked DBS samples

Drug-free DBSs were formed by pricking a finger of a healthy volunteer, collecting 20 μL of capillary blood into a glass capillary, and spotting the blood sample onto a pre-punched Whatman™ 903 DBS sampling disc in a snap-cap vial. The spot was dried up in the vial, which was placed in a zip-lock bag with a desiccant, at ambient temperature/humidity for 3 h.

Spiking the blood sample with warfarin was realized by pipetting 5 μL of warfarin standard solution into a 250 μL PCR vial, subsequent pipetting of 95 μL of capillary blood into the PCR vial and mixing the two liquids for 5 s. Four aliquots of the spiked blood were withdrawn with 20 μL glass capillaries, spotted onto pre-punched Whatman™ 903 DBS sampling discs in snap-cap vials and dried up at the above-reported conditions for 3 h. The four DBSs were prepared in about 60 s, i.e. before the blood started to clot and disabled its free flow into/from the 20 μL glass capillaries. The sampling procedure was identical to that described for the DBS sampling kit in chapters *Kit for the DBS self-sampling* and *DBS collection using the self-sampling kit,*

delivery to the laboratory and storage (in the main manuscript) except for the shipment to the laboratory.

Precision and accuracy of the 20 µL glass capillaries

The volumes withdrawn with the capillaries were examined with DI water and capillary blood. The capillaries were weighted before and after the withdrawal of the liquid samples and resulted in excellent accuracy (withdrawn volume) and precision (RSD values). The determined volumes (n = 5) were 20.10 ± 0.17 µL and 19.98 ± 0.26 µL for DI water and capillary blood, respectively. Subsequently, the withdrawn liquid samples were transferred to pre-punched DBS sampling discs from the capillaries and the transferred volumes were determined by weighting the discs before and after the liquid transfers. The determined volumes (n = 5) were 19.30 ± 0.33 µL and 19.76 ± 0.23 µL for DI water and capillary blood, respectively, and demonstrated a complete transfer of capillary blood to the sampling discs.

DBS drying before shipment to the laboratory

Although the rapid drying time using the desiccant in the zip-lock bag (3 h) might allow DBS formation during the transport, a concept of in-transport drying of capillary blood was rejected for two reasons. (i) Shipment of wet blood represents a biological hazard and would not be approved by the authorities. (ii) Wet blood samples shipment in different localities and seasons might result in improper drying process and samples might arrive to the laboratory frozen or wet if transported at freezing conditions. Such conditions are expected to be encountered during winter time in many localities around the world and would spoil the samples. For these reasons, the proposed concept involved the 3 h at-home drying time after capillary blood self-sampling, which ensured complete blood drying up and DBS formation. It did not add a significant extra

time to the entire transfer process and provided safe handling of blood samples by the mail/parcel delivery companies and the laboratory staff.

DBS elution

DBSs were eluted directly in the snap-cap vials with a specific volume of ACN and DI water. To minimize manipulation with the DBS sample and to avoid possible contamination of the DBS eluate, the Whatman™ 903 sampling disc was not removed from the vial after the DBS elution and the CE injection was performed directly from the free eluate above the disc. Two different elution processes were developed and DBS eluates were used on the day of elution only.

The manual elution process was performed by pipetting a given volume of ACN (80 – 400 µL) into the snap-cap vial with the DBS, the vial was closed with a PU cap and agitated at 1000 rpm for 1 min. Subsequently, a given volume of DI water (20 – 100 µL) was added to the vial, the vial was closed and agitated at 1000 rpm for another 5 min. The final optimized elution volumes were 80 µL of ACN and 20 µL of DI water (see chapter *Elution volume*).

The fully automated elution process was performed by loading the snap-cap vial with the DBS into the CE autosampler carousel and transferring ACN and DI water to the vial by the CE separation capillary. Using the optimized volumes, the vial was first filled with 80 µL of ACN and air bubbled for 1 min, followed by the addition of 20 µL of DI water with another air bubbling for 5 min. All liquid transfers and bubbling with the air were carried out by the CE internal pressurizing system and the procedure is detailed in chapter *Automated DBS elution/CE analysis* in the main manuscript and visualized in **Video S2**.

Figure S5. Optimization of the DBS drying time in a snap-cap vial at ambient temperature/humidity with and without a desiccant. Manual elution was carried out with 400 µL of ACN followed by 100 µL of DI water at the agitation speed of 1000 rpm for 5 and 5 min, respectively. CE conditions as for **Figure S1**; detection wavelength, 205 nm; warfarin concentration, 25 µg/mL; *n = 3*.

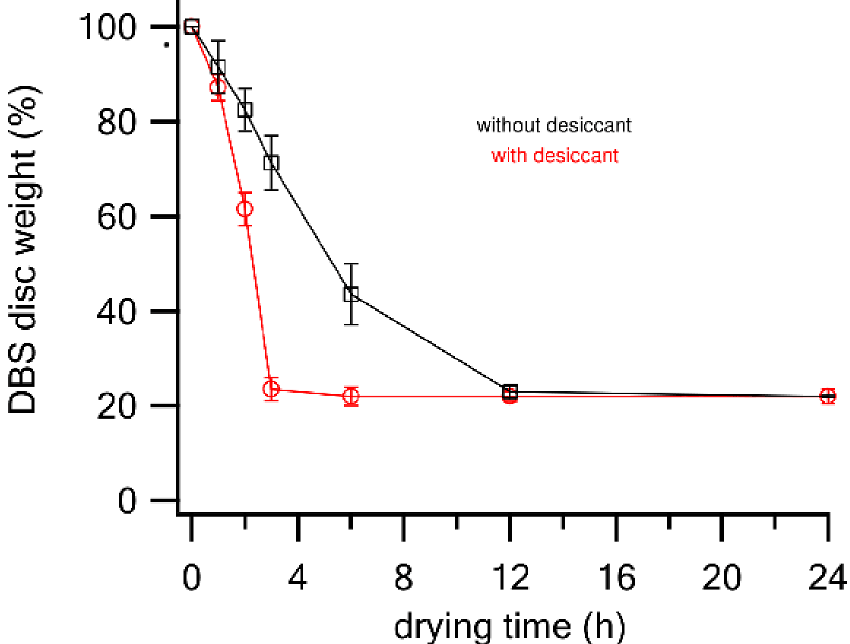
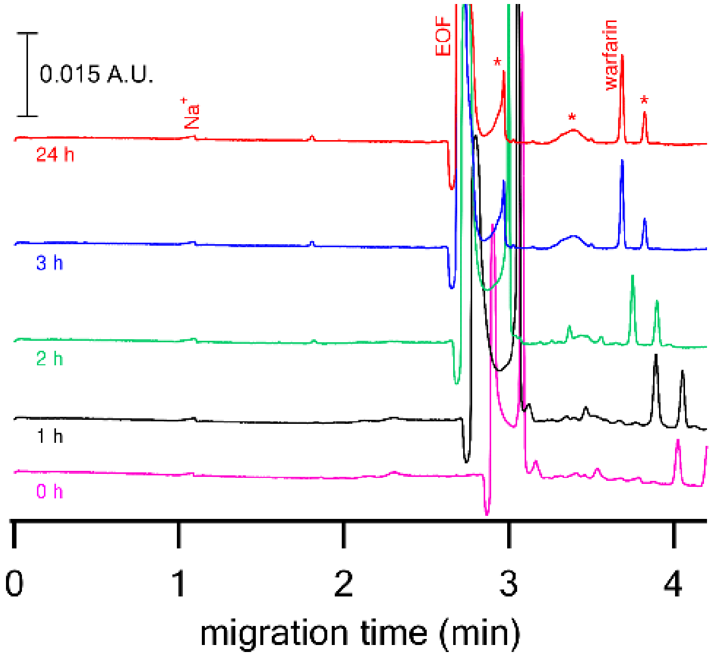


Figure S6. Optimization of DBS drying time in a snap-cap vial using a desiccant in the zip-lock bag. CE conditions as for **Figure S1**; detection wavelength, 205 nm. Elution conditions as for **Figure S5**; warfarin concentration, 25 µg/mL; * – unknown matrix compound; EOF – electroosmotic flow.



Manual DBS elution

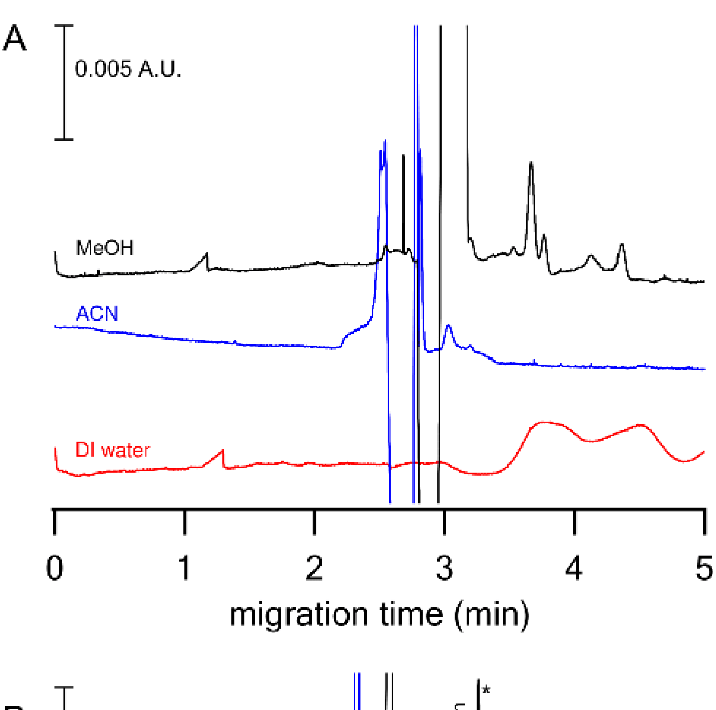
DBS elution is usually carried out with DI water, organic solvents, or aqueous mixtures of organic solvents.[4] DBS elution with organic or partially organic solvents might be especially useful when the matrix effects are to be suppressed and when the target analytes are hydrophobic compounds.[4] The spiked DBS samples (25 µg/mL of warfarin) were eluted with three pure elution solvents (DI water, MeOH, and ACN) and various aqueous/organic mixtures. The DBS elution process was first examined with 500 µL of the pure solvents agitated at 1000 rpm for 10 min. Electropherograms of the three eluates are shown in **Figure S7A**. Deteriorated

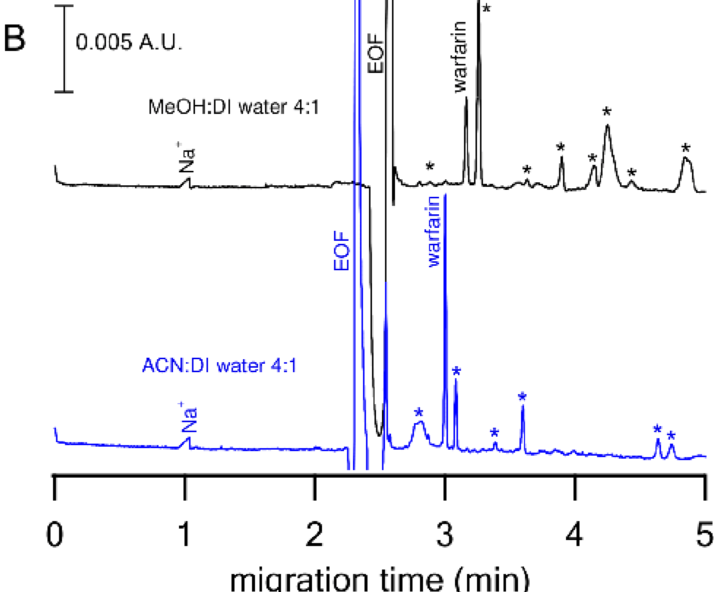
CE performance and no warfarin peak were observed for DBSs eluted with DI water due to the co-elution of high molecular-weight blood matrix components and their adsorption to the separation capillary wall. DBS elution with ACN eliminated most blood components while several trace components were detected in the MeOH-based eluates. Nevertheless, CE measurements of the purely organic eluates revealed no warfarin (in ACN) and only poorly detectable warfarin (in MeOH) due its hydrophilic and polar character. Thus, a two-step elution process was examined in the following protocol using DBS elution with an organic solvent (elimination of matrix components) followed by elution with DI water (warfarin release from the DBS due to the increase of the eluate polarity).

Composition of the elution mixture

For the sequential elution, 400 µL of MeOH or ACN was pipetted into the snap-cap vial with the DBS, the vial was closed and agitated at 1000 rpm for 5 min. Subsequently, 100 µL of DI water was added to the organic eluate, the vial was closed and agitated at 1000 rpm for another 5 min. The ability of the two-step procedure to eliminate matrix interferences (proteins, red blood cells, lipids, etc.) and to elute warfarin was characterized by CE analyses of the resulting eluates. Reduced elution of matrix components and improved elution of warfarin were achieved for the ACN:DI water mixture and electropherograms of the warfarin-spiked DBS samples eluted with MeOH:DI water and ACN:DI water are shown in **Figure S7B.**

Figure S7. Effect of elution solvent on elution of warfarin from DBS. CE conditions as for **Figure S1**; detection wavelength, 205 nm. (A) Elution was carried out with 500 µL of a pure solvent at the agitation speed of 1000 rpm for 10 min. (B) Elution was carried out with 400 µL of MeOH or ACN followed by 100 µL of DI water at the agitation speed of 1000 rpm for 5 and 5 min, respectively; warfarin concentration, 25 µg/mL; * – unknown matrix compound; EOF – electroosmotic flow.





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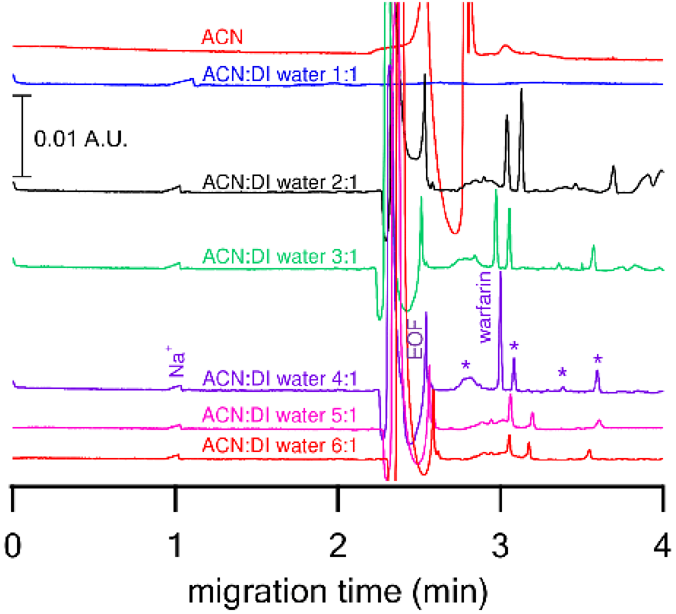
The two-step elution process was further examined at different ACN:DI water ratios (1:1, 2:1, 3:1, 4:1, 5:1 and 6:1, v/v) to obtain optimum conditions for the warfarin elution from the DBS samples. The resulting electropherograms are depicted in **Figure S8**. Elution of warfarin from DBSs was inefficient for the high ACN content (5:1, 6:1, v/v, and pure ACN) while co-elution of matrix components disabling warfarin determination was observed for the high DI water content (1:1, v/v). The highest efficiency for warfarin elution, minimum co-elution of matrix components, and optimal CE separation with best S/N ratio of warfarin were achieved with the ACN:DI water ratio of 4:1 (v/v).

Figure S8. Two-step elution of warfarin from DBS samples at different ACN:DI water ratios. CE conditions as for **Figure S1**; detection wavelength, 205 nm. Elution was carried out with different volumes of ACN and DI water at the specified ratios to achieve total elution volume 500 μL; agitation time, 5 min each; agitation speed, 1000 rpm; warfarin concentration, 25 μg/mL; * – unknown matrix compound; EOF – electroosmotic flow.



Elution time

DBS elution time is one of the crucial parameters, which determine the duration of the entire analytical process. The two-step elution process was, therefore, optimized to achieve exhaustive elution of warfarin from the DBSs with the highest possible repeatability in the shortest possible time. The first step, blood matrix elimination, was examined by agitating the vial with the DBS and 400 μL of ACN at 1000 rpm for 0, 1, 3, 5, 7, and 10 min. After the ACN agitation, 100 μL of DI water was added to the vial, and warfarin was eluted by agitating the vial at 1000 rpm for an additional 5 min. The 0 min ACN agitation time denotes pipetting DI water into the vial

immediately after ACN. The resulting eluates were analysed by CE and the effect of ACN agitation time on warfarin elution is depicted in **Figure 2** (red trace) in the main manuscript. ACN agitation time of 1 min was sufficient for an efficient DBS matrix elimination and longer ACN agitation did not essentially affect warfarin content in the eluate. The second step, warfarin elution with 100 µL of DI water, was optimized by agitating the ACN/DI water mixture at 1000 rpm for 0 – 10 min (blue trace in **Figure 2**) using the ACN agitation time of 1 min. The 0 min DI water agitation time denotes no agitation after DI water pipetting and immediate analysis of the elution mixture by CE. At short agitation times, the elution of warfarin was incomplete and the repeatability of the elution process was significantly compromised (RSD: 42.1%, 22.1% and 7.6% for 0, 1, and 3 min, respectively). Exhaustive elution of warfarin (99 – 100%) and improved repeatability (RSD ≤ 4.7%) were achieved for agitation times 5 – 10 min. The optimized two-step DBS elution process was, therefore, realized by ACN agitation for 1 min followed by addition of DI water/agitation of the mixture for another 5 min and resulted in an excellent elution efficiency (99 ± 4%).

Elution volume

Initially, DBSs were prepared by spotting 20 µL of warfarin-spiked capillary blood onto the sampling discs and these were eluted with 400:100 µL of ACN:DI water. Warfarin concentration in the spiked blood was 25 µg/mL to compensate for the 25-fold dilution during DBS elution and to ensure clearly detectable analytical signals of warfarin for the CE-UV analyses of the DBS eluates. Warfarin therapeutic range in blood is 1 – 3 µg/mL,[5] thus the analytical protocol required a further optimization to achieve sufficient CE-UV sensitivity at or even below the therapeutic concentrations.

The eluent volume was considered an important variable as the dilution factor directly influences the method sensitivity in terms of warfarin limit of detection/quantification (LOD/LOQ). The pre-punched 10 mm sampling disc with the DBS adsorbs approx. 30 µL of

the eluent. The remaining eluent in the vial forms a free solution above the disc and is used for the CE injection. The ratio ACN:DI water (4:1) was kept constant and the total eluent volume was reduced to 400, 200, 100, 75, and 50 µL. The lowest volume, which ensured repeatable DBS elution and reliable injection of the resulting eluate into the CE system, was 100 µL, corresponding to 5-fold dilution of the original capillary blood. The low dilution factor ensured adequate sensitivity of the CE-UV method and **Figure S9** depicts electropherograms of eluted DBSs prepared from drug-free capillary blood and the same blood spiked with 1 µg/mL of warfarin. A graphical scheme of the entire manual elution process is shown in **Figure S10**.

Figure S9. CE-UV determination of warfarin in DBS eluate of drug-free and warfarin-spiked capillary blood. CE conditions as for **Figure S1**; detection wavelength, 205 nm. Elution conditions: ACN (80 µL, 1 min agitation) and DI water (20 µL, 5 min agitation); agitation speed, 1000 rpm; warfarin concentration, 1 µg/mL; * – unknown matrix compound; EOF – electroosmotic flow.

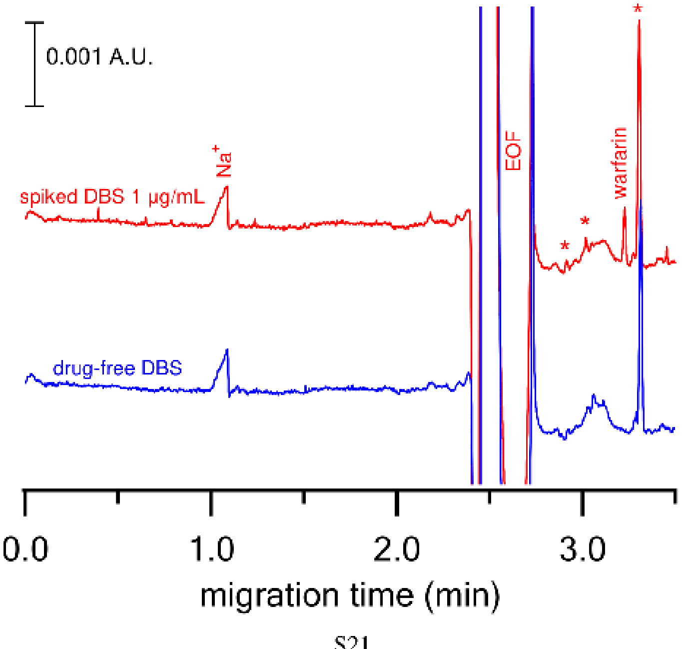


Figure S10. A graphical scheme of the manual DBS elution – CE analysis process. A – Pipetting of 80 µL of ACN, B – vial capping and transfer to the agitator, C – agitation at 1000 rpm for 1 min, D – transfer from the agitator, E – vial uncapping and pipetting of 20 µL of DI water, F – vial capping and transfer to the agitator, G – agitation at 1000 rpm for 5 min, H – transfer to the CE carousel, I – CE injection, J – CE/UV separation. Manual steps (A, B, D, E, F, H), automated steps (C, G, I, J).

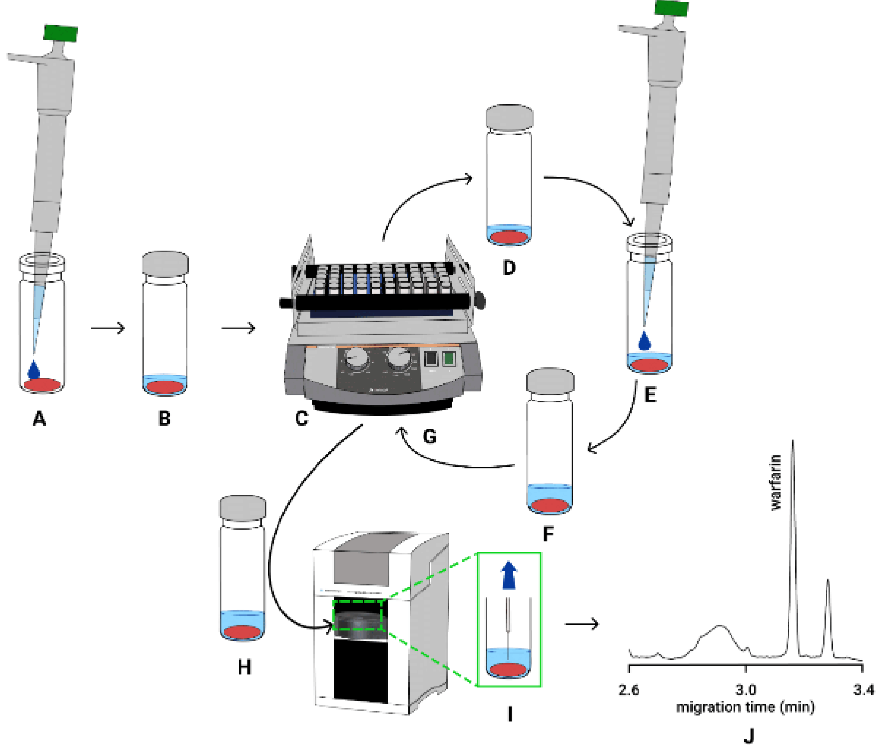
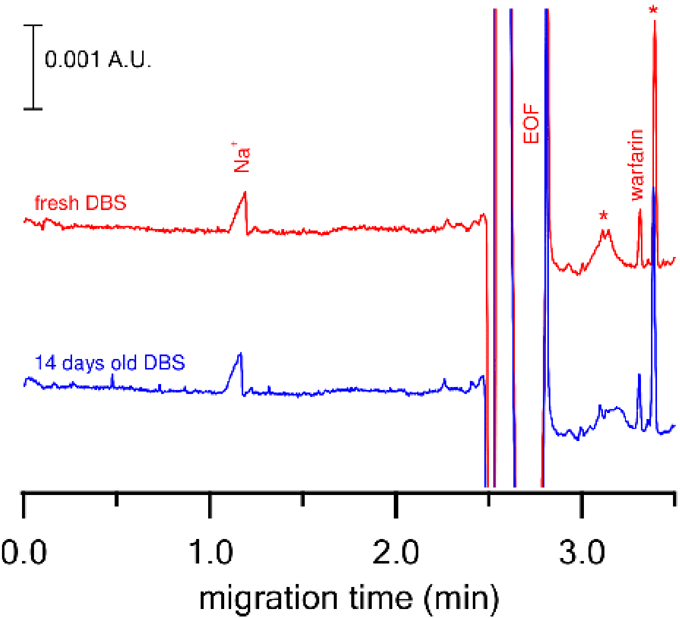


Figure S11. CE-UV determination of warfarin in eluates of fresh and aged DBSs. CE conditions as for **Figure S1**; detection wavelength, 205 nm. Elution conditions: ACN (80 µL, 1 min agitation) and DI water (20 µL, 5 min agitation); agitation speed, 1000 rpm; warfarin concentration, 1 µg/mL; haematocrit level – medium; * – unknown matrix compound; EOF – electroosmotic flow.



Total cost and time analysis

The cost estimates for the manual DBS elution followed by an automated CE-UV analysis and the fully automated DBS elution/CE-UV analysis are based on the actual prices for the products/services in Germany. Prices of reagents, solvents and consumables are from Sigma-Aldrich webpage, labour costs are based on an average salary of a laboratory assistant and electricity costs are based on an average cost of one kWh in 2019. The costs are estimated for the analysis of 96 DBS samples and are summarized in **Table S2**. DBSs are eluted with the same volumes of elution solvents (ACN and DI water) for both procedures. The manual DBS processing requires 192 micropipette tips for filling the 96 vials with ACN and DI water, whereas only 5 tips are needed for filling the elution solvents into CE vials for further handling by the CE instrument. The total processing time per sample (90 s for manual (see **Figure S10** for all manual steps) and 6 s for automated (just vial capping) analysis) requires 2.4 h of the laboratory staff labour for manual DBS processing vs. 10 min for the automated DBS processing/analysis. The costs of the flush (100 mM NaOH and DI water) and BGE solutions for CE analyses are same for both procedures. With the sample throughput of 6 and 3 samples/h for the manual vs. the automated DBS analysis, respectively, the total CE operational time (i.e. electricity consumption) for the analysis 96 DBS samples (including analysis of calibration and quality control standards) will be 24 vs. 48 hours, respectively. Additional costs of the agitator (required for the manual DBS elution) are not included in the estimation.

The sample throughput of the fully automated DBS elution/analysis is approximately 2-fold lower than that of the manual DBS elution/analysis. However, the automated method eliminates all manual steps, can be performed fully autonomously in a 24h/7 days regime and the total cost per sample is more than 7-fold lower as documented in **Table S2**. Besides, no external instrumentation is required and operator-induced errors are avoided, which further simplify and streamline the entire analytical workflow.

Table S2. Total cost-analysis for the manual DBS elution followed by an automated CE-UV analysis and the fully automated DBS elution/CE-UV analysis of 96 DBS samples.

Item	Manual DBS processing	Automated DBS processing
DBS solvents[a]	1.5 €	1.5 €
Consumables[b]	8 €	0.2 €
Staff labour[c]	48 €	3.3 €
CE solvents[d]	1 €	1 €
Electricity[e]	1.2 €	2.4 €
Total[f]	59.7 €	8.4 €

[a] 8 mL of HPLC grade ACN and 2 mL of DI water (https://www.sigmaaldrich.com)
[b] 192 vs. 5 micropipette tips for the manual vs. the automated DBS processing (https://www.sigmaaldrich.com)
[c] 2.4 hours vs. 10 min for the manual vs. the automated DBS processing (20 €/hour: https://www.stellenanzeigen.de/gehalt-vergleich/laborassistent-in/)
[d] 2 mL of 100 mM NaOH, 2 mL of DI water and 4 mL of BGE solution (https://www.sigmaaldrich.com)
[e] 24 hours vs. 48 hours CE operational time for the manual vs. the automated DBS processing (0.3 €/kWh : https://de.wikipedia.org/wiki/Strompreis; 4 kWh/day (PC + Agilent 7100 CE data sheet))
[f] additional costs of the agitator (required for the manual DBS elution) are not included

Table S3. Quantitative results for warfarin determination in DBS samples of a warfarin-treated subject using the fully automated DBS elution/analysis by CE-UV and the analysis of the resulting eluate by UHPLC-MS/MS, $n = 3$; concentrations in µg/mL.

Sample	CE-UV	UHPLC-MS/MS
DBS 1	2.41 ± 0.15	2.32 ± 0.05
DBS 2	2.46 ± 0.11	2.34 ± 0.04
DBS 3	1.62 ± 0.08	1.58 ± 0.03
DBS 4	0.45 ± 0.03	0.41 ± 0.01
DBS 5	n.d.	≤ LOQ

n.d. – not detected
LOQ – limit of quantification

Detailed sequence for the fully automated DBS elution and CE-UV determination of warfarin.

The sequence is exported from Agilent 7100 CE ChemStation software.

```
Data File  : C:\Chem32\1\Data\Lenka-warfarin\2020-06-30 09-44-53 DBS - LR -
1mg_1.D

Acq. Method: WAR_CE.M

The Acq. Method's Instrument Parameters for the Run were:

     Vialtable Information for Method:

        Vial#    Used in      Name
           1      METHOD      Waste
           2      METHOD      0.1 M NaOH
           3      METHOD      DI water
           5      METHOD      BGE flush
           6      METHOD      BGE run Inl
           7      METHOD      BGE run Out
          10      METHOD      DI water
          11      METHOD      Acetonitrile
          30      METHOD      Vial with the DBS
          48      METHOD      Empty vial

=====================================================================
                                 CE
=====================================================================

CE (G7150A)
===========

Replenish Execution Mode:                                  Serial
UseElectric:                                                  Yes

Cassette Temperature
Cassette Temperature Mode:                        Temperature set
Temperature:                                              25.0 °C

Pressure
Pressure Mode:                                                Off
Pressure:                                                       2

Inlet Vial:                                                     6

Outlet Vial:                                                    7


Power:                                                      6.0 W
Voltage:                                                  25.0 KV

Current
Current:                                                   150 µA

Current Lower Limit Alarm Mode:                               Off

Replenish Program
```

```
Preconditioning Program
Function            Parameter
-------------------------------------------------------------
Flush               180s (Inlet: 11 Outlet: 30)
Flush               60s (Inlet: 48 Outlet: 30)
Flush               140s (Inlet: 10 Outlet: 30)
Flush               300s (Inlet: 48 Outlet: 30)
Flush               60s (Inlet: 2 Outlet: 1)
Flush               90s (Inlet: 3 Outlet: 1)
Flush               120s (Inlet: 5 Outlet: 1)


Injection Program
Function            Parameter
---------------------------------------------------------------------
Apply Pressure      55mbar for 12s (Inlet: Injection Vial Outlet: 8)


Postconditioning Program


Stop Time
Stoptime Mode:                                              Time set
Stoptime:                                                   4.00 min

Post Time
Posttime Mode:                                                   Off

Timetable
Time Function            Parameter
------------------------------------------------------------------
0.00 Change Voltage      25 KV
```

Video S1. A video demonstrating the DBS self-sampling. The video includes: (i) envelope unpacking, (ii) removing the single components of the DBS self-sampling kit from the zip-lock bags, (iii) preparation for capillary blood collection, (iv) capillary blood collection into the end-to-end 20 µL glass capillary, (v) dispensing the collected blood onto the pre-punched DBS sampling disc, (vi) disposal of the 20 µL end-to-end glass capillary, (vii) packing the in-vial DBS sampling disc spotted with blood into the "Sample" zip-lock bag with desiccant and vial cap, (viii) packing the "Biological material" zip-lock bag, (ix) packing both zip-lock bags into the envelope for shipment.

Video S2. A time-lapse video (selected procedures 10-times faster) of the fully automated DBS elution inside the Agilent 7100 CE instrument. The video includes: (i) loading the vial with the DBS sample, (ii) filling the DBS sample vial with 80 µL ACN, (iii) loading the inlet vial with air, (iv) bubbling the eluate with air for 60 s, (v) loading the inlet vial with DI water, (vi) filling the DBS sample vial with 20 µL DI water, (vii) loading the inlet vial with air, (viii) bubbling the eluate with air for 300 s, (ix) loading CE operational vials.

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9.5 Příloha V

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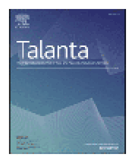

At-line coupling of hollow fiber liquid-phase microextraction to capillary electrophoresis for trace determination of acidic drugs in complex samples

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ABSTRACT

Direct analysis of complex samples is demonstrated by the at-line coupling of hollow fiber liquid-phase microextraction (HF-LPME) to capillary electrophoresis (CE). The hyphenation of the preparative and the analytical technique is achieved through a 3D-printed microextraction device with an HF located in a sample vial of a commercial CE instrument. The internal geometry of the device guides the CE separation capillary into the HF and the CE injection of the HF-LPME extract is performed directly from the HF lumen. The 3D-printing process ensures uniform dimensions of the devices, their constant position inside the sample vial, and excellent repeatability of the HF-LPME as well as the CE injection. The devices are cheap (~0.01 €) and disposable, thus eliminating any possible sample-carryover, moreover, the at-line CE analysis of the extract is performed fully autonomously with no need for operator's intervention. The developed HF-LPME/CE-UV method is applied to the determination of acidic drugs in dried blood spot and wastewater samples and is characterized by excellent repeatability (RSD, 0.6–9.6%), linearity (r^2, 0.9991–0.9999), enrichment (EF, 29–97), sensitivity (LOD, 0.2–3.4 μg/L), and sample throughput (7 samples/h). A further improvement of selected characteristics of the analytical method is achieved by the at-line coupling of HF-LPME to capillary isotachophoresis (ITP) with electrospray ionization-mass spectrometry (ESI-MS). The HF-LPME/ITP-ESI-MS system facilitates enhanced selectivity, matrix-free analytical signals, and up to 34-fold better sensitivity due to the use of ESI-MS detection and additional on-capillary ITP preconcentration of the HF-LPME extracts.

1. Introduction

The automation of analytical processes is an important quest for many scientific areas including environmental and clinical analysis. In some analytical processes, the automation can be readily implemented because the samples of interest have simple matrices and can be injected directly with no or minimum sample treatment. Nevertheless, many samples are highly complex, their matrix components may interfere with subsequent analytical methods, and a comprehensive sample treatment is usually required before analysis. Thus, the automated analysis of complex samples necessitates full automation of the sample treatment and the analytical procedure, moreover, it also requires a tool suitable for the direct coupling of the two procedures.

Complex samples can be processed by the traditional pretreatment techniques of liquid-liquid extraction (LLE) [1] or solid-phase extraction (SPE) [2]. Despite their distinct advantages, LLE and SPE also exhibit obvious limitations, such as high environmental impact, high costs, high consumption of samples/reagents, laborious processes, and low degree of automation. The incorporation of down-scaled extraction techniques into the analytical workflow has, therefore, attracted a great deal of attention over the past two decades [3,4]. From these sample pretreatment techniques, solid-phase microextraction (SPME) [5] and liquid-phase microextraction (LPME) [6] have paved the way for the application of new micro-preparative approaches in analytical chemistry [7,8]. The marriage of the new microextraction techniques with the subsequent analysis is more straightforward, moreover, it offers an additional benefit in terms of better suitability for the automation of the sample treatment and analysis.

Amongst the plethora of various LPME set-ups, single-drop microextraction (SDME) and supported liquid membrane (SLM) extraction

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were considered attractive due to their ease of operation and suitability for automation [8]. The volumes of the resulting extracts are in the μL range and are thus comparable with injection volumes in liquid chromatography (LC) and gas chromatography (GC) as well as in non-separation analytical techniques. The direct coupling of SDME or SLM extraction to those analytical techniques can, therefore, be achieved by transferring the entire extract into the injection system using a syringe or a peristaltic pump [9,10]. However, SDME is a two-phase extraction process [11,12], the resulting acceptor is usually organic and is compatible with GC only. Another drawback of SDME is the rather low stability of the hanging drop in agitated sample solutions, which is even more compromised for complex samples [13]. The stability of the organic phase can be improved by using polymeric support (e.g. porous polypropylene (PP)), which is impregnated with an organic solvent to form the SLM [6]. Besides, the use of the SLM enables a facile application of a two-phase or a three-phase extraction process and the resulting extracts are thus directly compatible with most analytical techniques, including GC, LC, MS, and capillary electrophoresis (CE) [9,10].

The most prominent technique exploiting the principles of SLM extractions is hollow fiber-liquid phase microextraction (HF-LPME) [14], which has become an established technique thanks to the simple extraction principle, instrumentation, and cheap/disposable consumables. Nevertheless, although HF-LPME offers high flexibility, excellent sample clean-up, high preconcentration power, and is supported by more than 20 years of research, subsequent analysis of the resulting acceptor solution is mostly carried out off-line [15–17]. Automation of the entire analytical process was achieved by the coupling of HF-LPME to flow through, LC, or GC technique and by transferring the entire extract into the analytical system. However, such coupling necessitated the application of external instrumentation, i.e. flow injection or sequential injection manifold, syringe pump, commercial autosampler, and transfer lines, and was rather complex [8]. In CE, the compatibility between the typical injection volume and the volume of the HF-LPME extract was achieved by reducing the extraction unit size [18] and on-capillary treatment of the extract [19]. In order to ensure simple transfer of the extract to the separation capillary, the HF-LPME/CE coupling was almost exclusively realized with lab-made CE instruments and required multiple manual operations. HF-LPME/CE automation was later achieved by a purpose-made modification of the separation capillary [20]. The modification procedure was rather delicate, had compromised repeatability, and was not suitable for commercial CE with tubular electrodes. Moreover, the same HF was applied for multiple HF-LPMEs and was prone to clogging and sample carry-over, especially for extractions of dirty complex samples. Recently, our group has introduced a simple and cost-effective set-up for at-line coupling of HF-LPME to commercial CE for the direct analysis of body fluids, which addressed most of the above-mentioned drawbacks [21]. The HF-LPME was carried out in a CE sample vial filled with a body fluid and the vial was fitted with a tailor-made disposable HF-LPME device, which enabled autonomous at-line CE injection of the extract directly from the HF lumen.

Despite the automation of the HF-LPME/CE process, manual preparation of HF holders and low selectivity/sensitivity of CE-UV can be the limiting factors for broadening the application range of the technique, and an improved HF-LPME/CE concept is presented in this contribution. 3D-printing technology was applied for repeatable batch production of HF holders with defined dimensions resulting in the exact positioning of the HF-LPME device inside the vial and excellent repeatability of the hyphenated HF-LPME/CE analytical process. Benefits of the autonomous HF-LPME/CE have been demonstrated by monitoring concentrations of orally administered acidic drugs in dried blood spot (DBS) samples. The minimally invasive collection of DBSs and the batch-wise DBS pretreatment/analysis have enabled blood sampling at a high frequency and reduced the DBS analysis time, and the proposed concept might thus be highly attractive for high-resolution pharmacokinetic analyses. Moreover, it has been shown that the proposed concept is also suitable for the direct analysis of samples with highly complex matrices and with trace concentrations of target analyses. This was achieved by the at-line injection of HF-LPME extracts of wastewater to CE operated in a free-acid isotachophoresis (ITP) mode coupled to electrospray ionization-mass spectrometric (ESI-MS) detection. The HF-LPME/ITP-ESI-MS coupling enabled excellent selectivity and matrix-free analytical signals due to the HF-LPME clean-up and the use of the ESI-MS detection, and improved sensitivity due to the synergistic preconcentration effect of HF-LPME and on-capillary ITP.

2. Experimental

2.1. Reagents, standard solutions, and real samples

Analytical reagent grade chemicals and deionized (DI) water with resistivity higher than 18 MΩ cm were used for the experiments. Stock solutions of warfarin, ibuprofen, naproxen, ketoprofen, and diclofenac (Sigma, Steinheim, Germany) were prepared by dissolving 1 mg of the drug in 1 mL of methanol (Lach-Ner, Neratovice, Czech Republic) and were stored in a deep-freezer at − 20 °C. Standard solutions for CE analyses of the drugs were prepared by diluting stock solutions of the drugs with DI water or NaOH solutions. Stock solutions of 1 M HCl, 1 M NaOH, and 1 M NaCl were prepared by dissolving concentrated HCl, NaOH pellets, and crystalline NaCl (all Lach-Ner) in DI water. Acceptor solutions for HF-LPMEs were prepared by diluting the NaOH stock solution with DI water and donor solutions were prepared by diluting stock solutions of the drugs, HCl, and NaCl with DI water. Organic solvents for SLM impregnation were dihexyl ether (DHE, 97%, Sigma), 4-nitrocumene (4-NC, ≥ 98%, Tokyo Chemical Industry, Tokyo, Japan), 1-ethyl-2-nitrobenzene (ENB, > 98%), 2-nitrophenyl octyl ether (NPOE, ≥ 99%), 1-octanol (≥99%), 1-nonanol (≥99%, all Fluka, Buchs, Switzerland) and various mixtures of the solvents. Background electrolyte (BGE) solutions for CE-UV were made from stock solutions of 500 mM acetic acid and 500 mM sodium acetate (prepared from the corresponding chemicals, Fluka and Sigma), acetonitrile (Lach-Ner), and DI water. Leading and terminating electrolyte solutions for ITP-ESI-MS were made by diluting stock solutions of 100 mM formic acid and 100 mM propionic acid (prepared from the corresponding acids, Sigma) with DI water.

DBS samples were formed by spotting 22 μL of capillary blood from a finger prick onto Whatman™ 903 Protein Saver (GE Healthcare Ltd, Cardiff, UK) sampling card and by drying the spots at laboratory temperature for 3 h. For spiked DBS samples, 22 μL of capillary blood was mixed with 1 μL of a standard drug solution in a PCR microvial (250 μL) and 22 μL of the mixture was transferred to the sampling card. Capillary blood samples were donated by volunteers at the Institute of Analytical Chemistry after signing written informed consent. The sampling cards with the DBSs were stored in zip-lock plastic bags with a desiccant (in a refrigerator at 4 °C) for up to three months. Wastewater samples were collected in August 2020 and February 2021 at the municipal wastewater treatment station (Brno-Modřice, Czech Republic), were deep-frozen immediately after collection and stored at − 20 °C for up to three months.

2.2. Capillary electrophoresis

Separation, detection, and quantification of the acidic drugs were carried out using a 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a UV–Vis diode array detector and an ESI-MS (6130 single quadrupole, Agilent Technologies) detector. BGE solution for CE-UV consisted of 30 mM sodium acetate, 30 mM acetic acid, and 30% (v/v) acetonitrile with apparent pH 5.2 [22] and was prepared daily. The separation voltage was +25 kV and was applied to the injection end of a fused silica separation capillary (75 μm i.d./375 μm o.d., L_{tot} = 45 cm and L_{eff} = 36.8 cm) supplied by Polymicro Technologies (Phoenix, AZ, USA). The capillary temperature was maintained

at 25 °C. Standard and acceptor solutions were injected hydrodynamically at 50 mbar for 5 s. Analytical signals were monitored at 200, 214, and 226 nm.

Free-acid ITP-ESI-MS of acidic drugs was reported earlier [23] and was carried out in 10 mM formic acid as the leading electrolyte and 10 mM propionic acid as the terminating electrolyte. The electrolytes were prepared daily. A separation voltage of − 20 kV was applied to the injection end of a fused silica separation capillary (100 μm i.d./375 μm o. d., $L_{tot} = L_{eff} = 100$ cm, Polymicro Technologies) with the additional pressure of − 10 mbar to compensate for the ESI suction. The capillary temperature was maintained at 20 °C. Standard and acceptor solutions were injected hydrodynamically at 50 mbar for 30 s. The CE-MS coupling was done via ESI using a CE-ESI-MS sprayer kit (Agilent Technologies, G1607A). A coaxial sheath liquid flow, composed of 1% (v/v) acetic acid in 50% (v/v) methanol, was employed during the ITP-ESI-MS analyses. It was supplied via splitter by using a 1200 series isocratic pump (Agilent Technologies) at a flow rate of 8 μL/min. The MS detector was operated in selected ion monitoring (SIM) mode using the following operation parameters: capillary voltage, 3500 V negative; nebulizer pressure, 10 psi; drying gas flow, 10 L/min; drying gas temperature, 200 °C. Fragmentor values of 130 and 150 were employed for MS detection at m/z 205, 229, 253, and m/z 250, respectively. Details on capillary equilibration procedures and capillary flushing between analyses for CE-UV and ITP-ESI-MS modes can be found in the Supplementary Data. The CE and MS instruments were controlled and data acquisition was performed by the ChemStation CE software (Agilent Technologies).

2.3. Hollow fiber liquid-phase microextraction

HF-LPME was carried out with a 3D-printed holder and a HF extraction unit. The holder (see Fig. 1A for details) was 3D-printed by a Photon Mono printer (Anycubic Technology Co. Ltd., Shenzhen, PRC) using the photopolymerization principle via LCD-based stereo-lithography initiated by UV light at 405 nm. A clear 3D-printing UV sensitive resin (Basic, UV wavelength 405 nm) was purchased from Anycubic Technology Co. Ltd. The extraction unit (9.5 mm long) was cut from a porous polypropylene HF (Accurel PP 300/1200, Membrana, Wuppertal, Germany, wall thickness = 300 μm; i.d. = 1200 μm; pore size = 0.2 μm) and was closed at the bottom by mechanical pressure and sealed by heat [21]. The resulting extraction unit was stretched over the narrow end of the holder to form a disposable HF-LPME device (Fig. 1B), which was used for one extraction only. Resin consumption per holder was 0.15 mL and the total cost of the complete HF-LPME device was ~0.01 €. We are also confident that with slight modifications, the proposed HF-LPME device might be applied to other analytical techniques, such as LC or GC, and broaden the application range of this concept.

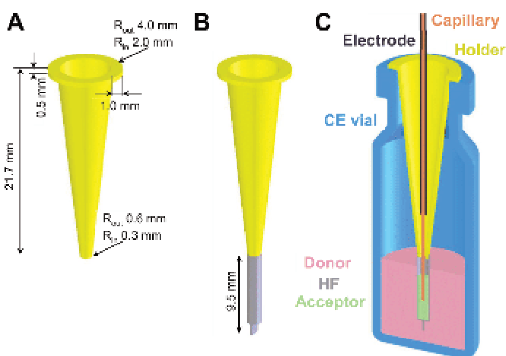

Fig. 1. A – Scheme and dimensions of the 3D-printed holder, B – scheme of the 3D-printed holder with an attached HF, C – scheme of the 3D-printed holder with the HF inside the CE vial depicting the tubular electrode and the separation capillary of the CE instrument.

The HF-LPME was performed according to the following steps. (i) A glass CE vial (Agilent Technologies, P/N 5182–9697) was filled with 550 μL of a donor solution. (ii) The HF of the HF-LPME device was impregnated with an organic solvent for 10 s and excess of the solvent was wiped off by a lint-free tissue. (iii) The lumen of the HF was filled with 5 μL of an acceptor solution by a 10 μL Hamilton syringe with a flat tip. (iv) The resulting HF-LPME device was inserted into the glass CE vial with the donor solution and the vial was closed with a polyurethane cap (Agilent Technologies, P/N 5181–1512). (v) The vial was placed in a vial holder (Agilent Technologies, P/N 9301–0722) secured to Vibramax 100 agitator (Heidolph Instruments GmbH, Schwabach, Germany) and the HF-LPME was initiated. Up to 50 samples were extracted simultaneously by agitation at 900 rpm for 30 min.

DBS samples were processed by punching out a 10 mm disc (containing the entire DBS) from the DBS sampling card and inserting the disc on the bottom of the glass CE vial. The vial was then filled with 550 μL of DBS eluent, completed with the HF-LPME device, closed with the cap, and extracted according to steps (i) – (v) described in the previous paragraph. The elution of dried capillary blood from the DBS and HF-LPME of analytes from the DBS eluate took place simultaneously during the vial agitation. The vial with the extracted DBS was transferred to CE autosampler after HF-LPME and the acceptor solution was injected directly from the HF lumen. The eluate and the DBS disc were retained in the vial during CE injection. HF-LPME of wastewater was realized by pipetting 550 μL of wastewater into the glass vial (step (i)) and by proceeding with steps (ii) – (v) reported in the previous paragraph.

2.4. Calculations

Extraction recovery (ER) and enrichment factor (EF) values were calculated according to Ref. [21] and corresponding equations are detailed in the Supplementary Data.

3. Results and discussion

3.1. CE at-line coupled to HF-LPME

The CE set-up was optimized for the at-line injection of the pre-treated complex sample directly from the HF-LPME device according to the requirements specified in our previous publication [21]. A short tubular electrode (Agilent Technologies, P/N G7100-60033) was installed at the injection side of the CE instrument and the separation capillary was pushed 8 mm out of the electrode. The novel 3D-printed HF holder ensured excellent repeatability of the HF position inside the CE vial and thus also excellent injection performance. The upper part of the holder was printed with a thin rim, which sat on the glass CE vial orifice and ensured constant height of the holder and thus of the HF inside the vial. The conical shape of the holder guided the electrode into the holder and the separation capillary into the HF lumen. During injection, approx. 3 mm of the separation capillary were immersed in the acceptor solution inside the HF and at least 3 consecutive CE injections could be performed from one HF-LPME device. A scheme of the HF-LPME device during CE injection is visualized in Fig. 1C. A standard solution of the five acidic drugs (10 mg/L) in 10 mM NaOH was used for the subsequent repeatability measurements. The repeatability of three consecutive injections from twenty unique HF-LPME devices was ≤4.9% (RSD, $n = 60$) and was comparable to injection repeatability from twenty glass CE vials filled with 600 μL of the standard solution (RSD ≤4.3%, $n = 60$).

3.2. HF-LPME optimization

Initial HF-LPME performance was investigated with a donor solution containing 250 μg/L of the acidic drugs, 10 mM HCl, and 150 mM NaCl. Acidic donor conditions were selected to promote the drugs' transfer (in neutral form) to the SLM and NaCl was included to simulate the conditions for HF-LPMEs of real samples. On the opposite side of the SLM, the transferred drugs were ionized and released into an alkaline acceptor solution consisting of 10 mM NaOH. The HF-LPME process was accelerated by agitation at 750 rpm for 10 min. Each variable affecting the HF-LPME performance was then comprehensively investigated using a one-at-the-time approach to achieve the most efficient and interference-free extraction of the drugs.

The most important variable for the HF-LPME selectivity and efficiency is the SLM composition. Six various solvents were used for the SLM impregnation and the resulting ER values are depicted in Fig. S1 in the Supplementary Data. In the first set of experiments, pure ENB, NPOE, 1-octanol, 1-nonanol, 4-NC, and DHE were examined. Best extraction efficiencies (ER = 35–48%) were achieved for DHE and 4-NC. Even better efficiencies (ER = 40–60%) were achieved in the second set of experiments for mixed SLM solvents (DHE:ENB (1:1 v/v) and DHE:4-NC (1:1 v/v)). However, SLMs containing nitrated organic solvents resulted in the transfer of traces of unknown compounds from the solvents into the acceptor solutions, which interfered with the CE-UV determination of some drugs. The interferences observed for the most efficient SLM (DHE:4-NC (1:1 v/v)) are depicted in Fig. S2 in the Supplementary Data and disabled proper quantification of diclofenac. Because this work aimed at the simultaneous HF-LPME of the entire group of model acidic drugs, DHE was selected as the SLM solvent for all subsequent experiments. Nevertheless, specific mixed-solvent SLMs might be preferred if more efficient extractions of particular analytes (which are not affected by the observed interferences, or the interferences are eliminated by the selective MS detection) are desirable.

The HF-LPME selectivity and efficiency are also influenced by the composition of the donor and the acceptor solution. At the beginning of the HF-LPME process, analytes are transferred from the donor solution to the SLM. This is achieved by converting the analytes of interest into their neutral forms. The target acidic drugs have pK_a values ~4–5 (https://go.drugbank.com/drugs/, last accessed on September 15th, 2021), are neutral at acidic conditions, and various donor solutions were thus tested without and with the addition of HCl (0–100 mM). Additionally, 150 mM NaCl was always added to the donor solution. The acceptor was a 10 mM NaOH solution to promote the release of the target analytes from the SLM. ER data are presented in Fig. S3 in the Supplementary Data. The donor solution with no addition of HCl exhibited poor extraction performance because the analytes were mostly ionized at the near-neutral conditions (pH ~ 6.4) and were not attracted by the SLM. The increased donor acidity eliminated analytes' ionization, improved the extraction performance, and resulted in ER values 38–49% for 25 mM HCl. A further improvement of ER values was observed for 50 mM HCl, nevertheless, it was not significant (except ibuprofen) and was accompanied by a slight reduction in repeatability (see the error bars). Acceptor solution was examined at 0–50 mM NaOH concentrations and the resulting ER values are presented in Fig. S4 in the Supplementary Data. Analytes were not efficiently released from the SLM at the near-neutral acceptor conditions (DI water only) and the best ER values were achieved for 10 and 25 mM NaOH acceptor solutions. A slight reduction of ER values and a significant broadening of CE-UV signals of the drugs were observed for 50 mM NaOH. The latter resulted from the mismatch between the extract and the BGE conductivity [21] and was partly observed also for 25 mM NaOH. Based on the above experiments, the most suitable donor and acceptor solutions for the HF-LPME of the five acidic drugs were 25 mM HCl and 10 mM NaOH, respectively.

The HF-LPME efficiency is also directly related to the availability of target analytes at the phase interface between the aqueous and the organic solution and agitation has a significant effect on the ER values. At no or too low agitation, the analytes' renewal at the phase interface is not efficient and at too high agitation, donor solution might not properly contact the HF due to the pronounced vortex in the vial [21]. Agitation time was increased to 30 min to ensure a sufficiently long extraction process and agitation speed was examined in the 600–1200 rpm range. Best extraction efficiencies (ER = 70–87%) for the actual HF-LPME set-up were achieved at 900 rpm and the ER data for all examined agitation speeds are shown in Fig. S5 in the Supplementary Data.

Extraction time plays a crucial role in HF-LPME because the system should ideally reach the equilibrium state before the HF-LPME can be terminated. Extraction time was examined for 0–30 min and corresponding curves demonstrating ER values at different extraction times are shown in Fig. 2. Ibuprofen, ketoprofen and diclofenac reached equilibrium after 15 min of extraction, nevertheless, further increase of ER was observed for warfarin and naproxen up to 30 min. Repeatability of the extraction process improved with time as is evidenced by the lowest standard deviation values for the 30 min extraction time. Since multiple samples were extracted simultaneously (Chapter 3.3.), the sample pretreatment time was not the limiting factor of the analytical procedure and an extraction time of 30 min was selected for all subsequent experiments.

3.3. Suitability for HF-LPME/CE of multiple samples

HF-LPMEs were carried out in a multiple-well plate format, which enabled simultaneous pretreatment of up to 50 samples. The CE autosampler carousel can accommodate 50 vials, however, several autosampler positions have to be reserved for BGE solutions, CE flushing solutions, and CE calibration standards. Thus, the autosampler offers approx. 40 positions, which can be loaded with sample vials for autonomous CE analyses. Forty standard donor solutions (spiked with 100 μg/L of the five acidic drugs) were simultaneously extracted at the previously optimized HF-LPME conditions and the stability of the achieved extraction equilibrium was examined. In a typical HF-LPME equilibrium, acidic drugs transferred to the alkaline acceptor solution are negatively charged, do not partition into the SLM again, and their acceptor concentrations should remain fairly stable. The 40 sample vials with the extracted donor solutions were loaded into the CE autosampler and a sequence was set up for autonomous CE analyses of the resulting extracts. The concentrations determined for the five acidic drugs in the acceptor solutions are depicted in Fig. S6 in the Supplementary Data. Injections 13, 27, and 29 failed and no analytical signals were recorded for the drugs. We assume that an air bubble was formed inside the HF lumen during manual filling with acceptor solution or the acceptor solution level has lowered during the HF-LPME resulting in no acceptor injection into the separation capillary. The remaining 37 injections were

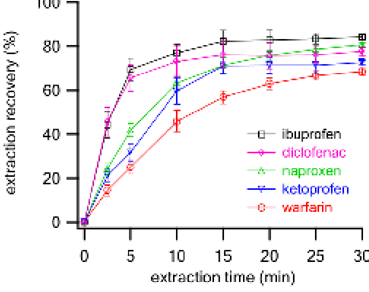

Fig. 2. Effect of extraction time on the HF-LPME of model acidic drugs. HF-LPME conditions: SLM, DHE; acceptor, 5 μL of 10 mM NaOH; donor, 550 μL of the five acidic drugs (250 μg/L) in 25 mM HCl and 150 mM NaCl; agitation speed, 900 rpm; *n* = 3.

evaluated and repeatability of the HF-LPME/CE-UV procedure was calculated for the five acidic drugs. RSD values ranged from 6.3% (ibuprofen) to 9.4% (warfarin) and confirmed the stability of the extraction equilibria and suitability of the proposed approach for the autonomous batch-wise analyses of acidic drugs in complex samples. The total time for the preparation (assembling the HF-LPME devices, pipetting donor solutions, impregnating SLMs, and filling with acceptor solutions) of the 40 sample vials was 80 min, extraction time was 30 min and CE analyses of the 40 samples took 240 min, offering a sample throughput of approx. 7 samples/h.

3.4. Analytical parameters

The analytical parameters were determined for donor solutions consisting of 25 mM HCl, 150 mM NaCl and various concentrations of the five acidic drugs. For validation purposes, two concentration levels of the drugs (10 and 100 μg/L) were selected. The acceptor solution was 10 mM NaOH, the SLM solvent was DHE, and the HF-LPME was carried out at 900 rpm for 30 min. Linearity was measured in the 5–250 μg/L calibration range with 7 calibration points. All analytical parameters are summarized in Table 1. ER values ranged from 64 to 89%, which corresponded to EFs of 71–97. The repeatability of the analytical process, which combines the repeatability of both HF-LPME and CE-UV techniques, was excellent with RSD values below 6.0%. Calibration curves were linear in the entire calibration range with coefficients of determination better than 0.9991. Limits of detection (LODs) were defined as analytical signals three times higher than background noise and ranged from 0.2 to 1.2 μg/L. HF-LPME/CE-UV analyses of a donor blank (25 mM HCl, 150 mM NaCl, no drugs) and three donor solutions after the addition of 5, 10, and 50 μg/L of the acidic drugs to the blank are depicted in Fig. S7 in the Supplementary Data.

3.5. Direct DBS analysis

Typical therapeutic concentrations of the five acidic drugs in blood samples range from 0.5 to 100 mg/L [24]. In the following experiments, drug-free DBS samples, spiked DBS samples, and DBS samples after oral administration of ibuprofen and naproxen were analyzed. Conventional DBS processing results in a considerable blood dilution (typically 10 to 100-fold) and drug concentrations in the DBS eluates might be too low for their determination by standard analytical techniques. Moreover, even the diluted blood matrix might be detrimental to the analytical process. In the proposed HF-LPME procedure, DBS elution, sample clean-up, and analyte preconcentration are achieved simultaneously since as soon as blood elutes from the DBS, blood components are released into the eluate and can partition into the SLM.

Two aspects, which differ from the HF-LPME of liquid donor solutions, were considered for DBS analyses: (i) the effect of blood buffering capacity on eluate pH and consequently on HF-LPME efficiency and (ii) the effect of extraction time on HF-LPME efficiency due to the need for the elution of analytes from the DBS before HF-LPME.

Drug-free capillary blood was spiked with 100 μg/L of the five acidic drugs and the resulting DBSs were eluted with DI water and 10–100 mM HCl as elution solutions. ER values are depicted in Fig. S8 in the Supplementary Data and demonstrate no HF-LPME at physiological conditions (DBSs eluted with DI water) and comparable extraction efficiencies at all acidic conditions. The acidic drugs were fully ionized at physiological conditions, did not partition into the SLM and acidic elution solvents were mandatory. The extraction time was examined between 5 and 30 min and the results were consistent with those reported earlier for standard solutions. ER values reached nearly a plateau after 15–20 min agitation and a slight increase of ER values was further achieved for warfarin and naproxen at 30 min. These results demonstrate that the elution of the dried blood has not affected the speed of the HF-LPME process. The analytes were gradually released from the DBS to the eluate, neutralized at acidic conditions, and transferred through the SLM to the acceptor solution. Based on the above-obtained data, DBSs were eluted with 25 mM HCl by agitation at 900 rpm for 30 min in all subsequent experiments.

Validation of the HF-LPME/CE-UV method for the determination of the five acidic drugs in DBS samples is summarized in Table 2. Most parameters show comparable data to those for standard solutions. ER values (49–70%) are slightly lower and can be explained by the interactions of the drugs with the blood matrix because all five acidic drugs exhibit ≥99% drug-protein binding [25] and were spiked directly into raw capillary blood. EFs ranged from 54 to 77 and RSD values were below 5.2%. Linearity measurements were carried out in the 10–250 μg/L calibration range, the calibration curves had 6 calibration points and coefficients of determination were better than 0.9991. LODs were between 0.3 and 1.7 μg/L. Representative electropherograms of a blank DBS (formed by spotting drug-free capillary blood) and spiked DBSs (spotted after spiking the same drug-free capillary blood with 10, 25, and 50 μg/L of the drugs) analyzed by the HF-LPME/CE-UV method are depicted in Fig. 3. Major blood interferents (such as salts, proteins, phospholipids, and other macromolecular compounds) were efficiently eliminated by the HF-LPME as was evidenced by the analytical signals of the blank DBS sample. A few blood matrix compounds were co-extracted into the acceptor solution and detected, nevertheless, they were baseline separated from the target analytes, their concentrations were minimal and did not interfere with the determination of target analytes.

The method LODs were suitable for the determination of all acidic drugs in blood samples and repeatability conformed with the requirements for the bioanalytical method validation (https://www.fda.gov/media/70858/download, last accessed on September 15th, 2021). The presented set-up used only 22 μL of capillary blood per sample and DBSs could be collected at virtually any interval because DBS collection required no phlebotomy and/or cannula insertion. The developed HF-LPME/CE-UV method was examined for the determination of

Table 1
Analytical parameters of the HF-LPME at-line coupled to CE for the determination of acidic drugs in physiological solutions.

Parameter	Warfarin	Ibuprofen	Naproxen	Ketoprofen	Diclofenac
ER (%), 10 μg/L	65.1	88.5	87.3	70.0	85.0
EF	71.6	97.4	96.0	77.0	93.5
RSD (%), 10 μg/L	5.4	6.0	5.3	5.6	4.1
ER (%), 100 μg/L	70.8	84.5	84.1	64.1	80.7
EF	77.9	93.0	92.5	70.5	88.8
RSD (%), 100 μg/L	1.1	1.8	2.6	1.4	2.3
R^2	0.9996	0.9991	0.9998	0.9998	0.9992
LOD (μg/L)	0.6	0.3	0.2	0.6	1.2
LOQ (μg/L)	1.9	1.1	0.7	2.1	3.8

Table 2
Analytical parameters of the HF-LPME at-line coupled to CE for the determination of acidic drugs in DBS samples.

Parameter	Warfarin	Ibuprofen	Naproxen	Ketoprofen	Diclofenac
ER (%), 10 μg/L	48.8	68.5	62.0	69.0	68.0
EF	53.7	75.4	68.2	75.9	74.8
RSD (%), 10 μg/L	2.9	4.5	5.2	4.8	4.2
ER (%), 100 μg/L	49.0	69.0	64.8	69.6	64.0
EF	53.8	75.9	71.3	76.6	70.4
RSD (%), 100 μg/L	3.6	5.2	1.5	0.6	1.9
R^2	0.9999	0.9991	0.9997	0.9997	0.9991
LOD (μg/L)	0.9	0.5	0.3	0.7	1.7
LOQ (μg/L)	2.9	1.5	1.0	2.4	5.5

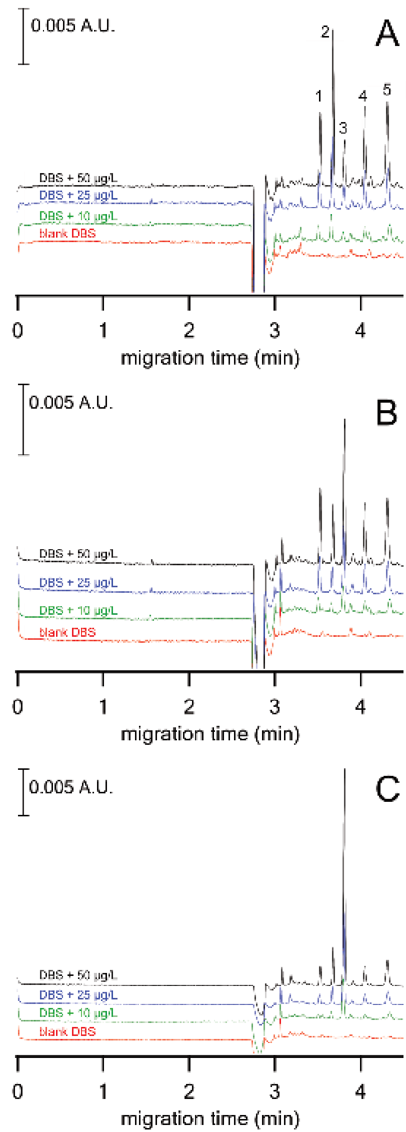

Fig. 3. At-line coupling of HF-LPME to CE-UV for the direct determination of model acidic drugs in DBS samples at (A) 200 nm, (B) 214 nm and (C) 226 nm. HF-LPME conditions: SLM, DHE; acceptor, 5 µL of 10 mM NaOH; donor, DBSs eluted with 550 µL of 25 mM HCl; agitation speed, 900 rpm; extraction time, 30 min. CE conditions: BGE solution, 30 mM sodium acetate, 30 mM acetic acid and 30% (v/v) acetonitrile at apparent pH 5.2; separation voltage, 25 kV; injection, 50 mbar for 5 s; detection wavelengths, 200, 214, and 226 nm; peak description, 1 – warfarin, 2 – ibuprofen, 3 – naproxen, 4 – ketoprofen, 5 – diclofenac.

ibuprofen and naproxen in DBSs after oral administration of Ibalgin 400 (400 mg of ibuprofen) and Nalgesin S (275 mg of naproxen) tablets, respectively. For the determination of ibuprofen, DBS samples were collected at 0, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 270, and 1440 min after the drug administration due to a rapid action of the drug and its short half-life ($t_{1/2}$ = 0.9–2.5 h) [24]. Due to a longer $t_{1/2}$ of naproxen (9–22 h) [24], DBS samples for the determination of naproxen were collected at 0, 30, 60, 90, 120, 150, 180, 240, 300, 360, 420, 600, 1080, and 1440 min after the drug administration. The resulting curves demonstrating the drug concentrations at different times after administration are depicted in Fig. S9 in the Supplementary Data. Maximum concentrations (ibuprofen, c_{max} = 27.5 mg/L and naproxen, c_{max} = 26 mg/L) were determined for DBSs collected 75 and 150 min (t_{max}) after the drug administration and were within the therapeutic concentration ranges reported earlier [24]. The resulting electropherograms showing the direct analyses of DBSs before (t_0) and after the drug administration (t_{max} at their c_{max}) are depicted in Fig. 4. The drug concentrations followed their predicted $t_{1/2}$ in a human body. Ibuprofen concentration was reduced to 5.4 mg/L at 270 min and ibuprofen was fully eliminated at 1440 min. Naproxen concentrations were reduced to 14.4 and 7.0 mg/L at 600 and 1440 min, respectively.

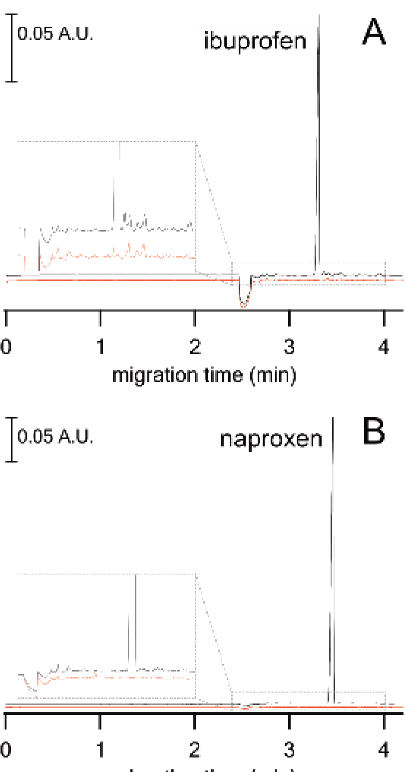

Fig. 4. Direct HF-LPME/CE-UV determination of ibuprofen (A) and naproxen (B) in DBSs collected at t_0 and t_{max} after oral administration of the two drugs. HF-LPME/CE-UV conditions as for Fig. 3; detection wavelength was 200 nm for ibuprofen and 226 nm for naproxen.

3.6. Direct wastewater analysis

Concentrations of acidic drugs in wastewater vary significantly and can be as high as several thousand μg/L [26]. Nevertheless, they are typically present at trace concentrations in wastewaters (ng/L to low μg/L range) [27] and their determination in untreated samples is rather difficult by most analytical methods due to the sample complexity and method sensitivity issues.

The four most abundant acidic drugs, ibuprofen, naproxen, ketoprofen, and diclofenac, were selected for the actual study because they are regularly controlled by authorities in wastewater and are among the emerging water pollutants. In order to eliminate the sample matrix and to increase the method sensitivity, wastewater samples were pretreated with the developed HF-LPME. The HF-LPME conditions were identical to those applied to HF-LPMEs of DBSs and the extraction equilibrium was achieved by the agitation of acidified wastewater samples (25 mM HCl) at 900 rpm for 30 min. Fig. S10 in the Supplementary Data shows the resulting electropherograms of pretreated influent wastewater samples (raw and spiked with 10 and 20 μg/L of the four acidic drugs) at-line injected to CE-UV. Wastewater samples contain solid particles and inorganic salts, which were efficiently eliminated during HF-LPME. Besides, they contain also a large number of organic compounds with physico-chemical properties similar to the acidic drugs. Because HF-LPME is not a species-selective technique, co-extraction of several matrix compounds was observed and the detected analytical signals confirmed the high complexity of the influent wastewater. Due to the non-selective character and lower sensitivity of UV detection, partial comigration of some analytes with the co-extracted organic matrix components was observed and the concentration of diclofenac in wastewater was below the method LOD, respectively. Fundamental analytical parameters of the HF-LPME/CE-UV method for the analysis of wastewater samples are summarized in Table S1 in the Supplementary Data.

The sensitivity and selectivity of CE for the determination of acidic drugs can be improved by the application of on-capillary ITP stacking and ESI-MS detection [23]. Because the CE-UV analyses of the pretreated wastewater samples showed some deficiencies in both aspects (sensitivity as well as selectivity), the HF-LPME was at-line coupled to the previously developed ITP-ESI-MS method. The original method was extended by the determination of two additional acidic drugs (naproxen and ketoprofen). The suitability of the HF-LPME acceptor for the ITP-ESI-MS analyses was examined by injections of standard solutions of acidic drugs prepared in DI water and 10 mM NaOH and revealed no measurable differences in the analytical performance. In the HF-LPME/ITP-ESI-MS, a short plug of the acceptor solution from the HF lumen was at-line injected right behind the leading electrolyte and ITP separation mode was realized by placing the capillary injection end into the terminating electrolyte. The negative pressure of − 10 mbar was applied to the inlet CE vial to compensate for the ESI suction. Analytical parameters of the HF-LPME/ITP-ESI-MS method for the determination of acidic drugs in wastewater samples are summarized in Table 3. The extraction efficiencies were slightly reduced in comparison to standard solutions and DBS samples and resulted in ER values of 28–70%. Wastewater samples contain large amounts of organic matter and solid particles and partial sorption of the drugs on these matrix components [28] is very likely the reason for the reduced ER values. Nevertheless, the hyphenated analytical method demonstrated sufficient EFs (31–77) and good repeatability (RSD ≤9.3%). Due to the higher sensitivity of the ITP-ESI-MS method, LODs improved by a factor of up to 34 in comparison to HF-LPME/CE-UV, were as low as 100 ng/L, and enabled trace determination of the drugs in wastewater samples. Moreover, the unique selectivity of the ESI-MS detection in the SIM mode resulted in matrix-free quantification of the drugs and improved reliability of the quantitative results. Representative electropherograms of the determination of naproxen and diclofenac in raw and spiked influent wastewater samples are depicted in Fig. 5 and quantitative results of the wastewater analyses are summarized in Table 3. A comparison with previous analytical methods for the determination of acidic drugs in complex samples is presented in Table 4. The proposed concept was comparable with the previous methods in terms of all analytical parameters (except for EF) and outperformed them in terms of sample pretreatment time and sample volume, moreover, it was operated in the automated mode.

Table 3
Analytical parameters of the HF-LPME at-line coupled to ITP-ESI-MS for the determination of acidic drugs in influent wastewater samples.

Parameter	Ibuprofen	Naproxen	Ketoprofen	Diclofenac
ER (%)	36.7	44.2	69.9	28.1
EF	40.4	48.6	76.9	30.9
RSD (%)	9.3	4.4	1.4	5.0
Concentration in sample 1 (μg/L)	13.2	19.9	4.6	1.9
Concentration in sample 2 (μg/L)	9.9	5.2	2.6	1.2
R^2	0.9997	0.9990	0.9993	0.9990
LOD (μg/L)	0.2	0.1	0.1	0.1
LOQ (μg/L)	0.6	0.3	0.3	0.3

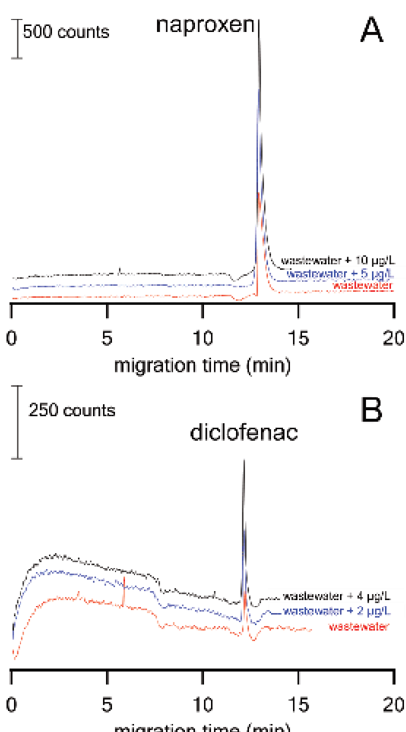

Fig. 5. Direct HF-LPME/ITP-ESI-MS determination of naproxen (A) and diclofenac (B) in influent wastewater. HF-LPME conditions as for Fig. 3 and ITP-ESI-MS conditions as in Experimental. Relative intensities of negative SIM signals were collected at *m/z* 229 for naproxen and *m/z* 250 for diclofenac.

Table 4
Analytical performance of the proposed method and previous methods reported in the literature for the determination of acidic drugs in complex samples using HF-LPME principles.

Analytes	Sample preparation	Analytical technique	ER (%)	EF	LOD (μg/L)	Sample volume (mL)	Preparation time/sample (min)	Automation	Reference
Ibu, Nap	EME	CE-UV	69–92	138–184	1–3	4	10	No	[29]
Ibu, Dic	HF-LPME	LC/MS-MS	n.r.	n.r.	0.1–0.3	50	15	No	[30]
Ibu, Ket, Nap, Dic	PHWE – HF-LPME	LC-ESI-MS	24–30	947–1213	0.4–3.7*	100	45 + 120	No	[31]
Ibu, Dic	HF-LPME	LC-UV	n.r.	n.r.	40–53	50	15	No	[32]
Ibu, Ket, Nap, Dic	HF-LPME	LC-UV	23–51	43–97	1.6–4.3	6.4	13	Yes	[33]
Ibu, Ket, Nap, Dic	HF-LPME	LC-UV	3–9	270–805	0.01–0.05	1000	45	Yes	[34]
Ibu, Ket, Nap, Dic, War	HF-LPME	CE-UV or ITP-ESI-MS	28–89	31–97	0.1–3.4	0.55	0.75	Yes	This work

EME electromembrane extraction, PHWE pressurised hot water extraction, n.r. not reported, * (ng/g).

4. Conclusions

Direct determination of model acidic drugs in complex samples is demonstrated by the at-line coupling of HF-LPME to CE. The front end of this hyphenated system serves for efficient sample treatment and is realized by the insertion of a disposable 3D-printed microextraction device with an HF directly into a CE vial with the complex sample. The application of the 3D-printing technology ensures superb device-to-device repeatability and constant position of the device inside the CE vial, thus providing excellent repeatability of the HF-LPME and CE injection. The 3D-printed device guides the CE separation capillary into the HF and the extract is at-line injected directly from the HF lumen. The samples are extracted in a multiple-well plate format enabling simultaneous pretreatment of up to 50 samples, which are subsequently loaded into an autosampler of a commercial CE instrument for fully autonomous CE analyses.

Benefits of the at-line HF-LPME/CE coupling are demonstrated by the direct analyses of DBS samples. DBS samples are fully compatible with the developed analytical method, are characterized by a simple collection, transport, and storage, and their attractiveness for biomedical analysis has been growing steadily in recent years. The proposed HF-LPME/CE concept enables a facile and rapid determination of therapeutic concentrations of model acidic drugs in DBSs and might be particularly attractive for high-resolution pharmacokinetic studies and biomedical applications. Moreover, the proposed instrumentation is also suitable for the direct analysis of highly complex samples with trace concentrations of target analytes, such as wastewater samples, by the at-line injection of the resulting extracts to the CE instrument operated in ITP-ESI-MS mode. The HF-LPME/ITP-ESI-MS hyphenation ensures matrix-free analytical signals due to the HF-LPME sample clean-up and selective ESI-MS detection and improved sensitivity due to the synergistic preconcentration effect of HF-LPME and on-capillary ITP and holds a great promise for trace analyses of various complex samples in the future.

Credit author statement

Blanka Miková: Methodology, Investigation, Writing- Original draft. Miloš Dvořák: Methodology, Supervision, Writing - review & editing. Lenka Ryšavá: Methodology, Investigation. Zdenka Malá: Methodology, Investigation. Petr Gebauer: Conceptualization, Methodology, Supervision. Pavel Kubáň: Conceptualization, Methodology, Supervision, Writing- Original draft, Writing - review & editing, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.123068.

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Supplementary Data for the manuscript

At-line coupling of hollow fiber liquid-phase microextraction to capillary electrophoresis with ultraviolet and mass spectrometric detection for trace analyses of complex samples

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Capillary equilibration.

CE-UV. Initial equilibration was performed by flushing the separation capillary with 100 mM NaOH for 10 min, deionized water for 10 min, and BGE solution for 10 min. The equilibration at the beginning of a working day included flushing the capillary with 100 mM NaOH for 5 min, deionized water for 5 min, and BGE solution for 5 min. Between two consecutive CE analyses, the capillary was flushed with BGE solution for 1 min. At the end of a working day, the capillary was flushed with 100 mM NaOH, deionized water, and air (5 min each). The capillary was pressurized at 950 mbar during all flushing procedures and the capillary cartridge temperature was maintained at 25 °C.

ITP-ESI-MS. Initial equilibration was performed by flushing the separation capillary with 100 mM NaOH for 60 min, deionized water for 30 min, 100 mM HCl for 60 min, and deionized water for 30 min. The equilibration at the beginning of a working day included flushing the capillary with deionized water for 30 min. Between two consecutive CE analyses, the capillary was flushed with leading electrolyte for 5 min. At the end of a working day, the capillary was flushed with deionized water for 30 min. The capillary was pressurized at 950 mbar during all flushing procedures and the capillary cartridge temperature was maintained at 20 °C.

Calculations. Extraction recovery (ER) and enrichment factor (EF) values of the HF-LPMEs were calculated according to equation (1) and (2), respectively:

$$ER\,(\%) = \frac{n_{a,\,final}}{n_{d,\,initial}} \times 100 = \frac{V_a}{V_d} \times \frac{C_{a,\,final}}{C_{d,\,initial}} \times 100 \qquad (1)$$

$$EF = \frac{C_{a,\,final}}{C_{d,\,initial}} \qquad (2)$$

where $n_{a,final}$ and $n_{d,initial}$ are the number of the analyte moles finally transferred into the acceptor solution and the number of the analyte moles initially present in the donor solution, respectively. V_a is the acceptor solution volume, V_d is the donor solution volume, $C_{a,final}$ is the final analyte concentration in the acceptor solution and $C_{d,initial}$ is the initial analyte concentration in the donor solution.

Figure S1. Effect of the SLM composition on ER (%) values of the acidic drugs. HF-LPME conditions: acceptor, 5 µL of 10 mM NaOH; donor, 550 µL of the five acidic drugs (250 µg/L) in 10 mM HCl and 150 mM NaCl; agitation speed, 750 rpm; extraction time, 10 min; *n = 3*.

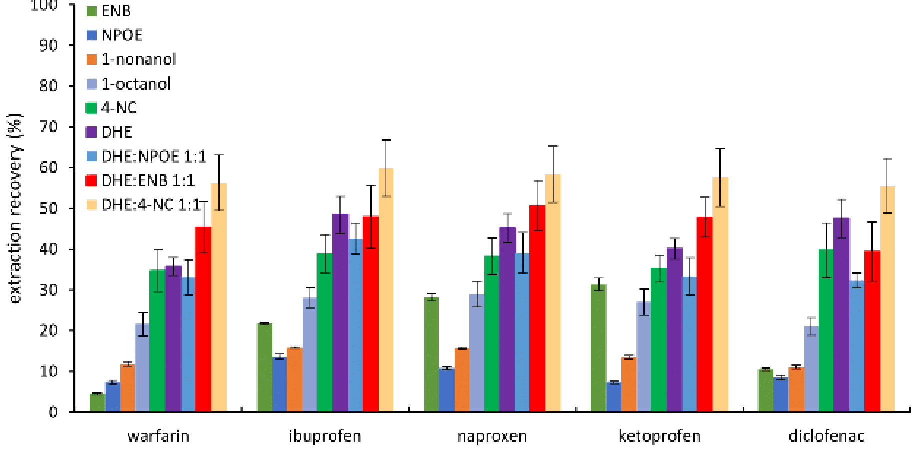

Figure S2. Interfering peaks observed for the HF-LPME/CE-UV of a standard solution extracted through mixed SLM consisting of DHE:4-NC (1:1, v/v). HF-LPME conditions as for Figure S1. CE conditions: BGE solution, 30 mM sodium acetate, 30 mM acetic acid and 30% (v/v) acetonitrile at apparent pH 5.2; separation voltage, + 25 kV; injection, 50 mbar for 5 s; detection wavelength, 200 nm; 1 – warfarin, 2 – ibuprofen, 3 – naproxen, 4 – ketoprofen; 5 – diclofenac; * – unknown interfering components.

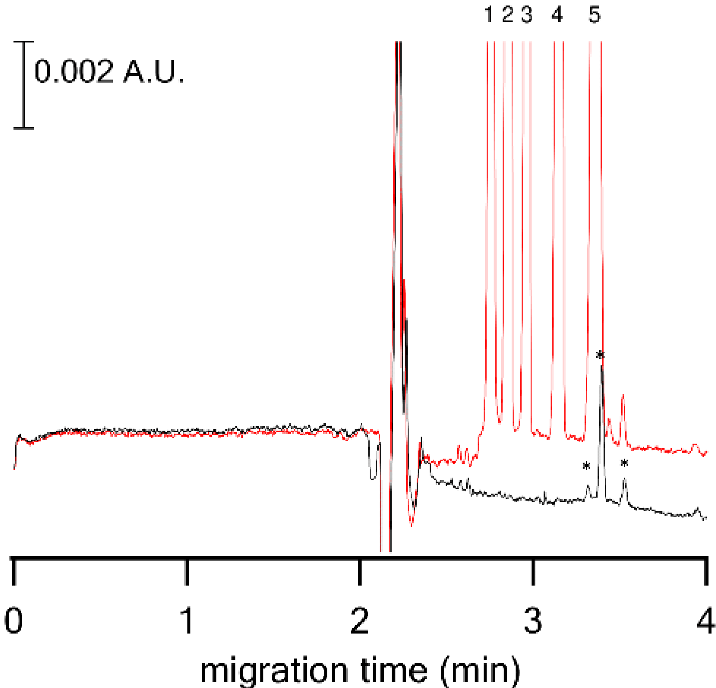

Figure S3. Effect of the donor solution composition on ER (%) values of the acidic drugs. HF-LPME conditions: SLM, DHE; acceptor, 5 μL of 10 mM NaOH; donor, 550 μL of the five acidic drugs (250 μg/L) in 150 mM NaCl; agitation speed, 750 rpm; extraction time, 10 min; *n* = *3*.

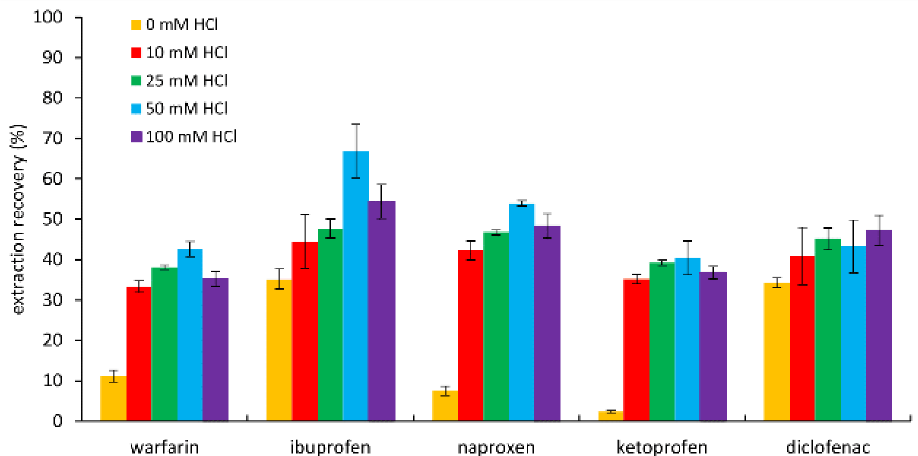

Figure S4. Effect of the acceptor solution composition on ER (%) values of the acidic drugs. HF-LPME conditions: SLM, DHE; acceptor, 5 μL; donor, 550 μL of the five acidic drugs (250 μg/L) in 150 mM NaCl and 25 mM HCl; agitation speed, 750 rpm; extraction time, 10 min; *n* = *3*.

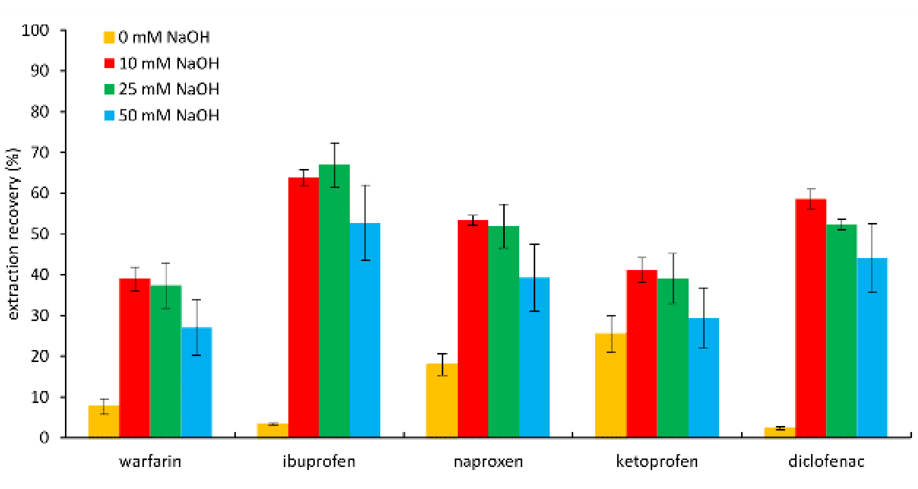

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Figure S5. Effect of the agitation speed on ER (%) values of the acidic drugs. HF-LPME conditions: SLM, DHE; acceptor, 5 µL of 10 mM NaOH; donor, 550 µL of the five acidic drugs (250 µg/L) in 25 mM HCl and 150 mM NaCl; extraction time, 30 min; $n = 3$.

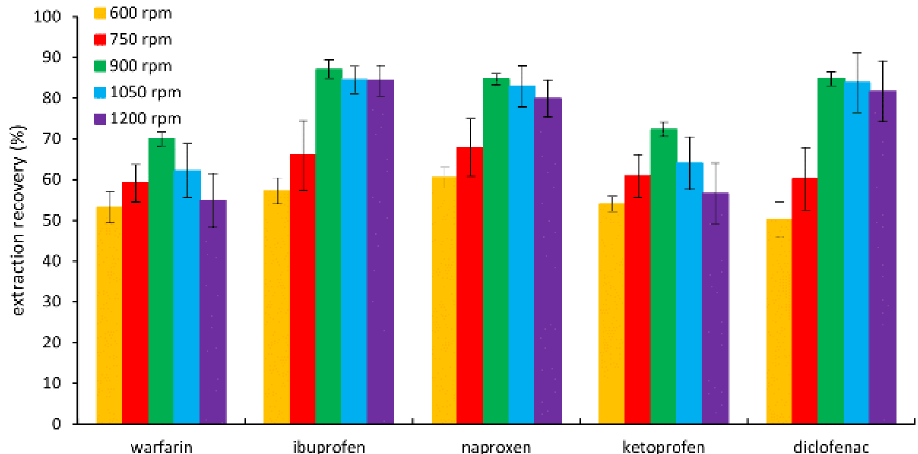
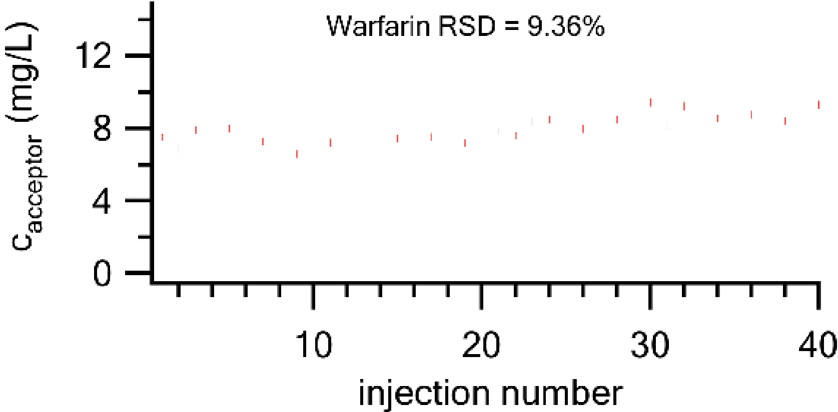
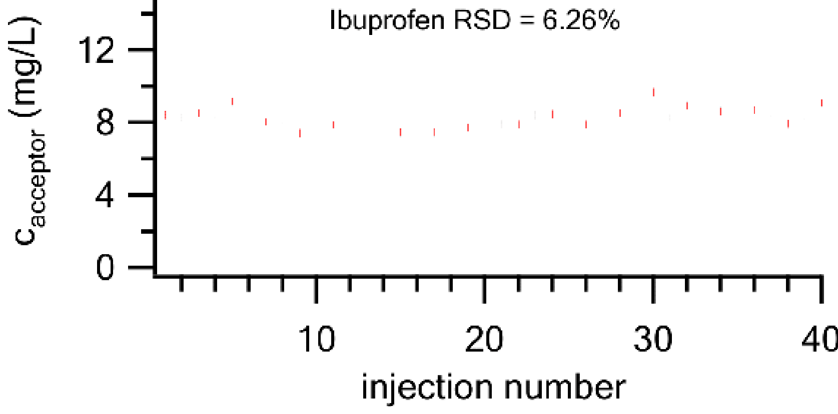


Figure S6. Repeatability of 40 consecutive HF-LPME/CE-UV analyses of the acidic drugs. HF-LPME conditions: SLM, DHE; acceptor, 5 μL of 10 mM NaOH; donor, 550 μL of the five acidic drugs (100 μg/L) in 25 mM HCl and 150 mM NaCl; agitation speed, 900 rpm; extraction time, 30 min. CE conditions as for Figure S2.





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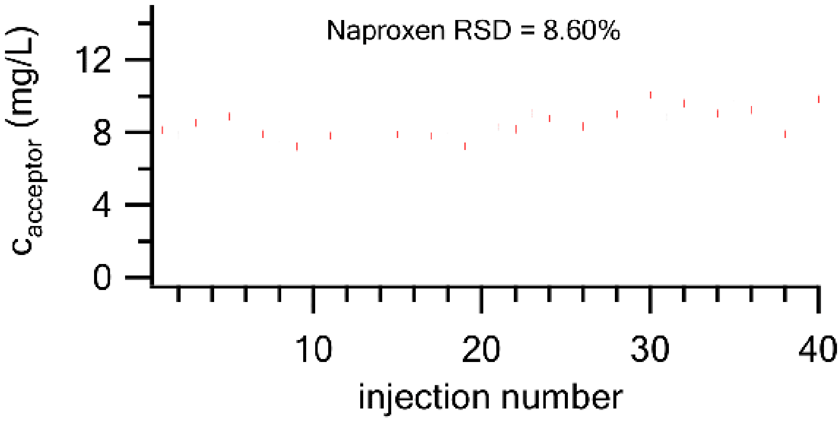
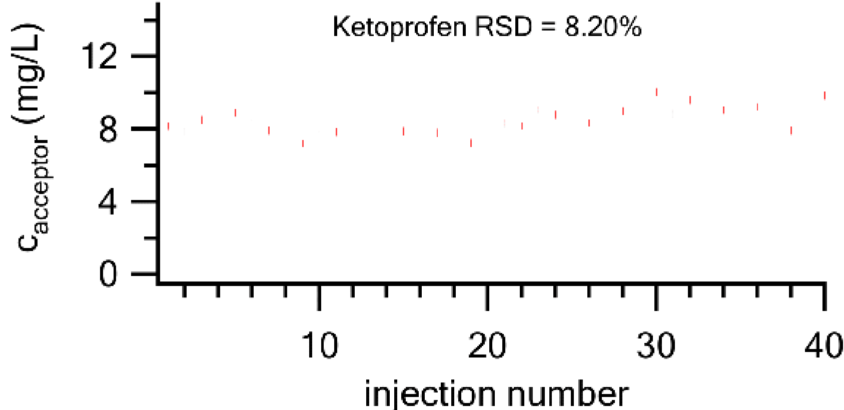
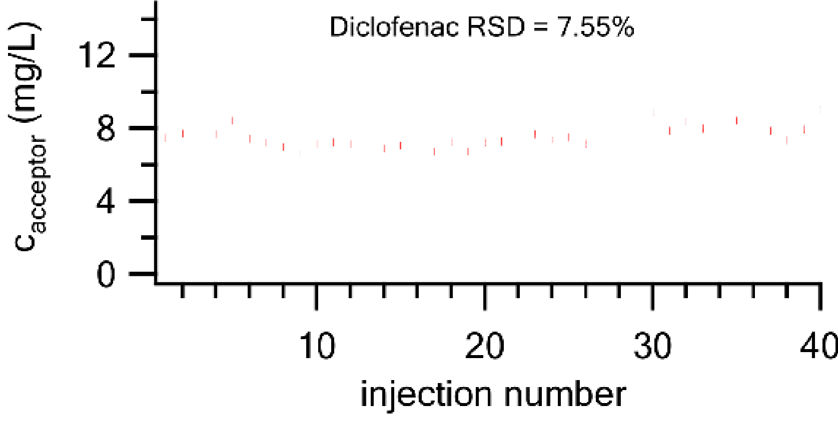
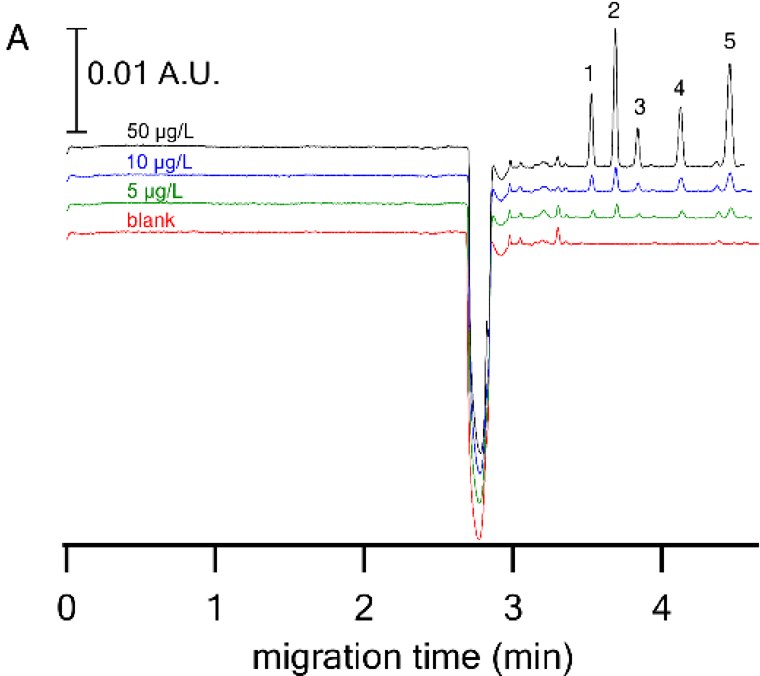


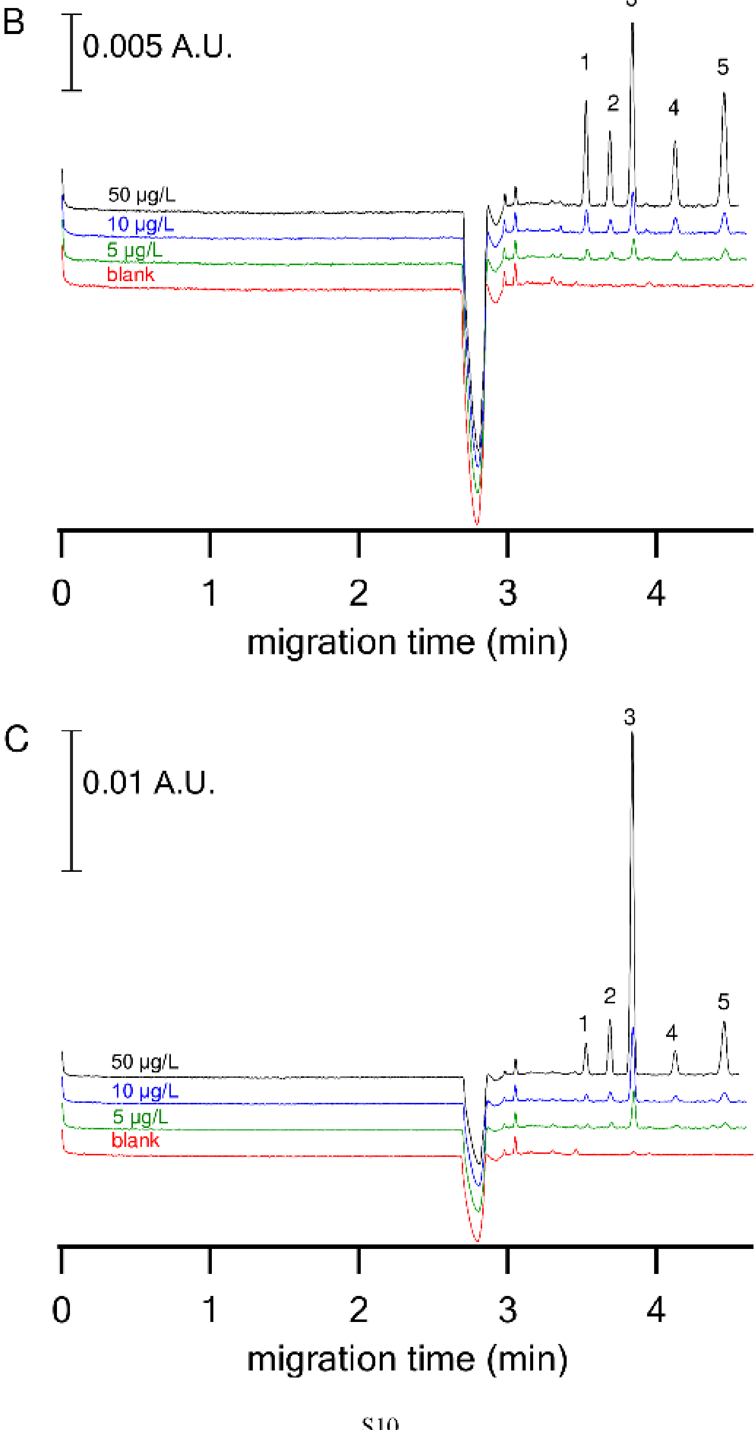




S8

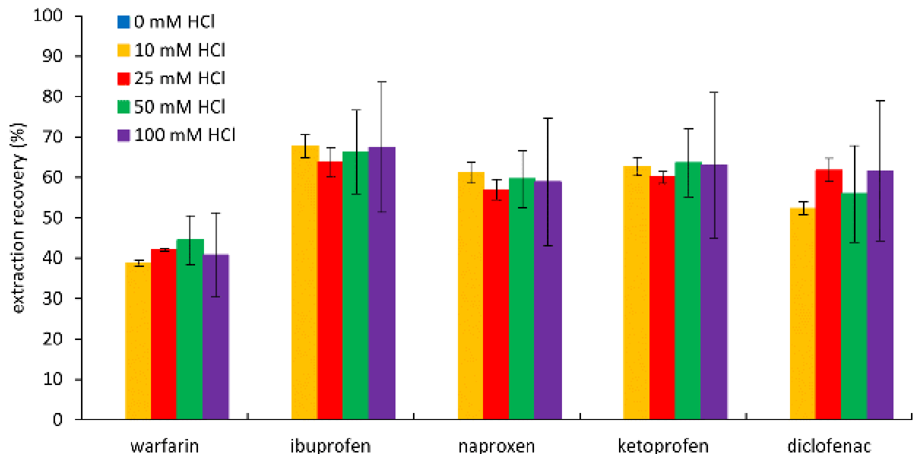
Figure S7. Electropherograms for the HF-LPME at-line coupled to CE-UV for the determination of the five acidic drugs in standard donor solutions at (A) 200 nm, (B) 214 nm and (C) 226 nm. HF-LPME conditions as for Figure S6 and CE conditions as for Figure S2.





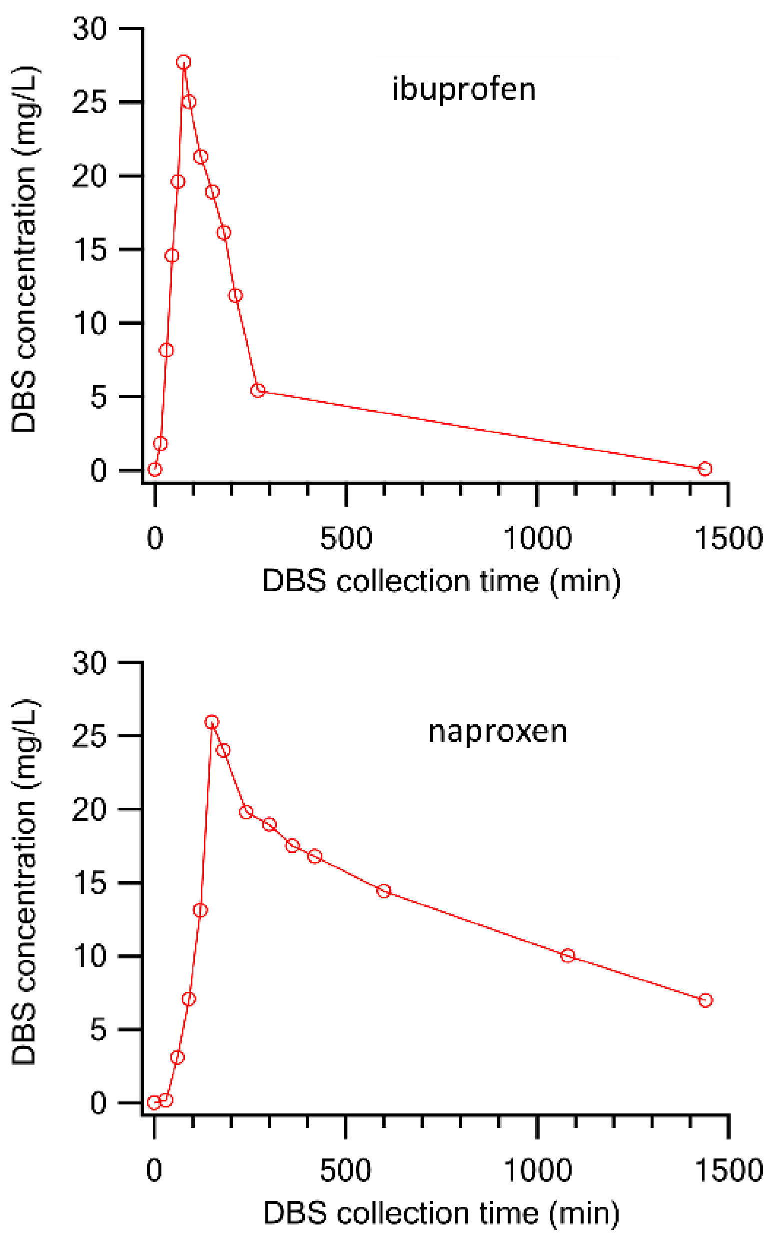
S10

Figure S8. Effect of the donor solution composition on ER (%) values of the acidic drugs extracted from DBSs. HF-LPME conditions: SLM, DHE; acceptor, 5 μL of 10 mM NaOH; donor, drugs-free capillary blood spiked with 100 μg/L of the drugs and the DBSs eluted with 550 μL of various HCl solutions; agitation speed, 900 rpm; extraction time, 30 min; $n = 3$.



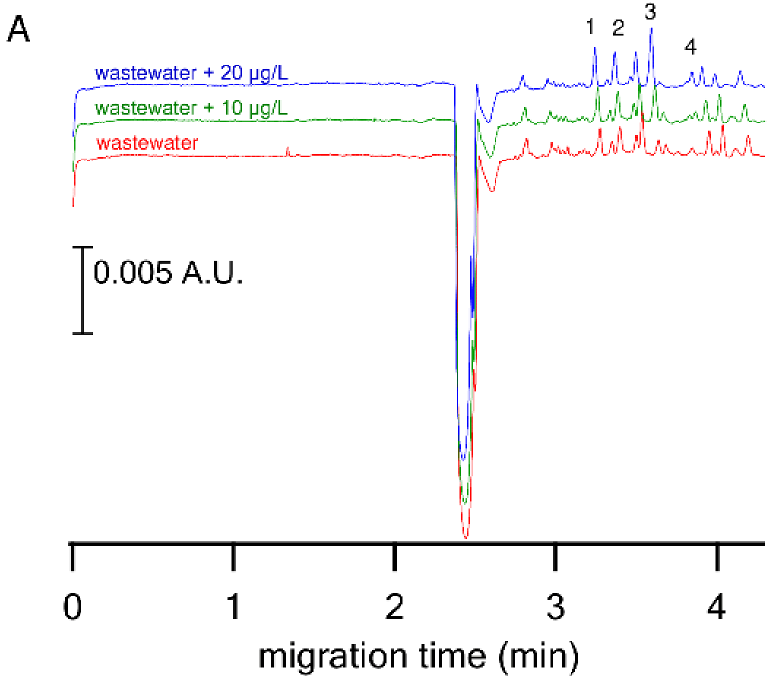
S11

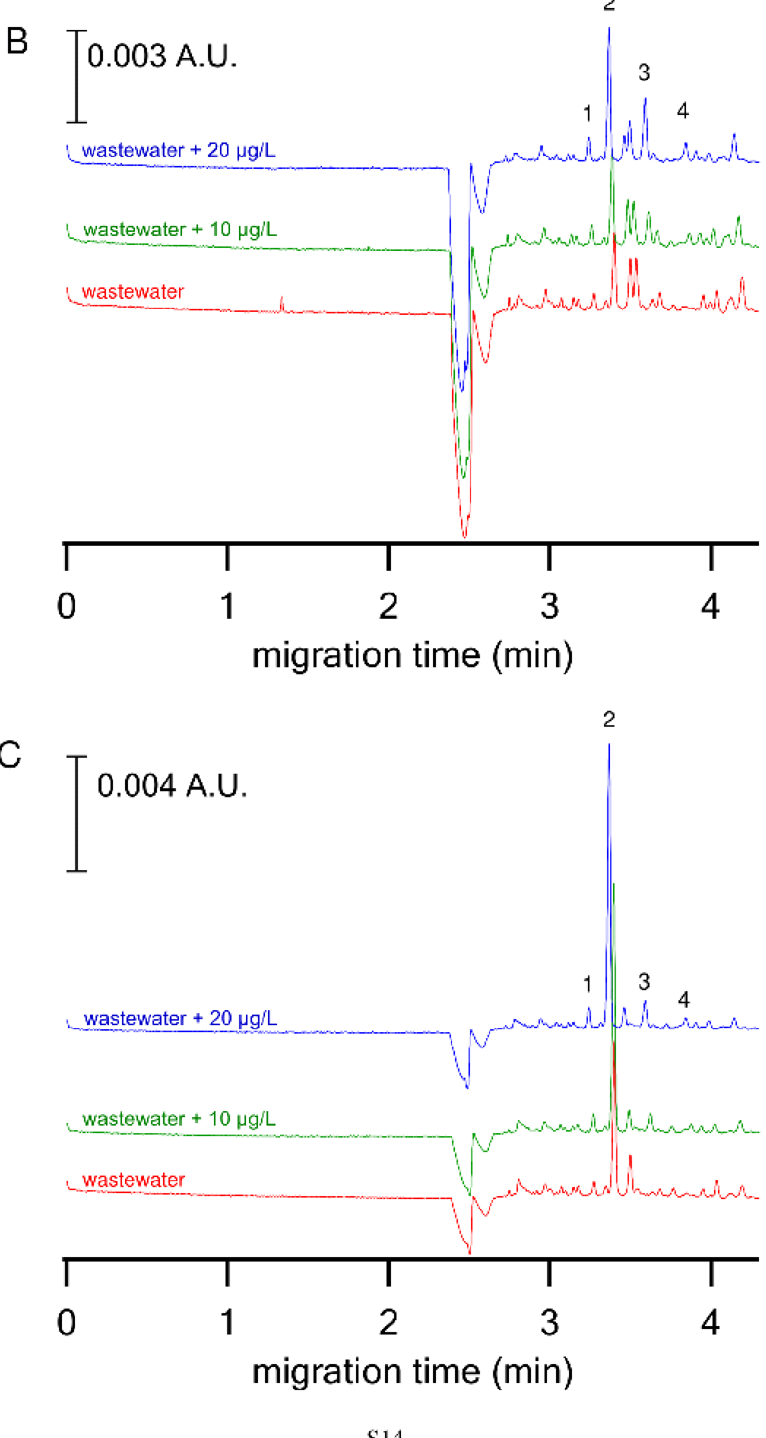
Figure S9. Pharmacokinetics of ibuprofen and naproxen in DBS samples determined by the at-line coupling of HF-LPME to CE-UV. HF-LPME conditions as for Figure S8 (DBSs were eluted with 25 mM HCl) and CE conditions as for Figure S2.



S12

Figure S10. Electropherograms for the HF-LPME at-line coupled to CE-UV for the determination of the five acidic drugs in influent wastewater at (A) 200 nm, (B) 214 nm and (C) 226 nm. HF-LPME conditions as for Figure S6 and CE conditions as for Figure S2. 1 – ibuprofen, 2 – naproxen, 3 – ketoprofen; 4 – diclofenac.





S14

Table S1. Analytical parameters of the HF-LPME at-line coupled to CE-UV for the determination of acidic drugs in influent wastewater samples.

Parameter	Ibuprofen	Naproxen	Ketoprofen	Diclofenac
ER (%)	34.8	44.4	62.5	26.4[a]
EF	38.3	48.8	68.8	29.0[a]
RSD (%)	8.7	1.9	8.2	9.6[a]
Concentration in sample 1 (μg/L)	13.7	20.4	4.7	n.d.
Concentration in sample 2 (μg/L)	9.4	4.7	2.6	n.d.
R^2	0.9991	0.9994	0.9997	0.9995
LOD (μg/L)	1.7	0.6	0.9	3.4
LOQ (μg/L)	5.7	1.9	2.9	11.4

[a] after spiking wastewater with 10 μg/L of diclofenac

n.d. – not detected

9.6 Příloha VI

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Fully soluble polymeric foams for in-vial dried blood spot collection and analysis of acidic drugs by capillary electrophoresis

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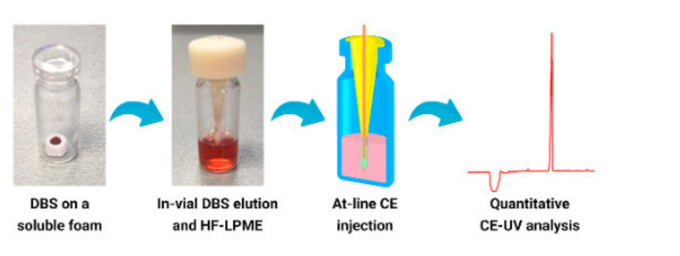
HIGHLIGHTS

- Fully soluble polymeric foams were developed for the collection of dried blood spots.
- The best performance was achieved with foams made of 6% polyvinylpyrrolidone.
- The foams outperformed standard sorbents in terms of processing time and efficiency.
- Nearly exhaustive in-vial elution and extraction were obtained for model acidic drugs.
- The drugs were at-line determined by capillary electrophoresis-ultraviolet detection.

GRAPHICAL ABSTRACT



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ABSTRACT

Polymeric foams tailor-made of polyvinylpyrrolidone (PVP) and carboxymethylcellulose/oxidized 6-carboxycellulose (CMC07/OC) composite were proposed as suitable sorbents for the collection and analysis of dried blood spots (DBSs). The PVP and CMC07/OC foams were easy to prepare, enabled collection of minute volumes of capillary blood, and blood drying at ambient temperature. The resulting foams were prepared as small porous discs with uniform dimensions (approx. 6 × 3 mm) and were fully soluble in aqueous solutions. The DBSs were formed in standard capillary electrophoresis (CE) vials fitted with the soluble foam discs and enabled the direct in-vial DBS processing and at-line analysis by CE. The DBSs were pretreated with a simple process, which involved a complete dissolution of the foam disc in an acidic solution and a simultaneous hollow fiber liquid-phase microextraction (HF-LPME) in one step. The complete solubility of the foam disc with the DBS served for a quantitative transfer of all blood components into the eluate and a nearly exhaustive HF-LPME of target analytes, whereas the blood matrix and the polymeric foam components were efficiently retained by the organic solvent impregnated in the walls of the HF. The suitability of the PVP and CMC07/OC foams for the collection and the direct analysis of DBSs was demonstrated by the HF-LPME/CE determination of model acidic drugs (warfarin, ibuprofen, naproxen, ketoprofen, and diclofenac) at therapeutically relevant concentrations. Repeatability of the analytical method was better than 8.1% (RSD), extraction recoveries ranged from 70 to 99%

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(for PVP foam), calibration curves were linear over two orders of magnitude (R^2 higher than 0.9991), and limits of detection were less than 44 μg/L (for concentrations in undiluted capillary blood). The soluble polymeric foams exhibited non-significant variations in analyte concentrations for DBSs prepared from blood samples with different hematocrit levels and for aged DBSs (less than 9.2%), moreover, they outperformed standard DBS sampling devices in terms of sample pretreatment time and extraction recovery.

1. Introduction

The key factors of the contemporary biomedical assays are simple collection, transport, and storage of biomedical samples, minimum manipulation with the samples, and facile sample pretreatment and analysis. Novel analytical concepts for biomedical assays have, therefore, been systematically investigated and have aimed at the elimination of various drawbacks associated with the biomedical use of liquid blood samples. Collection, processing, and analysis of liquid blood are usually burdened by the need for a trained phlebotomist, invasive draw process, demanding transport and storage conditions, laborious and time-extensive sample pretreatment, and high costs of analytical procedures. An alternative concept to the collection of venous blood, i.e. the collection of capillary blood from a finger or a heel prick in the form of a dried blood spot (DBS), has attracted considerable attention and has been generally accepted for specific clinical assays [1–3].

The capillary blood volumes are typically much smaller (<100 μL), the DBS collection is considerably less invasive and it is suitable for remote sampling and self-sampling even by individuals with no previous experience with blood microsampling [4–6]. Capillary blood is usually collected on a cellulose-based DBS sampling card, is dried out at the ambient temperature and the resulting DBS is thereafter suitable for simple transport and storage [2,3]. Despite the above-mentioned benefits, the actual DBS analyses have also certain limitations, which prevent their wider use and as a consequence, novel methods for the DBS collection, processing, and analysis are currently being investigated.

One of the main shortcomings of the actual methods is the DBS processing, which is mostly performed manually by a multiple-step process, is time-consuming, and laborious. As an alternative to the manual DBS processing, semi-automated and automated procedures were suggested recently, which offer a more convenient DBS elution, pretreatment, and analysis [2,7–11]. Moreover, capillary blood is usually sampled as a drop with an unknown volume and the distribution of analytes on DBS cards and the extraction efficiency may differ due to the different blood hematocrit levels and related chromatographic effects on sampling cards [12,13]. Their subsequent impact on DBS processing (manual as well as (semi)-automated) and quantitative DBS analysis might be enormous and various approaches were described for correction or elimination of these adverse effects. These are, for example, the application of perforated or precut DBS cards [14,15], or the use of a whole DBS punch [16] instead of the currently preferred DBS sub-punch. Nevertheless, the above-mentioned approaches are burdened by the need for sampling an exact blood volume to the DBS card and blood must be collected using a precise volumetric device or an end-to-end glass capillary [11,17,18]. Another alternative approach is the determination of unknown blood volume by the determination of potassium, sodium and/or chloride content in the DBS eluate [19–21], the measurement of the DBS eluate conductivity [21,22] or the non-contact prediction of hematocrit levels in DBS [23,24].

Collection of blood samples with unknown and/or uneven volumes can also be eliminated by the recently developed volumetric absorptive microsampling (VAMS), in which an exact volume of capillary blood is absorbed by a polymeric tip of a defined size [25]. However, the use of VAMS might be limited by the rather high cost of the device [26] and the use of the polymeric sorbent tip. Blood penetrates deeply into the VAMS 3D geometry and is fixed in the internal cavities of the sorbent, which might prolong the elution times and decrease the analyte yields during subsequent processing [27,28]. Moreover, the use of VAMS constitutes an environmental burden due to the necessary disposal of the plastic components of VAMS sets and the polymeric tips containing blood sample residues.

Indeed, commercial DBS sampling devices have several disadvantages and there is an urgent quest for novel DBS sampling concepts, which could outperform the actual state-of-the-art devices. Their development is, thus, at the forefront of the actual DBS research [18,25, 29,30]. One of the prominent alternatives to cellulose-cards and VAMS are the recently developed soluble DBS sampling sorbents based on alginate, chitosan, and carboxymethylcellulose [31,32]. The application of the soluble sorbents enables collection/transport/storage of capillary blood in a similar way as with the ordinary sampling devices, moreover, blood constituents might be released more easily into the DBS eluate due to the sorbents' solubility [31,32]. However, in comparison to cellulose cards, the production of alginate and chitosan DBS sampling sorbents requires rather sophisticated processes, is time-extensive, and uses multiple chemical reagents (foaming agents, gelling ions, pH modifiers, and plasticizers) [31]. Another disadvantage is the high cost of chitosan polymers and the lower solubility of chitosan [31] and carboxymethylcellulose sheets [32]. Moreover, the dissolved DBS sampling sorbents might not be directly compatible with the subsequent analysis, thus, pretreatment of the eluates is necessary, which is usually carried out manually and off-line [31,32].

Sample pretreatment might, thus, be necessary for most analytical applications of DBSs (collected on standard or alternative sorbents). The aim of the DBS pretreatment is to eliminate interfering compounds from the DBS matrix and to increase the final analyte concentration in the extract. This can be simultaneously achieved by extracting target analytes from a relatively large volume of the DBS eluate into a few microliters of extraction solvent or acceptor solution using a hollow fiber liquid-phase microextraction (HF-LPME) [33–35]. In HF-LPME, the HF is impregnated with a microliter volume of a water-immiscible solvent to form the so-called, supported liquid membrane (SLM) and filled with a microliter volume of an acceptor solution. The SLM enables a selective transfer of target analyte(s) from the DBS eluate to the acceptor solution and retains interfering components in the eluate. Recently, it was demonstrated that HF-LPME can be performed in a CE sample vial fitted with a tailor-made disposable microextraction device, which is suitable for at-line CE injection of the acceptor solution directly from the HF lumen [36]. Thus, the CE injection requires no operator's intervention and can be fully automated for batch-wise CE measurements. The developed setup is suitable for various complex samples, but it might be particularly attractive for DBS analysis since DBS elution and HF-LPME of target analytes are performed simultaneously during one common agitation cycle [36,37].

In this contribution, various polymeric foams were prepared by controlled lyophilization as new sorbents for DBS collection and analysis. The polymeric foams were cheap, easy to prepare, enabled collection of minute volumes of capillary blood, drying at ambient temperature, complete solubility in aqueous solutions, and direct DBS analysis by the at-line coupling of HF-LPME to CE. A small cylindric sorbent disc with exact dimensions (made of the developed polymeric foam) was placed inside a CE vial and the DBS was formed directly in the CE vial, thus eliminating further manual manipulations of the biological material. The DBS elution process involved a complete dissolution of the polymeric foam and the DBS and ensured an improved availability of all blood components in the eluates. Consequently, nearly exhaustive extraction of target analytes was achieved as was demonstrated by the

HF-LPME/CE determination of acidic drugs in DBSs collected on the tailor-made polymeric foams.

2. Experimental

2.1. Chemicals and solutions

Deionized (DI) water with resistivity higher than 18 MΩ cm was prepared by exchange of ions in a mixed-bed ion exchanger water purification system G7749 (Miele, Gütersloh, Germany). All chemicals were of analytical reagent grade. Warfarin, ibuprofen, naproxen, ketoprofen, and diclofenac sodium salt were from Sigma-Aldrich (Steinheim, Germany). The five acidic drugs were dissolved at a concentration of 1.000 mg/L in pure methanol (Lach-Ner, Neratovice, Czech Republic) and were stored at −20 °C. Standard solutions and donor solutions were prepared by dissolving appropriate volumes of the above-reported stock solutions in DI water and various HCl or NaOH solutions. The organic solvent for the SLM impregnation was dihexyl ether (DHE, 97%, Sigma-Aldrich). Stock solutions of 1 M HCl and 1 M NaOH were prepared by dissolving concentrated HCl (37%, Lach-Ner) and NaOH pellets (Lach-Ner) in DI water and were used for preparing operational solutions for HF-LPMEs and CE analyses. Stock solutions of 500 mM acetic acid and 500 mM sodium acetate were prepared from acetic acid (≥99.9%, Fluka, Buchs, Switzerland) and crystalline sodium acetate (≥99%, Sigma-Aldrich) in DI water. Background electrolyte (BGE) solutions for the CE determination of acidic drugs were prepared from these two stock solutions, acetonitrile (Lach-Ner) and DI water. Polymeric foams were made of gelatin (GEL, from bovine skin, type B, Sigma-Aldrich), polyvinyl alcohol (PVA, 99+% hydrolyzed, Sigma-Aldrich), carboxymethylcellulose sodium salt powder (CMC07, degree of substitution 0.7, Sigma-Aldrich), carboxymethylcellulose sodium salt cloth (CMC02, degree of substitution 0.2, Holzbecher, Česká Skalice, Czech Republic), oxidized 6-carboxycellulose (OC, Synthesia, Pardubice, Czech Republic), and polyvinylpyrrolidone (PVP, K 90, Sigma-Aldrich).

2.2. Preparation of soluble polymeric foams

Porous polymeric foams were prepared by freeze-drying their aqueous dispersions. Depending on their solubility, polymers were either dissolved or disintegrated in DI water to achieve the dispersion concentration ranges of 0.5–10% (w/v). PVA-based and PVP-based dispersions were prepared by dissolving PVA or PVP powder on a magnetic stirrer (Variomag Poly 15, ThemoFisher Scientific, Waltham, MA, USA) at room temperature by stirring at 400 rpm for 20 h. GEL dispersions were prepared by dissolving powdered gelatin biopolymer on the magnetic stirrer at 60 °C by stirring at 400 rpm for 1 h. CMC07 and OC dispersions and their mixtures were prepared by dissolving powdered biopolymers on the magnetic stirrer at room temperature by stirring at 400 rpm for 5 h. CMC02 dispersions were prepared by disintegrating (T 18, IKA Instruments, Staufen, Germany) finely cut cloth pieces at 6000 rpm for 5 min. CMC02/OC mixtures were prepared similarly by the disintegration of CMC02 and by mixing the resulting CMC02 dispersion with dissolved OC solution. Homogenous viscous dispersions of all polymers were pipetted into 96-well plates (plasma-treated, VWR, Radnor, PA, USA). Afterward, the 96-well plates filled with the polymeric dispersions were frozen in a freezer at −25 °C for 1 h and put in a freeze-drier (Christ Epsilon 2-10D LSCplus, Osterode am Harz, Germany) for lyophilization at −35 °C for 48 h.

2.3. Capillary blood collection and DBS processing

Details on the capillary blood collection using DBS cards and VAMS can be found in the Supporting Information. For blood collection onto soluble polymeric foams, a foam was placed in a CE glass vial (2 mL; Agilent Technologies, Waldbronn, Germany, P/N 5182–9697), 10 μL of blood was pipetted (details in the Supporting Information) onto the foam, the vial was placed in a plastic zip-lock bag with a desiccant, and the bag was closed. A DBS was formed within 3 h. The typical steps of the DBS collection on a 6% PVP soluble polymeric foam are depicted in Fig. 1A–D.

All collected DBSs were processed in the glass CE vials. Details on the processing of DBSs collected on DBS cards and VAMS devices can be found in the Supporting Information and followed the recommendations of their producers. For processing the DBSs on polymeric foams, the CE vial with the DBS was filled with 550 μL of an eluent, and target analytes were simultaneously eluted and extracted by agitation at 1050 rpm for 30 min using Vibramax 100 agitator (Heidolph Instruments GmbH, Schwabach, Germany). Fig. 1E–F represent the processing of a DBS collected on a 6% PVP soluble polymeric foam. After completion of the elution and extraction, the vial with the extracted sample was transferred to the CE autosampler carousel for at-line injection.

2.4. Hollow fiber liquid-phase microextraction

A comprehensive description of the HF-LPME instrumentation and procedure for the DBS pretreatment can be found in an earlier publication [37], is described in the Supporting Information, and involves the subsequent steps. (i) Placing the DBS into the glass CE vial, (ii) filling the vial with an eluent, (iii) impregnating the HF with an organic solvent, (iv) filling the HF lumen with an acceptor solution, (v) inserting the HF-LPME device into the vial, (vi) closing the vial, and (vii) agitating the vial.

2.5. Capillary electrophoresis

CE measurements of the DBS eluates pretreated by HF-LPME were carried out with a 7100 CE instrument (Agilent Technologies). Details on BGE solution composition, CE operational parameters, electrode selection and exact setting of the separation capillary injection end can be found in the Supporting Information and Fig. S1.

2.6. Calculations

Extraction recovery (ER) values were calculated according to Ref. [36] and the corresponding equation is specified in the Supporting Information.

2.7. Statistical analysis

At least three samples were measured for each experimental analysis throughout the study. The data are graphically presented with standard deviations. Statistical analyses were carried out using open-source Python scientific library SciPy [38]. Statistical significance was assessed using *t*-test and one-way ANOVA.

3. Results and discussion

3.1. Preparation of the polymeric foams

The aim of this study was to investigate the properties of tailor-made polymeric materials that could be applied as suitable alternatives to commercial devices for the collection of DBSs. The study focused on the development of novel polymeric foams, which would (i) enable simple preparation optimally from a single component only, (ii) high stability on contact with capillary blood, (iii) full dissolution of the foam sorbent as well as the resulting DBS in aqueous solutions, and (iv) highly efficient extraction of model analytes from the liquid eluate. The initial selection involved 2% PVA, 3% GEL, 1% CMC07 powder, 0.5% CMC02 cloth, 2% OC, and 6% PVP.

The preparation method has a significant influence on the pore size of the resulting foam and thus also on the absorption capacity of the sorbent and its solubility. The foams were prepared by pipetting 150 μL

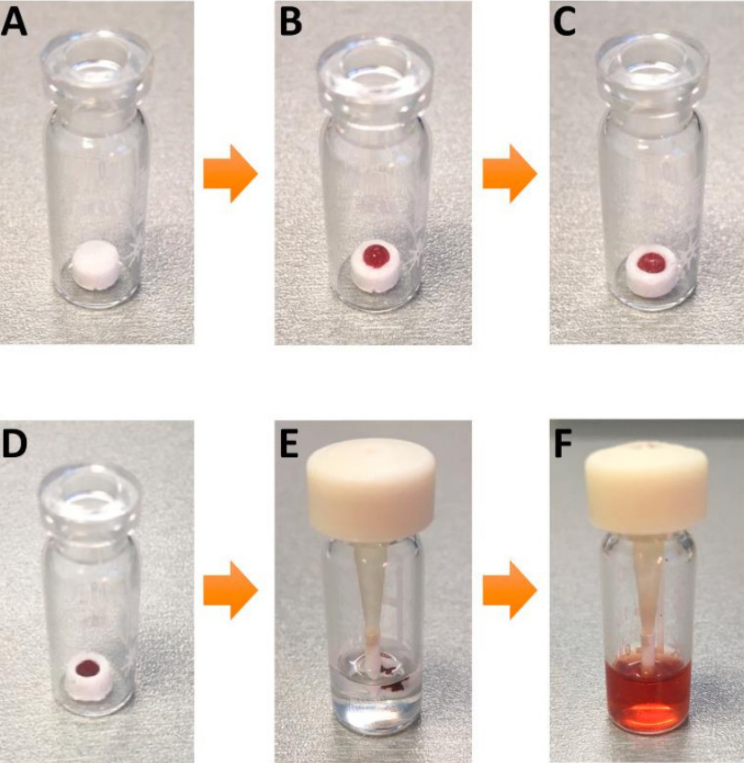


Fig. 1. Photographs depicting the DBS formation on the 6% PVP soluble polymeric foam with the subsequent DBS elution and extraction. A – inserting 6% PVP foam inside the CE vial; B – pipetting 10 μL of capillary blood onto the foam; C – absorption of blood by the foam; D – formation of the DBS on the foam; E – filling the CE vial with 550 μL of 25 mM HCl and a disposable HF-LPME device; F – translucent reddish solution after the foam/DBS dissolution.

of the dispersions into 96-well plates followed by freeze-drying (lyophilization). Direct lyophilization at −35 °C produced polymeric foams with structural defects and inconsistently sized pores, thus several different pre-lyophilization freezing approaches were examined to improve the pore uniformity and the structural integrity of the produced foams. Shock freezing using liquid nitrogen (−196 °C) produced variously shaped foams, that did not stay intact as one cylindrical disc. On the contrary, more structurally consistent foams with uniform pore distribution were obtained by pre-freezing the dispersions in a deep freezer at −25 °C for 1 h and this pre-freezing procedure was used for all subsequent experiments. The polymeric dispersions cool down gradually and at a rather slow rate by deep freezing as opposed to the use of liquid nitrogen or direct lyophilization (where the temperature change is immediate) and the milder temperature changes have a major effect on the structural stability of the foam [39,40].

3.2. Solubility of the polymeric foams

The resulting foams were removed from the well plates (see the Supporting Information), placed in the glass CE vials and their solubility was investigated by adding 550 μL of various aqueous solutions (DI water, 100 mM HCl, 100 mM acetic acid, and 100 mM NaOH) or organic solvents (methanol and acetonitrile) and agitating the vials at 1050 rpm for 30 min. All polymeric foams were insoluble in the organic solvents, while the polymeric foams made of 1% CMC07 powder, 2% OC, and 6% PVP were fully soluble in the aqueous solutions. The foam made of 0.5% CMC02 cloth dissolved partially in the aqueous solvents leaving small cloth debris in the solutions. The solubility difference between 1% CMC07 and 0.5% CMC02 foams was expected as it is mainly related to their degree of substitution. The lower the degree of substitution, the less soluble the CMC fibers are, the substitution degree 0.4 being the threshold value [41]. Polymeric foams prepared from PVA and GEL were insoluble in both, aqueous and organic solvents, and were thus excluded from further experiments.

Subsequent experiments were carried out with single component dispersions and polymeric foams were prepared from 0.5 to 3% CMC07, 0.5–1% CMC02, 2–4% OC and 4–10% PVP. The solubility of the PVP foams improved for lower PVP concentrations resulting in fully soluble (4 and 6%), partially soluble (8%), and insoluble (10%) foams. However, immediate dissolution occurred for the lowest PVP concentration and preliminary tests with capillary blood revealed that 4% PVP foams were not suitable for reliable DBS collection due to their nearly complete dissolution upon contact with the blood drop. Similar characteristics (i. e., very fast dissolution and poor ability for the collection of capillary blood) were also observed for low concentrations of CMC07 and OC. The solubility of the foams can be adjusted by mixing different polymeric components and a set of binary composite dispersions with various compositions and concentrations of CMC07, CMC02, and OC was subsequently prepared for further solubility testing. Their solubility characteristics are summarized in Table 1 and showed similar behavior to

Table 1
Solubility of polymeric foams.

Foam composition	pH	Elution solvent					
		H_2O	100 mM HCl	100 mM HAc	100 mM NaOH	MeOH	ACN
0.5% CMC07	7.00	✓	✓	✓	✓	×	×
1% CMC07	7.04	✓	✓	✓	✓	×	×
2% CMC07	7.08	✓	✓	✓	✓	×	×
3% CMC07	7.11	✓	✓	✓	✓	×	×
0.5% CMC02	7.00	/	/	/	/	×	×
1% CMC02	7.05	×	×	×	×	×	×
2% OC	6.49	✓	✓	✓	✓	×	×
3% OC	6.53	✓	✓	✓	✓	×	×
4% OC	6.64	✓	✓	✓	✓	×	×
4% PVP	7.19	✓	✓	✓	✓	×	×
6% PVP	6.19	✓	✓	✓	✓	×	×
8% PVP	5.11	/	/	/	/	×	×
10% PVP	4.96	×	×	×	×	×	×
0.5% CMC02/2% OC	6.55	/	/	/	/	×	×
1% CMC02/2% OC	6.50	/	/	/	/	×	×
0.5% CMC07/0.5% OC	6.90	✓	✓	✓	✓	×	×
1% CMC07/0.5% OC	6.64	✓	✓	✓	✓	×	×
1% CMC07/1% OC	6.68	✓	✓	✓	✓	×	×
1% CMC07/1.5% OC	6.63	✓	✓	✓	✓	×	×
1% CMC07/2% OC	6.63	✓	✓	✓	✓	×	×
1% CMC07/2.5% OC	6.51	×	×	×	×	×	×
1% CMC07/3% OC	6.56	×	×	×	×	×	×
1.5% CMC07/2% OC	6.62	×	×	×	×	×	×
2% CMC07/2% OC	6.60	×	×	×	×	×	×
2.5% CMC07/2% OC	6.71	×	×	×	×	×	×
3% CMC07/2% OC	6.67	×	×	×	×	×	×

HAc – acetic acid.
✓ – fully soluble.
/– partially soluble.
× – insoluble.

PVP, i.e. dissolution upon contact with the blood drop and poor solubility for low and high polymer concentrations, respectively. Two foams (the mixture of 1% CMC07/2% OC and 6% PVP) were selected for further experiments due to their ease of preparation, suitability for capillary blood collection, and full solubility in model aqueous solutions. Detailed electron scanning microscope images of internal structures of the two selected foams, and two foams rejected due to their too high (1% CMC07) and too low (1% CMC02) solubility are depicted in Fig. S2 in the Supporting Information.

3.3. *Polymeric foams as DBS sampling sorbents*

In a typical DBS collection, capillary blood is collected onto a solid (paper card, VAMS) sampling device and blood is dried up at ambient temperature for 2–3 h. The soluble polymeric foams behave differently from the solid sorbents on contact with blood, and their properties and the required drying period were examined in detail in the following experiments. A volume of 10 μL of capillary blood was accurately transferred onto the 6% PVP and 1% CMC07/2% OC foams prepared according to the previous procedure from 150 μL of the dispersions. During the contact with the polymeric foam, capillary blood was gradually absorbed by the inner structure of the foam (see Fig. S3 in the Supporting Information) and the top surface dissolved partially. The stability of the sampling foam was given by the volume of the dispersion used for lyophilization and was optimized in the next section. The open vial with the foam/blood was subsequently placed in a zip-lock bag with a desiccant, the bag was closed, and the DBS formation at ambient temperature was monitored by weighing the vial at 30–720 min (see Fig. S4 in the Supporting Information). The desiccant in the bag absorbed the liquid fraction evaporated from blood, and the DBSs were formed in 3 h for both foams; the in-bag drying can be used e.g. for patient-centric DBS collection and immediate shipment to a laboratory. Drying time of 3 h was also achieved for DBSs formed in open vials and dried at ambient temperature.

3.4. *Dissolution of polymeric foams with DBSs followed by HF-LPME*

The selected polymeric foams are fully soluble in most aqueous solutions regardless of their pH and composition. The resulting eluates contain dissolved polymers and DBS matrix components, which are difficult to handle with standard separation techniques and result in analytical interferences and/or analytical system poisoning. In the presented study, a three-phase HF-LPME was selected as the most suitable pretreatment method, because HF-LPME enables efficient clean-up of complex samples and maintains high enrichment capability. Moreover, HF-LPME can be at-line coupled to commercial CE, and the resulting acceptor solutions can be injected directly from the HF lumen. Initial operating conditions for the HF-LPME/CE of acidic drugs (acceptor solution, 10 mM NaOH; elution solvent, 25 mM HCl; agitation, 900 rpm; time, 30 min) were adapted from the previous publication [37], and were re-examined for DBS extractions from the soluble foams. The entire HF-LPME optimization is summarized in the Supporting Information and the only modified variable was the agitation speed (1050 rpm), which ensured faster and more efficient dissolution of the foams.

The volume of the polymeric dispersion pipetted to the 96-well plates was investigated first. The minimum volume that formed a liquid column in the well was 100 μL and the maximum volume the well could accommodate was 500 μL. However, after the lyophilization of 100 μL of the dispersions, the resulting foams deteriorated significantly after the 10 μL capillary blood sampling because blood saturated the entire foams. After the lyophilization of 500 μL of the dispersions, the resulting foams extended from the wells and the top parts had non-uniform shapes. Thus, a minimum and a maximum volume of the dispersions were selected, offering the testing range 150–400 μL. The volume of the dispersion determines the size of the foam and consequently, larger foams can adsorb larger volumes of capillary blood. On

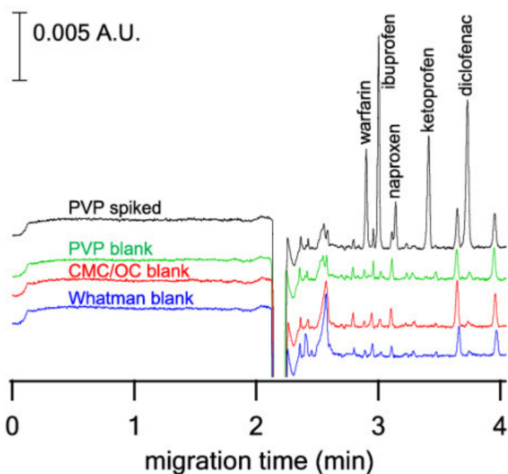


Fig. 2. Electropherograms for the determination of the five acidic drugs in blank and spiked DBSs. HF-LPME conditions: SLM, DHE; acceptor, 5 μL of 10 mM NaOH; donor, a DBS (blank or spiked with the five acidic drugs at 2750 μg/L) eluted with 550 μL of 25 mM HCl; agitation speed, 1050 rpm; extraction time, 30 min. CE conditions: BGE solution, 30 mM sodium acetate, 30 mM acetic acid, and 30% (v/v) acetonitrile at apparent pH 5.2; separation voltage, +25 kV; injection, 50 mbar for 5 s; detection wavelength, 200 nm.

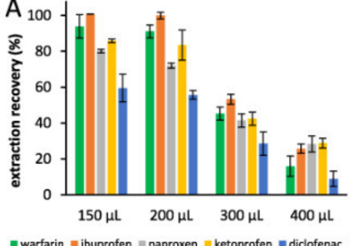
the other hand, larger foams result in more concentrated and viscous eluates, which might have a direct bearing on the HF-LPME efficiency and might also result in an increased co-extraction of the foam components. The volume of the elution solvent (25 mM HCl) was set at 550 μL offering the most convenient HF-LPME conditions [37]. DBSs were formed by pipetting 10 μL of drug-free capillary blood spiked with 2750 μg/L of the five acidic drugs to the foams (final concentrations of the drugs in DBS eluates were 50 μg/L). Overlaid electropherograms of a blank DBS collected on the 6% PVP, 1% CMC07/2% OC foams, and Whatman™ card and a spiked DBS collected on the 6% PVP foam after HF-LPME of the eluates are depicted in Fig. 2. Several compounds were co-extracted during the HF-LPME from blank and spiked DBS eluates. However, since they migrated at times different from the analytes, or their analytical signals were below the method limit of quantification (LOQ), or their signals could be attenuated by the selection of a species-specific detection wavelength (e.g. 214 nm for diclofenac and 226 nm for naproxen, see Fig. S5 in the Supporting Information), they did not interfere with the CE determination of the analytes.

ER values for HF-LPMEs of the eluates of the two polymeric foams were determined by at-line CE analyses of the resulting acceptor solutions and are depicted in Fig. 3A (6% PVP) and 3B (1% CMC07/2% OC). Comparable ER values were obtained for 6% PVP foams prepared from 150 to 200 μL of the dispersion. A considerable decrease in ERs was observed for larger PVP foams, which was attributed to the higher viscosity and complexity of the resulting eluates. Similarly, maximum ER values were achieved for 1% CMC07/2% OC foams prepared from 150 μL of the dispersion, while ERs considerably decreased for the larger CMC/OC foams. To achieve minimum consumption of chemicals, reliable DBS collection, and efficient HF-LPME, polymeric foams were prepared from 150 μL of the dispersions in all subsequent experiments. The resulting polymeric foams were discs with a diameter of 5.9 ± 0.05 mm (given by the i.d. of the 96-well plate) and a height of 2.6 ± 0.1 mm and 3.0 ± 0.1 mm for 6% PVP and 1% CMC07/2% OC, respectively, (n = 5, mean value ± SD).

3.5. *Minimum drying time and DBS stability*

The minimum drying time was further examined by dissolving the foams with spiked DBSs, extraction of the analytes by HF-LPME, and CE analysis. The elution solution was pipetted into the vial with the DBS immediately after the drying time (30–720 min) elapsed, the complete HF-LPME device was placed into the vial, and the DBS was dissolved/extracted at 1050 rpm for 30 min. The acceptor solution enriched with the analytes was at-line injected into CE-UV for the determination of the five acidic drugs. ER values for the two polymeric foams are depicted in Fig. S6 in the Supporting Information. A gradual increase of ER values was observed for drying times 30–120 min, ER values reached their maxima at 180 min and formed a plateau at 180–720 min. Consequently, the minimum drying time of 3 h experimentally achieved by gravimetry in Fig. S4 was confirmed by the actual HF-LPME/CE measurements and was consistent with drying times for standard DBS collection devices (paper cards and VAMS) [2,26]. Fig. S7 shows two photographs of the dissolved 6% PVP and 1% CMC07/2% OC foams with DBSs. Translucent reddish solutions were obtained, indicating a complete dissolution of the foam components and the DBS.

Solubility characteristics of the polymeric foams and stability of the acidic analytes in DBSs collected on the polymeric foams were investigated for four weeks. The foams with the DBSs were stored in CE vials in closed zip-lock bags with the desiccant – one set of DBSs was refrigerated at 4 °C and the other one was stored at room temperature. The foams were dissolved, extracted, and analyzed 3 h and then one, two, three, and four weeks after the DBS collection. Solubility of the polymeric foams was not affected by the storage temperature nor by the storage time and all foams were fully soluble in 550 μL of 25 mM HCl. The stability of the target analytes in DBSs collected on the foams was examined by HF-LPME/CE analyses of the resulting eluates and is summarized in Fig. S8 in the Supporting Information [37]. The ER values ranges were 66–95% and 37–93% for 6% PVP and 1%



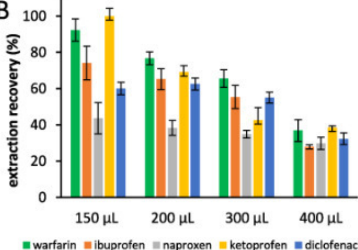


Fig. 3. The effect of the polymeric dispersion volume on the ER values of acidic drugs extracted from DBSs formed on the resulting foams. A – 6% PVP, B – 1% CMC07/2% OC. HF-LPME conditions as for Fig. 2 (spiked concentration, 5500 μg/L). The bars show a mean value ± SD (n = 3).

CMC07/2% OC, respectively, and did not differ by more than 7.8% for the various storage conditions and times. The results were evaluated by a *t*-test at $\alpha = 0.05$ level of significance and showed that the differences in ER values for both foams at the two storage conditions and the four storage times were statistically not significant; differences in ER values between the two foam materials were statistically significant.

3.6. Effect of blood hematocrit and DBS ageing

Former studies have demonstrated serious effects of blood hematocrit and prolonged DBS storage time on quantitative DBS analyses. These have mainly been affected by the different sizes of DBSs, different distribution of analytes in central/peripheral parts of DBSs, time-instability of some analytes in the dried matrices, and their storage time-dependent elution [11–13,18]. To verify the robustness of the proposed soluble foams for DBS collection, drug-free capillary blood samples with different levels of hematocrit (low/medium/high, 25/43/61%) were prepared according to Refs. [11,18], spiked with 550 μg/L of the five acidic drugs (final concentrations of the drugs in DBS eluates were 10 μg/L), and sampled on the 6% PVP and 1% CMC07/2% OC polymeric foams. The first set of DBSs was eluted, extracted, and analyzed immediately after collection and drying for 3 h, and the second set of DBSs was processed in the same way four weeks later (after being stored in a closed zip-lock bag with the desiccant at room temperature). ER values determined for DBSs at different hematocrit levels and different storage times are depicted in Fig. 4. The drugs concentrations extracted from eluates of DBSs with different hematocrit values were constant and the differences (defined as $ER_{max} - ER_{min}$) were 0.2–7.7% for 6% PVP and 0.7–9.2% for 1% CMC07/2% OC soluble foams. Similar differences were determined also for the fresh vs. the aged DBS samples; the differences were 0.4–8.5% for 6% PVP and 0.1–8.3% for 1% CMC07/2% OC. Statistical evaluation of the results (one-way ANOVA or *t*-test at $\alpha = 0.05$ level of significance) indicated a non-significant effect of the hematocrit levels and the DBS storage time on ER values. They also confirmed a statistically significant difference in ER values for the two foam materials.

3.7. Comparison of soluble polymeric foams, DBS cards, and VAMS

The major difference between the elution of DBSs from insoluble and soluble DBS sampling sorbents is the availability of blood compounds in the final eluates. In principle, blood is absorbed by the sorbent and penetrates into its internal structure, which is insoluble for cellulose-based cards and VAMS. The blood sample dries up inside the internal structure of the sorbent and various blood components (including target analytes) might be retained by the sorbent during DBS elution and, thus, not available for subsequent processing and analysis [27,28,31]. Careful optimization is, thus, needed to ensure robust and quantitative release of target analytes into the eluate. On the contrary, the application of soluble sampling sorbents ensures complete dissolution of the DBS sampling sorbent as well as all blood components, and thus their full availability in the DBS eluates. To examine the effect of the solubility of various sampling sorbents on the determination of acidic drugs in DBSs, drug-free capillary blood was spiked with 550 μg/L of the model drugs and a 10 μL volume was collected by the soluble polymeric foams, standard cellulose-based sampling cards, and VAMS devices. The DBS elution and HF-LPME of the drugs from the soluble foams were performed simultaneously according to the above-optimized procedure. DBSs collected on commercial DBS sampling devices were eluted in 550 μL of 25 mM HCl at elution conditions recommended by the producers (typically 1200 rpm and 60 min), and were followed by HF-LPMEs of the resulting eluates at 1050 rpm for 30 min. ER values are shown in Fig. 5 and demonstrate pronounced differences for various sampling sorbents. The highest extraction yields were obtained for DBSs eluted from 6% PVP soluble foams resulting in 70–99% ER values demonstrating nearly exhaustive extraction of all (except diclofenac (70%)) acidic drugs. Lower ER values (44–54%) were obtained for naproxen and diclofenac eluted from DBSs formed on 1% CMC07/2% OC foams. We assume that these acidic drugs interact with the cellulose-based polymers after the foam and the DBS are dissolved because recoveries of other drugs (e.g. warfarin and ketoprofen) were not affected and demonstrated their full availability in the resulting eluates.

For the DBSs collected on Whatman™ 903 cards, ER values comparable to 6% PVP were obtained for the determination of naproxen and diclofenac. However, ER values were 1.4, 1.8, and 1.9-fold lower for ibuprofen, ketoprofen, and warfarin, respectively. The other two insoluble sampling sorbents exhibited even more pronounced performance deterioration and resulted in up to 2.8-fold and 10.6-fold lower recoveries (in comparison to 6% PVP) for DBSs collected on VAMS

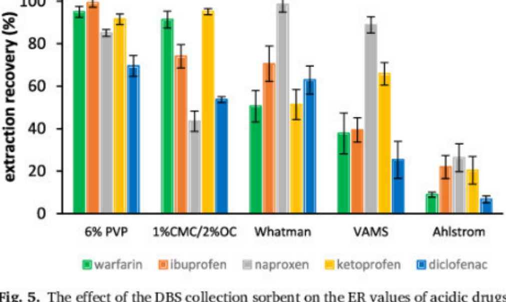


Fig. 5. The effect of the DBS collection sorbent on the ER values of acidic drugs extracted from DBSs formed on paper-based sampling cards, VAMS, and soluble foams. HF-LPME conditions as for Fig. 4. The bars show a mean value ± SD (n = 3).

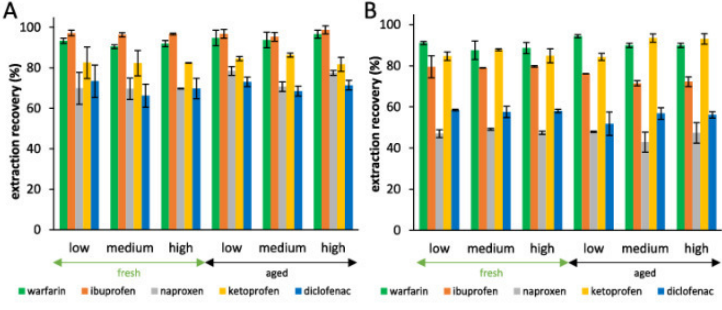


Fig. 4. The effect of DBS hematocrit levels and DBS storage time on the ER values of acidic drugs extracted from DBSs formed on soluble polymeric foams. A – 6% PVP, B – 1% CMC07/2% OC. HF-LPME conditions as for Fig. 3 except for spiked drug concentration (550 μg/L).
The DBSs were processed 3 h (fresh) and 4 weeks (aged) after collection; the hematocrit levels were 25, 43, and 61% for low, medium, and high, respectively. The bars show a mean value ± SD (n = 3).

Table 2
Analytical parameters of the HF-LPME at-line coupled to CE for the determination of model acidic drugs in DBSs collected on soluble polymeric foams. HF-LPME and CE conditions as for Fig. 5, n = 5. Concentrations, LODs and LOQs are expressed as the drug concentrations in the undiluted capillary blood before DBS collection and elution. Calibration range = 275–27500 μg/L.

Foam	Parameter	warfarin	ibuprofen	naproxen	ketoprofen	diclofenac
6% PVP	ER (%), 125 μg/L	97.6	101.8	80.4	87.5	62.6
	RSD (%), 125 μg/L	4.6	2.9	4.5	5.8	2.2
	ER (%), 275 μg/L	96.8	98.4	83.5	86.0	65.7
	RSD (%), 275 μg/L	5.8	4.2	2.2	1.8	2.1
	ER (%), 5500 μg/L	94.9	99.2	85.1	91.5	69.5
	RSD (%), 5500 μg/L	2.6	1.8	1.6	7.5	8.1
	ER (%), 27500 μg/L	97.1	97.6	84.5	85.4	70.4
	RSD (%), 27500 μg/L	4.3	3.1	2.1	4.8	2.8
	R^2	0.9995	0.9991	0.9991	0.9992	0.9994
	LOD (μg/L)	38	33	16	33	38
	LOQ (μg/L)	125	109	53	109	125
1% CMC/2% OC	ER (%), 125 μg/L	98.7	68.9	40.1	91.3	49.1
	RSD (%), 125 μg/L	5.4	6.2	1.6	2.0	1.0
	ER (%), 275 μg/L	94.8	72.5	42.5	93.4	50.5
	RSD (%), 275 μg/L	4.5	5.5	2.4	1.8	3.1
	ER (%), 5500 μg/L	91.3	74.1	43.5	95.1	53.7
	RSD (%), 5500 μg/L	3.9	5.5	4.8	1.4	1.3
	ER (%), 27500 μg/L	89.4	72.5	45.0	96.8	49.4
	RSD (%), 27500 μg/L	2.8	4.4	1.4	1.9	3.1
	R^2	0.9996	0.9996	0.9996	0.9993	0.9994
	LOD (μg/L)	38	44	33	33	44
	LOQ (μg/L)	125	145	109	109	145

(Mitra®) and Ahlstrom (GenCollect™ 2.0) devices, respectively.

3.8. Analytical performance

The analytical parameters for the determination of acidic drugs in DBS samples collected on soluble polymeric foams are summarized in Table 2. The analytical procedure involved the simultaneous dissolution of the polymeric foam, elution of DBS, and HF-LPME of the resulting eluate, which were achieved by agitating the DBS in 550 μL of 25 mM HCl at 1050 rpm for 30 min. The HF-LPME acceptor solution was 10 mM NaOH and was subsequently at-line injected into the CE-UV instrument for the determination and quantification of extracted acidic drugs. Blank DBS samples were formed by sampling drug-free capillary blood (see Fig. 2 and S5) onto the soluble foams. The drug-free capillary blood was spiked with 125, 275, 5500 and 27500 μg/L (4 quality control samples) of the five acidic drugs and repeatability of the analytical procedure (expressed as RSD values of peak areas) was evaluated for DBSs formed on the two soluble foams. Repeatability for the determination of acidic drugs in five DBS samples was within 1.6–8.1% and 1.3–5.5% for soluble foams consisting of 6% PVP and 1% CMC07/2% OC, respectively. Inter-day (three different days) and inter-sample (three different volunteers) repeatability was better than 8.0% and is summarized in Table S1 in the Supporting Information. The RSD values comply with the requirements on repeatability in bioanalytical method validation and demonstrate that the effect of the sample matrix on the extraction and analytical process was well within acceptance limits [42,43]. ER values were 70–99% and 44–95% for the analyses of DBSs formed on 6% PVP and 1% CMC07/2% OC soluble foams, respectively. The linearity of the technique was measured for a concentration range of 275–27.500 μg/L (6 calibrators at 275, 550, 1375, 2750, 5500, and 27500 μg/L in DBS matrix) and the obtained coefficients of determination (R^2) were 0.9991–0.9996. The concentrations of calibrators were back-calculated from the calibration curve, did not differ from the nominal values by more than 10.5%, and showed that the linear model can be accepted [42,43]. LODs and LOQs of the method were 16–44 μg/L and 53–145 μg/L, respectively, and were suitable for the determination of therapeutic concentrations of acidic drugs (1–50 mg/L) in clinical samples [44]. The LODs and LOQs were defined as the lowest concentrations of an analyte giving analytical signals three- and ten-times higher than baseline noise (S/N = 3, S/N = 10), respectively, and were calculated for the original undiluted capillary blood.

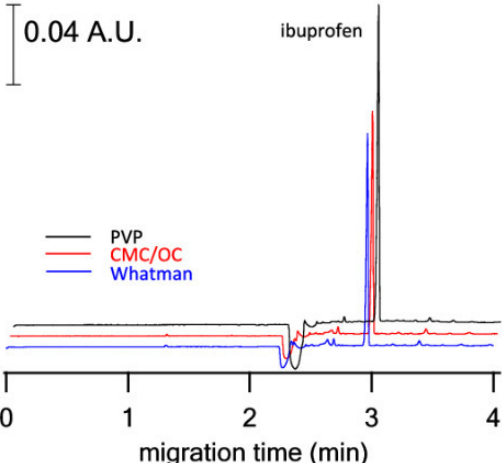


Fig. 6. At-line coupling of HF-LPME to CE-UV for the direct determination of ibuprofen in DBS samples collected on various DBS collection sorbents 90 min after ibuprofen administration. HF-LPME and CE conditions as for Fig. 2. Native ibuprofen concentration after drug ingestion, 29.3 mg/L.

3.9. Determination of ibuprofen therapeutic concentrations

The developed soluble polymeric foams were further tested for the collection of capillary blood and pretreatment/analysis of the resulting DBSs after oral administration of a tablet containing 400 mg of ibuprofen. Capillary blood was collected before and 90 min after the drug administration (ibuprofen's half-life in humans is 0.9–2.5 h) [44]. Blood was collected onto the two soluble foams and additionally also onto the Whatman™ collection card. Several minor compounds were detected in the blank samples as a result of their co-extraction from the

DBS eluates during HF-LPME. They do not interfere with the determination of ibuprofen, as has been verified by the HF-LPME/CE analysis of a spiked DBS sample collected on the 6% PVP (see Fig. 2), and their identification was not necessary. The electropherograms in Fig. 6 depict the determination of ibuprofen in capillary blood collected on the three different DBS sampling sorbents after the drug administration; the electropherograms were offset by 2 s for better readability. The results are consistent with the trends reported for DBS samples spiked with acidic drugs in the previous paragraph, demonstrating exhaustive HF-LPME of ibuprofen for the 6% PVP soluble foam. Ibuprofen concentration in the DBS sample was determined as 29.3 ± 0.9 mg/L (n = 3, mean value ± SD) and conforms with its typical therapeutic concentrations [44].

4. Conclusions

Various polymeric foams were prepared and their composition optimized for the collection and analysis of DBSs. The foams were prepared from PVP or a mixture of CMC07 and OC in a simple manner based on homogenization of an aqueous polymeric dispersion, pipetting a given volume of the dispersion into a 96-well plate, and gradual freezing/lyophilization of the dispersion. The resulting polymeric foams were porous discs of uniform dimensions (approx. 6 × 3 mm) with full solubility in standard aqueous solutions. The soluble polymeric foams enabled collection of minute volumes of capillary blood and blood drying in a standard CE vial, which were followed by an in-vial DBS pretreatment (elution and HF-LPME) and at-line CE analysis. The DBS elution process involved the simultaneous dissolution of the entire foam as well as the entire DBS and ensured a quantitative transfer of all blood components into the eluates. As a result of their full availability in the eluates, nearly exhaustive extractions of target analytes were achieved, which were demonstrated by the HF-LPME/CE determination of model acidic drugs in DBSs collected on the developed polymeric foams. The soluble polymeric foams exhibited low variations in analyte concentrations for DBSs prepared from blood samples with different hematocrit levels and for aged DBSs. The acidic drugs stored in the foam-based DBSs at ambient temperature were stable for at least four weeks. Blood collection on the soluble polymeric foams offered faster pretreatment times and improved ERs in comparison to standard DBS sampling devices. The new PVP and CMC07/OC foams, therefore, represent an attractive alternative to standard DBS collection devices and might constitute a new group of DBS sampling sorbents in the future. Moreover, since the HF-LPME operational conditions (i.e. SLM/donor/acceptor solution composition) can be fine-tuned for specific ionic species, the suggested concept might be applied to a broad range of analytes and various analytical and detection techniques.

CRediT authorship contribution statement

Lenka Ryšavá: Conceptualization, Investigation, Methodology, Writing – original draft, Funding acquisition. **Jana Dorazilová:** Conceptualization, Investigation, Writing – original draft. **Miloš Dvořák:** Supervision, Methodology, Writing – review & editing. **Petr Sedláček:** Supervision, Writing – review & editing. **Lucy Vojtová:** Supervision, Writing – review & editing, Funding acquisition. **Pavel Kubáň:** Conceptualization, Supervision, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition.

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.

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.2023.340793.

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SUPPORTING INFORMATION

Fully soluble polymeric foams for in-vial dried blood spot collection and analysis of acidic drugs by capillary electrophoresis

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Experimental

Blood sampling onto DBS cards, VAMS, and polymeric foams

Capillary blood was collected by volunteers at the Institute of Analytical Chemistry after signing a written informed consent. Collection of DBSs was approved by the Research Council of the Institute of Analytical Chemistry. Before pricking, the fingertip was wiped off with a lint-free cotton pad soaked with ethanol (Lach-Ner). Ethanol at the fingertip was left to evaporate to avoid blood precipitation. The fingertip was pricked using an automated skin puncture device (ACCU-CHEK FastClix, Roche, Mannheim, Germany) with disposable lancets, which were discarded after use. The first blood drop was wiped off with a dry cotton pad and the second drop was used for blood sampling. Exactly 10 µL of capillary blood was pipetted into a graduated polypropylene micropipette tip (Sorenson Bioscience Inc., Salt Lake City, UT, USA; One Touch 1 – 20 µL) using a fixed-volume micropipette (10 µL, Eppendorf, Hamburg, Germany). Blood from the tip was quantitatively dispensed onto a DBS sampling card (Whatman™ 903 Protein Saver, GE Healthcare Ltd, Cardiff, UK, or Ahlstrom GenCollect™ 2.0, Ahlstrom Germany GmbH, Bärenstein, Germany) and was air-dried at room temperature in a holder for 3 h to form the DBS. For blood sampling by VAMS, Mitra® Clamshell devices (blood capacity of 10 µL, Neoteryx, Torrance, CA, USA) were applied. Capillary blood flew spontaneously into the internal structure of the porous polymeric VAMS tip and the exact blood volume (given by the size of the tip) was absorbed [1]. After blood collection, the VAMS device was left in the dedicated box and was dried up for 3 h at room temperature. For blood sampling onto a soluble polymeric foam, the foam was removed from the 96-well plate. Some foams attached to the well wall during lyophilization and these were detached from the wall by a stainless pin. The released foam was carefully removed with a tweezer and placed in a CE glass vial (2 mL; Agilent Technologies, Waldbronn, Germany, P/N 5182-9697), see Figure 1A, 10 µL of capillary blood was quantitatively pipetted onto the

foam (Figure 1B), was allowed to soak into the internal foam structure (Figure 1C), and to dry up (Figure 1D).

DBS samples spiked with the drugs were prepared by mixing 90 μL of capillary blood with 10 μL of a standard drug solution in a PCR microvial (250 μL) and 10 μL of the mixture was pipetted onto the polymeric foam, onto the DBS sampling card or was absorbed by the VAMS device. All collected DBSs were processed in the glass CE vials. The DBS elution solution must meet certain criteria for the subsequent HF-LPME: (i) it must be aqueous to dissolve the polymeric foams, (ii) must be acidic to render the target analytes neutral, and (iii) must not contain organic solvent not to dissolve the SLM.

The DBS on a polymeric foam was formed directly in the CE vial and was used with no modifications. The CE vial with the DBS was filled with 550 μL of an eluent, and target analytes were simultaneously eluted and extracted by agitation at 1050 rpm for 30 min (Vibramax 100, Heidolph Instruments GmbH, Schwabach, Germany). Figures 1E – 1F represent the processing of a DBS collected on a 6% PVP soluble polymeric foam. The DBS formed on a DBS card was processed by punching out a 10 mm disc with the entire DBS from the card and by placing the disc into the CE vial. The CE vial with the DBS was filled with 550 μL of an eluent, DBS was eluted by agitation at 1200 rpm for 60 min and target analytes were extracted by agitation at 1050 rpm for 30 min. For processing the DBS formed by the VAMS device, the tip with the DBS was removed from the supporting rod and placed into the CE vial. The CE vial with the DBS was filled with 550 μL of an eluent, DBS was eluted by agitation at 1200 rpm for 60 min and target analytes were extracted by agitation at 1050 rpm for 30 min. After completion of the extraction, the vial with the extracted sample was transferred to the CE autosampler carousel for at-line injection.

Hollow fiber liquid-phase microextraction

HF holders for the at-line coupling of HF-LPME to CE were 3D printed from a clear UV sensitive resin (Basic, 405 nm) using a Photon Mono 3D printer (both Anycubic Technology Co. Ltd., Shenzhen, PCR). The dimensions of the conical holders were specified in our previous publication [2]. Each holder was fitted with a 9.5 mm long extraction unit cut from a porous polypropylene hollow fiber (Accurel PP 300/1200, Membrane, Wuppertal, Germany) with outer diameter (o.d.), internal diameter (i.d.), wall thickness, and pore size of 1800, 1200, 300, and 0.2 μm, respectively. The extraction unit was sealed by mechanical pressure and heat on one side (approx. 1 mm of the unit) and the open side of the unit was stretched over the narrower end of the holder.

A comprehensive description of the HF-LPME procedure for the DBS pretreatment can be found in publication [2] and is briefly summarized here. The DBS disc (punched from the card, VAMS, or foam) is placed into the glass CE vial and the vial is filled with 550 μL of an eluent using an Eppendorf micropipette. The HF is impregnated with an organic solvent (by dipping the HF for 10 s into the solvent and wiping-off the solvent excess by a lint-free tissue) and the HF lumen is filled with 5 μL of an acceptor solution using a Hamilton syringe with flat tip. The HF-LPME device is inserted into the glass vial and the vial is closed. Finally, the vial is placed on the Vibramax 100 agitator and agitated at 1050 or 1200 rpm as specified in the previous Section.

Capillary electrophoresis

Acidic drugs were detected by a built-in UV-Vis detector at 200 nm (warfarin, ibuprofen, and ketoprofen), 214 nm (diclofenac), and 226 nm (naproxen). The BGE solution for the determination of the acidic drugs was optimized previously [3] and consisted of 30 mM sodium acetate, 30 mM acetic acid, and 30% (v/v) acetonitrile. The BGE solution was prepared daily

and had an apparent pH of 5.2. CE separations were performed in a fused silica capillary (75 µm i.d./375 µm o.d., L_{tot} = 45 cm and L_{eff} = 36.8 cm), supplied by Polymicro Technologies (Phoenix, AZ, USA). The separation voltage applied to the inlet electrode was +25 kV, the temperature inside the CE cartridge was 25 °C, and injections were performed hydrodynamically at 50 mbar for 5 s. The inlet platinum electrode (Agilent Technologies, P/N G7100-60033) was 5 mm shorter than the outlet platinum electrode (Agilent Technologies, P/N G7100-60007) to avoid sample cross-contamination with BGE solution during the injection [2]. The injection end of the separation capillary protruded 8 mm from the short electrode, thus approx. 3 mm of the capillary were immersed in the acceptor solution during injection, and the acceptor solution was injected directly from the HF lumen. For an exact setting of the injection end length, the total length of the capillary exposed from the CE cartridge (at the injection side) was 49 mm and is visualized in Figure S1 in the Supporting Information.

Calculations

Extraction recovery (ER) values of the HF-LPME of dried blood spots (DBSs) eluted from soluble polymeric foams, DBS cards, and VAMS were calculated according to equation (1):

$$ER\ (\%) = \frac{n_{a,\ final}}{n_{d,\ initial}} \times 100 = \frac{V_a}{V_d} \times \frac{C_{a,\ final}}{C_{d,\ initial}} \times 100 \qquad (1)$$

where $n_{a,final}$ and $n_{d,initial}$ are the number of the analyte moles finally transferred into the acceptor solution and the number of the analyte moles initially present in the donor solution, respectively. V_a is the acceptor solution volume, V_d is the DBS eluate volume, $C_{a,final}$ is the final analyte concentration in the acceptor solution and $C_{d,initial}$ is the initial analyte concentration in the capillary blood divided by a factor of 55 (10 µL DBS diluted by 550 µL of the eluate).

Results and discussion

Hollow fiber liquid-phase microextraction optimization

Ten μL of drug-free capillary blood spiked with 5500 μg/L of the five acidic drugs was pipetted to the foams. The drugs were extracted from the resulting DBSs into a 10 mM NaOH acceptor solution. The acceptor solution composition was based on our previous publication [2] and ensured full ionization of acidic drugs at the SLM/acceptor solution phase interface. The pH of the eluent might be affected by the presence of blood components from DBS and polymeric components from the foams and because acidic drugs (with pK_a values between 4 and 5) were extracted, acidic eluate conditions were examined. Acidic conditions promote the transfer of the drugs (in their neutral form) to the SLM at the eluate/SLM phase interface. The results are depicted in Figure S8 and demonstrate low ER values for DI water (8 – 33%) and 10 mM HCl (14 – 48%), maximum ER values for 25 and 50 mM HCl (39 – 97%), and slightly reduced ER values for 100 mM HCl (22 – 90%). Soluble foams were thus eluted with 25 mM HCl in all subsequent experiments.

The extraction efficiency is considerably influenced by the presence of target analytes at the phase interface between the eluent and the SLM. In HF-LPME, efficient transfer of target analytes to the phase interface is achieved by the eluate agitation. Moreover, agitation is also crucial for rapid and efficient dissolution of the polymeric foam to release DBS components into the eluate. If the eluate is then insufficiently agitated, the distribution of analytes between the aqueous and the organic phase may not be sufficient and may induce low ER values. On the contrary, too vigorous agitation may form a pronounced vortex in the eluate and the eluate may not contact the HF properly, which consequently may again induce low ER values. The agitation speed was, therefore, examined in the range of 600 – 1350 rpm for 30 min and the corresponding results are depicted in Figure S9. Highest ER values were achieved for agitating 6% PVP foams at 1050 rpm (74 – 97 %) and 1% CMC07 / 2% OC foams at 900 rpm (46 –

100%). The slightly higher agitation speed required for HF-LPME of PVP-based foams might be explained by their slightly higher stability and lower solubility. A further increase of the agitation speed was not necessary because the HF-LPME efficiency was nearly constant (plateaus were observed or ERs slightly decreased) for speeds higher than 1050 rpm. Soluble foams were thus eluted at 1050 rpm in all subsequent experiments.

Another crucial factor in HF-LPME is the extraction time. The extraction system should ideally reach an equilibrium state in the final phase and due to the application of a soluble foam as the DBS sampling sorbent, the foam is supposed to completely dissolve in the elution solvent. The fully dissolved foam might consequently ensure exhaustive transfer of target analytes from the eluate since they are not retained in the sorbent. Extraction time was examined for 0 – 50 min and corresponding curves demonstrating ER values at different extraction times are shown in Figure S10. The 6% PVP and 1% CMC07 / 2% OC polymeric foams were fully dissolved in 10 and 7 min, respectively, and the transfer of the acidic drugs to the acceptor solution was completed in about 30 min. Nearly exhaustive extractions (ER = 76 – 98%) were obtained for all acidic drugs eluted from 6% PVP polymeric foams and for warfarin, ibuprofen, and ketoprofen eluted from 1% CMC07 / 2% OC polymeric foams. ER values for naproxen and diclofenac from 1% CMC07 / 2% OC polymeric foams at 30 min extraction time were slightly lower and were 49 and 56%, respectively. Because sample processing by HF-LPME can be simultaneously performed for multiple DBS samples, the 30 min extraction time is not limiting and was applied for all subsequent experiments.

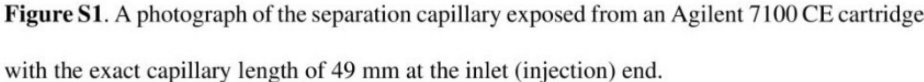


Figure S1. A photograph of the separation capillary exposed from an Agilent 7100 CE cartridge with the exact capillary length of 49 mm at the inlet (injection) end.

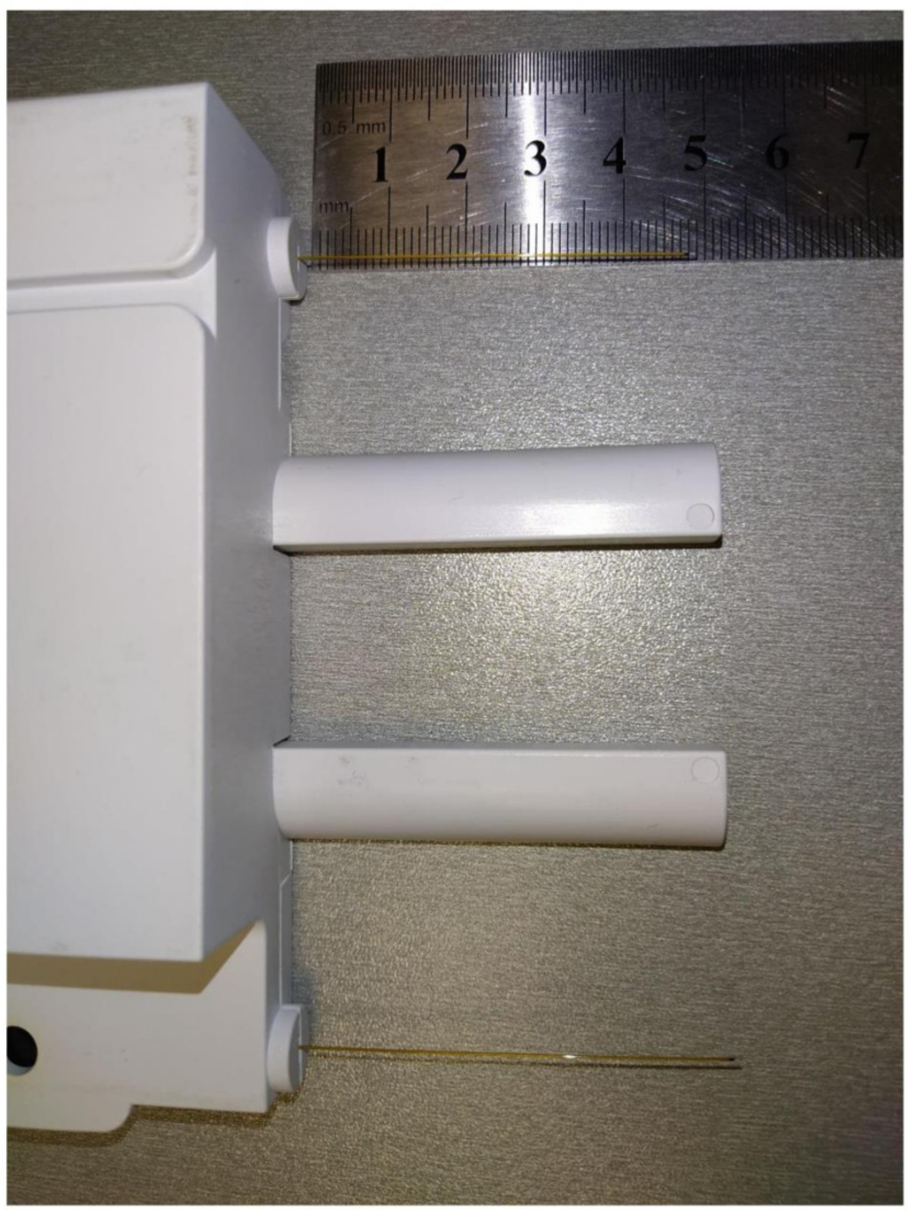


Figure S2. Electron scanning microscope (MIRA 3, Tescan, Brno, Czech Republic) images of the selected polymeric foams demonstrating optimum (6% PVP, 1% CMC07 / 2% OC), too high (1% CMC07) and too low (1% CMC02) porosity of the foams.

6% PVP

1% CMC07 / 2% OC

1% CMC07

1% CMC02

Figure S3. Gradual absorption of capillary blood by the polymeric foam made of 6% PVP. Blood volume, 10 μL. The foam was placed on a pin for better visualisation of the absorption.

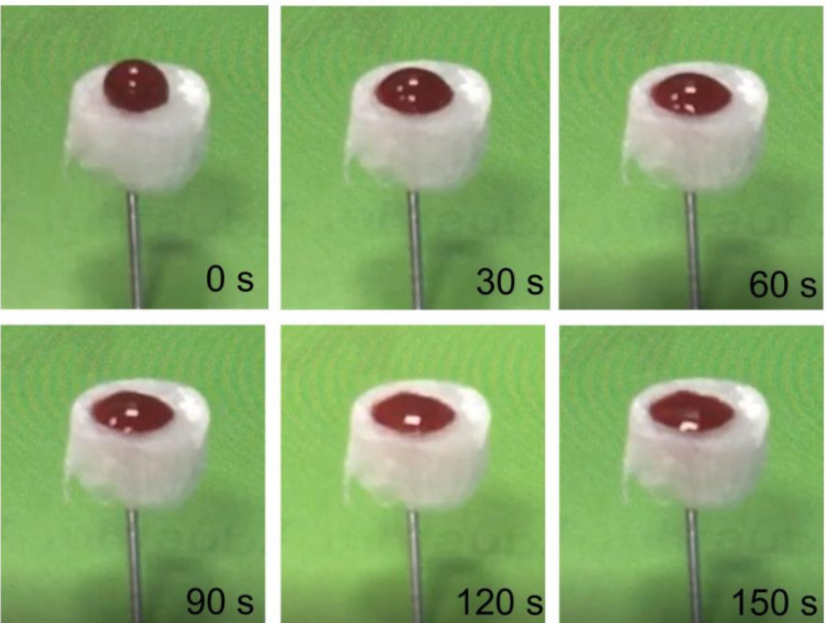


Figure S4. The effect of the drying time on the capillary blood sample weight. Capillary blood (10 μL) was sampled on polymeric foams made of 6% PVP and 1% CMC07 / 2% OC and the corresponding decrease in weight (due to the DBS formation) was determined gravimetrically. The points show a mean value ± SD (n = 3).

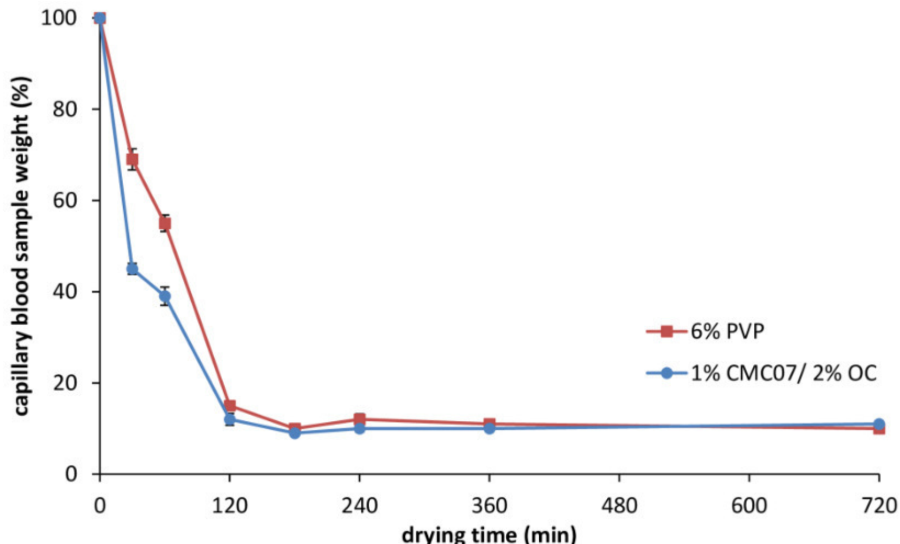


Figure S5. Electropherograms recorded at different detection wavelengths for the determination of compounds co-extracted by HF-LPME from a blank DBS. HF-LPME conditions: SLM, dihexyl ether (DHE); acceptor, 5 μL of 10 mM NaOH; donor, 6% PVP foam with a DBS (10 μL of capillary blood) eluted with 550 μL of 25 mM HCl; agitation speed, 1050 rpm; extraction time, 30 min. CE conditions: BGE solution, 30 mM sodium acetate, 30 mM acetic acid, and 30% (v/v) acetonitrile at apparent pH 5.2; separation voltage, + 25 kV; injection, 50 mbar for 5 s. (*) possible comigration with diclofenac (attenuated at 214 nm); (+) partial separation from naproxen (attenuated at 226 nm). All other compounds were fully separated from the analytes or were below the method LOQ.

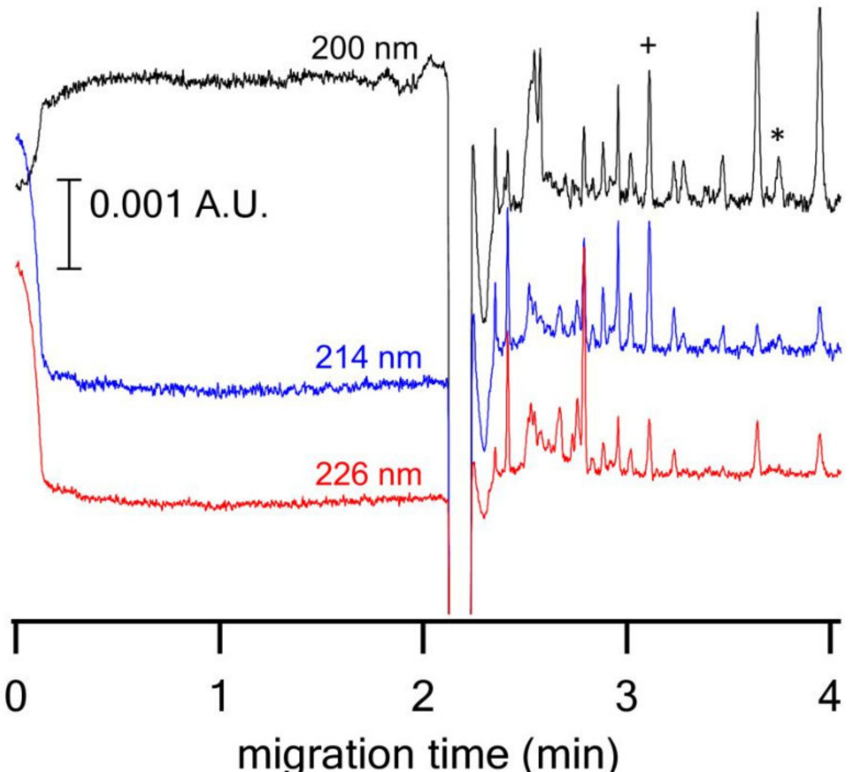


Figure S6. Optimization of the capillary blood drying time. Capillary blood (10 μL) was sampled on soluble polymeric foams made of (A) 6% PVP and (B) 1% CMC07 / 2% OC. The foam with the capillary blood was inside a glass CE vial, which was placed in a zip-lock bag with a desiccant and dried up at ambient temperature for 30 – 720 min. HF-LPME conditions as for Figure S5 (spiked concentration, 5500 μg/L). The points show a mean value ± SD (n = 3).

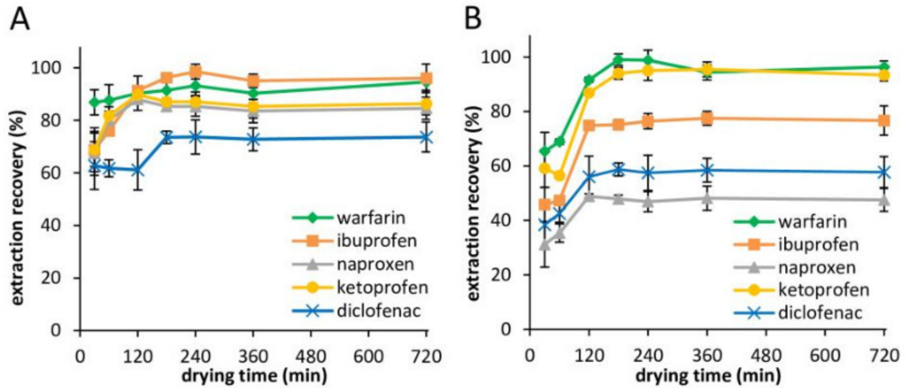
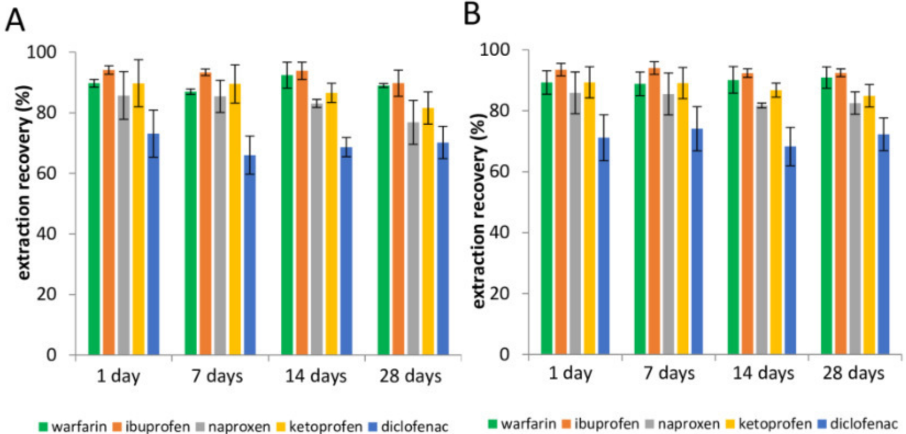


Figure S7. DBS eluate after dissolution of a 6% PVP (left) and 1% CMC07 / 2% OC (right) polymeric foam (with 10 μL DBS) in 550 μL of 25 mM HCl.

Figure S8. Stability of the acidic drugs in DBSs collected on soluble polymeric foams made of (A, B) 6% PVP and (C, D) 1% CMC07 / 2% OC stored at laboratory temperature (A, C) and in a refrigerator at 4 °C (B, D). HF-LPME conditions as for Figure S6. The bars show a mean value ± SD (n = 3).



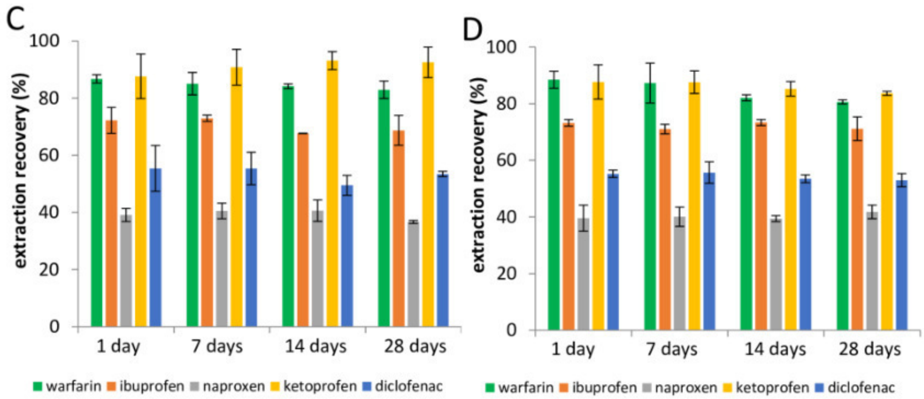


Figure S9. The effect of HCl concentration in donor solution (DBS eluate) on the ER values of the HF-LPME for the determination of model acidic drugs. A – 6% PVP, B – 1% CMC07 / 2% OC. HF-LPME conditions as for Figure S6, except HCl concentration. The bars show a mean value ± SD (n = 3).

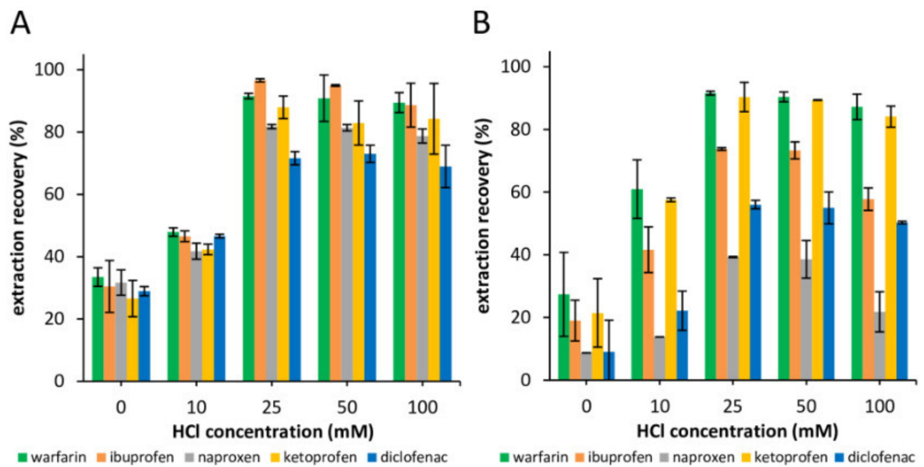


Figure S10. The effect of agitation on the ER values of the HF-LPME for the determination of model acidic drugs. A – 6% PVP, B – 1% CMC07 / 2% OC. HF-LPME conditions as for Figure S6, except agitation speed. The points show a mean value ± SD (n = 3).

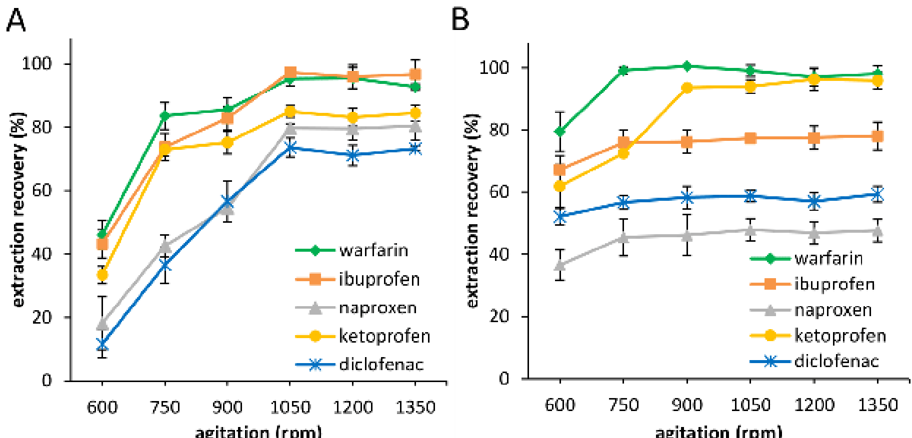


Figure S11. The effect of extraction time on the ER values of the HF-LPME for the determination of model acidic drugs. A – 6% PVP, B – 1% CMC07 / 2% OC. HF-LPME conditions as for Figure S6, except extraction time. The points show a mean value ± SD (n = 3).

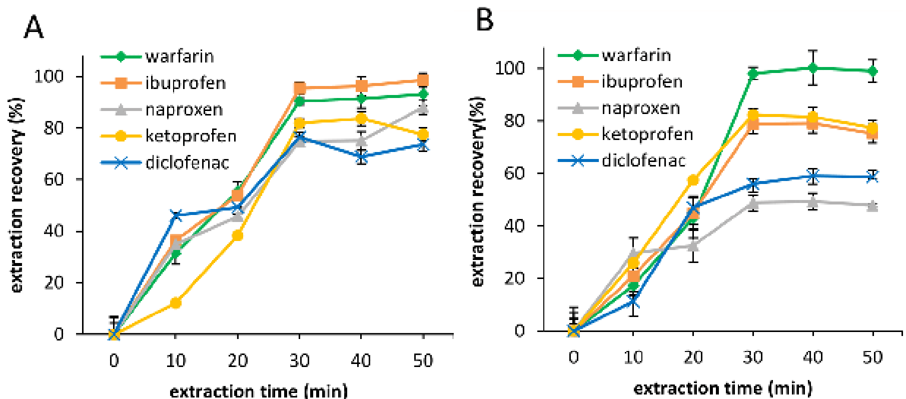


Table S1. Repeatability of the HF-LPME at-line coupled to CE for the determination of model acidic drugs in DBSs collected on soluble polymeric foams. HF-LPME conditions as for Figure S6. Concentrations are expressed as the drug concentrations in the undiluted capillary blood before DBS collection and elution. Inter-day data measured at three different days in one week; inter-sample data measured for DBSs of three different volunteers at one day; n = 3.

Foam	Parameter	Warfarin	Ibuprofen	Naproxen	Ketoprofen	Diclofenac
		Inter-day measurements				
6% PVP	RSD (%), 275 µg/L	3.8	2.5	3.4	3.9	4.2
	RSD (%), 5500 µg/L	2.5	6.3	8.0	4.6	4.1
	RSD (%), 27500 µg/L	5.9	3.7	4.2	4.8	5.0
1% CMC / 2% OC	RSD (%), 275 µg/L	3.3	2.1	2.9	3.0	6.5
	RSD (%), 5500 µg/L	2.8	2.3	4.0	3.6	3.8
	RSD (%), 27500 µg/L	6.0	5.5	6.7	4.4	3.8
		Inter-sample measurements				
6% PVP	RSD (%), 275 µg/L	3.9	5.7	3.4	5.6	4.4
	RSD (%), 5500 µg/L	1.7	2.7	3.9	2.3	3.4
	RSD (%), 27500 µg/L	4.6	3.8	2.2	2.3	3.7
1% CMC / 2% OC	RSD (%), 275 µg/L	4.1	2.7	4.4	7.2	7.3
	RSD (%), 5500 µg/L	2.6	2.4	2.6	4.3	3.2
	RSD (%), 27500 µg/L	4.3	5.2	4.7	5.3	6.0

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10 SEZNAM POUŽITÝCH ZKRATEK A SYMBOLŮ

BGE	základní elektrolyt
CE	kapilární elektroforéza
C^4D	kapacitně vázaná bezkontaktní vodivostní detekce
CMC07	karboxymetylcelulóza
DART-MS	hmotnostní spektrometrie s přímou analýzou v reálném čase
DBS	suchá krevní skvrna
DESI-MS	hmotnostní spektrometrie s desorpční elektrosprejovou ionizací
DMPK	metabolismus léčiv / farmakokinetika.
DNA	deoxyribonukleová kyselina
ESI	elektrosprejová ionizační technika
ER	extrakční výtěžnost
ESM	elektronová rastrovací mikroskopie
GC	plynová chromatografie
HF-LPME	mikroextrakce přes duté vlákno
HF	porézní duté vlákno
HPLC	vysokoúčinná kapalinová chromatografie
HRMS	hmotnostní spektrometrie s vysokým rozlišením
ICP-MS	hmotnostní spektrometrie s indukčně vázaným plazmatem
INR	mezinárodní normalizační poměr
ITP	izotachoforéza
LC-UV	kapalinová chromatografie s detekcí v ultrafialové oblasti
LLE	extrakce kapalina-kapalina
LOD	limit detekce
LOQ	limit kvantifikace
LPME	mikroextrakce do kapalné fáze
MS	hmotnostní spektrometrie
NBS	novorozenecký screening
OC	oxidovaná-6-karboxycelulóza
PAH	fenylalaninhydroxyláza
PP	polypropylen

PVP	polyvinylpyrrolidon
RSD	relativní standardní odchylka
R^2	koeficient determinace
SDME	mikroextrakce do jedné kapky rozpouštědla
SPE	extrakce na pevné fázi
SLM	kapalná fáze na membránovém nosiči
TDM	terapeutické monitorování léčiv
UV-VIS	spektrofotometrie s detekcí v ultrafialovo-viditelné oblasti
UHPLC	ultra-vysokoúčinná kapalinová chromatografie
VAMS	technika odměrného vzorkování mikro-objemů

11 PUBLIKAČNÍ ČINNOST AUTORKY

a) Publikace v recenzovaných časopisech s impaktovým faktorem:

- RYŠAVÁ, L.; DVOŘÁK, M.; KUBÁŇ, P. The effect of membrane thickness on supported liquid membrane extractions in-line coupled to capillary electrophoresis for analyses of complex samples. *Journal of Chromatography A*, 2019, roč. **1596**, s. 226-232. ISSN: 0021-9673. DOI: 10.1016/j.chroma.2019.02.067.
- DVOŘÁK, M.; RYŠAVÁ, L.; KUBÁŇ, P. Capillary electrophoresis with capacitively coupled contactless conductivity detection for quantitative analysis of dried blood spots with unknown blood volume. *Analytical Chemistry*, 2020, roč. **91**, č. 1, s. 1557-1564. ISSN: 0003-2700. DOI: 10.1021/acs.analchem.9b04845.
- MIKOVÁ, B.; DVOŘÁK, M.; RYŠAVÁ, L.; KUBÁŇ, P. Hollow fiber liquid-phase microextraction at-line coupled to capillary electrophoresis for direct analysis of human body fluids. *Analytical Chemistry*, 2020, roč. **92**, č. 10, s. 7171-7178. ISSN: 0003-2700.

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b) Mezinárodní konferenční příspěvky:

- RYŠAVÁ, L.; PŘIKRYL, J.; DVOŘÁK, M.; MALÁ, Z.; KUBÁŇ, P. Rapid fabrication of microextraction devices for direct determination of basic drugs in dried blood spots. *CECE 2018. 15th International Interdisciplinary Meeting on Bioanalysis.* Brno: Ústav analytické chemie AV ČR, v. v. i., 2018 - (Foret, F.; Křenková, J.; Drobníková, I.; Klepárník, K.; Přikryl, J.), s. 257-260. ISBN 978-80-904959-5-1.
- KUBÁŇ, P.; DVOŘÁK, M.; RYŠAVÁ, L.; LUČAJ, M. New trends in direct coupling of liquid phase microextraction to capillary electrophoresis for analyses of biological fluids. *ITP2018. Final Program.* Japan, Kyoto: Kyoto University at Katsura, 2018.
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- KUBÁŇ, P.; RYŠAVÁ, L.; DVOŘÁK, M. Capillary electrophoresis for the automated extraction and analysis of dried blood spots in clinical analysis. *LACE 2021.* Virtual event 2021. s 55.

12 ŽIVOTOPIS

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Vzdělání

2018 – 2023	Fakulta chemická, Vysokého učení technického v Brně	
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2016 – 2018	Fakulta chemická, Vysokého učení technického v Brně	
	Studijní obor:	Chemie pro medicínské aplikace – magisterské studium
	Diplomová práce:	Aplikace polymerních membrán v mikroextrakčních technikách pro analýzy biologických vzorků
2013 – 2016	Fakulta chemická, Vysokého učení technického v Brně	
	Studijní obor:	Chemie pro medicínské aplikace – bakalářské studium
	Bakalářská práce:	Kyselina octová jakožto vedlejší produkt metabolismu bakterií produkující PHA

Pracovní stáže a zkušenosti

2017 – 2023	Ústav analytické chemie Akademie věd ČR, v. v. i., Oddělení elektromigračních metod
2017	Synthon s.r.o – Oddělení ARG
2016	Teva Czech Industries s.r.o. – Oddělení RD - laboratoř GC, TAPI
2015	Vinařská laboratoř Ing. Alice Becková, Mikulov – Oddělení analytických metod
2012	Zdravotnické laboratoře - Ifcor 99, Synlab, Helios - cytologie Damier

Dovednosti

Anglický jazyk B1

Přehled řešených projektů a aktivit:

ŘEŠENÉ PROJEKTY:

2022 – 2023	Příspěvek k rozvoji moderních potravinářských věd (FCH-S-22-7961)
2021 – 2023	Spolupráce na mezinárodním grantu: Fluidic analytical platforms for assessing environmental and human exposure to emerging contaminants (PID2020-117686RB-C33 (MCIN/AEI))
2021 – 2022	Vývoj a využití progresivních postupů při zpracování, analýze a hodnocení potravin, potravinářských surovin a odpadů (FCH-S-21-7483)
2021 – 2022	Řešitel KING - Soluble materials for dried blood spot self-sampling and automated analysis (FCH-K-21-7023)
2020 – 2021	Aplikace progresivních metod a postupů v rámci moderních potravinářských věd (FCH-S-20-6316)
2019 – 2020	Využití pokročilých metod a postupů v rámci moderních potravinářských věd (FCH-S-19-5983)
2018 – 2020	Využití kapilární elektroforézy při analýze suchých krevních skvrn (GA ČR 18-13135S)
2017 – 2018	Nové mikroextrakční postupy pro úpravu, prekoncentraci a analýzu vzorků s komplexními matricemi (GA ČR 16-09135S)
2016 – 2018	Studium vztahu mezi strukturou, transportními a tokovými vlastnostmi biopolymerních gelů na mikro i makroskopické úrovni (TA ČR LD 15047)
2016 – 2018	Využití koncepce biorafinerie k valorizaci odpadní biomasy dřevozpracujícího průmyslu (LD 15031)

ÚČAST NA AKCÍCH:

- 2020 – 2022 - Absolvování kurzu pedagogického minima na Institutu celoživotního vzdělávání VUT

- 5 – 13.5. 2020 Kurz time managementu a řízení lidských zdrojů

- 3. – 4.12.2020 Kurz prezentování AV ČR, Brno: kurz zaměřený na rétoriku a veřejné vystupování

- 18. – 22. 11.2019 - Kurz základů vědecké práce, Ústav analytické chemie AV ČR, Brno, ČR: kurz zaměřený na techniky a metodiku vědecké práce.

- Pedagogická praxe – 1. a 3. ročník DSP asistence při vedení diplomantů a bakalářů při praktické části jejich závěrečných prací. Podíl na výuce praktik z instrumentální a strukturní analýzy – úlohu kapilární elektroforéza, tj. instrumentace, která je hlavní užitou analytickou technikou dizertace.

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To whom it might concern

A confirmation of the international collaboration of MSc. Lenka Ryšavá (Brno University of Technology)

Hereby, I confirm that I am the principal investigator of the research project *Fluidic analytical platforms for assessing environmental and human exposure to emerging contaminants* proposal granted by the Spanish Ministry of Science and Innovation (MCIN) and the Spanish State Research Agency (AEI) through project PID2020-117686RB-C33 (MCIN/AEI)

and that RNDr. Pavel Kubáň, Ph.D. Dsc. from the Institute of Analytical Chemistry of the Czech Academy of Sciences (supervisor of MSc. Ryšavá) is a cooperating subject in this grant proposal. MSc. Ryšavá and the two teams have carried out a scientific research within this grant project, and the results of this international collaboration have been presented on a scientific conference (Latin-American Symposium on Biotechnology, Biomedical, Biopharmaceutical, and Industrial Applications of Capillary Electrophoresis and Microchip Technology – LACE 2021, Sao Paulo, Brazil) thus demonstrating the collaboration among the Ph.D. student, my research group (FI-TRACE at the University of the Balearic Islands, www.fitrace.es), and the research group of the collaborator (RNDr. Kubáň).

Yours sincerely,

M. Miró

Manuel Miró