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Nové postupy v metabolické analýze biotekutin  
New procedures in metabolomic analysis in biofluids

Disertační práce

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Jménem dalších spoluautorů prohlašuji, že Mgr. Lucie Řimnáčová přispěla ke vzniku předložené publikace 3 (její podíl 20 %) a její účast na tomto článku byla následující: pomoc při vývoji metody, část experimentální činnosti na GC-MS, pomoc při zjišťování struktury derivátů a přípravě rukopisu.

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## Abstrakt

Předmětem této práce bylo studium reaktivity protických metabolitů s chlormravenčany a jejich využití v metabolomické GC-MS analýze biotekutin. Tento výzkum byl veden ve třech samostatných studiích a jeho výsledkem jsou tři nové, originální metody pro GC-MS stanovení protických nízkomolekulárních metabolitů v biologickém materiálu, především biotekutinách.

První studie zkoumá objev rychlé derivatizace alicyklických hydroxylových skupin fluoralkylchlormravenčany (FCF) v bezvodém prostředí [1]. Působením FCF dochází k okamžité přeměně této hydroxylové skupiny na karbonát a tento krok lze snadno spojit s mikroextrakcí kapalina-kapalina (LLME) vznikajících derivátů do organické fáze. Reakce alicyklické hydroxy skupiny s FCF byla testována celkem na 12 klinicky významných steroidech a 4 tokoferolech. Byly popsány analytické vlastnosti zkoumaných analytů a metoda byla validována pro stanovení 6 diagnostických sterolů a 4 tokoferolů v lidském séru a plodové vodě. Nová metoda byla dále úspěšně použita k identifikaci a stanovení sterolů a tokoferolů ve tkáních hmyzu, ruměnice pospolné (*Pyrrhocoris apterus*) [2].

Druhá studie byla zaměřena na zkoumání reaktivity protických, zejména kyselých močových metabolitů s FCF, především s heptafluorbutylchlormravenčanem (HFBCF). Technikami GC-MS a LC-HRMS byly podrobně zkoumány reakční produkty 153 močových metabolitů a dvou vnitřních standardů. Nový postup byl validován pro stanovení 132 metabolitů v lidské moči a úspěšně ověřen pomocí GC-MS analýzy certifikovaného vzorku moči se známými koncentracemi diagnosticky významných organických kyselin a na analýze vzorků moči získaných od 100 zdravých dobrovolníků. Objem 25  $\mu$ l moči umožnil přímé stanovení 112 fyziologických metabolitů v tomto souboru vzorků [3]. Analytický protokol popsání metody byl dále připraven pro kapitolu v knize [4].

Třetí studie popisuje rychlou GC-MS metodu pro stanovení kyselých močových biomarkerů expozice průmyslovým zdravím škodlivým látkám v lidské moči. Byly zkoumány reakce biomarkerů expozice benzenu, toluenu, styrenu, xylenů, alkoxyethanolů, sirouhlíku, furalu a *N,N*-dimethylformamidu s různými alkychlormravenčany (RCF). Většina uvedených biomarkerů poskytovala jeden předpokládaný produkt, některé však poskytovaly s RCF ještě další neobvyklý produkt. Jejich struktura byla jednoznačně potvrzena pomocí techniky LC-HRMS a derivatizačních činidel značených stabilními izotopy. Na základě získaných poznatků byla vypracována a validována nová GC-MS metoda pro současné stanovení 14 biomarkerů expozice v lidské moči a ověřena pomocí analýzy vzorku certifikované referenční moči.

Nové poznatky získané při výzkumu reaktivity alicyklické hydroxylové skupiny a dalších protických funkčních skupin metabolitů s chlormravenčany v souhrnu vedly k vypracování 3 nových analytických postupů přípravy biologických vzorků s perspektivním uplatněním především v oblasti GC-MS metabolomické analýzy.

## Abstract

The aim of this thesis has been the research on reactivity of protic metabolites with chloroformates and its application for GC-MS analysis of biofluids. The research was conducted in three separate studies and the results are three new, original methods for GC-MS determination of low-molecular protic metabolites in biological material especially in biofluids.

The first study explores the discovery of fast derivatization of alicyclic hydroxyl groups by fluoroalkyl chloroformates (FCFs) under anhydrous conditions [1]. FCF fully converts the hydroxyl group into a corresponding carbonate and the step can easily be coupled with liquid-liquid microextraction (LLME) of the arising derivatives into organic phase. The reaction of the alicyclic OH group with FCFs was tested on 12 clinically relevant steroids and 4 tocopherols. The analytical properties of determined analytes were described and the method was validated for the GC-MS determination of 6 diagnostic sterols and 4 tocopherols in human serum and amniotic fluid. The new method was further successfully used for determination of sterols and tocopherols in tissues of the bug *Pyrrhocoris apterus* [2].

The second study was focused on the reactions of protic, particularly urinary metabolites with FCFs, mainly heptafluorobutyl chloroformate (HFBCF). Reaction products of 153 urinary metabolites with HFBCF and two internal standards were investigated in detail by GC-MS and LC-HRMS. The new procedure was validated for 132 metabolites in human urine and was successfully evaluated by GC-MS analysis of a certified urine sample containing known concentrations of diagnostic organic acids and by analysis of urine samples obtained from 100 healthy volunteers. A urine sample volume of 25  $\mu\text{l}$  allows direct determination of 112 metabolites in the sample set [3]. The analytical protocol was further prepared for the chapter in the book [4].

The third study describes a rapid GC-MS method for determination of acidic urinary biomarkers arising after human exposure to industrial pollutants. The exposition-biomarkers of benzene, toluene, styrene, xylenes, alkoxyalcohol, carbon disulfide, fural, and *N,N*-dimethylformamide were examined after the reaction with different alkyl chloroformates (RCFs). Whereas most of the analytes provided a single, expected product, some provided another, unusual product. Their structure was clearly confirmed by LC-HRMS and by derivatization with isotopically labelled agents. The method was validated for determination of 14 biomarkers of exposure in human urine and successfully evaluated by the analysis of a reference urine material.

New knowledge, acquired by the research of the reactivity of alicyclic hydroxyl group and other protic function groups with alkyl chloroformates, enables perspective use particularly in the field of GC-MS based metabolomics.

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# 1 Úvod

## 1.1 Biotekutiny a jejich metabolický výzkum

Distribuce metabolitů i cizorodých látek (xenobiotik) je v každém organismu zajišťována především prostřednictvím cirkulace biologických roztoků (biotekutin) obsahujících nesmírně pestré zastoupení organických i anorganických látek. Metabolity představují chemické struktury s často naprosto rozdílnými fyzikálně-chemickými vlastnostmi, jejich zastoupení se liší až o několik řádů. Navíc některé metabolity jsou zapojeny do velkého počtu metabolických drah, zatímco jiné pouze do několika z nich [5]. Analýza kompletního souboru všech metabolitů v organismu – metabolomu – proto představuje mimořádně náročný úkol, který lze realizovat pouze pomocí uceleného souboru analytických metod [5].

Hlavní biotekutinou je v lidském těle krev, k dalším patří lymfa, tkáňový mok, trávicí šťávy. Řada metabolitů je z těla vylučována prostřednictvím moče a potu. Hlavním předmětem zájmu je zkoumání metabolomu odstředěné krve ve formě krevní plasmy nebo séra, na druhé straně výzkum metabolomu látek vylučovaných v moči, tzv. urinomika.

Metabolomickou analýzu dnes reprezentuje celá řada analytických přístupů. Nejčastěji se provádí tzv. metabolické profilování, kde se analyzuje, identifikuje a částečně kvantifikuje vybraný počet metabolitů, který náleží do třídy chemicky podobných sloučenin (např. aminokyseliny, sacharidy nebo polární lipidy), nebo které jsou společně zapojené do určité metabolické dráhy.

Z analytického hlediska je důležité dělení metabolomické analýzy na necílenou a cílenou. První přístup představuje systematický screening látek v měřeném vzorku, kdy hledáme rozdíly v zastoupení metabolitů ve srovnávání kontrolního vzorku se vzorkem, který je předmětem našeho výzkumu. Pokud jsou nalezeny statisticky prokazatelné rozdíly a podaří se vytvořit vhodnou hypotézu k pozorovaným jevům, pokračuje výzkum analýzou zacílenou na konkrétní metabolity vykazující změny v zastoupení pomocí klasické kvantitativní analýzy, která umožňuje podrobněji posoudit hladiny zastoupení konkrétních analytů [6].

Při komplexním výzkumu metabolismu je velká poptávka po nových, citlivých a robustních analytických metodách umožňujících široké pokrytí metabolomu v konkrétní matici. Hlavní výzkumné nástroje dnes představují separační metody ve spojení s hmotnostní spektrometrií (MS) a nukleární magnetická rezonance (NMR). Přednosti a omezení těchto instrumentálních technik jsou shrnuty v tabulce 1 [7].

Tabulka 1: Srovnání analytických metod využívaných při studiu metabolomu [7].

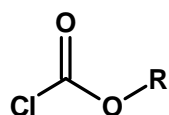
Technika	Výhody	Nevýhody
NMR	<ul style="list-style-type: none"> <li>• nedestruktivní technika</li> <li>• minimální příprava vzorku před analýzou</li> <li>• snadná identifikace neznámých sloučenin</li> <li>• možnost měření <i>in vivo</i></li> </ul>	<ul style="list-style-type: none"> <li>• nízká citlivost</li> <li>• vyšší požadavky na objem vzorku</li> </ul>
MS	<ul style="list-style-type: none"> <li>• rychlý "screening" metabolitů</li> <li>• vysoká citlivost</li> <li>• minimální příprava vzorku před analýzou</li> <li>• doporučeno pro identifikaci neznámých sloučenin</li> </ul>	<ul style="list-style-type: none"> <li>• identifikace metabolitů všeobecně vyžaduje MS/MS</li> <li>• efekty spojené s matricí</li> <li>• nekompatibilní s vysokou iontovou silou</li> </ul>
GC-MS	<ul style="list-style-type: none"> <li>• vysoká separační účinnost</li> <li>• ideální pro analýzu složitých směsí</li> <li>• umožňuje souběžnou analýzu různých tříd metabolitů</li> <li>• snadné rozhraní mezi GC a MS</li> <li>• reprodukovatelnost</li> </ul>	<ul style="list-style-type: none"> <li>• neschopnost analyzovat termolabilní metabolity</li> <li>• vyžaduje derivatizace polárních netěkavých metabolitů</li> <li>• nesnadná identifikace neznámých sloučenin po derivatizaci</li> </ul>
LC-MS	<ul style="list-style-type: none"> <li>• vysoká citlivost</li> <li>• umožňuje analýzu termolabilních metabolitů</li> <li>• průměrné chromatografické rozlišení</li> </ul>	<ul style="list-style-type: none"> <li>• efekty spojené s matricí vzorku</li> <li>• někdy nutnost odsolování</li> <li>• omezené informace o struktuře</li> </ul>
CE-MS	<ul style="list-style-type: none"> <li>• minimální potřeba vzorku i základního elektrolytu</li> <li>• vysoké rozlišení</li> <li>• užitečná pro analýzu složitých směsí</li> </ul>	<ul style="list-style-type: none"> <li>• obtížné rozhraní CE s MS, nekompatibilita základních elektrolytů</li> <li>• složitá metodika a kvantifikace</li> <li>• nízká citlivost</li> </ul>

Plynová chromatografie spojená s hmotnostní detekcí (GC-MS) patří mezi často používané, nákladově nejméně náročné techniky v metabolomickém výzkumu. Je vhodná především pro analýzu těkavých a termostabilních nízkomolekulárních sloučenin. V organismu nicméně převažují metabolity s polárními funkčními skupinami, které bývá nezbytné chemicky modifikovat některou z derivatizačních metod a zvýšit tak jejich těkavost. Dvě hlavní derivatizační metody v metabolomice představují silylace, (jejímiž hlavními produkty jsou trimethylsilyl a *tert*-butyldimethylsilyl deriváty) v kombinaci s oximací [8, 9] a dále pak derivatizace pomocí chlormravenčanů [10], díky níž lze efektivně přeměnit aminoskupiny na karbamáty, karboxyly na estery a OH, SH skupiny na příslušné karbonáty [11]. Zásadní předností tohoto postupu je, že probíhá velice rychle s vysokými výtěžky ve vodném prostředí a dá se často aplikovat *in situ*, tedy přímo v biologickém vzorku [12].



## 1.2 Derivatizace chlormravenčany (chlorformiáty)

Alkylchlormravenčany (struktura uvedena na Obr 1) jsou v organické chemii známy od začátku 19. století pro jejich schopnost tvořit smíšené anhydridy [13]. V analytické chemii začaly být používány od sedmdesátých let 19. století k chránění amino a fenolických skupin [14 – 16] a pro tvorbu intermediátů s aktivovanou karboxylovou skupinou [17, 18].



Obr. 1: Struktura chlormravenčanů (R – alkyl).

Chlormravenčany jsou vysoce reaktivní činidla, která reagují s kyselinami za přítomnosti alkoholu a pyridinu za vzniku esterů [19], s thioskupinou za vzniku thiokarbonátů, s hydroxyskupinou (alkoholovou či fenolickou) za vzniku karbonátů a s aminoskupinou za vzniku karbamátů [11]. Hlavními přednostmi derivatizace RCF ve srovnání se silylacemi jsou vedle reakce ve vodném prostředí, že vznikají stabilní produkty, dojde k vyčerpání činidel a současně lze provádět mikroextrakci kapalina-kapalina přidávkem nemísitelného organického rozpouštědla. Organickou fází lze pak přímo dávkovat do injektoru plynového chromatografu (GC). Reakce probíhá do 5 sekund s vysokým výtěžkem. Určité omezení lze pozorovat pouze u méně reaktivních alifatických a alicyklických hydroxylových skupin, které zůstávají nezreagované, například v serinu a threoninu [20], nebo ve 3- a 4-hydroxykarboxylových kyselinách [21].

Derivatizace s RCF byla poprvé použita pro GC analýzu 44 organických kyselin [22] nebo pro stanovení karboxylových, keto, hydroxy a amino kyselin, které jsou užitečné pro diagnózu nemoci javorového sirupu (leucinózy) [23]. V těchto prvních studiích se ukázalo, že u kyselin jantarové a glutarové dochází k vnitřní cyklizaci a ke vzniku nestabilních anhydridů. Esterifikace širokého spektra di- a trikarboxylových kyselin s methylchlormravenčanem (MCF) a ethylchlormravenčanem (ECF) byla vyřešena opakovaným přidáním reakčního činidla v přítomnosti hydroxidu sodného [24]. Tento postup umožnil stanovení širokého spektra amino a karboxylových kyselin v různých biologických matricích [25 – 34].

Výhodné analytické vlastnosti RCF vedly k přípravě chlormravenčanů s fluorovanými alkyly o různé délce řetězců. Vincenti a kol. [35 – 38] syntetizovali fluoralkylchlormravenčany s dlouhým řetězcem a využily je pro GC-MS analýzu malých, velmi polárních vedlejších produktů používaných při dezinfekci vody. Skupina Abeho a kol. [39, 40] zkoušela trifluorethylchlormravenčan (TFECF) pro chirální separaci sady aminokyselin. Analytické vlastnosti chlormravenčanů s pentafluorpropylovou

(PFPCF) nebo heptafluorobutylovou (HFBCF) skupinou byly rozsáhle studovány Hucem a spolupracovníky [41 – 43].

Na rozdíl od běžných RCF jsou tyto fluorované analogy reaktivnější a jsou schopny transformovat cílové protické funkční skupiny za katalýzy pyridinem bez přítomnosti alkoholu. Fluorované chlormravenčany s pěti (PFPCF) nebo sedmi atomy fluoru (HFBCF) představují nová, efektivní činidla v GC-MS analýze, zejména organických kyselin a aminokyselin, včetně jejich chirální separace [42 – 44]. Např. HFBCF činidlo se osvědčilo pro chirální separaci aminokyselin v lidském séru [45, 46]. Přípravu vzorku lze provést ve třech jednoduchých krocích: (a) uvolnění vázaných thiolů redukčním činidlem 2,3-dimerkapropanolsulfonátem, (b) následované sražením bílkovin kyselinou chloristou a (c) přímou derivatizací supernatantu HFBCF za katalýzy pyridinem. Tímto způsobem byly získány mimořádně čisté extrakty, které umožnily GC-MS analýzu metabolitů v plazmě či séru [45]. Připravené deriváty aminokyselin vykazovaly výborné separační vlastnosti na chirální stacionární fázi Chirasil-Val, kde bylo rozděleno více než 35 aminokyselinových enantiomerů, s výjimkou D a L enantiomerů argininu, cystinu a prolinu [46].

I přes rostoucí poznatky ve více jak dvacetiletém výzkumu analytických vlastností chlormravenčanů ve vodném prostředí je stále velmi málo známo o reaktivitě protických funkčních skupin a vznikajících produktech u důležitých skupin metabolitů, jaké představují steroly, steroidy, tokoferoly nebo organické kyseliny [22, 47].

## 2 Cíle práce

1. Studium reaktivity protických funkčních skupin s alkylchlorformiáty ve vybraných skupinách metabolitů, v první řadě alicyklické hydroxylové skupiny steroidů a tokoferolů a uplatnění nových poznatků v metabolomické GC-MS analýze.
2. Výzkum vznikajících reakčních produktů heptafluorbutylchloroformu se 153 protickými močovými metabolity pro optimalizaci podmínek přípravy vzorků moči a jejich GC-MS metabolomickou analýzu.
3. Studium xenobiotik v lidské moči po expozici organismu organickým rozpouštědly benzenem, toluenem, styrenem, xyleny, alkoxyethanolů, sirouhlíkem, furalu a *N,N*-dimethylformamidem a jejich derivatizace alkylchlorformiáty a GC-MS stanovení.

Výzkum byl realizován ve třech samostatných studiích a na základě nových poznatků byly vypracovány tři nové originální postupy analýzy protických metabolitů v biotekutinách technikou GC-MS.

### 3 Výsledky jednotlivých studií

#### 3.1 Nová metoda derivatizace hydroxylových skupin pomocí fluoralkylchlormravenčanů a její reálné využití pro stanovení sterolů a tokoferolů technikou GC-MS.

##### 3.1.1 Úvod

Organické sloučeniny steroidní povahy obsahující hydroxylovou skupinu je často výhodné analyzovat pomocí GC. Problémem je, že aktivní vodík v hydroxylové skupině interaguje s jinými protickými funkčními skupinami (účinkem intermolekulárních sil) nejčastěji v kontaminovaném injektoru nebo na velkém měrném povrchu stacionární fáze v kapilární koloně. Převedení steroidu do plynné fáze působením vysoké teploty může vyvolat dehydrataci labilní alifatické nebo alicyklické hydroxylové skupiny. Proto je pro toto stanovení žádoucí derivatizace, díky níž lze dosáhnout zlepšení analytických, separačních a detekčních vlastností steroidů při GC-MS analýze [8].

Vhodným modelem pro studium reakce RCF se steroly představuje cholesterol (Chol). Jde o významný metabolit, který se v hojném množství vyskytuje v membránách eukaryotických buněk [48]. Chol lze relativně jednoduše měřit technikou GC-MS v derivatizované formě. Na kvalitní, deaktivované kapilární GC koloně lze současně sledovat nativní analyt s volnou hydroxylovou skupinou, a tak např. sledovat výtěžek reakce.

Předmětem první práce bylo studium reakce hydroxylové skupiny cholesterolu s různými chlormravenčany. Nejprve byla tato reakce studována ve vodném prostředí za podmínek optimalizovaných pro stanovení amino- a karboxylových kyselin v lidských biotekutinách [41, 45, 46] a v průmyslových vodách [36, 37, 49]. V dalším kroku byly testovány alkylované a fluorované chlormravenčany a jejich reaktivita s hydroxylovou skupinou Chol v bezvodém prostředí. Po nalezení optimálních podmínek reakce byl soubor stanovovaných analytů rozšířen o zástupce dalších sterolů, dále vybraných steroidů a tokoferolů. Nová metoda byla porovnána se silylační metodou a byly porovnány a vyhodnoceny výhody a omezení obou GC-MS metod.

Tento nový postup byl nakonec validován pro stanovení šesti diagnostických sterolů (desmosterol, lathosterol a dihydrocholesterol – ukazatelé biosyntézy cholesterolu, kampesterol,  $\beta$ -sitosterol a stigmasterol – ukazatelé absorpce cholesterolu) a čtyř tokoferolů ( $\alpha$ -,  $\beta$ -,  $\gamma$ - a  $\delta$ -tokoferol – v tucích rozpustné antioxidanty ze skupiny E vitamínů) v lidském séru a plodové vodě. Prvním krokem ke stanovení byla saponifikace vzorku, aby došlo k uvolnění vázaných sterolů a tak k analýze obou jejich forem tzv. celkových sterolů. Krevní séra byla získána od žen před a po porodu. Získané výsledky byly porovnány s publikovanými výsledky z dřívějších studií [1].

Metoda byla také využita pro sledování sezonních změn koncentrací 3 sterolů ( $\beta$ -sitosterol, kampesterol a cholesterol) a 2 tokoferolů ( $\gamma$ - a  $\delta$ -) v membránách hmyzu – ruměnice pospolné (*Pyrrhocoris apterus*) [2].

### **3.1.2 Testování reaktivity hydroxylové skupiny s chlormravenčany**

#### **3.1.2.1 Experimentální část**

##### **3.1.2.1.1 Použité chemikálie a činidla**

Cholesterol, dihydrocholesterol,  $\alpha$ -tokoferol,  $\beta$ -tokoferol,  $\gamma$ -tokoferol,  $\delta$ -tokoferol, ethanol, isooktan, pyridin, kyselina chlorovodíková, uhličitan sodný, dimethylformamid, MCF, ECF, isobutylchlormravenčan (iBCF), hexylchlormravenčan (HCF), 1-(trimethylsilyl)imidazol (TMSI) a *N,N'*-dicyklohexylkarbodiimid (DCC) byly pořízeny u Sigma-Aldrich (Praha, Česká Republika). Lathosterol, kampesterol, stigmasterol,  $\beta$ -sitosterol, desmosterol, testosteron, estradiol, estron, 24S-hydroxycholesterol a 22S-hydroxycholesterol byly od firmy Steraloids (Newport, RI, USA). *n*-Hexan byl koupen u firmy Merck (Praha, Česká Republika), acetonitril a methanol byly pořízeny u Fisher Scientific (Pardubice, Česká Republika) a TFECF a HFBCF byly připraveny v Laboratoři analytické biochemie a metabolomiky (České Budějovice, Česká Republika) ve spolupráci s Dr. V. Pejchalem (Univerzita Pardubice, Česká Republika), které dodává firma Pragolab, s.r.o (Praha, Česká republika). Zásobní roztoky všech použitých standardů byly připraveny v ethanolu o koncentraci 1 mg/ml. Při manipulaci s chlormravenčany je důležité používat ochranné pomůcky (brýle, gumové rukavice) a všechny reakce provádět v dobře odvětrávané digestoři.

##### **3.1.2.1.2 Vybavení laboratoře a GC-MS analýza**

Derivatizace byly prováděny v mikro reakčních nádobkách s víčkem a teflonovým septem (Supelco, PA, USA). Pokud se derivatizace dělala v bezvodém prostředí, byl použit speciální uzávěr vybavený Restec miniert<sup>®</sup> ventilem (Chromservis, Praha, Česká Republika), který umožňoval přidávek činidel stříkačkou bez otevření lahvičky, a tím bylo zajištěno, že vzorek přišel do minimálního styku se vzdušnou vlhkostí. Na dávkování vzorků a reakčních činidel byly použity stříkačky Hamilton (Hamilton, Bonaduz, Švýcarsko). Míchačka (50-2400 rpm) byla od firmy P-Lab (Praha, Česká Republika), vialky a 10  $\mu$ l stříkačky do automatického injektoru byly od Thermo Fisher Scientific (Praha, Česká Republika). Pro sušení reakčních rozpouštědel byla použita molekulová síta (10-20) od Sigma-Aldrich (Praha, Česká Republika).

Pro analýzu všech studovaných reakčních produktů byl použit GC vybavený teplotně programovatelným nástřikem (PTV) spojený s MS Trace DSQ vybaveným kvadrupólovým analyzátozem a elektronovou ionizací (EI) (Thermo Fisher Scientific, San Jose, USA). Pro zpracování dat byl použit software Xcalibur (verze 2.1, Thermo Fisher Scientific). Na kolonu byl dávkován 1  $\mu$ l vzorku ve splitless módu, použitý skleněný Siltec deaktivovaný baffle liner byl pořízen u firmy Restek (PA, USA).

Teplota nástřiku byla držena na 280°C, byla použita kapilární chromatografická kolona DB-1HT, 15m × 0,25 mm i. d., s tloušťkou filmu stacionární fáze 0,1 μm (Agilent Technologies, CA, USA). Teplotní program byl následující: 180°C po 1 min, pak zvýšení po 30°C/min na 330°C (drženo 2 min). Jako nosný plyn se používalo He a jeho průtok byl 1,1 ml/min. Teplota iontového zdroje – 250°C, teplota GC-MS rozhraní – 300°C, EI mód (70 eV) ve skenovacím rozsahu 100 – 700 Da. Hmotnostní spektra derivátů byla měřena také pomocí chemické ionizace (CI), kde byl jako ionizační plyn vyzkoušen isobutan a amoniak jak v pozitivním, tak v negativním modu. Nastavení GC bylo stejné, jako při použití EI. Teploty u MS byly následující: iontový zdroj – 200°C a GC-MS rozhraní – 250°C. Průtok ionizačního plynu byl 1,2 ml/min. Skenovací rozsah 100 – 700 Da.

### 3.1.2.1.3 Vývoj metody

Reaktivita alicyklické OH skupiny Chol s chlormravenčany v analytickém měřítku byla nejprve zkoumána ve vodném a bezvodém prostředí. Zásobní roztok Chol o objemu 3 μl byl nadávkován do reakční nádoby a rozpouštědlo bylo odpařeno do sucha proudem dusíku. Byly postupně otestovány různé chlormravenčany (MCF, ECF, iBCF, HCF, TFECF a HFBCF).

V první fázi byla zkoušena derivatizace s RCF ve vodném prostředí. K odpařenému Chol bylo přidáno 100 μl destilované vody a 10 μl pyridinu a směs byla krátce zamíchána. Poté bylo přidáno 30 μl příslušného alkoholu (methanol, ethanol, hexanol nebo isobutyl alkohol, v analogii dle použitého chlormravenčanu) a 12 μl chlormravenčanu (MCF, ECF, HCF nebo iBCF) ve 100 μl isooktanu, směs byla opět krátce zamíchána a nakonec bylo přidáno 50 μl kyseliny chlorovodíkové o koncentraci 1 mol/l a směs bylo mícháno asi 20 s. Část horní organické fáze byla odebrána do vialky a 1 μl vzorku byl dávkován do GC-MS. Pro HCF byla vyzkoušena modifikace reakce dle metody Vincentiho [36, 37, 49] a do reakce byl přidán DCC rozpuštěný v pyridinu. V tomto případě se postup derivatizace lišil pouze v bodě přidání pyridinu, místo 10 μl pyridinu se přidalo 10 μl roztoku pyridinu s DCC o koncentraci 400 mg/ml.

V dalším kroku byly zkoušeny fluorované chlormravenčany. Odparek Chol byl smíchán se 150 μl vodného roztoku uhličitanu sodného o koncentraci 0,05 mol/l a s 50 μl směsí obsahující isooktan – TFECF (resp. HFBCF) o poměru 5:3 (v/v). Reakční směs byla míchána asi 10 s a poté bylo přidáno 50 μl uhličitanu sodného o koncentraci 0,1 mol/l s přidáním pyridinem (5:1, v/v) a opět bylo mícháno asi 10 s. Nakonec bylo přidáno 50 μl isooktanu a po zamíchání byla odebrána část horní organické vrstvy do vialky a 1 μl vzorku byl dávkován do GC-MS. Pro případ derivatizace s TFECF byl také otestován přídavek DCC v pyridinu (koncentrace 400 mg/ml), podobně jako v případě s RCF.

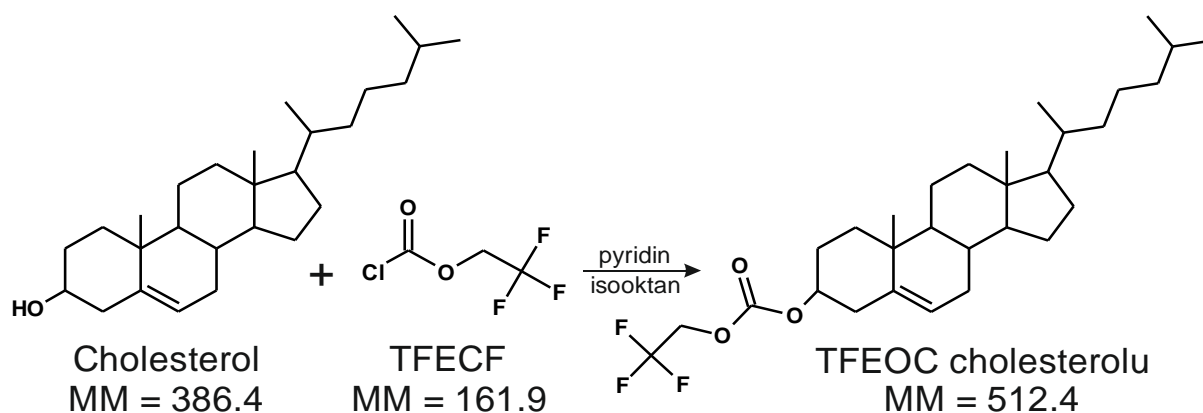
V druhé fázi byla testována derivatizace hydroxylové skupiny Chol pomocí různých chlormravenčanů v bezvodém prostředí. Pro tyto reakce byla použita organická rozpouštědla, která byla vysušena molekulovými sítý. K vysušenému vzorku Chol v reakční nádobce se speciálním víčkem

(viz. kapitola 3.1.2.1.2) bylo přidáno 50  $\mu\text{l}$  směsi isooktan – pyridin (9:1, v/v) a po krátkém míchání bylo přidáno 100  $\mu\text{l}$  acetonitrilu. Poté bylo přidáno 100  $\mu\text{l}$  směsi obsahující opět isooktan a příslušný chlormravenčan (byl testován MCF, ECF, iBCF, HCF, TFECF a HFBCF) v poměru 95:5 (v/v) a obsah byl míchán po dobu asi 20 s. Po vyčeření na dvě nemísitelné fáze byla do vialky odebrána část horní isooktanové vrstvy a 1  $\mu\text{l}$  byl dávkován na kolonu do systému GC-MS. Postup s TFECF byl pak aplikován na několik dalších sterolů, dva hydroxysteroly, na hlavní androgenní steroidní hormon – testosteron, na dva stěžejní hormony ze skupiny estrogenů – estron a estradiol a čtyři tokoferoly, důležité v tucích rozpustné vitamíny, které patří mezi antioxidanty a jsou užitečné při prevenci vzniku kardiovaskulárních onemocnění a některých typů rakovin [50, 51], jmenovitě – steroly: desmosterol, dihydrocholesterol, lathosterol, kampesterol,  $\beta$ -sitosterol a stigmasterol, hydroxy steroly: 22S-hydroxycholesterol a 24S-hydroxycholesterol, steroidy: estron, estradiol a testosteron a tokoferoly:  $\alpha$ -tokoferol,  $\beta$ -tokoferol,  $\gamma$ -tokoferol a  $\delta$ -tokoferol.

Pro porovnání analytických vlastností derivatizační produktů byl standard Chol podroben silylaci s TMSI činidlem, aby se dala porovnat reaktivita a retenční chování běžného trimethylsilyl derivátu s novým typem karbonátu vzniklého po reakci s hydroxylovou skupinou Chol a příslušného chlormravenčanu. Postup derivatizace s TMSI, který je naší skupinou běžně používán k derivatizaci sacharidů [52, 53], byl následující: vysušený vzorek Chol byl znovu rozpuštěn ve 100  $\mu\text{l}$  dimethylformamidu a poté bylo přidáno 30  $\mu\text{l}$  TMSI. Vzorek byl uzavřen víčkem s teflonovým septem a zahříván na 80°C po dobu 30 minut. Po ochlazení na laboratorní teplotu bylo ke vzorku přidáno 100  $\mu\text{l}$  isooktanu a po zamíchání a opětném vyčeření byla odebrána část horní vrstvy, jejíž 1  $\mu\text{l}$  byl dávkován do GC-MS. Tento postup byl pak aplikován na stejný set látek, jako v případě TFECF.

### 3.1.2.2 Výsledky a diskuse

Pro studium reaktivity alicyklické hydroxylové skupiny s různými chlormravenčany byl vybrán jako modelová sloučenina Chol. Reakční schéma acylace Chol s TFECF je uvedeno na Obr. 2.



Obr. 2: Reakční schéma cholesterolu s trifluorethylchlormravenčanem (TFECF) a výsledný produkt – trifluorethoxy karbonát (TFEOC) cholesterolu.

Reaktivita hydroxy skupiny byla v první fázi zkoušena ve vodném prostředí, byly testovány již zaběhlé metodiky, které byly použity již dříve, například pro stanovení polárních metabolitů v lidských biotekutinách [41, 43, 45, 46, 54] nebo v průmyslových vodách [37, 49]. Chol byl podroben derivatizaci s různými chlormravenčany (MCF, ECF, iBCF, HCF, TFECF a HFBCF) a výsledné karbonáty byly měřeny na GC-MS. Pro vyhodnocení derivatizační účinnosti byl na GC-MS také měřen nezreagovaný Chol. Porovnání derivatizační účinnosti všech zkoušených chlormravenčanů ve vodném prostředí je uvedeno v Tab. 2 A. Z výsledků je patrné, že reaktivita hydroxylové skupiny Chol s RCF ve vodném prostředí je nízká, výtěžky reakce mezi 4 – 10 %, a že stoupá se zavedením fluorovaných chlormravenčanů (výtěžek 41 % a 48 %). Dále se ukázalo, že vliv na reaktivitu má nahrazení samotného pyridinu za roztok pyridinu s DCC. Zde se výtěžek reakce zvedl z 5 % na 53 % u použití HCF a ze 41 % na 93 % při použití TFECF. Avšak ani u jednoho z použitých postupů se příslušný karbonát Chol nepřiblížil 100% výtěžku. Tudíž byla do studie zařazena další série pokusů, kde se testované reakce převedly do bezvodého prostředí.

V bezvodém prostředí byla použita sušená rozpouštědla a speciální uzávěr na reakční nádoby, který umožňoval přidávání reakčních činidel stříkačkou skrz septum a tudíž bylo zamezeno zbytečnému styku reakčních směsí se vzdušnou vlhkostí. Ukázalo se, že reakční výtěžky jsou vyšší v bezvodém prostředí, ale ke kompletnímu vzniku požadovaného derivátu vedla pouze cesta při použití fluorovaných chlormravenčanů (TFECF a HFBCF). V této fázi byl pro porovnání také připraven trimethylsilyl ether Chol, kde došlo k 100% konverzi Chol na derivát. Kompletní výsledky jsou uvedeny v Tab. 2 B. TIC GC-MS chromatogram Chol a jeho derivátů vzniklých po reakci s různými chlormravenčany a TMSI v bezvodém prostředí je uveden na Obr. 3.



Tabulka 2: EI odezvy nederivatizovaného Chol a jeho alkyloxykarbonáty (ROC) měřeny jednotnými podmínkami na GC-MS. A: Příprava vzorku ve vodném prostředí. B: Příprava vzorku v bezvodém prostředí.

**2A: Vodné prostředí**

Analyt	Derivatizační činidlo	MM <sup>e</sup>	RT (min)	Chol PA <sup>h</sup>	Chol-ROC PA	Relativní EI odezva Chol-ROC (%)
Chol	žádné	386,4	4,23	16212150	0	0
Chol - MOC <sup>a</sup>	MCF	444,4	4,78	15746879	1185249	7
Chol - EOC <sup>b</sup>	ECF	458,4	4,93	17052785	1894754	10
Chol - iBOC <sup>c</sup>	iBCF	486,4	5,28	19333588	805566	4
Chol - HOC <sup>d</sup>	HCF	514,4	5,81	20074728	1056565	5
Chol - HOC	HCF + DCC <sup>i</sup>	514,4	5,81	9842676	11099187	53
Chol - TFEOC <sup>e</sup>	TFECF	512,4	4,52	13746024	9552321	41
Chol - TFEOC	TFECF + DCC	512,4	4,52	1758047	23356924	93
Chol - HFBOC <sup>f</sup>	HFBCF	612,3	4,61	11416233	10538060	48

**2B: Bezvodé prostředí**

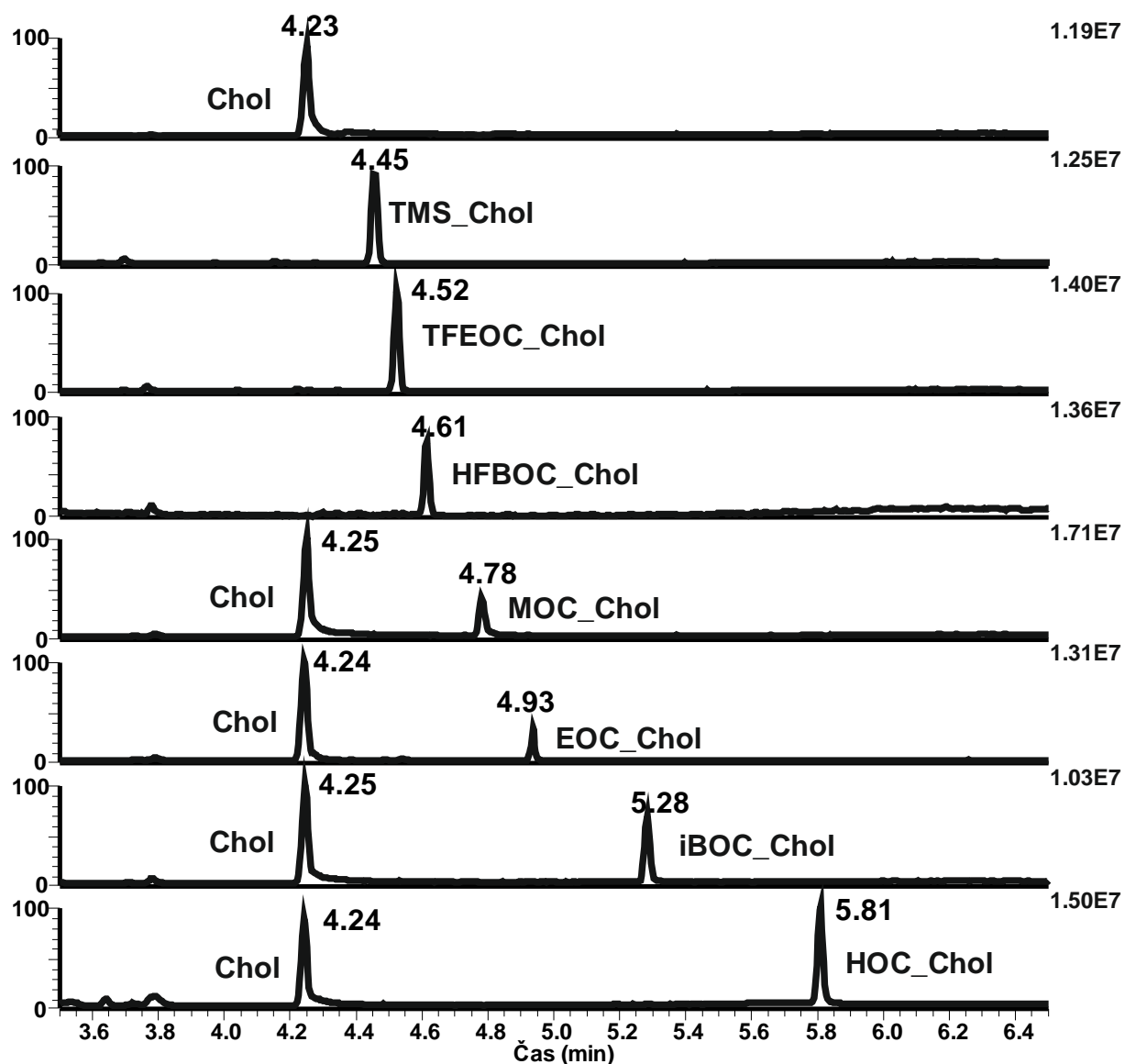
Analyt	Derivatizační činidlo	MM	RT (min)	Chol PA	Chol-ROC PA	Relativní EI odezva Chol-ROC (%)
Chol	žádné	386,4	4,23	18762260	0	0
Chol - TMS	TMSI	458,4	4,45	0	21106631	100
Chol - MOC	MCF	444,4	4,78	14838147	6359205	30
Chol - EOC	ECF	458,4	4,93	14427267	4809089	25
Chol - iBOC	iBCF	486,4	5,28	12896723	6944388	35
Chol - HOC	HCF	514,4	5,81	11656353	10345633	47
Chol - HOC	HCF + DCC	514,4	5,81	3602825	17590266	83
Chol - TFEOC	TFECF	512,4	4,52	0	21477150	100
Chol - HFBOC	HFBCF	612,3	4,61	0	20847372	100

EI MS odezva byla spočítána z TIC plochy píku (hmotový rozsah = m/z 100-700 Da)

<sup>a</sup>methoxykarbonát, <sup>b</sup>ethoxykarbonát, <sup>c</sup>isobutoxykarbonát, <sup>d</sup>hexyloxykarbonát,

<sup>e</sup>trifluoroethoxykarbonát, <sup>f</sup>heptafluorobutoxykarbonát, <sup>g</sup>monoisotopická hmotnost, <sup>h</sup>plocha píku,

<sup>i</sup>*N,N'*-dicyklohexylkarbodiimid



Obr. 3: TIC GC-MS chromatogram cholesterolu (Chol) a jeho derivátů; nederivatizovaný Chol, jeho trimethylsilyl-ether (TMS\_Chol) a karbonáty vzniklé po reakci Chol s různými chlormravenčany za bezvodých podmínek. Trifluoroethoxy karbonát – TFEOC\_Chol, heptafluorobutoxy karbonát – HFBOC\_Chol, methoxy karbonát – MOC\_Chol, ethoxy karbonát – EOC\_Chol, isobutoxy karbonát – iBOC\_Chol, hexyloxy karbonát – HOC\_Chol.

Ukázalo se, že pro derivatizaci hydroxylové skupiny Chol je vhodná reakce s fluorovanými chlormravenčany v bezvodém prostředí. Tato nová metoda je velmi jednoduchá a zahrnuje pouze přidání směsi isooktanu s fluorovaným chlormravenčanem do vysušeného vzorku. Při vývoji optimální metodiky pro tuto reakci byly zkoušeny různé poměry chlormravenčanu a organického rozpouštědla. Zkoušené poměry byly v rozmezí 2 – 10 % (v/v) chlormravenčanu a jako optimální byl vybrán 5% obsah chlormravenčanu v isooktanu. V první fázi vývoje metody se po přidání pyridinu a příslušného

chlormravenčanu tvořila bílá sraženina pyridin hydrochloridu, která se následně rozpouštěla přidáním 100 µl kyseliny chlorovodíkové o koncentraci 250 mmol/l v posledním kroku. Další vývoj metody vedl k tomu, že byl krok s přidáním kyseliny chlorovodíkové vynechán a nahrazen přidavkem acetonitrilu již ve druhém kroku, který vznikající sraženinu ihned rozpouští a navíc stejně jako vodná fáze odstraní polárnější nečistoty, tudíž dochází v jednom kroku k derivatizaci a k mikroextrakci na bázi kapalina – kapalina.

Jak již bylo uvedeno výše, pro aplikaci metody na další sloučeniny byla vybrána nákladově nejpříznivější reakce s TFEFCF. Aby bylo dokázáno, že derivatizace je účinná i pro další skupiny látek, byl soubor analytů rozšířen o další živočišné a rostlinné steroly, o zástupce hydroxycholesterolů a steroidních hormonů a tokoferolů. Struktura všech zkoumaných standardů je uvedena na Obr.S.1.

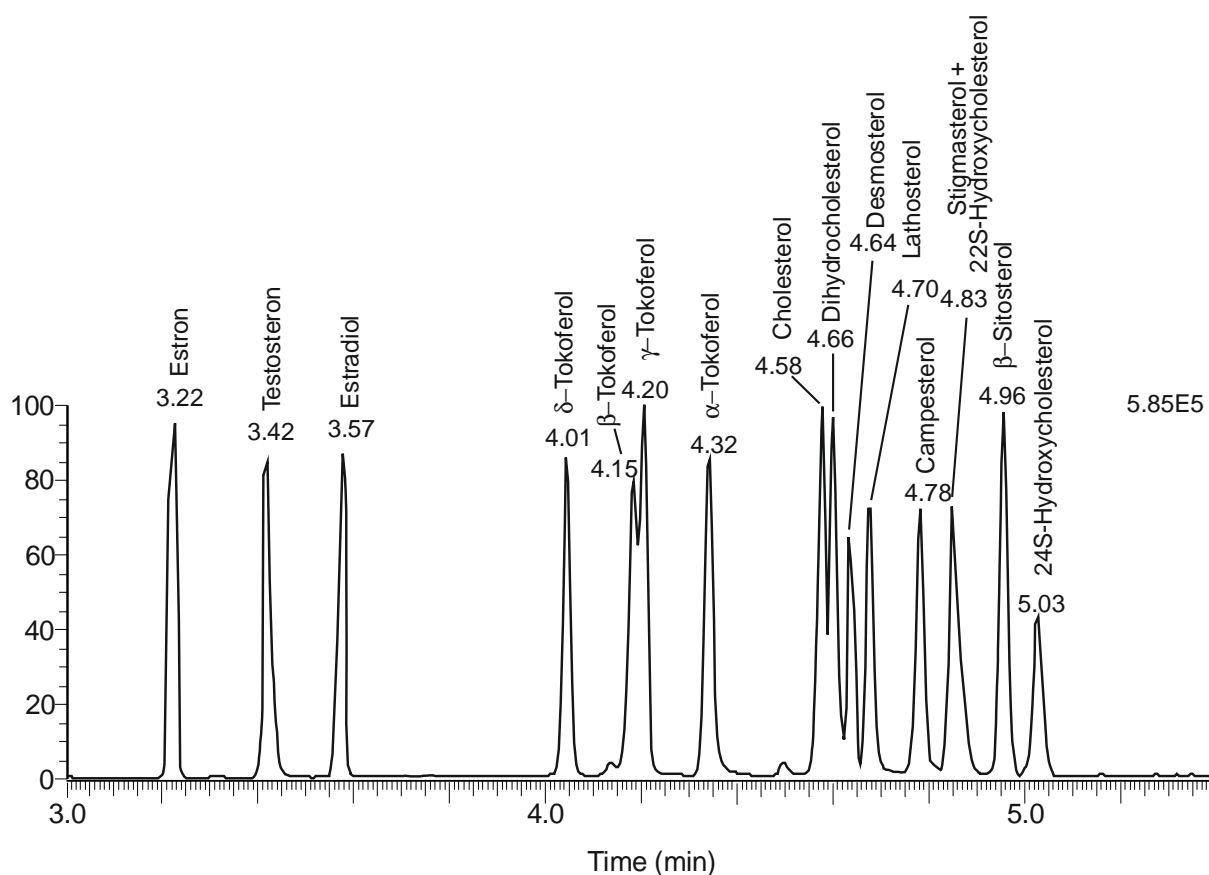
Ukázalo se, že vybrané modelové látky reagují obdobně za vzniku příslušných karbonátů, které v systému GC-MS poskytují jeden pík a dobře definovaná EI MS spektra. Druhá hydroxylová skupina, obsažená ve struktuře hydroxycholesterolů a některých steroidů také podléhá derivatizaci, stejně jako fenolický hydroxyl u tokoferolů. Velká přednost nové metody oproti derivatizacím se silylačními činidly spočívá ve skutečnosti, že keto skupina, je-li ve struktuře látky přítomna, zůstává po reakci nedotčena. U silylací je pro chránění keto skupiny potřebná její oximace.

Pro separaci těchto analytů se ukázala jako nejvhodnější nepolární kapilární kolona a 16 karbonátů bylo separováno během pěti minut v rozmezí teplot 240 – 300°C. TIC GC-MS chromatogram je uveden na Obr. 4, a všechny retenční charakteristiky, zahrnující monoisotopickou hmotnost, retenční čas a diagnostické EI fragmenty jsou v shrnuty Tabulce 3.

Tabulka 3: Analytické charakteristiky vyšetřovaných TFEOC derivátů

<b>Analyt</b>	<b>RT (min)</b>	<b>MM<sup>a</sup> TFEOC der.</b>	<b>Kvantifikační ion</b>	<b>Konfirmační ion</b>
Estron	3,22	396,1	396	352
Testosteron	3,42	414,2	372	124
Estradiol	3,57	524,1	524	236
δ-Tokoferol	4,01	528,3	263	528
β-Tokoferol	4,15	542,4	277	542
γ-Tokoferol	4,20	542,4	277	542
α-Tokoferol	4,32	556,4	165	556
Cholesterol	4,58	512,3	368	353
Dihydrocholesterol	4,66	514,4	499	514
Desmosterol	4,64	510,3	366	253
Lathosterol	4,70	512,4	399	512
Kampesterol	4,78	526,4	382	213
Stigmasterol	4,83	538,4	394	255
22S-Hydroxychoelsterol	4,83	654,3	510	366
β-Sitosterol	4,96	540,4	396	381
24S-Hydroxychoelsterol	5,03	654,3	510	366

<sup>a</sup>MM = monoisotopická hmotnost



Obr. 4: TIC GC-MS separace 16 vyšetřovaných analytů na 15 m × 0.25 mm i.d. DB-1HT kapilární chromatografické koloně.

El hmotnostní spektra všech vyšetřovaných látek jsou shrnuta na Obr. S.2. Nahrazením vodíku v hydroxylové skupině za TFEOC skupinu je molekulová hmotnost zvýšena o 126 Da. Pro derivát Chol je charakteristická neutrální ztráta trifluoroethyl hydrogenkarbonátové části [TFEOCOOH,  $M^{+•} - 144$ ] při čemž dojde ke stabilizaci náboje a ke vzniku kation-radikálu na Chol skeletu ( $M^{+•} = 368,2$ ). Jak je patrné z hmotnostního spektra, molekulární ion je u sterolů velmi slabý, intenzita se zvyšuje u derivátů lathosterolu a dihydrocholesterolu. Je to patrné způsobeno rozdílnou stabilitou sterolového skeletu díky nepřítomnosti dvojně vazby na druhém jádře ve sterolovém skeletu mezi uhlíky na pozici 5 a 6. Pokud je přítomna druhá hydroxylová skupina ve sterolovém skeletu (v případě hydroxycholesterolů a estradiolu), dochází opět k charakteristické ztrátě TFEOCOHOH [ $M^{+•} - 144$ ] za vzniku fragmentu  $m/z$  510 doprovázeného následnou ztrátou druhé TFEOCOHOH skupiny (fragment  $m/z$  366).

U tokoferolů je fragmentace v EI spektru rozdílná díky přítomnosti aromatického jádra, které stabilizuje výskyt zřetelného  $M^{+•}$  iontu. Charakteristické hlavní fragmentové ionty jsou  $M^{+•} - 225$  a  $M^{+•} - 224$ , které reprezentují postupné štěpení alkylového řetězce s přenosy vodíku, doprovázené dále ztrátou isoprenoidové skupiny (ion  $M^{+•} - 265$ ) [55]. V souhrnu všechny zkoumané deriváty steroidů

s TFECF poskytují EI spektra s vysoce charakteristickými fragmentovými ionty vhodnými pro jejich identifikace a citlivé stanovení na GC-MS.

U zkoumaných derivátů byla dále proměřena spektra pozitivní (PICI) a negativním (NICI) chemické ionizace. Jako ionizační plyn byl testován isobutan a amoniak. V isobutanových PICI spektrech sterolů byl pozorován jako nejintenzivnější ion po ztrátě trifluoroethylhydrogenkarbonátové skupiny. Experimenty ukázaly, že karbonátová skupina je velmi labilní a dochází k jejímu odštěpení i za nižších ionizačních podmínek. V PICI spektrech při použití amoniaku byl pozorován pouze málo intenzivní ion  $[M+NH_4^+]$ .

PICI spektra cholesterolu jsou uvedena na Obr. S.3.

NICI spektra testovaných látek obsahovala vesměs málo použitelné fragmenty u nižších  $m/z$ , proto bylo od jejich dalšího studia upuštěno.

### **3.1.3 Aplikace metody pro stanovení sterolů a tokoferolů v lidském séru a plodové vodě a její validace.**

#### **3.1.3.1 Experimentální část**

##### **3.1.3.1.1 Chemikálie**

5- $\alpha$ -Cholestan a cholesteryl palmitát byly pořízeny u Sigma-Aldrich (Praha, Česká Republika), hydroxid draselný byl koupen u firmy Merck (Praha, Česká Republika). Dihydrocholesterol,  $\alpha$ -tokoferol,  $\beta$ -tokoferol,  $\gamma$ -tokoferol,  $\delta$ -tokoferol, ethanol, isooktan, pyridin, TFECF, lathosterol, kampesterol, stigmasterol,  $\beta$ -sitosterol, desmosterol, *n*-hexan, acetonitril, methanol – viz. sekce 3.1.2.1.1.

##### **3.1.3.1.2 Biologický materiál**

Soubor dvaceti reálných vzorků lidského séra matek před a po porodu (deset matek, každá 10 vzorků před porodem a 10 vzorků po porodu) a pět vzorků plodové vody byly poskytnuty Dr. Martinem Hillem (Endokrinologický ústav, Praha, Česká Republika) a uchovány při teplotě  $-80^\circ\text{C}$ . Pro vývoj metody bylo použito sérum, které bylo získáno od pěti zdravých dobrovolníků a směsná plodová voda, která byla připravena smícháním z obdržených vzorků.

##### **3.1.3.1.3 Vybavení laboratoře**

Pro odpařování vzorků do sucha po saponifikaci byla použita vakuová odparka Thermo Fisher Scientific Jouan RC 10.10 (Trigon Plus, Praha, Česká Republika).

Zbýlé vybavení viz. sekce 3.1.2.1.2.

##### **3.1.3.1.4 Příprava zásobních a kalibračních roztoků**

Zásobní roztoky sterolových a tokoferolových standardů, cholesteryl palmitátu a 5- $\alpha$ -cholestanu, který byl použit jako interní standard (I.S.) byly připraveny v ethanolu o koncentraci 1 mg/ml. Kalibrační roztoky standardů sterolů (6 koncentračních hladin) byly připraveny postupným ředěním zásobního roztoku ethanolem na koncentrace v rozmezí 0,25 – 50  $\mu\text{g/ml}$  (0,25, 0,5, 2,5, 5, 25a

50 µg/ml). Tokoferolové kalibrační roztoky byly naředěny stejným způsobem, ale na koncentrace v rozmezí 0,75 – 150 µg/ml (0,75, 1,5, 7,5, 15, 75 a 150 µg/ml). Roztok I.S. byl naředěn ethanolem na koncentraci 50 µg/ml.

### 3.1.3.1.5 Kvantifikace a validace metody, příprava reálných vzorků

Pro kvantitativní stanovení vybraných analytů v biotekutinách byla vybrána derivatizační metoda s TFECF (viz. sekce 3.1.2.1.3) a deriváty byly měřeny na GC-MS za stejných podmínek jak již bylo popsáno výše (viz. sekce 3.1.2.1.2). Analyty byly ale měřeny v režimu SIM (selected ion monitoring) skenovacím módu. Pro každý analyt byl vybrán kvantifikační a konfirmační ion (viz. Tabulka 3) a detekován v hmotovém okně 1,2 Da v čase 25 ms.

Obsah všech sterolů (volných i vázaných) a tokoferolů byl stanoven po saponifikaci každého vzorku pomocí hydroxidu draselného o koncentraci 2 mol/l v 90% ethanolu. Obsah byl zahříván na teplotu 60°C po dobu 120 min [56]. Účinnost saponifikace byla ověřena dvěma metodami:

1) První ověření bylo provedeno pomocí cholesteryl palmitátu. 1 µl standardního roztoku cholesteryl palmitátu byl smíchán s 250 µl hydroxidu sodného o koncentraci 2 mol/l v 90% ethanolu a hydrolyzován při 60°C po 120 minut. Směs byla po vychladnutí extrahována 500 µl *n*-hexanu a 300 µl hexanové části bylo odebráno do derivatizační nádoby. Po odpaření do sucha byl obsah derivatizován metodou s TFECF. Pokus byl opakován třikrát.

2) Vybrané sérum a plodová voda (každý 3×) bylo saponifikováno a nepolární složky byly extrahovány do *n*-hexanu stejným způsobem, jako je popsáno v bodu 1). Po odpaření hexanové fáze byl vzorek rozpuštěn 300 µl methanolu a byla sledována přítomnost triglyceridů a diglyceridů na HPLC-MS na RP-koloně, Gemini C18, 110 A, 150 x 2 mm i.d., 3 µm (Phenomenex, CA, USA). Tato metoda byla podrobně popsána v publikaci [57].

Příprava vzorků biotekutin byla následující: 100 µl každé biotekutiny (sérum či plodová voda) bylo smícháno s 10 µl zásobního roztoku I.S. v derivatizačních nádobkách a po zamíchání bylo přidáno saponifikační médium (250 µl hydroxidu draselného ( $c = 2 \text{ mol/l}$ ) v 90% ethanolu) a tato směs byla zahřívána na 60°C po dobu 120 minut. Po vychladnutí byl obsah extrahován 500 µl *n*-hexanu, poté bylo odebráno 300 µl hexanové vrstvy do nové derivatizační nádoby a obsah byl odpařen do sucha na vakuové odparce. K suchému odparce bylo přidáno 50 µl směsi isooktan/pyridin (9:1, v/v), mícháno asi 10 s a po přidání 100 µl acetonitrilu a 100 µl směsi isooktanu/TFECF (95:5, v/v) bylo obsahem mícháno asi 20 s. Po reakci bylo odebráno 50 µl horní isooktanové vrstvy do vialky a 1 µl vzorku byl dávkován na kolonu GC-MS. Schematický postup je znázorněn na Obr. 5 a zahrnuje saponifikaci vzorku, extrakci sterolů a tokoferolů do hexanu, odpaření rozpouštědla, derivatizaci s TFECF a GC-MS analýzu.

Kalibrace byla provedena metodou standardního přidavku. Ke kontrolnímu séru a kontrolní plodové vodě bylo přidáno 10 µl standardního roztoku I.S. a 20 µl kalibračního roztoku, který obsahoval

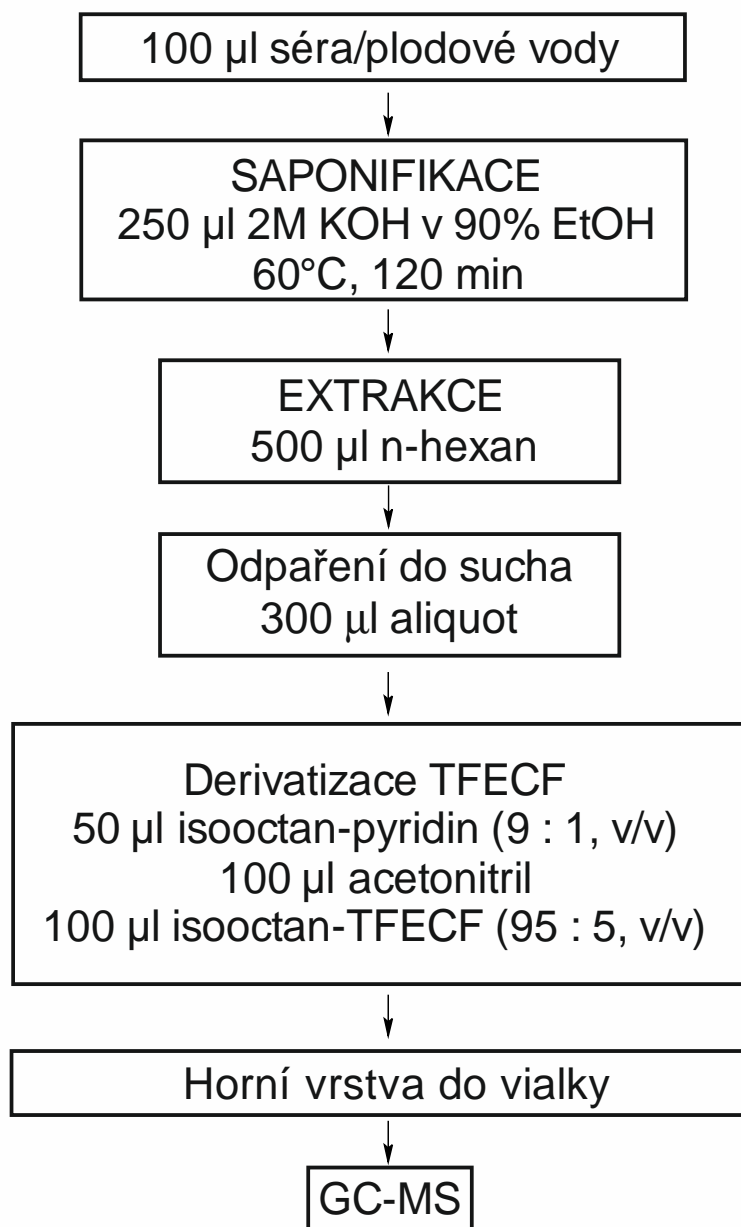
stanovované steroly a tokoferoly v šesti koncentračních hladinách. Finální přidané koncentrace sterolů a tokoferolů v matrici byly následující: 0,05, 0,1, 0,5, 1, 5 a 10 µg/ml pro steroly a 0,15, 0,3, 1,5, 3, 15 a 30 µg/ml pro tokoferoly. Vzorky byly dále saponifikovány a poté derivatizovány s TFECF a měřeny na GC-MS, jak je již popsáno výše. Každý analyt byl posléze vyhodnocen jako poměr ploch analytu a I.S. (5- $\alpha$ -cholestan, finální koncentrace v biotekutinách byla 5 µg/ml) a byla vynesena kalibrační závislost poměru ploch na koncentraci. Vzorky séra a plodové vody se standardními přídávky na nejvyšší, střední a nejnižší hladině (0,05, 1 a 10 µg/ml pro steroly a 0,15, 3 a 30 µg/ml pro tokoferoly) byly použity pro určení dalších validačních parametrů vyvinuté metody.

Mezi validační parametry patří vyhotovení kalibrační závislosti, určení limitu detekce (v případě metody standardního přídávku určení tzv. nízkého limitu stanovení = LLOQ – lower limit of quantification), preciznosti (anglický ekvivalent = precision), přesnosti (anglický ekvivalent = accuracy), výtěžnosti (anglický ekvivalent = recovery) a stability. Všechny tyto parametry byly stanoveny v souladu s doporučeními [58]. Což znamená, že LLOQ bylo stanoveno jako nejnižší bod kalibrační přímky po analýze vzorku s nejnižším standardním přídávkem na GC-MS a tento bod musel být změřen se shodností 20 % a s přesností v rozmezí 80 – 120 % (n = 5). Preciznost a přesnost byla stanovena analýzou kontrolních vzorků v jedné sérii (within-run) a ve 14-ti denní periodě (between-run) (n=5). Návratnost byla také určena z kontrolních vzorků a to tak, že nejprve byla určena přírodní koncentrace každého sterolu a tokoferolu a pak změřena koncentrace přidaná. Tyto dvě hodnoty byly porovnány a návratnost byla spočtena podle vzorce: (naměřená koncentrace – přírodní koncentrace)/přidaná koncentrace  $\times$  100. Stabilita TFEOC derivátů byla vyhodnocena také z kontrolních vzorků a to pouze z těch, kde byla přidána střední koncentrační hladina. Každý pokus byl připraven ve třech provedeních. V prvním případě byl vzorek po derivatizaci uchován při pokojové teplotě a v druhém případě v lednici o teplotě 4°C. Stabilita derivátů byla měřena v pravidelných intervalech po dobu 72 hodin. Koncentrace analytů byla navíc porovnána se vzorky, které byly vždy připraveny každý den čerstvě.

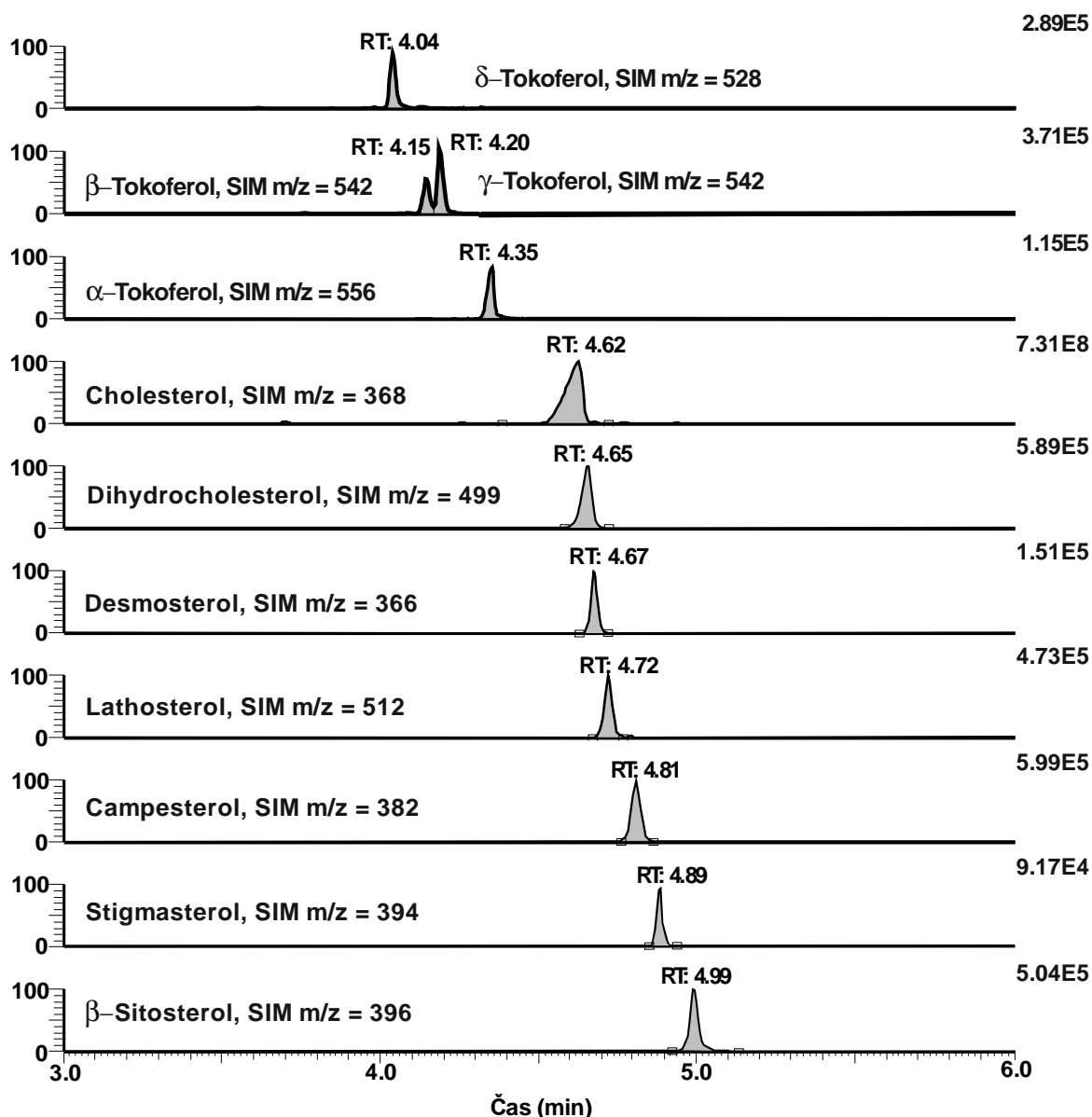
### 3.1.3.2 Výsledky a diskuse

Nová analytická metoda popisována v této studii byla použita pro stanovení 10 sterolů a tokoferolů v lidském séru a v plodové vodě. Vzorový chromatogram všech stanovovaných analytů v lidském séru je vyobrazen na Obr. 6. Před aplikací na reálné vzorky byla tato metoda validována. Steroly se v biotekutinách vyskytují jak ve volné formě, tak ve formě vázané, tudíž byl každý vzorek před derivatizací podroben saponifikaci [56]. Funkčnost saponifikačního procesu byla ověřena dvěma metodami, jejichž postup je popsán v experimentální části. U prvního ověření bylo prokázáno, že se všechny cholesteryl palmitát přemění na volný cholesterol (měřeno na GC-MS) a tedy, že saponifikace byla úspěšná a u druhého prověření se zkoumala přítomnost triglyceridů a diglyceridů ve vzorku (měřeno na HPLC-MS) a jejich nepřítomností bylo potvrzeno, že saponifikace proběhla úplně.





Obr. 5: Postup přípravy vzorku pro kvantitativní stanovení šesti sterolů a čtyř tokoferolů v lidském séru a v plodové vodě.



Obr. 6: Charakteristické SIM GC-MS chromatogramy sterolů a tokoferolů detekovaných v séru těhotné ženy (vzorek Wb4).

Všechna validační kritéria byla splněna. Kalibrační data zahrnující kalibrační rovnice, rozsah kvantifikace, hodnotu regresního koeficientu a LLOQ jsou uvedeny v Tabulce 4. Preciznost měřená v jedné sérii (within-run) byla v rozsahu 0,9 – 19,5 % a ve 14-ti denní periodě (between-run) byla v rozsahu 0,2 – 19 %. Výsledky pro jedno sériové měření (within-run) přesnosti byly v rozmezí 80 – 115 % a pro měření ve 14-ti denní periodě (between-run) byly v rozsahu 82 – 122 %. Výtěžnost byla v rozmezí 90 – 110 % v lidském séru a v rozmezí 92 – 110 % v plodové vodě. Pro lidské sérum jsou tyto výsledky podrobně uvedeny v Tab. S.1. TFEOC deriváty byly shledány stabilní, odchylka jednotlivých měření byla  $\pm 12\%$  a to je v tolerančním limitu.

Tabulka 4: Kalibrační data pro GC-MS stanovení kontrolního séra a plodové vody.

Analyt-TFEOC der.	RT (min)	Rozsah kvantifikace (µg/ml)	Regresní rovnice (sérum)	R2 (sérum)	Regresní rovnice (plodová voda)	R2 (plodová voda)	LLOQ* (µg/ml)
5-α-Cholestan	3,69	5			Interní standard (I.S.)		
δ-Tokoferol	4,01	0,15 - 30	0,0075x-0,0008	0,9907	n.d. <sup>a</sup>	n.d.	0,15
β-Tokoferol	4,15	0,15 - 30	0,0163x-0,0022	0,9986	n.d.	n.d.	0,15
γ-Tokoferol	4,20	0,15 - 30	0,0220x+0,0113	0,9531	n.d.	n.d.	0,15
α-Tokoferol	4,32	0,15 - 30	0,0141x-0,0030	0,9796	n.d.	n.d.	0,15
Dihydrocholesterol	4,64	0,05 - 10	0,0216x+0,0011	0,9983	0,0234x-0,0027	0,9995	0,05
Desmosterol	4,66	0,05 - 10	0,0400x+0,0409	0,9746	0,0364x-0,0081	0,9959	0,05
Lathosterol	4,68	0,05 - 10	0,0644x+0,0101	0,9992	0,0418x-0,0030	0,9991	0,05
Kampesterol	4,77	0,05 - 10	0,0932x+0,0123	0,9994	0,1015x-0,0152	0,9994	0,05
Stigmasterol	4,83	0,05 - 10	0,0472x-0,0012	0,9998	0,0797x-0,0058	0,9997	0,05
β-Sitosterol	4,93	0,05 - 10	0,0352x+0,0095	0,9978	0,1005x-0,0117	0,9997	0,05

<sup>a</sup>n.d.= nestanoveno; \* LLOQ je nejnižší bod kalibrační přímky a je stejný pro sérum i plodovou vodu

Po úspěšné validaci byla metoda aplikována na reálné vzorky. Jednalo se o soubor dvaceti vzorků krevního séra deseti matek, vždy před a po porodu a pěti vzorků plodové vody (viz. experimentální část). Nalezené koncentrace stanovovaných celkových sterolů a tokoferolů se shodovaly s hodnotami, které byly naměřeny v dřívějších studiích [59, 60, 61 – 72]. Koncentrace prekursorů cholesterolu – dihydrocholesterolu a lathosterolu se pohybovala v rozmezí 0,08 – 32,4 µg/ml, byla vyšší před porodem. Desmosterol byl nalezen v rozmezí 0,3 – 10,5 µg/ml a měl podobné koncentrační hladiny v séru před i po porodu. Také rostlinné steroly nejevily významné rozdíly před a po porodu. Kampesterol se vyskytoval v rozmezí 1,4 – 6,9 µg/ml, β-sitosterol mezi 0,7 – 5,7 µg/ml a stigmasterol v rozmezí 0,07 – 1,7 µg/ml. Tento trend je v souladu s dříve publikovanými výsledky. Výsledky těchto měření jsou podrobně uvedeny v Tab. 5.

Hladiny tokoferolů se pohybovaly v rozmezí popsaných v dřívějších pracech [64 – 72], (Tab. 5). Koncentrace stanovovaných analytů v plodové vodě byla nižší, než u séra, pro dihydrocholesterol v rozmezí 0,29 – 1,8 µg/ml, pro lathosterol mezi 0,7 – 1,3 µg/ml, desmosterol 0,3 – 0,7 µg/ml, kampesterol kolem 0,2 µg/ml, pro β-sitosterol okolo 0,5 µg/ml a pro stigmasterol kolem 0,1 µg/ml (Tab. 6). V plodové vodě nebyly tokoferoly detekovány.

Tabulka 5: Koncentrační hladiny ( $\mu\text{g/ml}$ ) měřených sterolů a tokoferolů v séru deseti zdravých žen před porodem (Wb) a po porodu (Wa). Výsledky vyjádřeny jako střední hodnota ( $n = 3$ )  $\pm$  směrodatná odchylka.

	Dihydrocholesterol	Desmosterol	Lathosterol	Kampesterol	Stigmasterol	$\beta$ -Sitosterol	$\alpha$ -Tokoferol	$\beta$ -Tokoferol	$\gamma$ -Tokoferol	$\delta$ -Tokoferol
<sup>a</sup> Wb1	32,38 $\pm$ 1,53	0,60 $\pm$ 0,01	7,44 $\pm$ 0,21	5,14 $\pm$ 1,09	1,73 $\pm$ 0,31	5,72 $\pm$ 0,09	9,90 $\pm$ 0,28	0,91 $\pm$ 0,10	0,44 $\pm$ 0,02	n.d. <sup>c</sup>
<sup>b</sup> Wa1	2,64 $\pm$ 0,22	0,55 $\pm$ 0,01	5,42 $\pm$ 0,11	2,51 $\pm$ 0,03	0,06 $\pm$ 0,00	1,53 $\pm$ 0,06	3,19 $\pm$ 0,08	0,80 $\pm$ 0,07	0,07 $\pm$ 0,00	n.d.
Wb2	1,73 $\pm$ 0,41	0,85 $\pm$ 0,06	4,57 $\pm$ 0,35	4,85 $\pm$ 0,24	0,06 $\pm$ 0,01	2,85 $\pm$ 0,15	14,10 $\pm$ 0,11	0,92 $\pm$ 0,09	0,11 $\pm$ 0,01	n.d.
Wa2	0,70 $\pm$ 0,19	0,64 $\pm$ 0,06	2,12 $\pm$ 0,19	3,95 $\pm$ 0,14	0,07 $\pm$ 0,01	1,94 $\pm$ 0,05	2,52 $\pm$ 0,05	0,64 $\pm$ 0,02	n.d.	n.d.
Wb3	12,42 $\pm$ 1,49	0,81 $\pm$ 0,10	2,47 $\pm$ 0,20	2,08 $\pm$ 0,42	0,40 $\pm$ 0,10	1,85 $\pm$ 0,32	9,33 $\pm$ 0,31	1,18 $\pm$ 0,27	0,32 $\pm$ 0,02	n.d.
Wa3	0,48 $\pm$ 0,08	0,90 $\pm$ 0,05	1,57 $\pm$ 0,03	1,35 $\pm$ 0,07	0,04 $\pm$ 0,00	0,77 $\pm$ 0,07	7,18 $\pm$ 1,05	0,61 $\pm$ 0,09	n.d.	n.d.
Wb4	5,94 $\pm$ 0,02	0,26 $\pm$ 0,02	4,82 $\pm$ 0,01	6,93 $\pm$ 0,04	0,03 $\pm$ 0,00	4,85 $\pm$ 0,04	0,40 $\pm$ 0,02	0,52 $\pm$ 0,01	1,15 $\pm$ 0,21	0,72 $\pm$ 0,14
Wa4	0,94 $\pm$ 0,01	0,36 $\pm$ 0,01	0,82 $\pm$ 0,01	6,73 $\pm$ 0,23	0,24 $\pm$ 0,01	3,87 $\pm$ 0,13	0,24 $\pm$ 0,04	0,52 $\pm$ 0,01	1,23 $\pm$ 0,92	0,74 $\pm$ 0,02
Wb5	13,45 $\pm$ 2,71	0,65 $\pm$ 0,01	1,90 $\pm$ 0,45	1,26 $\pm$ 0,19	0,29 $\pm$ 0,08	0,78 $\pm$ 0,16	12,20 $\pm$ 1,10	0,66 $\pm$ 0,04	n.d.	n.d.
Wa5	4,33 $\pm$ 0,74	0,70 $\pm$ 0,03	0,08 $\pm$ 0,01	1,55 $\pm$ 0,16	0,05 $\pm$ 0,00	0,85 $\pm$ 0,01	1,22 $\pm$ 0,03	0,35 $\pm$ 0,03	n.d.	n.d.
Wb6	4,32 $\pm$ 0,62	10,50 $\pm$ 2,21	3,57 $\pm$ 0,32	3,64 $\pm$ 0,04	1,45 $\pm$ 0,14	3,72 $\pm$ 0,08	12,30 $\pm$ 0,41	1,50 $\pm$ 0,02	1,52 $\pm$ 0,61	0,66 $\pm$ 0,01
Wa6	2,57 $\pm$ 0,04	0,96 $\pm$ 0,11	1,81 $\pm$ 0,11	5,70 $\pm$ 0,21	0,82 $\pm$ 0,09	2,84 $\pm$ 0,11	6,19 $\pm$ 0,16	0,67 $\pm$ 0,01	1,09 $\pm$ 0,09	0,74 $\pm$ 0,03
Wb7	3,16 $\pm$ 0,18	0,46 $\pm$ 0,04	2,61 $\pm$ 0,08	3,59 $\pm$ 0,13	0,61 $\pm$ 0,02	2,70 $\pm$ 0,05	1,29 $\pm$ 0,50	0,26 $\pm$ 0,01	0,70 $\pm$ 0,07	0,67 $\pm$ 0,01
Wa7	1,04 $\pm$ 0,08	0,30 $\pm$ 0,01	0,84 $\pm$ 0,06	3,81 $\pm$ 0,02	0,68 $\pm$ 0,01	2,28 $\pm$ 0,25	1,42 $\pm$ 0,05	0,25 $\pm$ 0,03	0,68 $\pm$ 0,01	0,66 $\pm$ 0,00
Wb8	2,05 $\pm$ 0,17	0,71 $\pm$ 0,02	3,13 $\pm$ 0,12	4,66 $\pm$ 0,07	0,61 $\pm$ 0,03	2,64 $\pm$ 0,19	4,19 $\pm$ 0,08	0,38 $\pm$ 0,02	0,79 $\pm$ 0,05	0,71 $\pm$ 0,01
Wa8	1,67 $\pm$ 0,04	0,50 $\pm$ 0,01	1,30 $\pm$ 0,04	2,86 $\pm$ 0,12	0,56 $\pm$ 0,01	1,97 $\pm$ 0,03	3,00 $\pm$ 0,01	0,55 $\pm$ 0,06	0,98 $\pm$ 0,11	0,81 $\pm$ 0,00
Wb9	2,45 $\pm$ 0,03	0,56 $\pm$ 0,04	3,37 $\pm$ 0,31	4,20 $\pm$ 0,03	0,49 $\pm$ 0,05	2,56 $\pm$ 0,02	1,94 $\pm$ 0,05	0,29 $\pm$ 0,01	0,74 $\pm$ 0,02	0,67 $\pm$ 0,02
Wa9	1,98 $\pm$ 0,21	0,48 $\pm$ 0,01	2,16 $\pm$ 0,01	2,66 $\pm$ 0,11	0,34 $\pm$ 0,01	2,01 $\pm$ 0,09	5,04 $\pm$ 0,22	0,35 $\pm$ 0,02	0,85 $\pm$ 0,05	0,68 $\pm$ 0,10
Wb10	5,80 $\pm$ 0,18	0,56 $\pm$ 0,01	3,52 $\pm$ 0,20	2,63 $\pm$ 0,08	0,49 $\pm$ 0,05	4,12 $\pm$ 0,61	5,75 $\pm$ 0,11	0,80 $\pm$ 0,04	1,20 $\pm$ 0,18	0,64 $\pm$ 0,05
Wa10	3,91 $\pm$ 0,26	0,47 $\pm$ 0,02	1,32 $\pm$ 0,07	5,25 $\pm$ 0,20	0,62 $\pm$ 0,02	3,53 $\pm$ 0,07	2,00 $\pm$ 0,05	0,43 $\pm$ 0,01	0,86 $\pm$ 0,01	0,66 $\pm$ 0,08

<sup>a</sup>Wb1 = vzorek žena 1 před porodem, <sup>b</sup>Wa1 = vzorek žena 1 po porodu, <sup>c</sup>n.d.= nedetekováno

Tabulka 6: Koncentrační hladiny ( $\mu\text{g/ml}$ ) sterolů a tokoferolů v plodové vodě získané od pěti žen v posledním trimestru těhotenství. Výsledky vyjádřeny jako střední hodnota ( $n = 3$ )  $\pm$  směrodatná odchylka.

	Dihydrocholesterol	Desmosterol	Lathosterol	Kampesterol	Stigmasterol	$\beta$ -Sitosterol	$\alpha$ -Tokoferol	$\beta$ -Tokoferol	$\gamma$ -Tokoferol	$\delta$ -Tokoferol
<b>W1<sup>a</sup></b>	0,32 $\pm$ 0,01	0,38 $\pm$ 0,03	1,09 $\pm$ 0,17	0,19 $\pm$ 0,01	0,08 $\pm$ 0,00	0,15 $\pm$ 0,01	n.d. <sup>b</sup>	n.d.	n.d.	n.d.
<b>W2</b>	0,40 $\pm$ 0,08	0,36 $\pm$ 0,01	1,31 $\pm$ 0,15	0,17 $\pm$ 0,00	0,08 $\pm$ 0,00	0,14 $\pm$ 0,01	n.d.	n.d.	n.d.	n.d.
<b>W3</b>	1,82 $\pm$ 0,14	0,67 $\pm$ 0,02	16,49 $\pm$ 0,69	0,23 $\pm$ 0,00	0,10 $\pm$ 0,01	0,23 $\pm$ 0,00	n.d.	n.d.	n.d.	n.d.
<b>W4</b>	0,32 $\pm$ 0,03	0,33 $\pm$ 0,01	0,85 $\pm$ 0,04	0,17 $\pm$ 0,01	0,08 $\pm$ 0,00	0,16 $\pm$ 0,02	n.d.	n.d.	n.d.	n.d.
<b>W5</b>	0,29 $\pm$ 0,03	0,34 $\pm$ 0,02	0,70 $\pm$ 0,04	0,18 $\pm$ 0,01	0,09 $\pm$ 0,01	0,15 $\pm$ 0,01	n.d.	n.d.	n.d.	n.d.

<sup>a</sup>W1 = vzorek žena 1, <sup>b</sup>n.d. = nedetegováno.

### 3.1.4 Aplikace metody pro stanovení sterolů v membránách hmyzu *Pyrrhocoris apterus*.

#### 3.1.4.1 Experimentální část

##### 3.1.4.1.1 Chemikálie

<sup>13</sup>C<sub>2</sub> Cholesterol byl od firmy Cambridge Isotopes Laboratories, Inc. (Andover, USA), dichlormethan a chloroform byl zakoupen od firmy Merck (Praha, Česká republika) a ethyl-acetát od Sigma-Aldrich (Praha, Česká republika). Acetonitril, *n*-hexan,  $\gamma$ -tokoferol,  $\delta$ -tokoferol, isooktan, pyridin, kampesterol,  $\beta$ -sitosterol, cholesterol, TFECE a methanol – viz. sekce 3.1.2.1.1.

##### 3.1.4.1.2 Biologický materiál

Dospělí jedinci hmyzu ruměnice pospolné (*Pyrrhocoris apterus*) byli ručně sbíráni ve volné přírodě v okolí Českých Budějovic v pravidelném období v roce 2011. Sledované vzorky zahrnovaly soubor přezimující generace a pak 1. a 2. generace rodící se během roku. Detailně popsáno v tabulce 7.

Tabulka 7: Popis vývojového/reprodukčního stavu dospělé *P. apterus* použitého pro analýzy.

Generace hmyzu	Datum sběru <sup>a</sup>	Vývojový/reprodukční stav
Přezimující	18. 1.	Konec reprodukční diapauzy, post-diapauzní klid
	10. 3.	Post-diapauzní klid
	20. 5.	Reprodukce (pářící páry)
1.	20. 6.	Reprodukce (pářící páry, z volné přírody)
	28. 7.	Reprodukce (pářící páry, venkovní klec)
1. a 2.	17. 9.	Udržování reprodukční diapauzy, teplá aklimatizace
	1. 11.	Udržování/ukončení reprodukční diapauzy, studená aklimatizace
	7. 12.	Ukončení reprodukční diapauzy, studená aklimatizace

<sup>a</sup> Všechny hmyz byl sbírán během roku 2011.

##### 3.1.4.1.3 Vybavení laboratoře

SPE kolonky aminopropyl silica Bond Elut LRC-NH<sub>2</sub> od Agilent Technologies (CA, USA). Zbylé vybavení viz. sekce 3.1.2.1.2 a 3.1.3.1.3.

##### 3.1.4.1.4 Příprava zásobních roztoků

<sup>13</sup>C<sub>2</sub> Cholesterol, který se používal jako I.S., byl rozpuštěn v isooktanu na koncentraci 2.5 nmol/ $\mu$ l.

##### 3.1.4.1.5 Příprava reálných vzorků

Hmyzí jedinec byl rozdělen na dvě části, na tzv. thorax (obsahující svalovou tkáň) a na tzv. břišní tuk. Každý vzorek obsahoval tkáň ze třech jedinců a byl připraven ve čtyřech opakováních. Všechny lipidy byly ze vzorků extrahovány použitím směsi chloroform : methanol (2 : 1, v/v) dle zavedené Folchovy metody [73, 74]. Poté byl obsah odpařen do sucha pod proudem dusíku, bylo přidáno

2,5 nmol I.S., 2 ml extrakční směsi acetonitril : voda (8 : 2, v/v) a 1 ml *n*-hexanu. Vzorkem bylo mícháno po dobu 30 sekund. Pro analýzu nepolárních lipidů byla odebrána hexanová fáze, která byla dále frakcionována pomocí SPE. Obdržené frakce byly následující: 1. frakce obsahovala steryl estery (eluent byl *n*-hexan), 2. frakce obsahovala triglyceridy (eluentem byla směs *n*-hexan : dichlormethan, 8 : 2, v/v) a třetí frakce obsahovala tokoferoly, steroly a diglyceridy (eluent byla směs *n*-hexanu a ethyl-acetátu, 7 : 3, v/v). Po rozdělení byla dále zpravována 3. frakce, která byla odpařena na vakuové odparce do sucha a poté podrobena derivatizaci s TFECF. Postup byl následující: 50 μl reakčního media obsahujícího směs pyridinu a isooktanu v poměru 9 : 1 (v/v) bylo přidáno k vysušenému vzorku a vzorkem bylo krátce zamícháno, po přidání 100 μl acetonitrilu, 100 μl směsi TFECF s isooktanem v poměru (5 : 95, v/v) a promíchání byla odebrána část horní isooktanové vrstvy do vialky a 1 μl vzorku byl dávkován na kolonu na GC-MS. Podmínky nastavení GC-MS viz. kapitola 3.1.2.1.2. Semikvantitativní odhad množství sterolů a tokoferolů byl spočítán jako poměr plochy píku analytu a plochy píku I.S. o známé koncentraci dle vzorce:  $c(\text{analytu}) (\text{nmol/vzorek}) = (\text{plocha píku analytu} / \text{plocha píku I.S.}) * 2,5$ . Konečné výsledky byly vyjádřeny v mol% jako poměr počtu mol analytu na počet mol fosfolipidů\*100. Stanovení fosfolipidů nebylo součástí této práce a přesný postup je uveden v publikaci [2].

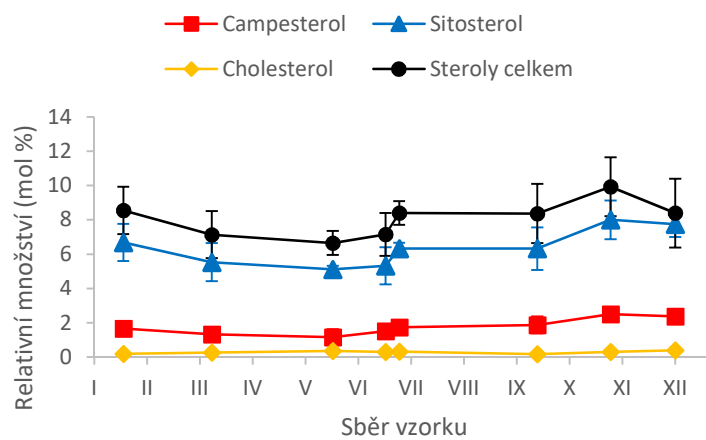
#### 3.1.4.2 Výsledky a diskuse

Ve vzorcích byly identifikovány tři různé steroly, cholesterol, β-sitosterol a kampesterol. Sezonní změny v relativním zastoupení jednotlivých sterolů jsou ukázány na Obr. 7.

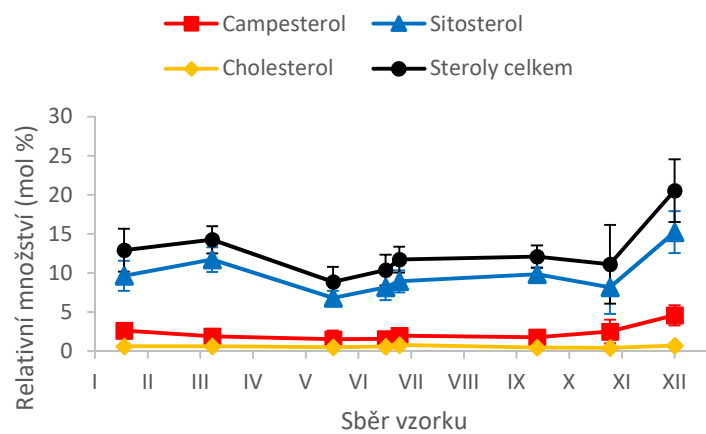
Obsah cholesterolu byl relativně nízký, v rozsahu 0,2 – 0,4 mol% ve svalové tkáni a mezi 0,5 – 0,8 mol% v tukové tkáni. Rostlinné steroly (β-sitosterol a kampesterol) byly obsaženy ve vyšším množství, kampesterol v rozmezí 1,2 – 2,5 mol% ve svalové tkáni a 1,5 – 4,6 mol% v tukové části, β-sitosterol mezi 5,1 – 8,0 mol% ve svalové tkáni a 6,8 – 15,2 mol% v tukové tkáni. Zatímco zastoupení cholesterolu byla během roku téměř stejné u obou tkání, oba rostlinné steroly vykazovaly podobné sezonní trendy, minimum během jara a léta a maximum během podzimu a zimy.

Ve vzorcích byly dále nalezeny dva izomery tokoferolu. δ-Tokoferol se vyskytoval v rozmezí 0,1 – 1,2 mol% ve svalech a 0,3 – 5,5 mol% v tukové tkáni a γ-tokoferol mezi 3,0 – 8,6 mol% ve svalové části a 9,1 – 31,3 mol% v tukové části. Přehled změn obsahu tokoferolů během kalendářního roku zachycuje obrázek 8.

(A)



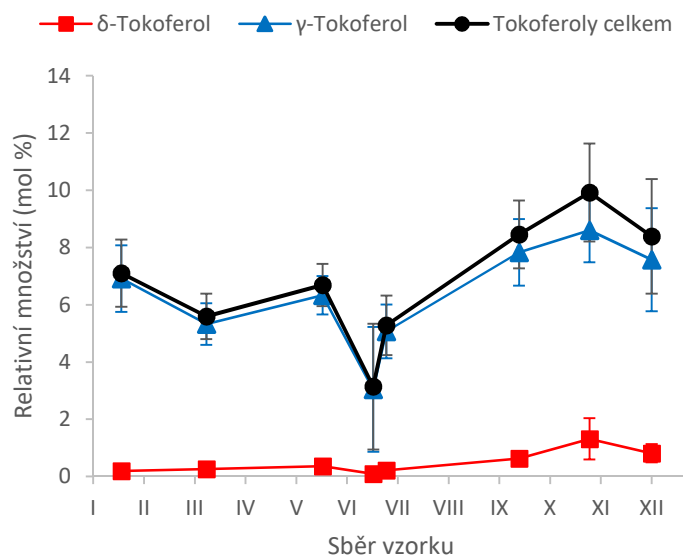
(B)



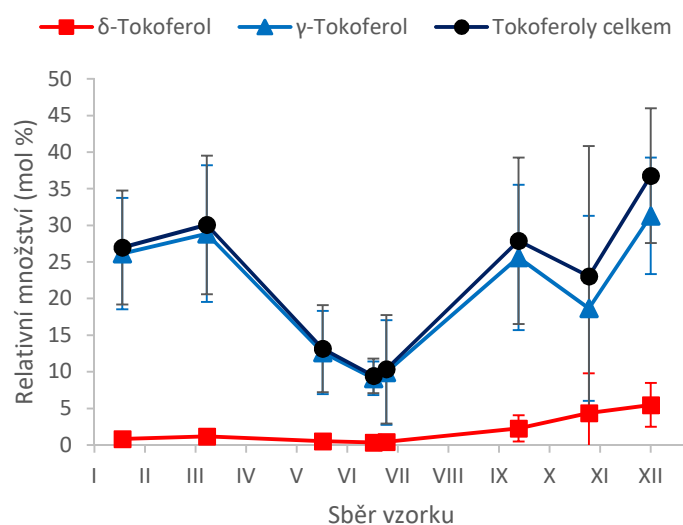
Obrázek 7: Sezonní změny obsahu sterolů ve svalových tkáních (A) a v tukové části (B) dospělců *Pyrrhocoris apterus*. Na ose x jsou jednotlivé měsíce roku, každý bod je průměrem ze čtyř nezávislých vzorků (každý vzorek obsahuje tkáň ze třech jedinců) [2].



(A)



(B)



Obrázek 8: Sezonní změny obsahu tokoferolů ve svalových tkáních (A) a v tukové části (B) dospělců *Pyrrhocoris apterus*. Na ose x jsou jednotlivé měsíce roku, každý bod je průměrem ze čtyř nezávislých vzorků (každý vzorek obsahuje tkáň ze třech jedinců) [2].

Hlavní potravou testovaného hmyzu jsou lipová semínka, avšak příležitostně se živí mrtvými bezobratlými živočichy, což vysvětluje přítomnost cholesterolu v jeho tkáních. V tukové tkáni bylo nalezeno vyšší množství sterolů než ve svalové části, což je v souladu s očekávanými výsledky, protože tuková tkáň slouží jako zásobárna lipidů, včetně sterolů [75, 76]. Tokoferoly představují minoritní, ale nezbytnou složkou buněčných membrán, jejich koncentrace u bezobratlých se pohybuje

mezi 0,1 – 1,0 mol% [77]. V této studii jsme pomocí nové metody detekovali nezvykle vysoké zastoupení  $\gamma$  a  $\delta$ -tokoferolů, a stejně jako v případě rostlinných sterolů, jejich vyšší hladiny byly nalezeny v tukových tkáních. Zdrojem těchto tokoferolů jsou patrně lipová semínka, kde jsou tyto dva tokoferoly hojně zastoupeny [2]. Nová metoda pro stanovení tokoferolů tak přispěla k lepšímu poznání sezonních sezónních metabolických změn v buněčných membránách modelového druhu hmyzu, ruměnice pospolné (*Pyrrhocoris apterus*).

## **3.2 Zkoumání reaktivity protických metabolitů s heptafluorbutylchlor-mravenčanem a jejich GC-MS analýza v moči.**

### **3.2.1 Úvod**

Lidská moč je komplexní biotekutina, která kromě vody, anorganických solí, močoviny, těkavých látek a organických sloučenin iontové povahy, obsahuje také velké množství malých polárních metabolitů s protickými funkčními skupinami. Obsah těchto látek v moči odráží celkový stav organismu. Jelikož je odběr moči neinvazivní, je analýza moči široce využívána v diagnostice a jsou vyvíjeny stále nové metody rozšiřující metabolomické stanovení. Mezi nejčastější patří techniky separační spojené s hmotnostní spektrometrií, které se zabývají cílenou metabolomickou analýzou i metabolickým profilováním [6, 78, 79].

V této studii byla vyvinuta nová GC-MS metoda pro cílené stanovení protických metabolitů v moči. Byly zde studovány reakční produkty a analytické vlastnosti více než 150 známých metabolitů, zejména s amino a karboxylovou skupinu. Vyvinutá metoda zahrnuje okamžitou konverzi disulfidických můstků na příslušné thioly pomocí *tris*-(3-hydroxypropyl) fosfinu (THP) následovanou derivatizací s HFBCF katalyzovanou pyridinem. Deriváty poté obsahují vysoce nepolární heptafluorbutylovou část a během reakce jsou současně extrahovány od isooktanové nemísitelné vrstvy (tzv. liquid-liquid mikroextrakce, LLME), kterou lze přímo analyzovat technikou GC-MS [3].

### **3.2.2 Experimentální část**

#### **3.2.2.1 Vzorky moči**

Vzorky moči byly získány od zdravých dospělých osob, které pravidelně navštěvovaly preventivní prohlídky ve Fakultní nemocnici v Ostravě. Informovaný souhlas byl získán od každého subjektu a jeho součástí bylo prohlášení účastníků, že netrpí žádnou zjevnou nemocí a poruchou metabolismu. Koncentrace kreatininu v moči byla měřena Jaffeho kolorimetrickou metodou [80], která se běžně používá v klinické laboratoři. Vzorky byly dodány v uzavřených plastových lahvičkách se známou koncentrací kreatininu, s informací o věku subjektu, skladovány při teplotě 4°C a zpracovány do tří dnů od obdržení.

#### **3.2.2.2 Chemikálie a reakční média**

Roztoky proteinových aminokyselin a další amino-karboxylové metabolity byly pořízeny od firmy Merck (Sigma-Aldrich, Praha, Česká Republika). 2-Methylcitronová kyselina (racemát) byla obdržena od firmy C/D/N Isotopes (Quebec, Kanada). Další méně běžné karboxylové kyseliny a konjugáty glycinu byly od Dr. D. Bruneta, oddělení Organické chemie, Universita Autónoma v Madridu (Madrid, Španělsko). Lyofilizovaná moč s certifikovanou hladinou diagnostických organických kyselin (ORG-1) byla získána od ERNDIM Foundation (<http://cms.erudimqa.nl>, Winterswijk, Holandsko). THP

redukční činidlo a isooktan byly koupeny od firmy Merck KGaA (Darmstadt, Německo). Heptafluorbutanol (HFBOH) byl od Fluorochem Ltd. (Derbyshire, Velká Británie). HFBCF byl připraven v Laboratoři analytické biochemie a metabolomiky (České Budějovice, Česká Republika) ve spolupráci s Dr. V. Pejchalem (Univerzita Pardubice, Česká Republika), které dodává firma Pragolab, s.r.o (Praha, Česká republika). Další organická rozpouštědla a chemikálie byly od firmy Merck (Sigma-Aldrich).

Umělá moč byla připravena dle protokolu [81] tak, že ve vodě byly rozpuštěny následující chemikálie o výsledné koncentraci: 1% močovina, 8 mmol/l kreatinin, 7 g/l NaCl a 3 g/l K<sub>2</sub>SO<sub>4</sub>. Vodný roztok redukčního činidla THP byl použit o koncentraci 0,5%, 5% zásobní roztok byl skladován v mrazničce.

Organické reakční médium obsahovalo směs isooktan-HFBOH-HFBCF v poměru 15 : 1 : 4. Katalytické médium obsahovalo 4% pyridinu v 1 mol/l vodném roztoku NaOH. Roztoky uvedených pracovních médií byly připravovány týdně.

### **3.2.2.3 Zásobní a pracovní roztoky**

Zásobní roztoky většiny standardů byly rozpuštěny v roztoku uhličitanu sodného o koncentraci 100 mmol/l. Výjimku tvořily proteinové aminokyseliny, homocystein a zkoumané aminy, ty byly rozpuštěny v HCl o koncentraci 50 mmol/l. 153 zkoumaných močových metabolitů bylo rozředěno ve vodě nebo v umělé moči na výslednou koncentraci 10 μmol/l.

Roztoky kyseliny 4-fenylmásečné a homofenylalaninu (tyto standardy byly použity jako vnitřní standardy) byly rozpuštěny ve vodném roztoku uhličitanu sodného o koncentraci 100 mmol/l a výsledná koncentrace byla 200 μmol/l.

Pracovní roztoky močových metabolitů o různých koncentracích byly připraveny odpovídajícím ředěním roztoků zásobních. Zásobní roztoky byly ředěny umělou močí.

### **3.2.2.4 Vybavení**

Skleněné zkumavky o rozměrech 6 x 50 mm (Kimble-Kontes, Vineland, NJ) se používaly pro derivatizaci moči, nastavitelné pipety (50 – 100 μl rozsah objemu) se skleněnými kapilárami byly použity pro pipetování organické fáze s reakčními činidly a byly od firmy Merck (Darmstadt, Německo), pro odebrání organické fáze po derivatizaci se používaly 25 mm špičky na pipety od VWR International (Praha, Česká Republika). Reakční směs byla míchána na míchače REAX 1 od Heidolph (Schwabach Německo) a nemísitelné fáze po derivatizaci byly odděleny na centrifuze od firmy Labmark (Praha, Česká Republika).

### **3.2.2.5 Příprava vzorků**

Do skleněné zkumavky bylo nadávkováno 25 μl moči a postupně bylo přidáváno 25 μl roztoku obsahující vnitřní standardy, 25 μl 0,5 % THP redukčního činidla a po promíchání se zkumavka nechala stát 1 minutu (nebo déle). Dále pak bylo postupně přidáno 50 μl reaktivního organického media a

25 µl katalytického media a obsah byl zamíchán po dobu 2-3 sekund. Po dalším přidání 25 µl katalytického media byl obsah zkumavky míchán po dobu cca 5 sekund, dokud se nerozdělená mléčně zabarvená organická fáze nevyčeřila zpět do dvou fází. Na konec bylo přidáno 50 µl isooktanu a 25 µl HCl o koncentraci 1 mol/l, směs byla míchána po dobu 2-3 sekund a po krátké centrifugaci (3000 rpm) byla odebrána horní organická fáze do autosamplerové vialky. 1 µl vzorku byl nadávkován do GC-MS systému.

U některých analytů po reakci s HFBCF nevznikly očekávané produkty, popřípadě byl sledován kromě očekávaného derivátu vznik dalšího produktu. Pro objasnění těchto struktur byla měřena každá z problematických látek zvlášť a byla využita i LC-MS analýza. Pro LC-MS byla úprava vzorku stejná jako u metodiky GC-MS, pouze byl vynechán poslední krok, tj. přidání 1 M HCl, odebraná organická fáze byla odpařena do sucha pod proudem dusíku a odparek znovu rozpuštěn v mobilní fázi. Po objasnění reakčních produktů po derivatizaci s HFBCF byly výsledky konzultovány s literaturou, hlavně s databází HMDB (Human Metabolome Data Base).

Pro kalibraci byla skupina metabolitů rozdělena do čtyř skupin a byla připravena pětibodová kalibrační přímka. Každá z kalibračních hladin L1, L2, L3, L4 a L5 byla proměřena třikrát. Hladina L1 (L2) odpovídala 10 krát (2,5 krát) naředěné hladině L3, hladina L4 (L5) byla 2,5 krát (10 krát) koncentrovanější, než hladina L3. Pro několik metabolitů byla měřena ještě hladina L6 (25 krát vyšší koncentrace než u hladiny L3), aby byly pokryty i náhodně se vyskytující vyšší koncentrační hladiny, zejména při výskytu diagnostických dědičných metabolických poruch.

### **3.2.2.6 GC-MS analýza reakčních produktů**

Plynový chromatograf 7890A spojený s hmotnostním spektrometrem 5975C (Agilent Technologies, Santa Clara, CA) byl využit pro GC-MS analýzu. Separace analytů probíhala na kapilární koloně ZB-XLB (30 m × 0,25 mm × 0,25 µm) (Phenomenex, Torrance, CA). Průtok He byl nastaven na 1,2 ml/min a teplotní program byl následující: od 60°C do 240°C po 5°C/min, a poté do 320°C po 20°C/min (celkový čas analýzy byl 38 minut). Teploty EI iontového zdroje, kvadrupólu a GC-MS rozhraní byly 230, 150 a 250°C. EI energie byla 70 eV. Teplota injektoru byla nastavena na 220°C, objem vzorku dávkovaný na kolonu ve splitless módu byl 1 µl. EI spektra derivatizovaných produktů byla studována po měření ve full-skenu v rozsahu 40 – 1050 Da (skenovací rychlost = 2,7 skenů/s).

Pro metabolické profilování a cílenou kvantifikaci byl měřen také full-sken ale v rozsahu 53 – 600 Da (4,2 skenů/s) nebo takzvaný SIM mód (selected ion monitoring mód), při kterém se sledovaly dva diagnostické fragmentové ionty. SIM parametry byly následující: 35 časových úseků, maximum 12 iontů ve skupině, elektronový násobič měl napětí 1318 V. Pro vyhodnocení dat byl použit program MSD ChemStation (verze E.02, Agilent).

Pro objasnění nečekaných struktur bylo také využito chemické ionizace v pozitivním módu (PICI). Zde byl použit plynový chromatograf spojený s kvadrupólovým hmotnostním spektrometrem Trace DSQ vybaveným chemickou ionizací (Thermo Fisher Scientific, San Jose, USA). Vyšetřované metabolity byly dávkovány ve splitless módu, objem 1  $\mu$ l, při teplotě 240°C. GC-kolona byla Agilent VF-1MS, 30 m  $\times$  0,25 mm  $\times$  0,25  $\mu$ m (HPST s.r.o., Praha, Česká Republika). Teplotní program byl následující: 50°C bylo drženo po 1,5 minuty, a pak byla teplota zvyšována po 20°C/min do 170°C a pak po 30°C/min do 300°C a držena po 1 minutu. Průtok helia byl 1,1 ml/min, iontový zdroj byl zahříván na 180°C a GC-MS rozhraní na 250°C. PICI spektra byla měřena v hmotnostním rozsahu 90 – 1150 Da, 2,5 skenů/s. Jako ionizační plyn byl použit isobutan, jehož průtok byl 1,5 ml/min. Pro zpracování dat byl použit software Xcalibur (verze 2.1, Thermo Fisher Scientific).

### **3.2.2.7 LC-MS analýza reakčních produktů**

Struktura některých derivátů byla ověřena pomocí LC-MS. Byl použit kapalinový chromatograf Accela 600 spojený s LTQ-XL hmotnostním spektrometrem (Thermo Fisher Scientific, San Jose, USA). Pro separaci byla použita kolona Kinetex C 18, 150  $\times$  2,1 mm, 5  $\mu$ m (Phenomenex, Torrance, USA), která byla temperována na 25°C. Mobilní fáze byla namíchána z methanolu s vodou obsahující 5 mM mravenčan amonný (pH = 4,5). Počáteční koncentrace methanolu byla 30 obj. % a byla lineárně zvyšována do 100 obj. % během 10 minut a poté držena na 100 % po dobu 3 minut. Průtok mobilní fáze byl 250  $\mu$ l/min a injektovaný objem byl 5  $\mu$ l. Elektrosprej v pozitivním módu byl nastaven následovně: teplota kapiláry 275°C, desolvatační teplota 150°C, zdrojové napětí 4 kV, napětí na kapiláře 40 V, dusík byl použit jako sušící plyn. Pro zpracování dat byl použit software Xcalibur (verze 2.1, Thermo Fisher Scientific).

## **3.2.3 Výsledky a diskuse**

### **3.2.3.1 Reakční produkty metabolitů po derivatizaci s HFBCF**

Soubor metabolitů byl vybrán na základě studia současné literatury a metabolických databází. Je uveden v tabulce S.2. Jedná se hlavně o aminokyseliny (56 aminokyselin a jejich konjugátů) a karboxylové kyseliny (86), biogenní aminy (9) a dva interní standardy (kyselina 4-fenylmásečná a homofenylalanin). Reakční produkty každého analytu (1 – 10 nmol) byly studovány ve vodném prostředí a v umělé moči a jejich EI spektra a PICI hmotnostní spektra byla prozkoumána. Když reakční produkt nebyl pozorován nebo byl přítomen v nízkém výtěžku, organická fáze byla odpařena, obsah byl znovu rozpuštěn v mobilní fázi a změřen na LC-MS. Navrhované struktury pro všechny zkoumané analyty jsou uvedeny v tabulce S.2. U většiny testovaných metabolitů docházelo ke vzniku jednoho definovaného derivátu a tento derivát poskytoval jeden pík v TIC GC-MS chromatogramu (120 analytů, 78 %).

Heptafluorbutyl ester kyseliny pyrohroznové (analyt číslo 1) byl detegován, ale jeho diagnostický fragment  $m/z$  43 byl překryt vysokým EI signálem píku rozpouštědla. Relativní TIC EI odezva pozorovaných minoritních vedlejších produktů, většinou s neúplně zreagovanými funkčními skupinami, byla menší než 4 % ve srovnání s píky očekávaných majoritních reakčních produktů. Méně reaktivní hydroxylové skupiny ve 3-hydroxykarboxylových kyselinách (metabolity číslo 4, 9, 24 a 79), hydroxyaminokyselinách (threonin, číslo 57, hydroxyprolin, číslo 89 a 94) a histidinu (číslo 114), zůstaly částečně nezreagované a vznikaly dva produkty, v tabulce označeny s příponou *a* a *b*.

Funkční skupiny cystinu (číslo 137) a homocystinu (číslo 145) byly pomocí HFBCF derivatizovány kompletně. Disulfidické můstky v obou aminokyselinách byly redukovány a vzniklý cystein a homocystein byl derivatizován. Tudíž změřený obsah cysteinu (resp. homocysteinu) odpovídal sumě cystin-cystein (resp. homocystin-homocystein).

U biogenních aminů putrescinu (číslo 105a) a kadaverinu (číslo 123a) kromě předpokládaného karbamátu vznikal ještě vedlejší produkt – isokyanát-karbamát (čísla 105b a 123b). V případě kyseliny 2-ketoglutarové (číslo 42a, 42b) a lysinu (číslo 119a, 119b) byly pozorovány dva izomery. U některých acylovaných aminokyselin, N-acetylglycinu (číslo 37), N-hexanoylglycinu (číslo 39) a kyseliny hippurové (číslo 78) docházelo k cyklizaci za současné ztráty vody.

U polyfunkčních organických kyselin (číslo 21, 60 a 68) nebyly výtěžky hlavních vznikajících produktů úplné. Na příklad u kyseliny citronové, která obsahuje tři stericky stíněné karboxylové skupiny a hydroxy skupinu, dochází ke vzniku tří produktů. Jedním z nich je kyselina *cis*-akonitová (číslo 60c). U glutaminu kromě očekávaného derivátu dochází ke vzniku derivátů pyroglutamové (číslo 69) a glutamové kyseliny (minoritně).

Poslední studovanou skupinou jsou metabolity číslo 147-155, které reagovaly s činidlem HFBCF málo, nebo omezeně. Kyselina mevalonová (číslo 147) neposkytuje vyhovující GC pík. Jak ukázala pozdější LC analýza, dochází u ní pouze k esterifikaci karboxylové skupiny a 3- a 5-hydroxy skupina se neacyluje. Podobně, amino-karboxylové funkční skupiny u citrulinu (číslo 148) a argininu (číslo 149a) jsou derivatizovány pouze v malé míře, přičemž guanidinová (u argininu) a ureido skupina (u citrulinu) zůstávají nezreagované. LC-MS analýza prokázala, že guanidinová skupina částečně zreaguje (číslo 149b) avšak je tak silně polární a/nebo termo labilní, že produkt argininu není na GC-MS detekovatelný. LC-MS analýza také ukázala, že močovina (číslo 151) a kyselina močová (číslo 152), které jsou v moči obsaženy ve vysoké koncentraci, s HFBCF nereagují a nepřecházejí do organické fáze během LLME.

Kyseliny 3,4-dihydroxyfenyloctová a 3,4-dihydroxymandlová (číslo 153 – 154) se ukázaly jako částečně labilní. Kyselina oxaloctová (číslo 155) se rychle rozkládá ve vodném prostředí [82].

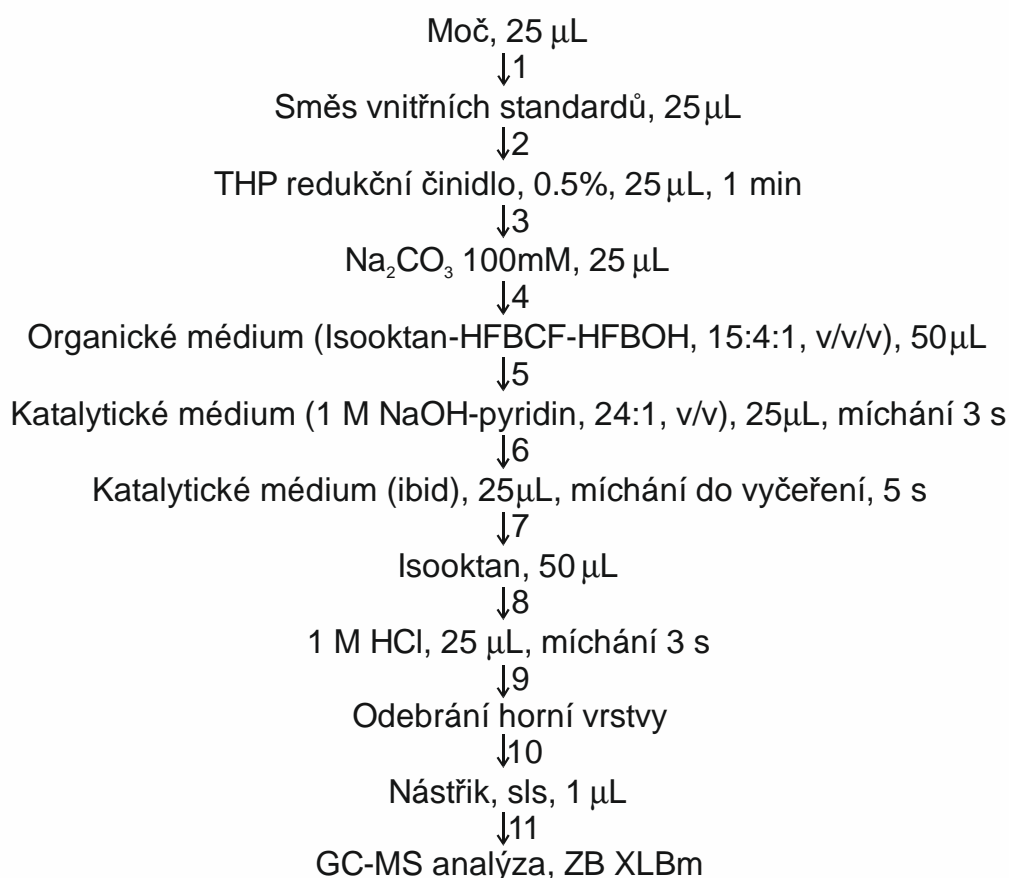
Biogenní aminy metanefrin (číslo 136) a normatanefrin (číslo 140) poskytovaly nízké reakční výtěžky a jsou v moči ve velmi nízké koncentraci, tudíž nebyly do dalšího studia zahrnuty.

### 3.2.3.2 Příprava vzorku moči ke GC-MS analýze

Nový postup zahrnuje redukci thiolových vazeb pomocí THP, úpravu pH, derivatizaci metabolitů pomocí směsi HFBCF-isooktan (organické medium) a NaOH-pyridin, (katalytické medium, přidává se ve dvou krocích z důvodů zlepšení výtěžku reakce), LLME, odstranění nečistot pyridinu pomocí HCl, odebrání horní organické vrstvy do autosamplerové vialky a GC-MS analýzu, viz Obr. 9

Pokud není zapotřebí stanovovat cystin a homocystin, je možné vynechat přidání THP. Pracovní postup pak dále zůstává beze změn. Během reakce dochází ke vzniku tří fází: 1) horní organická fáze, která obsahuje deriváty metabolitů, 2) dolní vodná fáze, která obsahuje pyridinové soli a další anorganické látky a za 3) ke vzniku oxidu uhličitého, který vzniká při rozkladu HFBCF během reakce.

Příprava vzorku je jednoduchý proces, při kterém je zapotřebí pouze 25  $\mu\text{L}$  moči. Vyvinutý proces je rychlejší, méně pracný a pokrývá širší škálu močových metabolitů, než dříve popsané techniky využívající RCF [83, 84].



Obr. 9: Postup přípravy vzorku pro GC-MS profilování protických metabolitů v lidské moči.



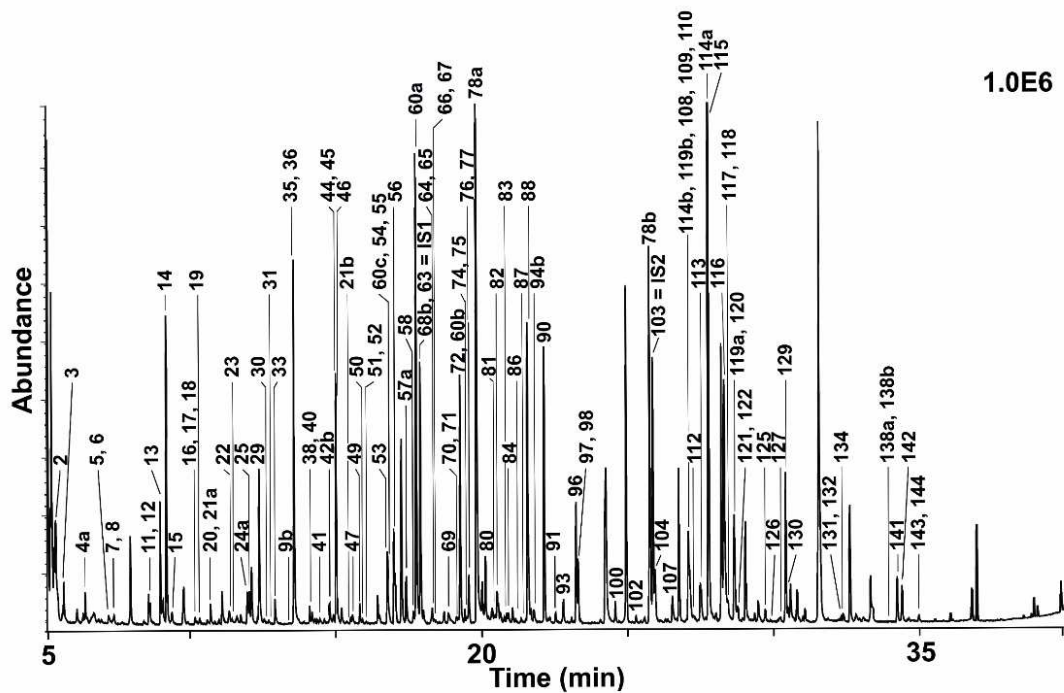
### 3.2.3.3 Kvantitativní stanovení metabolitů v lidské moči

Podrobné studium EI a PICI spekter zkoumaných metabolitů ukázalo jejich užitečnost při strukturní analýze metabolitů a množství diagnostických fragmentových iontů vhodných pro kvantitativní analýzu, viz Tab.1 v publikacích [3, 4].

Typický chromatografický záznam GC-MS analýzy směsné moči (smícháno 10 ženských a 10 mužských vzorků moči získaných od zdravých dobrovolníků) po současné derivatizaci s HFBCF a LLME je na obrázku 10. Metoda byla finálně upravena na GC-MS analýzu v SIM modu. K vývoji metody bylo použito 50 ženských a 50 mužských vzorků moči obdržených od zdravých jedinců. Kalibrace byly provedeny v umělé moči s pomocí 2 interních standardů. Bylo by žádoucí použít strukturně podobný (nejlépe izotopicky značený) interní standard pro každá analyt, ale při takto vysokém množství analytů je tento model těžko realizovatelný. Proto byly vybrány a pro kvantifikaci většiny organických a amino-karboxylových kyselin použity dva interní standardy, kyselina 4-fenylmásečná (číslo 63) a homofenylalanin (číslo 103). Kalibrační závislosti byly proměřeny v rozmezí třech řádu okolo střední hladiny L3. Kalibrační data zahrnují parametry kalibrační závislosti, koeficient determinace  $R^2$ , mez detekce LOD a spodní mez stanovení LLOQ (hladina L1), viz. kalibrační data v tabulce 3 v práci [3].

Koncentrace metabolitů naměřené v moči byly porovnány s publikovanými daty uvedenými v HMDB databázi. Nalezené hodnoty s hodnotami v HMDB databázi dobře korelují a je patrné, že 25  $\mu\text{l}$  moči je plně dostačující ke kvantifikaci 112 metabolitů v koncentračním rozsahu 0,2 – 400  $\mu\text{mol}/\text{mmol}$  kreatininu. Dvacet validovaných metabolitů nebylo v moči detekováno, jejich přítomnost v moči byla pod limitem detekce metody. Nicméně, při některé metabolické poruše může dojít ke zvýšení koncentrační hladiny některého z metabolitů a pak by příslušný metabolit mohl být kvantifikován. Kyselina hippurová (číslo 78), kyselina citronová (číslo 60) a histidin (číslo 114) byly detekovány ve vyšší koncentraci, než 100  $\mu\text{mol}/\text{mmol}$  kreatininu. V reakčním mediu je takový nadbytek derivatizačního činidla (10  $\mu\text{l}$ , tj. přibližně 60  $\mu\text{mol}$ ), že pro objem moči 25  $\mu\text{l}$  není problém derivatizovat všechny metabolity i ve vyšších koncentračních hladinách.

Popsané výsledky dokazují, že vyvinutá metoda je vhodným nástrojem pro cílenou GC-MS analýzu protických metabolitů v lidské moči.



Obrázek 10: TIC chromatogram (full scan) močových metabolitů ve směsné moči (smícháno 20 vzorků moči, 10 mužských a 10 ženských).

### **3.3 Stanovení biomarkerů expozice průmyslovým látkám pomocí alkylchlormravenčanů a jejich kvantifikace v lidské moči technikou GC-MS**

#### **3.3.1 Úvod**

Lidé jsou ve svém životním i pracovním prostředí exponováni velkým množstvím chemických látek, které jim mohou být potenciálně zdraví škodlivé. Je odhadováno, že člověk je během svého života vystaven 1 – 3 milionům xenobiotik [85]. Je proto nutno míru expozice kontrolovat a udržet v přijatelné výši, k tomu je třeba mít k dispozici vhodné analytické postupy. Existují dva základní analytické přístupy: v prvním případě – stanovení škodlivin přímo ve vnějším prostředí anebo tzv. biologické monitorování. Biologické monitorování znamená stanovení chemických polutantů nebo jejich metabolitů přímo v organismu nebo v definované biotekutině (nejčastěji v krvi či moči). V oboru hygieny práce je biologické monitorování uznávaným nástrojem pro hodnocení profesionálních expozic. Pro několik desítek chemických škodlivin byly zavedeny tzv. biologické limity, hladiny biomarkerů v biologickém materiálu (nejčastěji moči), jejichž nepřekračování by mělo zaručit ochranu zdraví pracujících osob při každodenní 8 hodinové expozici. Biologické limity průmyslových polutantů jsou zavedeny v mnoha státech. Mezi nejvíce respektované patří limity uvedené v American Conference of Governmental Industrial Hygienists (ACGIH) [86] a Germany Deutsche Forschungsgemeinschaft (DFG) [87]. Tyto organizace prezentují biologické limity pod specifickými názvy: ACGIH – Biological exposure indices (BEI) a DFG – Biologische Arbeitsstoff-Toleranz-Werte (BAT). Porovnání BEI a BAT biologických limitů pro vybrané metabolity, kterými se zabývá tato studie, je uvedeno v Tabulce 8.

Mnoho významných organických průmyslových škodlivin (rozpouštědla, monomery) je z organismu vylučováno močí ve formě karboxylových kyselin, které lze stanovit jako biomarkery expozice. Mezi nejčastěji sledované biomarkery patří metabolity benzenu, toluenu, styrenu, xylenu a alkoxyethanolů. Jejich metabolické dráhy jsou stručně uvedeny na Obr. 11 a 12. Biologické limity uvedených biomarkerů se pohybují v rozmezí od desítek  $\mu\text{g}$  do tisíců  $\text{mg}$  na litr moči, resp. na g kreatininu (viz. Tabulka 8).

Tabulka 8: Vybraná xenobiotika, jejich metabolity a porovnání biologických limitů.

Xenobiotikum	Metabolit	Zkratka	Biologický limit	
			Hodnota	Zdroj
benzen	kyselina <i>t,t</i> -mukonová	<i>t,t</i> -MA	500 µg/g kreat.	ACGIH
	kyselina <i>S</i> -fenylmerkapturová	<i>S</i> -PMA	25 µg/g kreat.	ACGIH
toluen	kyselina hippurová	HA	1600 mg/g kreat.	DFG, ACGIH
	kyselina <i>S-p</i> -tolylmerkapturová	<i>S</i> -TMA	-	-
	kyselina <i>S</i> -benzylmerkapturová	<i>S</i> -BMA	-	-
<i>o-,m-,p</i> -xyleny	kyselina <i>o-,m-,p</i> -methylhippurová	MHA	2000 mg/L moči*	DFG
styren	kyselina mandlová	MA	400 mg/g kreat.**	DFG
	kyselina fenylglyoxylová	PGA	600 mg/g kreat.**	ACGIH
alkoxyethanoly	kyselina methoxyoctová	MAA	1 mg/g kreat.	ACGIH
	kyselina ethoxyoctová	EAA	100 mg/L moči	ACGIH
			50 mg/L moči	DFG
	kyselina butoxyoctová	BAA	150 mg/g kreat.	DGF
sirouhlík	kyselina 2-thiothiazolidin-4-karboxylová	TTCA	0.5 mg/g kreat.	ACGIH
fural	kyselina furan-2-karboxylová	FCA	200 mg/L moči	ACGIH
<i>N,N</i> -dimethylformamid	kyselina <i>S</i> -( <i>N</i> -methylkarbamoyl)merkapturová	AMCC	40 mg/L moči	ACGIH

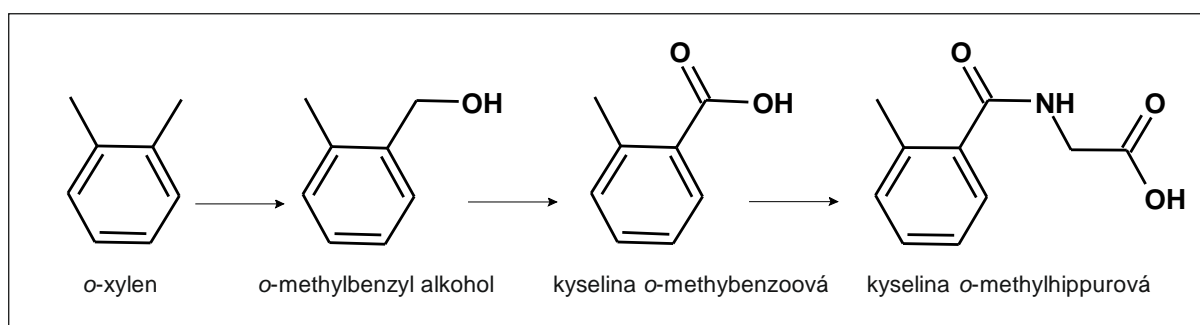
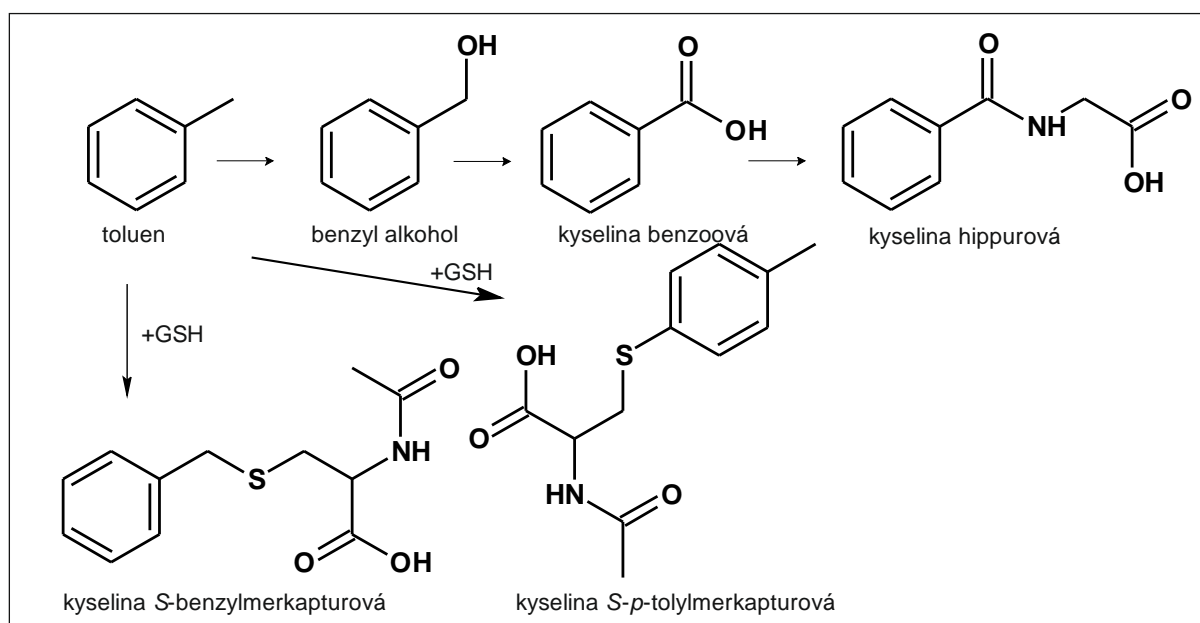
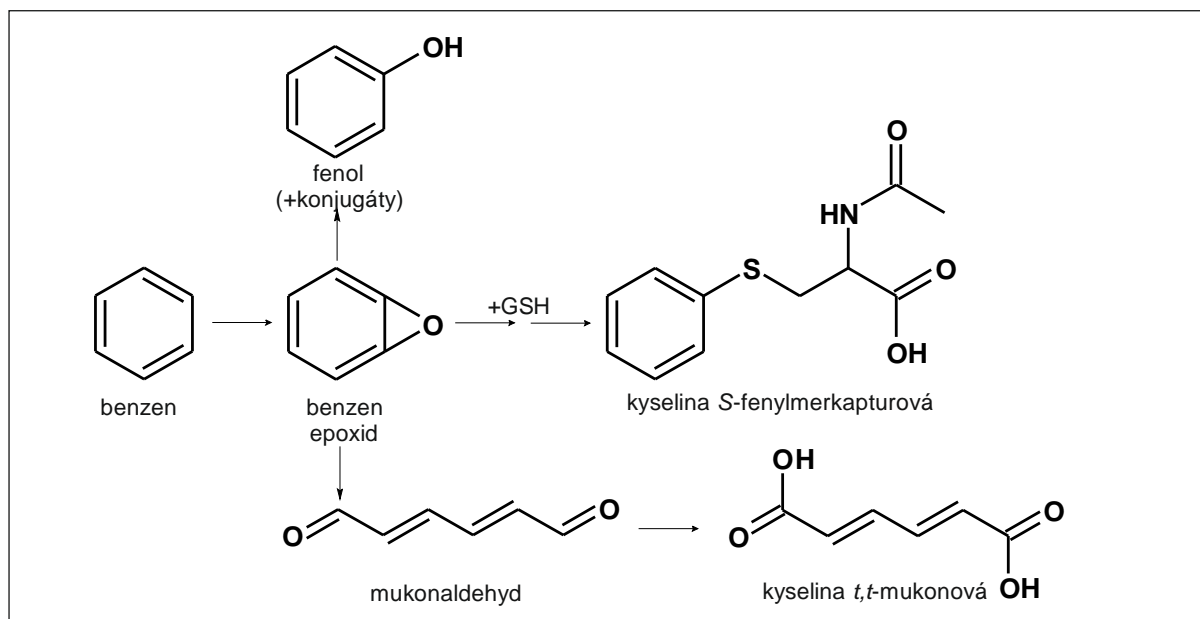
\* = suma kyselin methylhippurových, \*\* = suma kyseliny fenylglyoxylové a mandlové, kreat. = kreatinin

ACGIH = American Conference of Governmental Industrial Hygienists [86], DFG = Germany Deutsche Forschungsgemeinschaft [87]

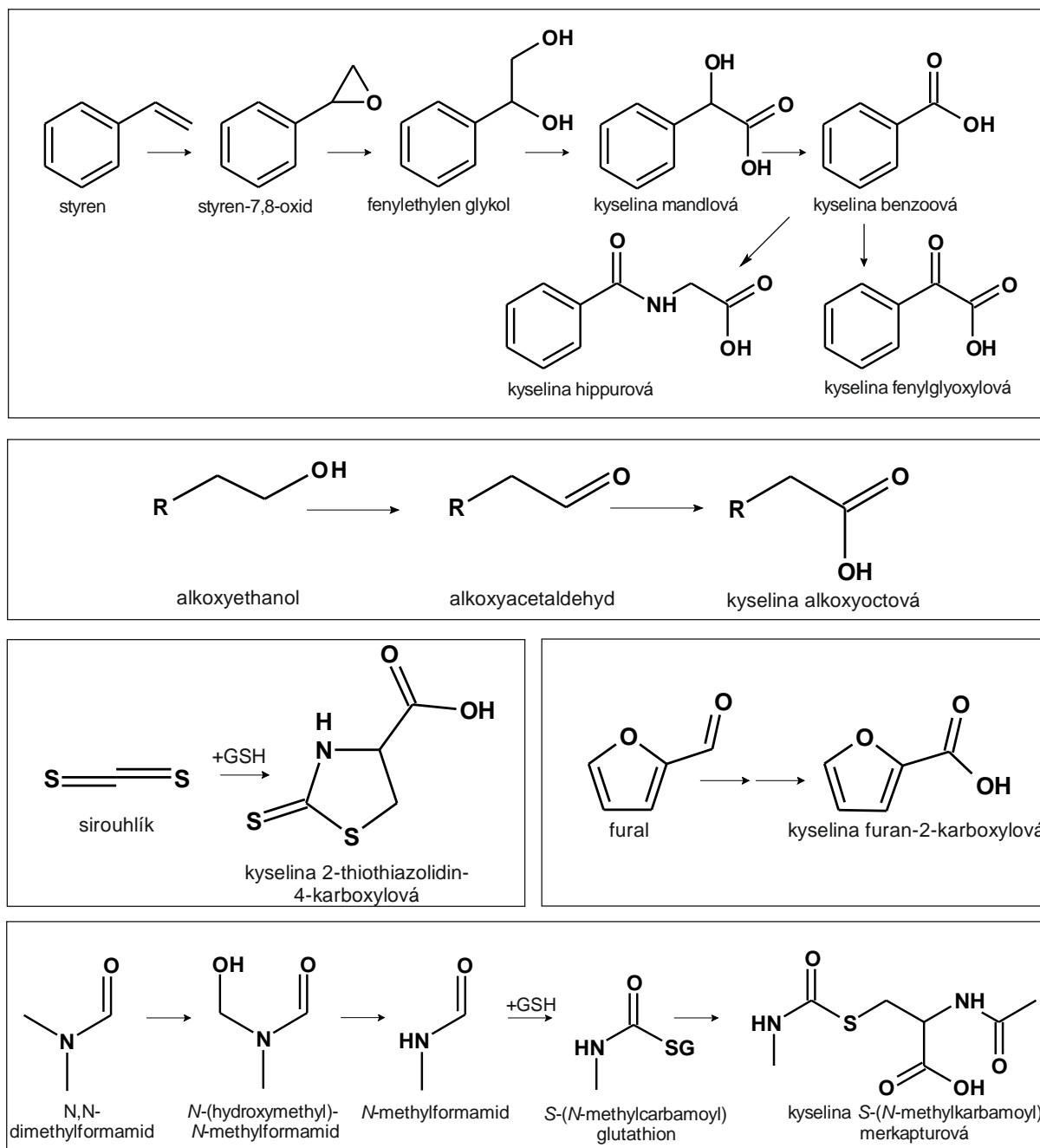
V současné době jsou dominantními metodami pro stanovení uvedených biomarkerů v moči techniky HPLC a GC, většinou dnes již v kombinaci s MS detekcí. Volné kyseliny lze stanovit pomocí HPLC [88]. GC-MS stanovení vyžaduje při přípravě vzorku použití některého z derivatizačních postupů; nejčastěji se používá methylace [89 – 94] nebo silylace [95 – 98]. Mezi novější práce, které se zabývají touto problematikou, patří například studie stanovení *S*-PMA v lidské moči pomocí HPLC-MS/MS [99 – 101] nebo metody stanovující v lidské moči *t,t*-MA [102, 103]. Stále se hledají nové postupy, které zjednodušují a zrychlují analýzu hledaných xenobiotik, např. Zhao a kolektiv [104] stanovují HA v lidské moči pomocí iontové chromatografie s vodivostní detekcí, nebo Cosnier a kol. [105] popsali stanovení MA, PGA a HA v myši moči pomocí GC-FID po předchozí derivatizaci s 2-propanolem v HCl. Metodu stanovení HA a kreatininu za suché skvrny moči pomocí HPLC/UV popisuje studie Autunese a kol. [106], čímž odpadá transport sbírané moči. Pro stanovení *t,t*-MA v lidské moči [107] byla použita SPE na speciálním sorbentu s následnou derivatizací extraktu pomocí ECF a GC-MS analýzou.

Zavedené normy v rámci hygienického dozoru obvykle vyžadují stanovení (předem známé) dominantní škodliviny. Vypracované pracovní postupy byly proto optimalizovány pro stanovení jednotlivých biomarkerů, i když expozice může nastat několika škodlivinám současně. Laboratoře tak musí udržovat a periodicky validovat řadu analytických metod.

Jako perspektivní alternativa se nabízí stanovení co největšího počtu biomarkerů jednotným analytickým postupem – tzv. profiling. To bylo motivací k systematickému studiu reakcí souboru biomarkerů uvedených v Tabulce 8 s RCF a jejímu využití k současnému stanovení 16 hlavních biomarkerů expozice v moči v programech biologického monitorování.



Obrázek 11: Metabolismus expozice benzenu [108], toluenu [109] a *o*-xylenu [110] v lidském organismu. GSH = glutathion.



Obrázek 12: Metabolické dráhy expozice styrenu [111], alkoxy ethanolů (R = 2-methoxy, 2-ethoxy a 2-butoxy) [112], sirouhlíku [113], furalu a *N,N*-dimethylformamidu v lidském organismu. GSH = glutathion

### 3.3.2 Experimentální část

#### 3.3.2.1 Chemikálie

Pyridin, kyselina 2-fenylmáslná, 2-fenoxyoxazol-5(4*H*)-on, kyselina chlorovodíková, roztok kyseliny chlorovodíkové v sušeném ethanolu (koncentrace 1,25mol/l), kyselina mravenčí, ethanol a mravenčan amonný byly pořízeny od firmy Merck (Praha, Česká republika). ECF, chloroform, <sup>13</sup>C<sub>2</sub>-

ethanol, D<sub>3</sub>-ethanol, benzylmerkaptan, kyselina adipová-1,6-<sup>13</sup>C<sub>2</sub> (AdA-1,6-<sup>13</sup>C<sub>2</sub>), kyselina pentafluorfenoxyoctová (PFPA), kyselina *S-p*-fluorfenylmerkapturová (*S*-FPMA) a standardy 16 organických kyselin uvedených v tabulce 8 byly pořízeny od firmy Sigma-Aldrich (Praha, Česká republika), hydroxid sodný od Lachemy (Brno, Česká republika). Methanol a acetonitril byly od firmy Fisher Scientific (Pardubice, Česká republika). Kyselina *D*-azetidin-2-karboxylová byla koupena od Calbiochem (Los Angeles, USA). Sirovodík byl vyroben v Kippově přístroji. D<sub>3</sub>-ethylchloromravenčan (D<sub>3</sub>-ECF) a <sup>13</sup>C<sub>2</sub>-ethylchloromravenčan (<sup>13</sup>C<sub>2</sub>-ECF) byly připraveny ve spolupráci s Dr. V. Pejchalem (Univerzita Pardubice, Česká Republika).

Lidská moč, použitá na testování a následnou validaci metody, byla obdržena od zdravých dobrovolníků. Kontrolní moč IP45, která obsahovala známé množství MAA, EAA, BAA, PGA *t,t*-MA a všechny izomery MHA, byla získána od Prof. Dr. Med. H. Drexlera (Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine of the Friedrich-Alexander-University Erlangen-Nuremberg, Německo), referenční materiál RM6009, obsahující certifikované množství PGA a MA, a dále moč dobrovolníka, který byl vystaven expozici ethoxyethanolem, byly dodány Dr. Jaroslavem Mrázem (Státního zdravotnický ústav, Praha, Česká Republika).

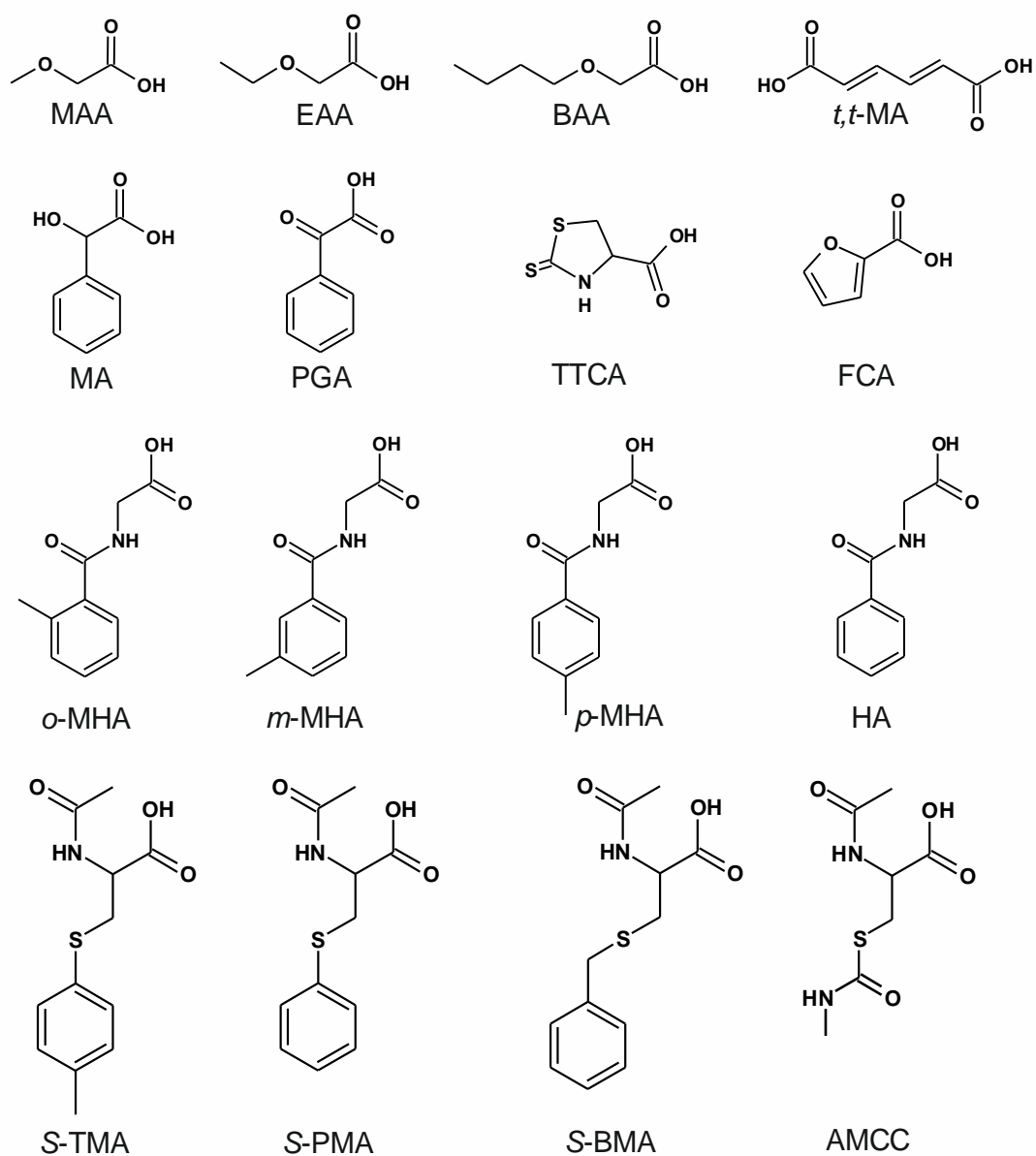
### 3.3.2.2 Studium reakce sledovaných biomarkerů s RCF

Byly připraveny roztoky standardů stanovovaných kyselin o finální koncentraci 0,01 mol/l a roztoky vnitřních standardů také o koncentraci 0,01 mol/l. Standardy byly rozpuštěny v destilované vodě. Přehled struktur stanovovaných látek je na Obr. 13.

Jako derivatizační činidlo byl použit ECF. Pro identifikaci vedlejších produktů byla využita reakce s izotopicky značeným ECF (D<sub>3</sub>-ECF a <sup>13</sup>C<sub>2</sub>-ECF).

Derivatizační postup byl následující: 100 µl moči (nebo vody) s 1 µl každého ze standardů (o koncentraci 0,01 mol/l) bylo smícháno se 75 µl směsi ethanol (D<sub>3</sub>-ethanol, <sup>13</sup>C<sub>2</sub>-ethanol) – pyridin (4 : 1, v/v) a reakční směs byla krátce promíchána. V dalším kroku bylo přidáno 75 µl směsi obsahující chloroform – ECF (D<sub>3</sub>-ECF, <sup>13</sup>C<sub>2</sub>-ECF) (9 : 1, v/v), pak 75 µl hydroxidu sodného o koncentraci 1,5 mol/l a po zamíchání znovu 75 µl směsi chloroform – ECF (D<sub>3</sub>-ECF, <sup>13</sup>C<sub>2</sub>-ECF) (9 : 1, v/v). Posledním krokem bylo přidání 75 µl kyseliny chlorovodíkové o koncentraci 3 mol/l a po promíchání byla odebrána část dolní organické vrstvy do vialky a pak 1 µl injektován do GC-MS.





Obrázek 13: Přehled struktur všech vyšetřovaných analytů.

### 3.3.2.3 Syntéza ethylesterů *o*-MHA, S-BMA a S-TMA

100 mg *o*-MHA (S-BMA, S-TMA) bylo naváženo do 5 ml skleněné vialky, byl přidán 1 ml reakčního media, tj. 1,25 M roztok kyseliny chlorovodíkové v sušeném ethanolu a směs byla míchána po dobu 5 hodin. Po reakci bylo rozpouštědlo odpařeno proudem dusíku a produkt byl znovu rozpuštěn v 1 ml chloroformu. Tato směs byla 3 krát extrahována proti 1 ml 0,1 M roztoku hydroxidu sodného a 3 krát proti 1 ml destilované vody. Poté bylo rozpouštědlo znovu odpařeno do sucha a výsledný produkt byl zvážen. Jeho čistota byla ověřena pomocí LC-MS analýzy (viz dále).

#### **3.3.2.4 LC-HRMS analýza**

LC-HRMS analýza byla využita pro zjištění elementárního složení derivátů, pro objasnění struktury vedlejších produktů reakce a pro kontrolu čistoty syntetizovaných produktů. Tyto analýzy byly provedeny na Q-Exactive hmotnostním spektrometru s vysokým rozlišením přímo spojeným s Dionex Ultimate 3000 a Dionex autosamplerm (Thermo Fisher Scientific, USA). K ionizaci byl použit elektrosprej v pozitivním módu s nastavením: zdrojové napětí 3.0 kV, teplota sprejovací kapiláry 350°C, sušící plyn 40 au, desolvatační teplota 350°C. Hmotnostní spektrometr byl kalibrován na hmoty 279,15909 Da a 391,284429 Da. Pro vyhodnocení a zpracování dat byl využit Xcalibur Core software (verze 2,0). Chromatografická separace byla provedena na koleně Kinetex (150mm x 3,0 mm i. d., 2,6 µm, Phenomenex, USA), gradient mobilní fáze byl: 0 min, 30 % A + 70 % B; 10.0 min, 100 % A; 11.0 min, 100 % A; 11.1 min, 30 % A + 70 % B; 14.5 min, 30 % A + 70 % B; přičemž A obsahoval 5 mmol/l mravenčan amonný v methanolu a B obsahoval 5 mmol/l vodný roztok mravenčanu amonného, průtok mobilní fáze byl 400 µl/min, dávkovací smyčka o objemu 5 µl, teplota kolony 35°C, teplota v autosampleru 10°C.

#### **3.3.2.5 GC-MS analýza**

GC-MS DSQ hmotnostní spektrometr (Thermo Electron, San José, CA, USA) vybavený elektronovou (EI) i chemickou (CI) ionizací a injektorem s programovatelnou teplotou (PTV) byl použit pro analýzu derivatizovaných vzorků. Xcalibur Core software, verze 2.0, sloužil k vyhodnocení a zpracování dat, na kolonu byl dávkován 1 µl vzorku ve splitless modu. Teplota injektoru byla nastavena na 50°C a po 0,25 minutě zvyšována po 10°C/s na výsledných 200°C. Kapilární chromatografická kolona Varian VF-17ms, 30 m x 0,25 mm i. d., s tloušťkou filmu 0,15 µm (Agilent, Middelburg, Nizozemí) měla počáteční teplotu 50°C po dobu 2 min, teplota byla zvyšována po 15°C/min do 170°C a pak po 35°C/min do výsledných 310°C. Tato teplota byla držena po dobu 1 min. Nosným plynem bylo helium o průtoku 1,2 ml/min, iontový zdroj měl teplotu 200°C a GC-MS rozhraní 250°C. Detekce probíhala v EI modu (70 eV) ve skenovacím rozsahu 40 – 400 Da. Ve validované metodě byl pak využit režim SIM, kde byly sledovány charakteristické diagnostické ionty pro každou látku. Pro potvrzení struktury vedlejších produktů reakce byla využita CI detekce v pozitivním módu, kde se měřilo ve skenovacím rozsahu 100 – 560 Da, jako ionizační plyn byl uplatněn isobutan o průtoku 1,6 ml/min, teplota iontového zdroje byla 160°C a teplota GC-MS rozhraní 250°C.

#### **3.3.2.6 Stanovení biomarkerů expozice v moči a validace metody**

Byla připravena tzv. kontrolní moč, kde bylo smícháno několik vzorků moči, které byly získány od zdravých dobrovolníků. Dle doporučení [58] byly validovány následující parametry: kalibrační závislost, limit detekce (LOD) a limit stanovení (LOQ), preciznost (anglický ekvivalent = precision), přesnost (anglický ekvivalent = accuracy), výtěžnost (anglický ekvivalent = recovery) a stabilita.

Kalibrace byla provedena metodou kalibrační křivky a přidavku jednotlivých analytů (s výjimkou kyseliny hipurové). Analyty byly rozděleny na skupiny a k nim byl vybrán strukturálně podobný vnitřní standard (viz. Tabulka 9).

Přesný postup úpravy vzorků byl následující: 75  $\mu$ l moči, 15  $\mu$ l směsi standardů a 10  $\mu$ l směsi interních standardů (aby výsledný objem odpovídal 100  $\mu$ l) bylo smícháno v reakční zkumavce. K této směsi bylo přidáno 75  $\mu$ l směsi ethanol : pyridin (4 : 1, v/v) a pak reakční směs ECF : chloroform (75  $\mu$ l, poměr 9 : 1, v/v), po promíchání 75  $\mu$ l hydroxidu sodného o koncentraci 1,5 mol/l a znovu 75  $\mu$ l směsi ECF : chloroform. Vzorek byl míchán po dobu asi 10 sekund a následovalo přidání 75  $\mu$ l kyseliny chlorovodíkové o koncentraci 3 mol/l. Po promíchání bylo odebráno 50  $\mu$ l dolní chloroformové vrstvy a k této části bylo přidáno 50  $\mu$ l ethanolu, obsah byl promíchán a 1  $\mu$ l vzorku bylo dávkováno do GC-MS. Bylo připraveno 6 koncentračních hladin a rozsah koncentrací byl vybrán v souladu s biologickými limity pro každou látku (viz. Tabulka 8). Každý vzorek byl derivatizován pětkrát. Analyty byly vyhodnoceny jako poměr plochy píku analytu a vnitřního standardu a byla vynesena kalibrační závislost poměru ploch na koncentraci. Kalibrační přímka každého analytu byla použita k určení směrnice, úseku, korelačního koeficientu a pro stanovení koncentrace látky u kontrolních vzorků referenčních materiálů. Kontrolní vzorky moči s přidavky na nejvyšší, střední a nejnižší hladině byly použity pro určení dalších validačních parametrů vyvinuté metody. LOD a LOQ byly vypočteny z pěti měření na nejnižší kalibrační hladině a to tak, že LOD odpovídal koncentraci, kde byl poměr signál k šumu 3 : 1 a LOQ koncentraci pro poměr signál k šumu 10 : 1. Preciznost a přesnost byly stanoveny analýzou vzorků (nejnižší, střední a nejnižší kalibrační hladina) v jedné sérii a ve 14-ti denní periodě (n = 5). Výtěžnost byla také určena také z těchto vzorků a to tak, že byla změřena koncentrace přidaná a určen poměr naměřené hodnoty ke skutečnému přidavku, spočtena dle vzorce:  $\text{naměřená koncentrace} / \text{přidaná koncentrace} * 100$ . Stabilita derivátů byla vyhodnocena ze vzorků, kde byla přidána střední koncentrační hladina (n = 3). V prvním případě byl vzorek po derivatizaci uchován při pokojové teplotě a v druhém případě v lednici o teplotě 4°C. Stabilita derivátů byla měřena v pravidelných intervalech po dobu 72 hodin. Koncentrace analytů byla navíc porovnána se vzorky, které byly připraveny každý dne čerstvě.

Tabulka 9: Přehled stanovovaných metabolitů xenobiotik a rozdělení do skupin dle použitých vnitřních standardů (IS).

---

<b>IS1 = Kyselina pentafluorfenoxyoctová</b>
Kyselina methoxyoctová
Kyselina ethoxyoctová
Kyselina butoxyoctová

---

<b>IS2 = Kyselina adipová <sup>13</sup>C<sub>2</sub></b>
Kyselina <i>t,t</i> -mukonová

---

<b>IS3 = Kyselina <i>D</i>-azetidin 2-karboxylová</b>
Kyselina 2-thiothiazolidin-4-karboxylová

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<b>IS4 = Kyselina 2-fenylmáselná</b>
Kyselina <i>o</i> -methylhippurová
Kyselina <i>m</i> -methylhippurová
Kyselina <i>p</i> -methylhippurová
Kyselina furan-2-karboxylová
Kyselina fenylglyoxylová
Kyselina mandlová

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<b>IS5 = Kyselina <i>S-p</i>-fluorfenylmerkapturová</b>
Kyselina <i>S</i> -benzylmerkapturová
Kyselina <i>S-p</i> -tolylmerkapturová
Kyselina <i>S</i> -fenylmerkapturová

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### 3.3.2.7 Ověření vypracované GC-MS metody měřením koncentrace metabolitů xenobiotik v referenčních materiálech.

K dispozici byly dva typy referenčních materiálů:

- 1) **Moč IP 45**, která obsahovala známé množství MAA, EAA, BAA, PGA, *t,t*-MA, *o*-, *m*- a *p*-MHA.
- 2) **Moč RM 6009**, která obsahovala známé množství PGA a MA.

Postup přípravy těchto vzorků byl následující: 90 µl moči a 10 µl směsi interních standardů bylo smícháno v reakční zkumavce a vzorek byl dále derivatizován s ECF viz. postup 3.3.2.3. Analyty byly vyhodnoceny jako poměr plochy píku analytu a vnitřního standardu a z kalibrační rovnice analytu byla vypočtena koncentrace. Každý vzorek byl měřen pětkrát.

### 3.3.2.8 Porovnání vypracované metody se silylační metodou pro stanovení EAA v moči u lidí exponovaných ethoxyethanolem.

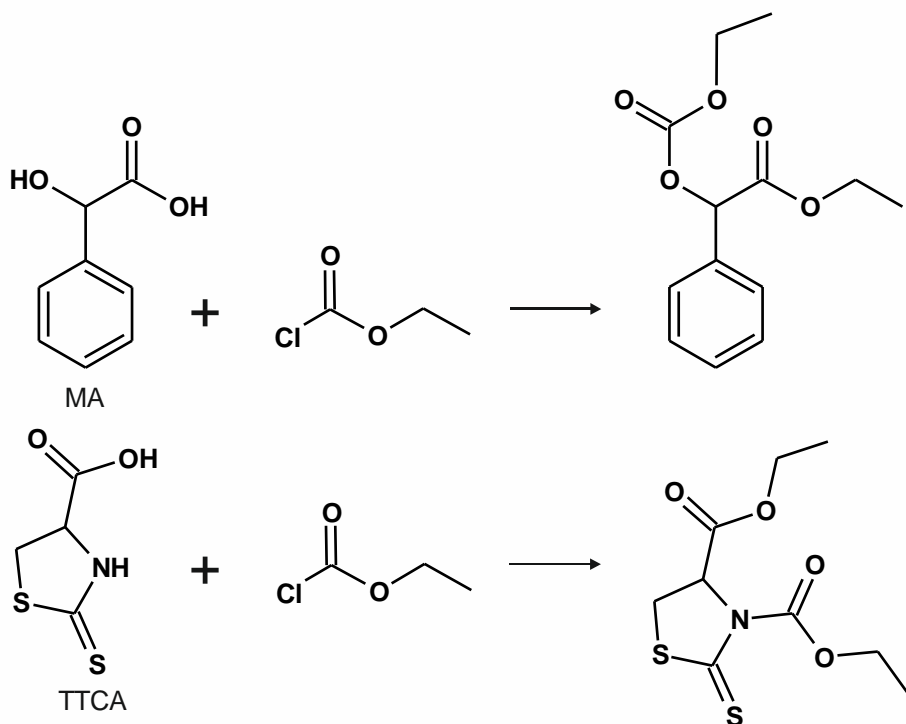
Byla provedena studie, kdy byl zdravý dobrovolník vystaven expozici ethoxyethanolu po dobu 4 hodin. Koncentrace ethoxyethanolu ve vzduchu byla 20 mg/m<sup>3</sup>, což je přípustný expoziční limit. Po expozici byly vzorky moči sbírány v pravidelných intervalech od času (*t* = 0) před expozicí a pak po dobu 188 hodin během a po expozici. V moči byl u každého vzorku stanoven metabolit ethoxyethanolu – EAA a každý vzorek byl měřen třikrát, v jedné sérii byl derivatizován ECF a v druhé sérii *N*-(tertbutyldimethylsilyl)-*N*-methyl-trifluoroacetamidem (MTBSTFA). Jako vnitřní standard byla v obou

případech použita kyselina pentafluorfenoxyoctová. Postup derivatizace s ECF byl stejný jako v sekci 3.3.2.3. Koncentrace byla vypočtena z dříve vyhotovené kalibrační závislosti (viz. sekce 3.3.2.3). Postup derivatizace s MTBSTFA byl popsán ve studii [114]. K 1 ml moči byl přidán vnitřní standard a byla provedena extrakce ethylacetátem, tento extrakt se odpařil do sucha a bylo přidáno derivatizační činidlo (MTBSTFA). Reakce probíhala po dobu 30 min při 80°C. Po derivatizaci byl vzorek opět extrahován ethylacetátem a 1 µl dávkován do GC-MS. Pro metodu s MTBSTFA byla vyhotovena kalibrační závislost, ze které byla určena koncentrace EAA v reálných vzorcích.

### **3.3.3 Výsledky a diskuse**

#### **3.3.3.1 Reakční produkty biomarkerů expozice s RCF**

Testování několika RCF ukázalo, že metoda založená na derivatizaci s ECF je nejvhodnější pro stanovení všech šestnácti vyšetřovaných analytů. U většiny testovaných biomarkerů docházelo ke vzniku jednoho očekávaného produktu s jasně definovaným hmotnostním spektrem. Monoethylester vznikl u MAA, EAA, BAA, FCA, PGA a dva vnitřní standardy PBA a PFPAA, diethylester vznikl pro *t,t*-MA a její vnitřní standard AdA-1,6-<sup>13</sup>C<sub>2</sub>, ethylester-ethylkarbonát pro MA a ethylester-ethylkarbamát pro TTCA (Obr. 14) a její vnitřní standard ACA. U metabolitů kyselin hippurových a merkapturových docházelo ke vzniku dvou produktů, vedle očekávaného ethylesteru vznikl ještě jeden produkt. Zajímavé vlastnosti měl jediný produkt reakce ECF s AMCC, kde naměřená hmotnostní spektra indikovala jinou strukturu. U neznámých vznikajících produktů byly proto provedeny další experimenty k objasnění jejich struktury (viz dále).

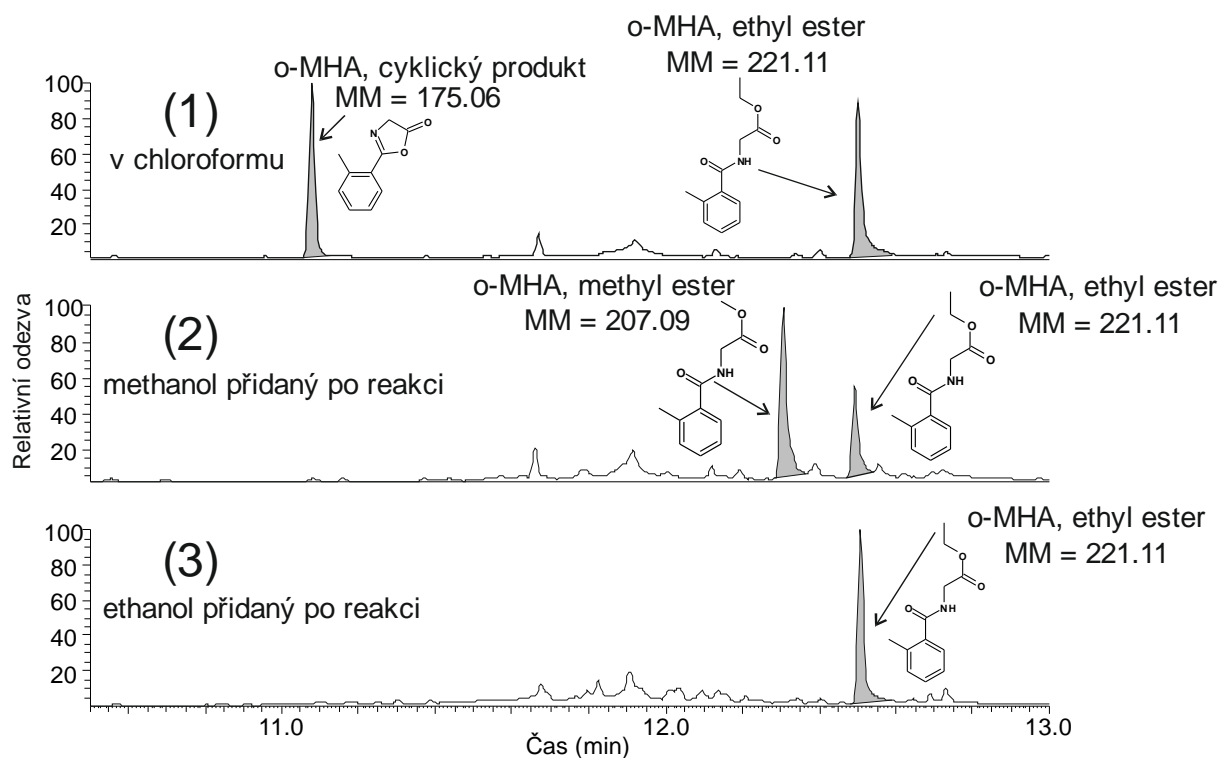


Obrázek 14: Reakční schéma kyseliny mandlové a kyseliny thiothiazolidin-4-karboxylové s ECF.

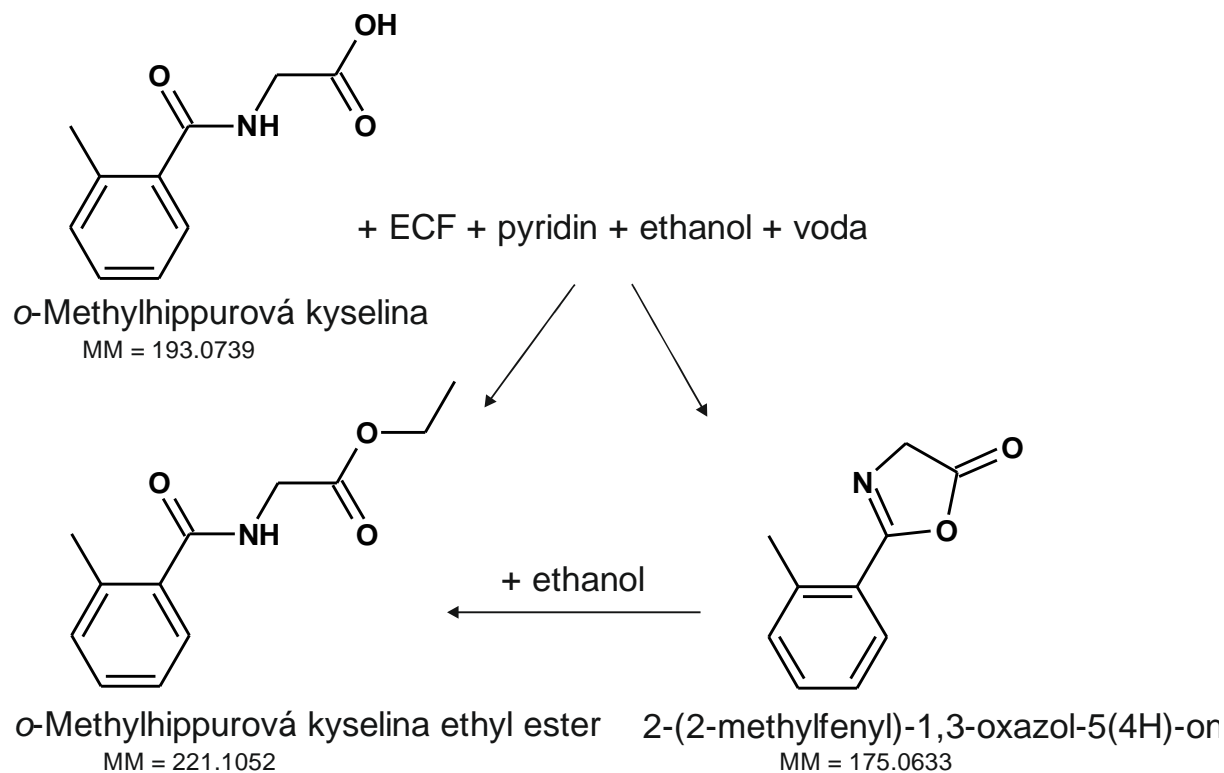
### 3.3.3.2 Deriváty hippurových kyselin

Hippurové kyseliny (HA, *o*-, *m*- a *p*-MHA) poskytují po derivatizaci s ECF dva produkty přibližně v poměru 2 : 1. Pomocí EI a PICI naměřených spekter se podařilo přiřadit pík s vyšší retencí očekávanému ethylesteru kyseliny, zatímco struktura píku s nižší retencí byla určena, též s pomocí literatury [115], jako dehydratační produkt hippurové kyseliny, 2-fenyloxazol-5(4*H*)-on. Struktura tohoto derivátu byla jednoznačně potvrzena porovnáním analytických vlastností s dostupným standardem 2-fenyloxazol-5(4*H*)-onu.

Další experimenty vyloučily, že cyklický produkt vzniká v injektoru GC. Byl připraven ethylester kyseliny *o*-MHA a GC-MS analýza potvrdila přítomnost pouze ethylesteru. Je známo [115], že oxazolony mají tendenci k rozkladu po přidání alkoholu. To se potvrdilo i v tomto případě, jak je ukázáno na obrázku 15, přidavek 50  $\mu$ l MeOH nebo EtOH ke stejnému extraktu vedl ke vzniku příslušného esteru a zastoupení cyklického produktu se výrazně zmenšilo. Pokud byl přidán místo alkoholu chloroform, poměry obou produktů každého hippurátu zůstaly zachovány. Z tohoto důvodu byl do postupu přípravy vzorku zařazen další krok - přidání 50  $\mu$ l ethanolu. Na Obr. 16 je uvedeno schéma reakce *o*-MHA s ECF.



Obrázek 15: GC-MS chromatogramy porovnávající reakční produkty *o*-MHA, (1) bez přidavku alkoholu, (2) po přidavku methanolu a (3) po přidavku ethanolu po derivatizaci s ECF.



Obrázek 16: Schéma finální přípravy vzorku pomocí reakce ECF s kyselinou *o*-methylhippurovou

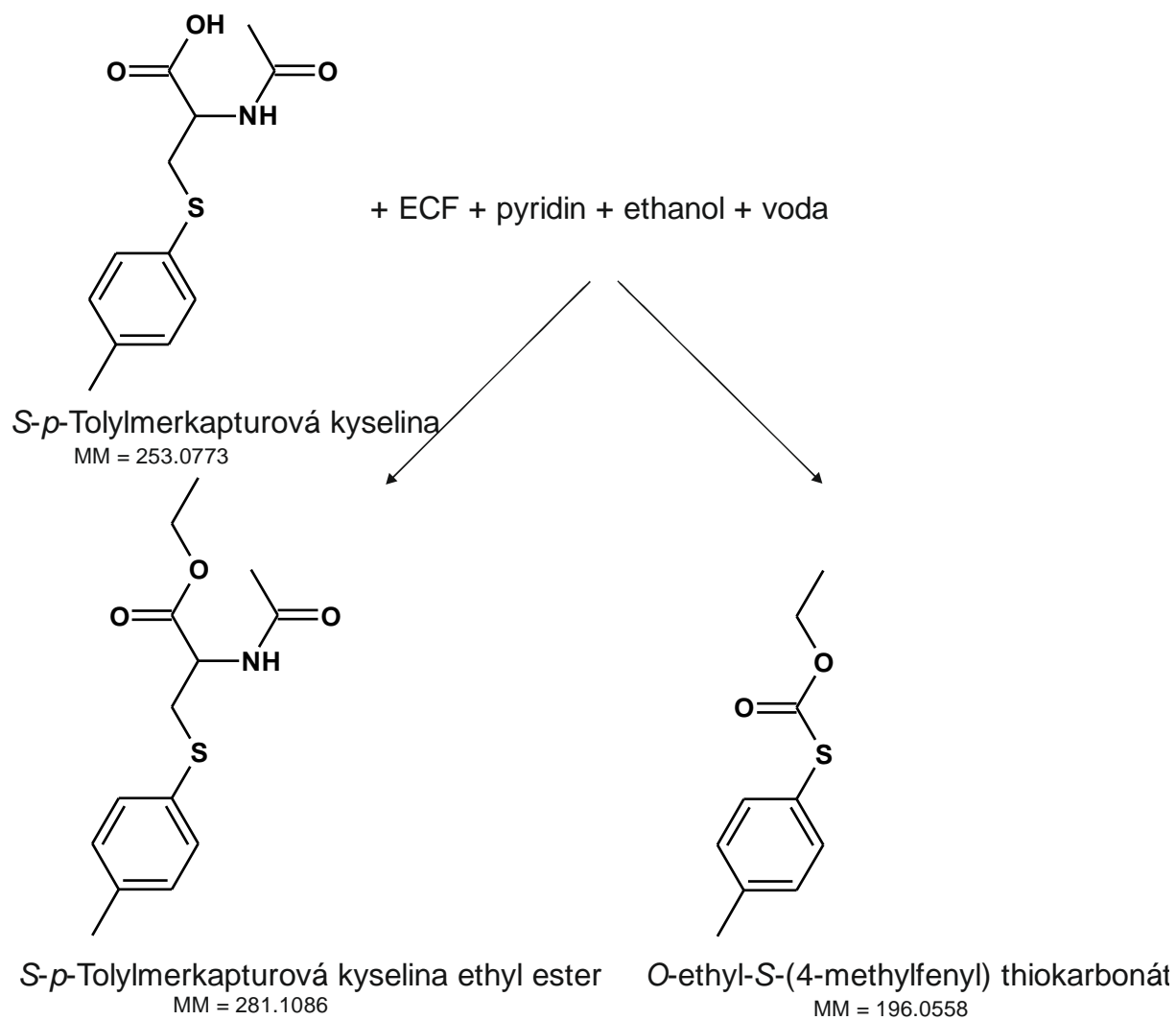
### 3.3.3.3 Deriváty merkapturových kyselin

Reakce *S*-PMA, *S*-TMA a *S*-FSPMA s ECF vede také ke vzniku dvou produktů, v poměru 1 : 1. Dříve eluující pík byl neznámý produkt, později eluující pík očekávaný ethylester. *S*-BMA dávala také dva produkty v poměru 2 : 5, opět dříve eluující pík byl neznámý produkt, a druhý pík ethylester.

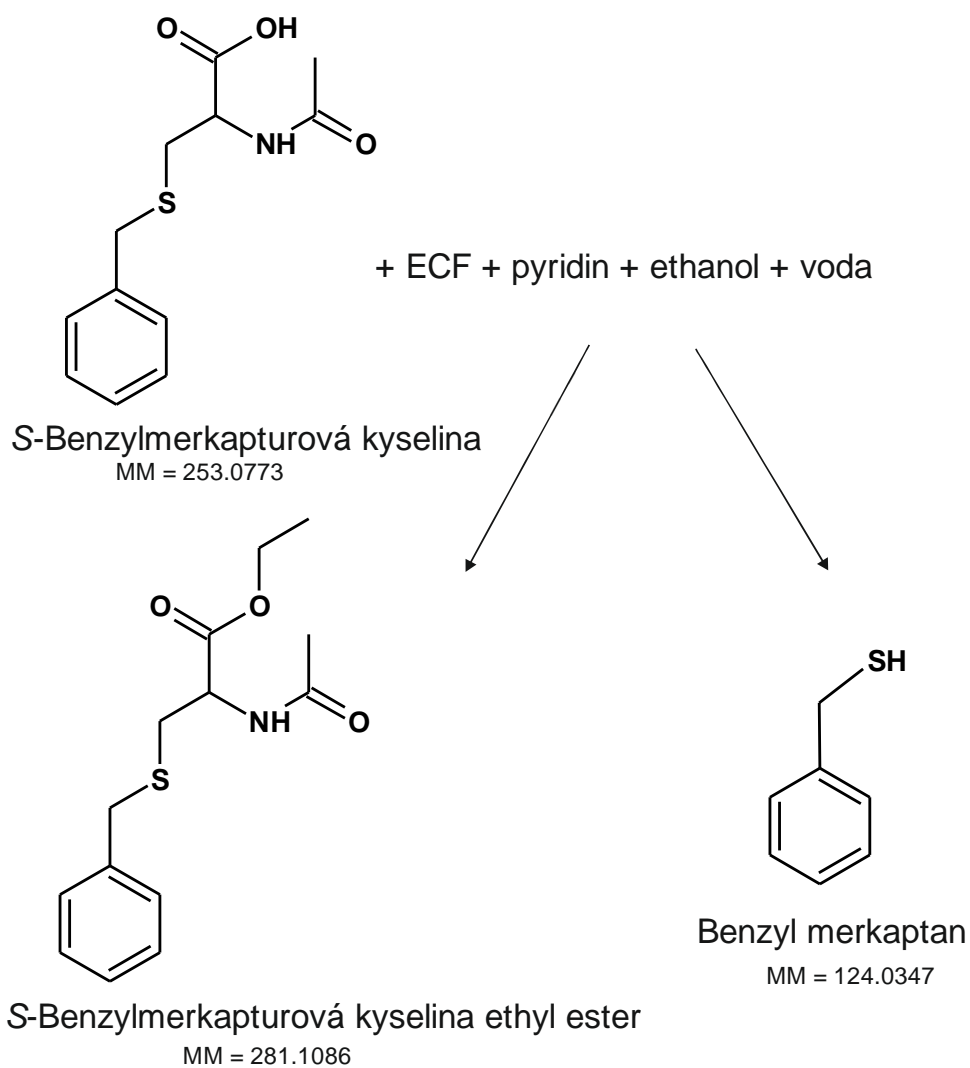
Naměřená EI spektra naznačila, že vedlejší produkt *S*-PMA, *S*-TMA a *S*-FSPMA vzniká analogicky, zatímco vedlejší produkt *S*-BMA vzniká rozdílnou cestou. K objasnění struktur byly připraveny ethylestery *S*-TMA a *S*-BMA. GC-MS analýza těchto ethylesterů ukázala, že *S*-TMA poskytuje pouze jeden pík, ale *S*-BMA píky dva. Je evidentní, že druhý produkt *S*-TMA vzniká během reakce s chlormravenčanem, kdežto druhý produkt *S*-BMA v GC-MS systému patrně rozkladem v injektoru. K určení struktury byly použity ethyl chlormravenčany ( $D_3$ -ECF a  $^{13}C_2$ -ECF) značené stabilními izotopy a změřena jejich EI i PICl spektra. *S*-PMA, *S*-TMA a *S*-FSPMA kromě předpokládaného ethylesteru tvoří v souladu s literaturou [116] také thiokarbonáty. Proces vzniku příslušných produktů s *S*-TMA znázorňuje Obr. 17.

Pro první eluovaný produkt *S*-BMA byla pro navržena struktura benzylmerkaptanu a potvrzena porovnáním s komerčně dostupným standardem benzylmerkaptanu. Reakční schéma *S*-BMA s ECF je uvedeno na Obr. 18.





Obrázek 17: Schéma reakce S-*p*-tolylmercapturové kyseliny s ECF.

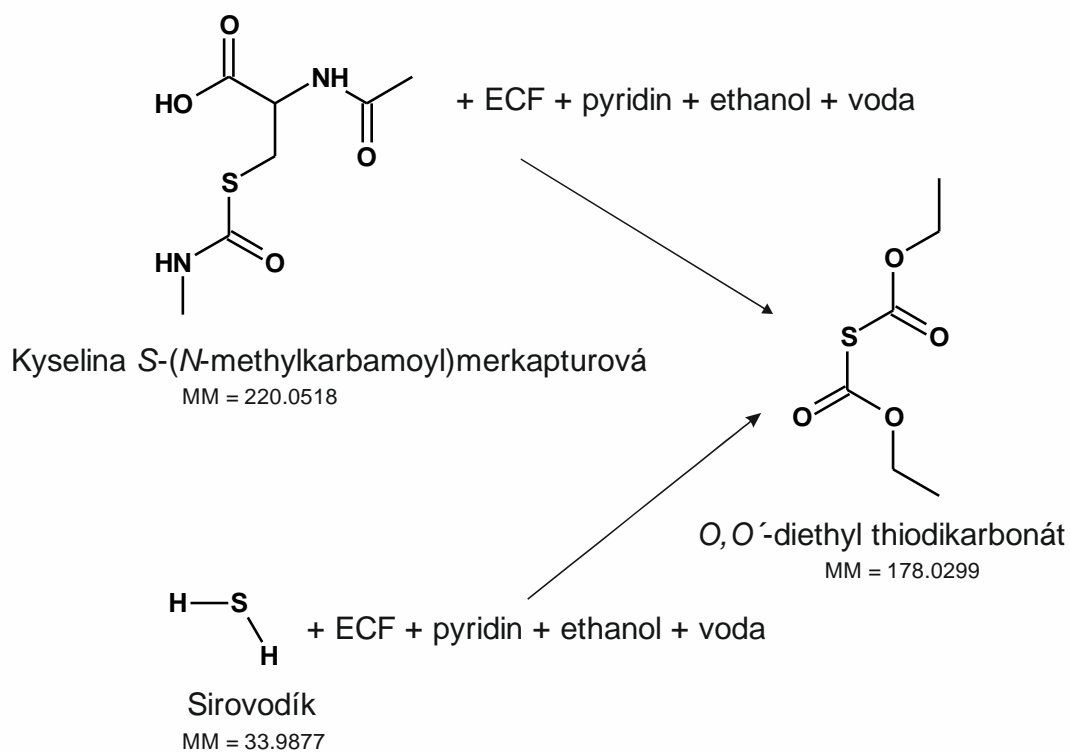


Obrázek 18: Schéma reakce S-benzylmercapturové kyseliny s ECF.

Produkt thiokarbonátu byl využit pro stanovení S-PMA a S-TMA, protože jeho pík méně chvostuje než ethylester. Ethylester byl naopak vhodnější analyt pro stanovení v případě S-BMA. Jako vnitřní standard byl využit pík thiokarbonátu FSPMA pro S-PMA a S-TMA a ethylesteru S-FSPMA pro S-BMA.

Jediný produkt s ECF neznámé struktury poskytovala AMCC. Derivatizace s izotopicky značenými ECF naznačila, že během reakce vzniká sirovodík, který reaguje s ECF. Příprava sirovodíku v Kippově přístroji a jeho následná reakce s ECF potvrdila totožnost derivátu H<sub>2</sub>S a produktu AMCC. Schéma reakce AMCC s H<sub>2</sub>S je znázorněno na Obr. 19.

Produkt sirovodíku vznikající po derivatizaci AMCC s ECF se bohužel ukázala jako málo stabilní, tudíž ho nebylo možno využít ke kvantitativnímu stanovení.



Obrázek 19: Schéma reakce kyseliny *S*-(*N*-methylkarbamoyl)merkapturové s ECF.

### 3.3.3.4 LC-HRMS analýza

K potvrzení struktury bylo změřeno elementární složení zkoumaných metabolitů biomarkerů expozice technikou LC-HRMS po derivatizaci s ECF a  $^{13}\text{C}_2$ -ECF. *t,t*-MA, TTCA, HA, směs izomerů MHA, *S*-PMA, *S*-TMA a *S*-BMA byly detekovány a měření potvrdilo dále správnost navržených struktur. Výsledky těchto měření jsou shrnuty v Tabulce 10.

Tabulka 10: Přehled derivátů zkoumaných biomarkerů technikou LC-HRMS pro detekované deriváty po reakci s ECF a  $^{13}\text{C}_2$ -ECF.

Metabolit	RT [min]	Přírodní	$^{13}\text{C}_2$	Přírodní	$^{13}\text{C}_2$	m/z Exp (Da)	$\Delta$ ppm	N pravidlo	m/z Exp (Da)	$\Delta$ ppm	N pravidlo
		m/z Teor (Da)	m/z Teor (Da)	Molekulový vzorec Ethyl	Molekulový vzorec Ethyl						
Kyselina <i>t,t</i> -mukonová	7,51	199,0965	203,1099	$\text{C}_{10}\text{H}_{15}\text{O}_4$	$\text{C}_6[13]\text{C}_4\text{H}_{15}\text{O}_4$	199,0966	0,525	ANO	203,1100	0,273	ANO
Kyselina mandlová	7,97	253,1071	257,1205	$\text{C}_{13}\text{H}_{17}\text{O}_5$	$\text{C}_9[13]\text{C}_4\text{H}_{17}\text{O}_5$	253,1071	0,039	ANO	257,1205	0,042	ANO
Kyselina 2-thiothiazolidin-4-karboxylová	5,74	264,0359	268,0493	$\text{C}_9\text{H}_{14}\text{NO}_4\text{S}_2$	$\text{C}_5[13]\text{C}_4\text{H}_{14}\text{O}_4\text{NS}_2$	264,0358	-0,136	ANO	268,0493	0,019	ANO
Kyselina hippurová	4,71	208,0968	210,1035	$\text{C}_{11}\text{H}_{14}\text{NO}_3$	$\text{C}_9[13]\text{C}_2\text{H}_{14}\text{O}_3\text{N}$	208,0968	-0,095	ANO	210,1036	0,146	ANO
Kyselina <i>o</i> -methylhippurová	5,06	222,1125	224,1192	$\text{C}_{12}\text{H}_{16}\text{NO}_3$	$\text{C}_{10}[13]\text{C}_2\text{H}_{16}\text{O}_3\text{N}$	222,1125	0,001	ANO	224,1193	0,359	ANO
Kyselina <i>m</i> -methylhippurová	5,70	222,1125	224,1192	$\text{C}_{12}\text{H}_{16}\text{NO}_3$	$\text{C}_{10}[13]\text{C}_2\text{H}_{16}\text{O}_3\text{N}$	222,1125	0,226	ANO			ANO
Kyselina <i>p</i> -methylhippurová	5,66	222,1125	224,1192	$\text{C}_{12}\text{H}_{16}\text{NO}_3$	$\text{C}_{10}[13]\text{C}_2\text{H}_{16}\text{O}_3\text{N}$	222,1125	0,271	ANO			ANO
Kyselina <i>S</i> -fenylmerkapturová (1)	8,52	183,0474	185,0541	$\text{C}_9\text{H}_{11}\text{O}_2\text{S}$	$\text{C}_7[13]\text{C}_2\text{H}_{11}\text{O}_2\text{S}$	183,0476	1,11	ANO	185,0543	0,614	ANO
Kyselina <i>S</i> -fenylmerkapturová (2)	6,78	268,1002	270,1069	$\text{C}_{13}\text{H}_{18}\text{NO}_3\text{S}$	$\text{C}_{11}[13]\text{C}_2\text{H}_{18}\text{O}_3\text{NS}$	268,1001	-0,413	ANO	270,1068	-0,445	ANO
Kyselina <i>S-p</i> -tolylmerkapturová(1)	9,36	197,0631	199,0698	$\text{C}_{10}\text{H}_{13}\text{O}_2\text{S}$	$\text{C}_8[13]\text{C}_2\text{H}_{13}\text{O}_2\text{S}$	197,0633	1,081	ANO	199,0699	0,57	ANO
Kyselina <i>S-p</i> -tolylmerkapturová (2)	7,67	282,1158	284,1226	$\text{C}_{14}\text{H}_{20}\text{NO}_3\text{S}$	$\text{C}_{12}[13]\text{C}_2\text{H}_{20}\text{O}_3\text{NS}$	282,1157	-0,463	ANO	284,1224	-0,599	ANO
Kyselina <i>S</i> -benzylmerkapturová	7,16	282,1158	284,1226	$\text{C}_{14}\text{H}_{20}\text{NO}_3\text{S}$	$\text{C}_{12}[13]\text{C}_2\text{H}_{20}\text{O}_3\text{NS}$	282,1157	-0,605	ANO	284,1224	-0,67	ANO

### 3.3.3.5 Kvantitativní stanovení biomarkerů expozice v moči a validace nové metody

Vypracovaná metoda byla validována pro zkoumané biomarkery uvedené v tabulce 9, kde jsou uvedeny i použité vnitřní standardy pro každou skupinu. GC-MS analýza byla prováděna v režimu SIM, retenční data a příslušné diagnostické ionty jsou uvedeny v tabulce 11.

Byla získána kalibrační závislost, mez detekce (LOD) a mez stanovitelnosti (LOQ). Všechny validované parametry dosáhly uspokojivých hodnot, kromě LOQ pro S-PMA (0,07 mg/l), která obvykle vyžaduje měření s biologickým limitem > 0,05 mg/l. Všechny validované parametry jsou shrnuty v Tab. 12. Preciznost byla v rozmezí 0,2 – 14,9 %, přesnost mezi 80 – 120 % a výtěžnost mezi 91 – 108 %. Všechny deriváty použité ke stanovení v moči vykazovaly také uspokojivou stabilitu ( $\pm 8$  %).

Tabulka 11: Přehled biomarkerů stanovených v moči vypracovanou GC-MS metodou

RT (min)	Analyt	MM <sup>a</sup>	Diagnostické m/z ionty <sup>b</sup>		
			m/z (100 %)	m/z <sup>1</sup>	m/z <sup>2</sup>
4.28	Kyselina methoxyoctová	118	45	88	42
4.84	Kyselina ethoxyoctová	132	59	88	43
6.55	Kyselina butoxyoctová	160	57	41	88
6.69	Kyselina furan-2-karboxylová	140	95	112	96
7.07	Kyselina S-benzylmerkapturová (1)	180	91	124	90
7.91	Kyselina pentaflurfenoxyoctová (IS <sup>c</sup> )	270	270	167	184
8.27	Kyselina S-(N-methylkarbamoyl)merkapturová	178	78	106	45
9.14	Kyselina 2-fenylmáselná (IS)	192	91	119	118
9.48	Kyselina adipová-1,6- <sup>13</sup> C <sub>2</sub> (IS)	204	113	159	129
9.64	Kyselina S-p-fluorfenylmerkapturová (1) (IS)	200	128	127	83
9.96	Kyselina fenylglyoxylová	178	105	77	51
10.01	Kyselina S-fenylmerkapturová (1)	182	110	109	182
10.20	Kyselina t,t-mukonová	198	97	153	125
10.38	Kyselina D-azetidin 2-karboxylová (IS)	201	128	56	127
10.77	Kyselina S-p-tolylmerkapturová (1)	196	91	124	196
11.82	Kyselina mandlová	252	107	79	206
12.39	Kyselina hippurová	207	105	77	134
12.49	Kyselina o-methylhippurová	221	118	119	91
12.71	Kyselina m-methylhippurová	221	119	91	221
12.76	Kyselina p-methylhippurová	221	119	91	221
13.04	Kyselina S-p-fluorfenylmerkapturová (2) (IS)	285	226	43	285
13.20	Kyselina 2-thiothiazolidin-4-karboxylová	263	263	118	117
13.23	Kyselina S-fenylmerkapturová (2)	267	208	135	43
13.46	Kyselina S-p-tolylmerkapturová (2)	281	222	149	43
13.59	Kyselina S-benzylmerkapturová (2)	281	91	222	43

<sup>a</sup> MM = nominální molekulová hmotnost derivátu

<sup>b</sup> Diagnostické m/z ionty vhodné pro identifikace derivátu, m/z (100 %) je iont s nejvyšší intenzitou ve spektru, m/z<sup>1</sup> a m/z<sup>2</sup> dva následující.

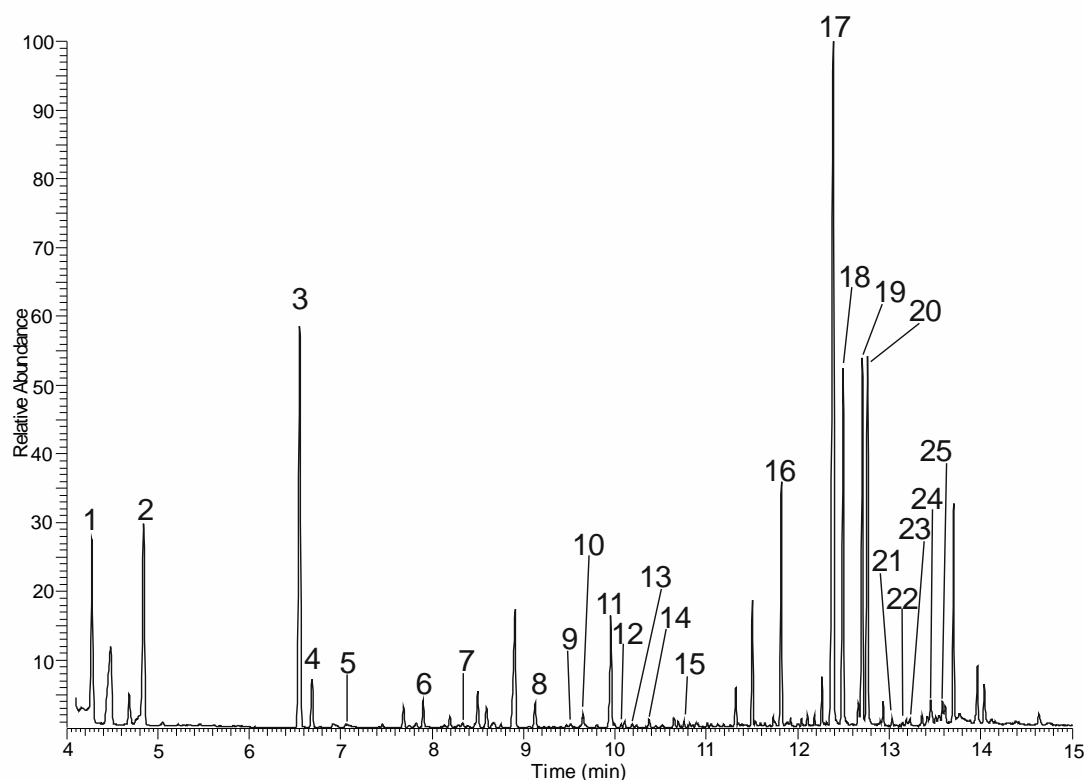
<sup>c</sup> IS = vnitřní standard

Tabulka 12: Validační parametry GC-MS metody pro stanovení zkoumaných biomarkerů v moči

Metabolit	R <sup>2</sup>	LOD (mg/l)	LOQ (mg/l)	Rozsah kvantifikace (mg/l)	Regresní rovnice	Biol. limit (mg/l)
Kyselina methoxyoctová	0.9999	0.02	0.07	0.29 - 288	0.2661x + 0.1581	1
Kyselina ethoxyoctová	0.9998	0.07	0.22	0.33 - 333	0.2123x + 0.0312	50
Kyselina butoxyoctová	0.9988	0.01	0.05	0.42 - 422	0.2430x + 0.1013	150
Kyselina furan-2-karboxylová	0.9998	0.01	0.04	0,15 - 605	0.0508x - 0.0032	200
Kyselina fenylglyoxylová	0.9985	0.03	0.11	0.15 - 600	0.0748x - 0.0029	400*
Kyselina <i>S</i> -fenylmerkapturová (1)	0.9998	0.02	0.07	0.48 - 19.1	1.7656x + 2,2128	0.025
Kyselina <i>t,t</i> -mukonová	0.9984	0.01	0.04	0.19 - 7.55	0.4293x + 0.3821	0.5
Kyselina <i>S-p</i> -tolylmerkapturová (1)	0.9991	0.05	0.16	0.51 - 20.24	1.1551x - 0.1521	–
Kyselina mandlová	1	0.01	0.02	0.27 - 1094	0.3446x + 0,0602	400*
Kyselina <i>o</i> -methylhippurová	0.9935	0.01	0.03	0.34 - 1351	0.5118x - 0,1229	2000**
Kyselina <i>m</i> -methylhippurová	0,9944	0.004	0.01	0.34 - 1351	0.3581x + 0,0980	2000**
Kyselina <i>p</i> -methylhippurová	0,999	0.04	0.14	0.34 - 1351	0.5107x - 0,0165	2000**
Kyselina 2-thiothiazolidin-4-karboxylová	0.9995	0.08	0.25	0.52 - 52.16	0.0057x + 0.0035	0.5
Kyselina <i>S</i> -benzylmerkapturová (2)	0.9939	0.08	0.26	0.05 - 20.24	0.7765x - 0.2201	–

\* = suma kyselin fenylglyoxylové a mandlové, \*\* = suma kyselina methylhippurových

Celkový obrázek GC-MS analýzy biomarkerů průmyslových škodlivin přidaných společně s vnitřními standardy do vzorku moči zdravého dobrovolníka po jeho přípravě vypracovaným postupem znázorňuje obrázek 20.



Obrázek 20: TIC GC-EI-MS chromatogram vzorku moči s přidanými standardy metabolitů xenobiotik (nejvyšší testovaná kalibrační hladina, viz. tabulka 12) po derivatizaci s ECF. Chromatografické podmínky viz sekce 3.3.2.4, 1 – kyselina methoxyoctová, 2 – kyselina ethoxyoctová, 3 – kyselina butoxyoctová, 4 – kyselina furan-2-karboxylová, 5 – kyselina *S*-benzylmerkapturová (1), 6 – kyselina pentafluorofenoxyoctová, 7 – kyselina *S*-(*N*-methylkarbamoyl)merkapturová, 8 – kyselina 2-fenylmáselná, 9 – kyselina adipová-1,6-<sup>13</sup>C<sub>2</sub>, 10 – kyselina *S-p*-fluorofenylmerkapturová (1), 11 – kyselina fenylglyoxylová, 12 – kyselina *S*-fenylmerkapturová (1), 13 – kyselina *t,t*-mukonová, 14 – kyselina *D*-azetidin 2-karboxylová, 15 – kyselina *S-p*-tolylmerkapturová (1), 16 – kyselina mandlová, 17 – kyselina hippurová, 18 – kyselina *o*-methylhippurová, 19 – kyselina *m*-methylhippurová, 20 – kyselina *p*-methylhippurová, 21 – kyselina *S-p*-fluorofenylmerkapturová (2), 22 – kyselina 2-thiothiazolidin-4-karboxylová, 23 – kyselina *S*-fenylmerkapturová (2), 24 – kyselina *S-p*-tolylmerkapturová (2), 25 – kyselina *S*-benzylmerkapturová (2).

### 3.3.3.6 Analýza referenčního materiálu a vzorků moči po expozici ethoxyethanolem

Validovaná GC-MS metoda byla ověřena na stanovení známého množství kyselin v moči u dvou typů referenčních materiálů. První referenční vzorek (IP 45) obsahoval známé množství kyselin MAA, EAA, BAA, PGA, *t,t*-MA, *o*-, *m*- a *p*-MHA a druhý (RM 6009) obsahoval známou koncentraci PGA a MA. Jak je patrné z Tab. 13, naměřené hodnoty byly v dobré shodě s hodnotami referenčními.

Tabulka 13: Porovnání výsledků stanovení koncentrace vybraných metabolitů xenobiotik v referenčních materiálech RM6009 a IP45.

Analyt	Referenční materiál	Referenční hodnota (mg/l)	Měřená hodnota (n=5) (mg/l)
Kyselina methoxyoctová	IP45	14.86	18.02±1.44
Kyselina ethoxyoctová	IP45	66.3	72.97±2.68
Kyselina butoxyoctová	IP45	90.8	87.00±0.65
Kyselina fenylglyoxylová	IP45	270.5	304.46±9.66
	RM6009	220±19.1	229±11.2
Kyselina <i>t,t</i> -mukonová	IP45	1.82	1.97±0.21
Kyselina mandlová	RM6009	326±11.4	311±8.9
Kyseliny methylhippurové	IP45	427.7	436.05±5.25

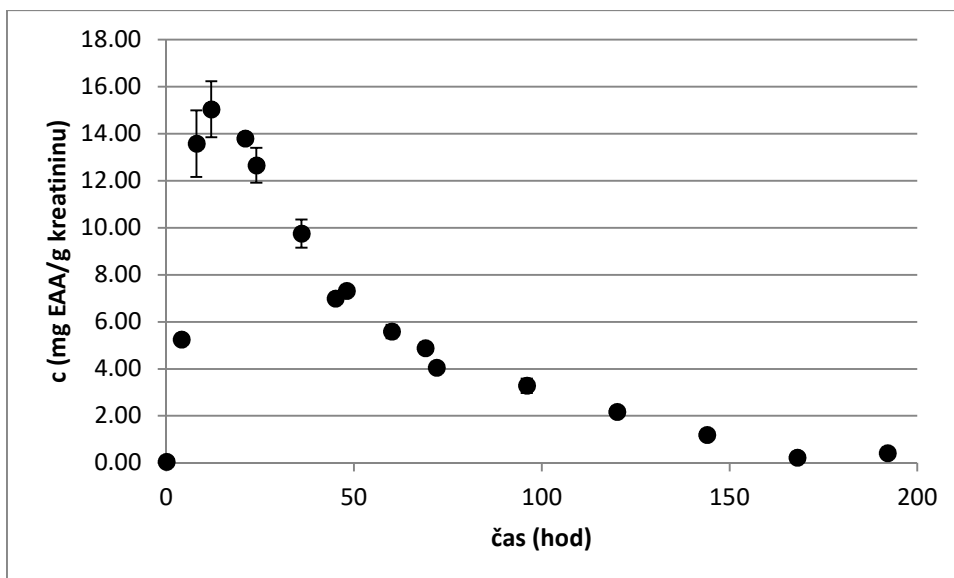
Nová metoda byla dále využita k analýze vzorků moči zdravého dobrovolníka, který byl vystaven po dobu 4 hodin expozici ethoxyethanolu a v jehož moči byl sledován výskyt tohoto biomarkeru – EAA v závislosti na čase. Metoda byla porovnána s klasickou silylační derivatizační metodou stanovení EAA [114]. Nalezené koncentrace EAA v moči pro obě metody jsou uvedeny v Tab.



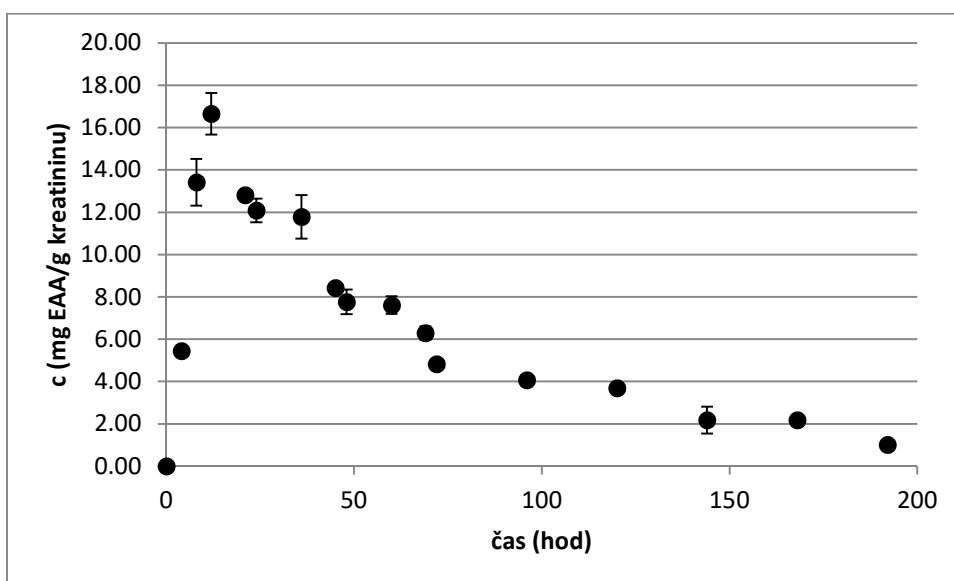
Tabulka 14: Porovnání výsledků stanovení EAA dvěma metodami derivatizace v moči exponované ethoxyethanolem.

čas (hod)	ECF		MTBSTFA	
	c (mg EAA/g kreatininu)	smdoch	c (mg EAA/g kreatininu)	smdoch
0	0.05	0.00	0.00	0.00
4	5.25	0.19	5.44	0.24
8	13.58	1.41	13.42	1.11
12	15.04	1.19	16.66	0.98
21	13.80	0.07	12.81	0.23
24	12.66	0.74	12.08	0.56
36	9.75	0.60	11.78	1.03
45	6.98	0.07	8.43	0.22
48	7.31	0.04	7.77	0.59
60	5.59	0.29	7.61	0.42
69	4.88	0.23	6.29	0.32
72	4.04	0.10	4.83	0.08
96	3.28	0.30	4.07	0.24
120	2.16	0.19	3.69	0.03
144	1.19	0.02	2.18	0.63
168	0.23	0.01	2.18	0.01
192	0.41	0.04	1.01	0.01

Grafy na Obr. 21 a 22 ukazují časovou závislost expozice biomarkeru EAA vylučovaného ve sbírané moči. Koncentrace EAA v moči nejprve narůstala a po dosažení maxima, cca po 8 hodinách začala klesat. Z výsledků je patrné, že EAA je v moči detekovatelná ještě 188 hodin po ukončení expozice. Ukázalo se, že nová derivatizace s ECF poskytuje výsledky velice podobné těm, získaným postupem přípravy vzorku pracnějším a zdlohavějším klasickou silylací MTBSTFA, přičemž nová metoda probíhá v jednom kroku a umožňuje stanovit současně až 16 biomarkerů v moči.



Obrázek 21: Graf závislosti koncentrace EAA v moči po expozici ethoxyethanolem na čase. V čase  $t = 0$  začal čas čtyřhodinové expozice ethoxyethanolem. Vzorky moči byly derivatizovány ECF ( $n = 3$ ).



Obrázek 22: Graf závislosti koncentrace EAA v moči po expozici ethoxyethanolem na čase. V čase  $t = 0$  začal čas čtyřhodinové expozice ethoxyethanolem. Vzorky moči byly derivatizovány MTBSTFA ( $n = 3$ ).

## 4 Závěr

Byly vypracovány tři nové metody pro GC-MS stanovení protických metabolitů v biotekutinách (lidská moč, lidské sérum a plodová voda) založených na in situ derivatizaci s alkylchlormravenčany a jejich fluorovanými analogy.

První z metod (kap. 3.1.) [1] popisuje novou, efektivní derivatizaci sekundární alicyklické hydroxylové skupiny ve sterolech a steroidech. Jako modelová sloučenina pro vývoj metody byl použit cholesterol a byly prověřeny různé alkylované chlormravenčany i jejich fluorované analogy pro reakci ve vodném i v bezvodém prostředí. Ke stoprocentní přeměně hydroxylové skupiny na očekávaný karbonát docházelo při použití činidel TFEFCF a HFBCF v bezvodém prostředí. Acylační reakce byla kombinována s mikroextrakcí kapalina-kapalina mezi isooktanovou a acetonitrilovou fází. Vyvinutá metoda byla úspěšně testována pro GC-MS analýzu 12 modelových sterolů a steroidů a 4 tokoferolů. Každý z analytů poskytoval jeden pík s výbornými separačními vlastnostmi a dobře definovaná EI spektra obsahující diagnostické ionty vhodné pro kvantitativní analýzu. Po těchto experimentech byla metoda validována pro stanovení 6 diagnostických sterolů a 4 tokoferolů v lidském séru a plodové vodě a aplikována pro stanovení sterolů a tokoferolů v reálných vzorcích séra a plodové vody. Obdržené výsledky dobře korelovaly s výsledky popsány v dřívějších studiích [68 – 72]. Metoda derivatizace byla také použita pro stanovení sterolů a tokoferolů u hmyzu – ruměnice pospolné (*Pyrrhocoris apterus*). [2]

Ve druhé studii (kap. 3.2.) [3] byla vyvinuta, popsána a validována GC-MS metoda založená na derivatizaci protických skupin pomocí HFBCF a současné mikroextrakci kapalina-kapalina, díky níž je možno stanovit profil 132 metabolitů v lidské moči. Vzorek moči (25  $\mu$ l) byl podroben derivatizaci a následné analýze technikou GC-MS. Bylo celkem sledováno 153 klinicky významných močových metabolitů (56 aminokyselin a jejich konjugátů, 84 organických kyselin, 9 biogenních aminů a 4 další polární analyty). 120 metabolitů poskytovalo (78 %) jeden produkt, 25 (16 %) produkty dva a kyselina 2-methylcitronová a kyselina citronová produktů více. Ze zkoumaného souboru 5 metabolitů nebylo možno detekovat a 3 metabolity byly vhodné pouze pro LC-MS analýzu. Po validaci na GC-MS byla metoda aplikována na reálné vzorky lidské moči a na moč obsahující certifikované množství vybraných metabolitů. Výsledky ukázaly, že vypracovaná metoda je vhodná pro cílenou metabolomickou analýzu lidské moči.

Třetí studie (kap. 3.3.) se zabývala výzkumem rychlé a jednoduché metody pro stanovení xenometabolitů v lidské moči. Jednalo se o metabolity benzenu, toluenu, xylenů, styrenu, alkoxyalkoholů, sirovodíku, furalu a *N,N*-dimethylformamidu. Metabolity byly derivatizovány ECF in situ a získaný extrakt přímo podroben GC-MS analýze. Většina analytů poskytovala jeden předpokládaný produkt, jen ve skupině hippurových a merkapturových kyselin byl současně pozorován

další produkt, jejichž struktura byla objasněna pomocí LC-HRMS a izotopicky značeným ECF. Vyvinutá metoda byla validována pro 14 běžných biomarkerů expozice a úspěšně otestována na certifikovaném referenčním materiálu, porovnána se zavedenou silylační metodou [114] a bylo dokázáno, že je použitelná pro stanovení uvedených metabolitů v laboratorní praxi.

## 5 Seznam zkratek

ACGIH – American Conference of Governmental Industrial Hygienists  
AdA-1,6-<sup>13</sup>C<sub>2</sub>–kyselina adipová-1,6-<sup>13</sup>C<sub>2</sub>  
AMCC – kyselina *S*-(*N*-methylkarbamoyl)merkapturová  
BAA – kyselina butoxyoctová  
BAT – Biologische Arbeitsstoff-Toleranz-Werte  
BEI – Biological exposure indices  
BSTFA – bis-(trimethylsilyl) trifluoroacetamidu  
CE – kapilární elektroforéza  
CI – chemická ionizace  
CoA – koenzym A  
DCC – *N,N'*-dicyklohexylkarobodiimid  
DFG – Germany Deutsche Forschungsgemeinschaft  
EAA – kyselina ethoxyoctová  
ECF – ethylchlormravenčan  
EI – elektronová ionizace  
EOC – ethoxykarbonát  
FCA – kyselina furan-2-karboxylová  
FCF – fluoralkylchlormravenčany  
GC – plynová chromatografie  
GC-FID – plynová chromatografie s plamenově ionizačním detektorem  
GC-MS – plynová chromatografie s hmotnostní detekcí  
GSH – glutathion  
HA – kyselina hippurová  
HCF – *n*-hexylchlormravenčan  
HFBCF – 2,2,3,3,4,4,4-heptafluorobutylchlormravenčan  
HFBOC – heptafluorobutoxykarbonát  
HFBOH – heptafluorbutanol  
HMDB – Human Metabolome Data Base  
HOC – hexyloxykarbonát  
HPLC – vysokoúčinná kapalinová chromatografie  
HPLC-MS/MS– vysokoúčinná kapalinová chromatografie s tandemovou spektrometrií  
HPLC-UV – vysokoúčinná kapalinová chromatografie s ultrafialovou detekcí  
Chol – cholesterol  
I.S. – vnitřní standard  
iBCF – isobutylchlormravenčan  
iBOC – isobutoxykarbonát  
LC-MS – kapalinová chromatografie s hmotnostní detekcí  
LLME – liquid-liquid mikroextrakce  
LLOQ – lower limit of quantification, nízký limit stanovení  
LOD – mez detekce  
LOQ – mez stanovení  
MA – kyselina mandlová  
MAA – kyselina methoxyoctová

MCF – methylchlormravenčan  
MM – monoisotopická hmotnost  
*m*-MHA – kyselina *m*-methylhippurová  
MOC – methoxykarbonát  
MS – hmotnostní spektrometrie  
MTBSTFA – *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamid  
n.d. – nedetekováno  
NICI – negativní chemická ionizace  
NMR – nukleární magnetická rezonance  
*o*-MHA – kyselina *o*-methylhippurová  
PA – peak area, plocha píku  
PCPCF – 2,2,3,3,3-pentafluoropropylchlormravenčan  
PFPA – kyselina pentafluorfenoxyoctová  
PGA – kyselina fenylglyoxylová  
PICI – pozitivní chemická ionizace  
*p*-MHA – kyselina *p*-methylhippurová  
PTV – programovatelný nástřik u GC  
RCF – alkylchlormravenčany  
ROC – alkyloxykarbonáty  
RP – reverzní fáze  
RSD – relativní směrodatná odchylka  
RT – retenční čas  
S/N - poměr signál k šumu  
S-BMA – kyselina *S*-benzylmerkapturová  
S-FPMA – kyselina *S-p*-fluorfenylmerkapturová  
SIM – selected ion monitoring  
SPE – solid phase extraction  
S-PMA – kyselina *S*-fenylmerkapturová  
S-TMA – kyselina *S-p*-tolylmerkapturová  
*t,t*-MA – kyselina *t,t* - mukonová  
TBDMS – *tert*-butyldimethylsilyl  
TFECF – 2,2,2-trifluoroethylchlormravenčan  
TFEOC – trifluoroethoxykarbonát  
THP – *tris*-(3-hydroxypropyl) fosfinu  
TIC – total ion current  
TMCS – trimethylchlorsilan  
TMS – trimethylsilyl  
TMSI - 1-(trimethylsilyl) imidazol  
TTCA – kyselina 2-thiothiazolidin-4-karboxylová  
UV – ultrafialový

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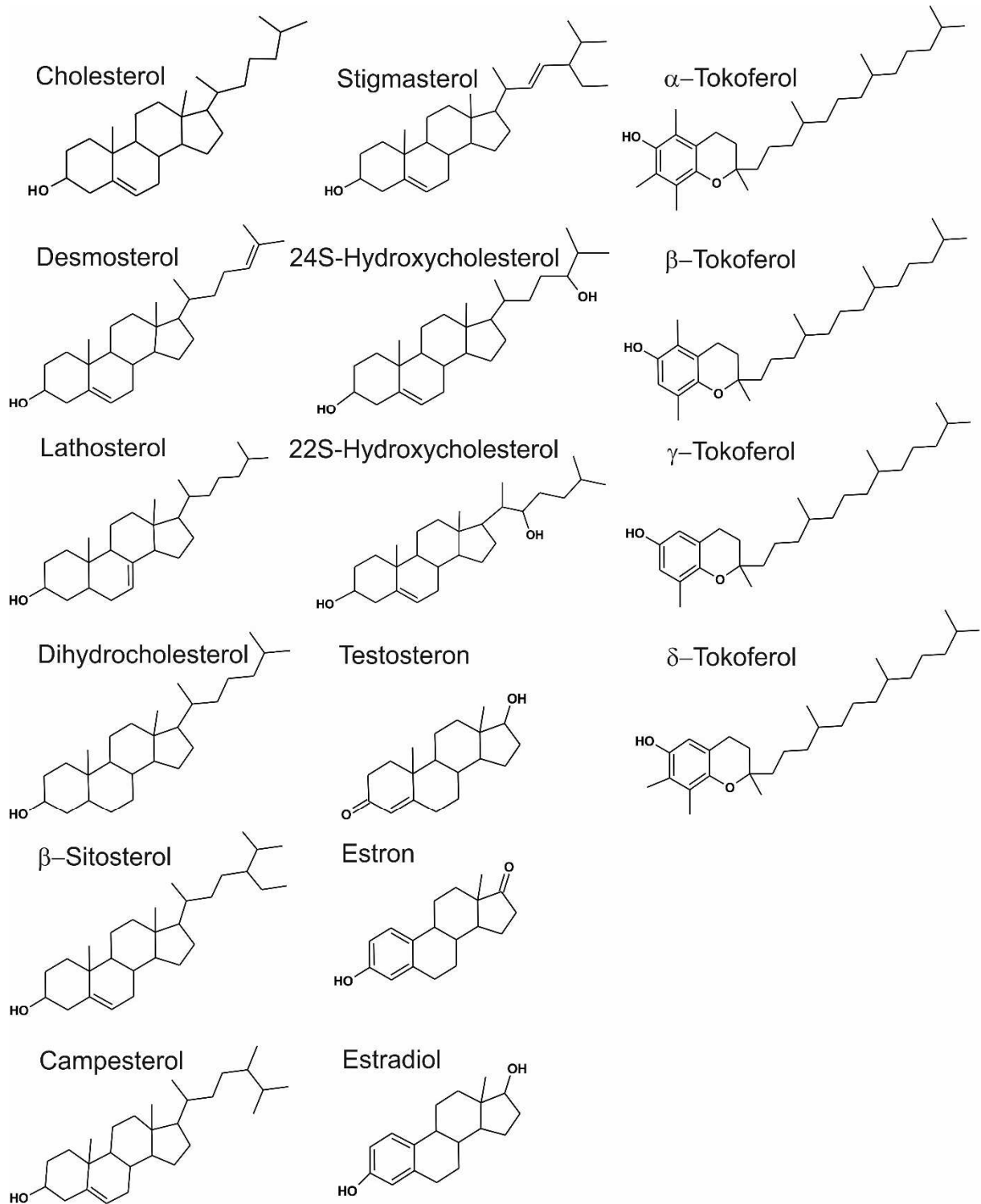
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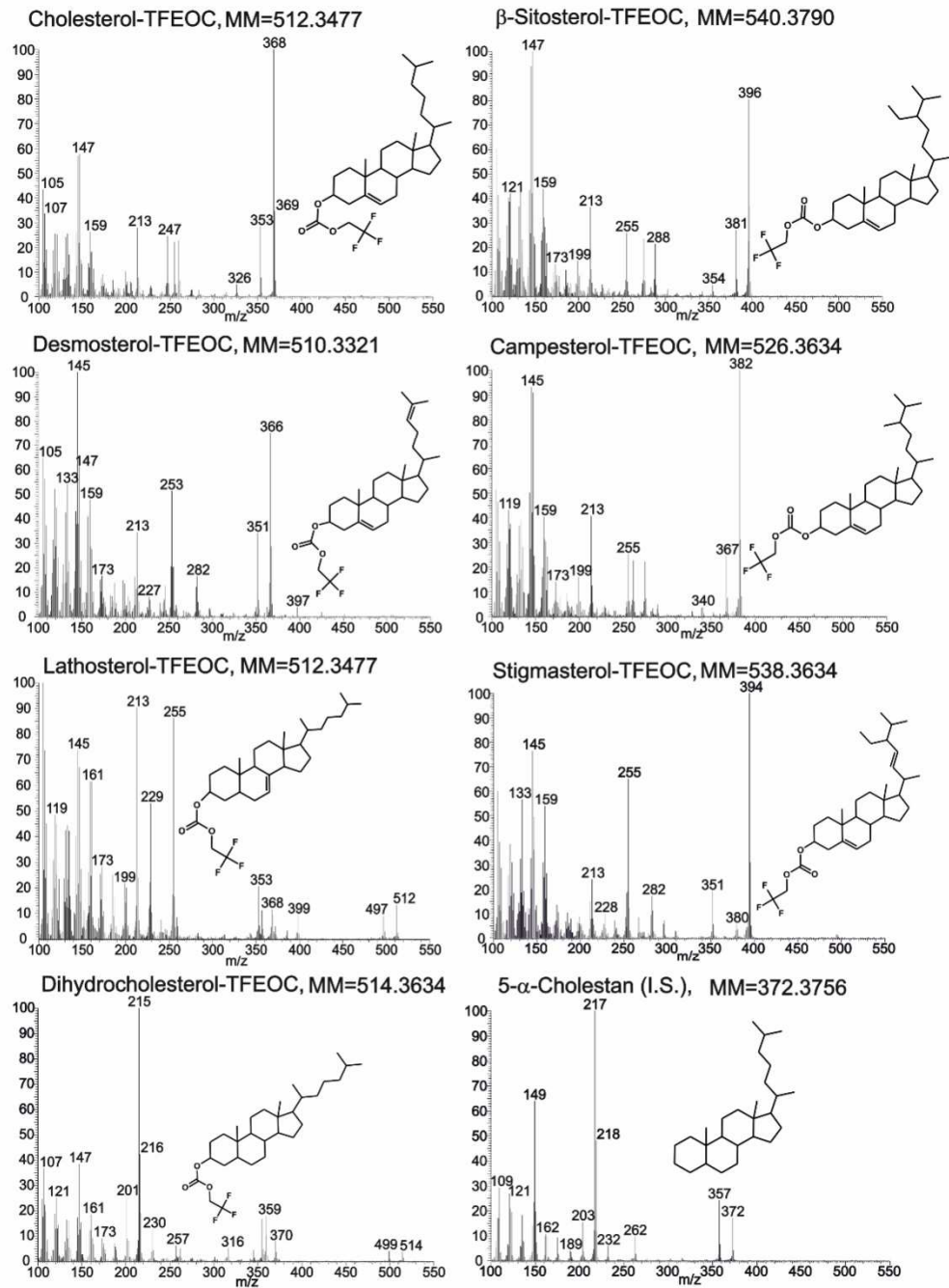
## 7 Přílohy

Obr. S.1: Struktury vyšetřovaných sterolů, steroidů a tokoferolů.

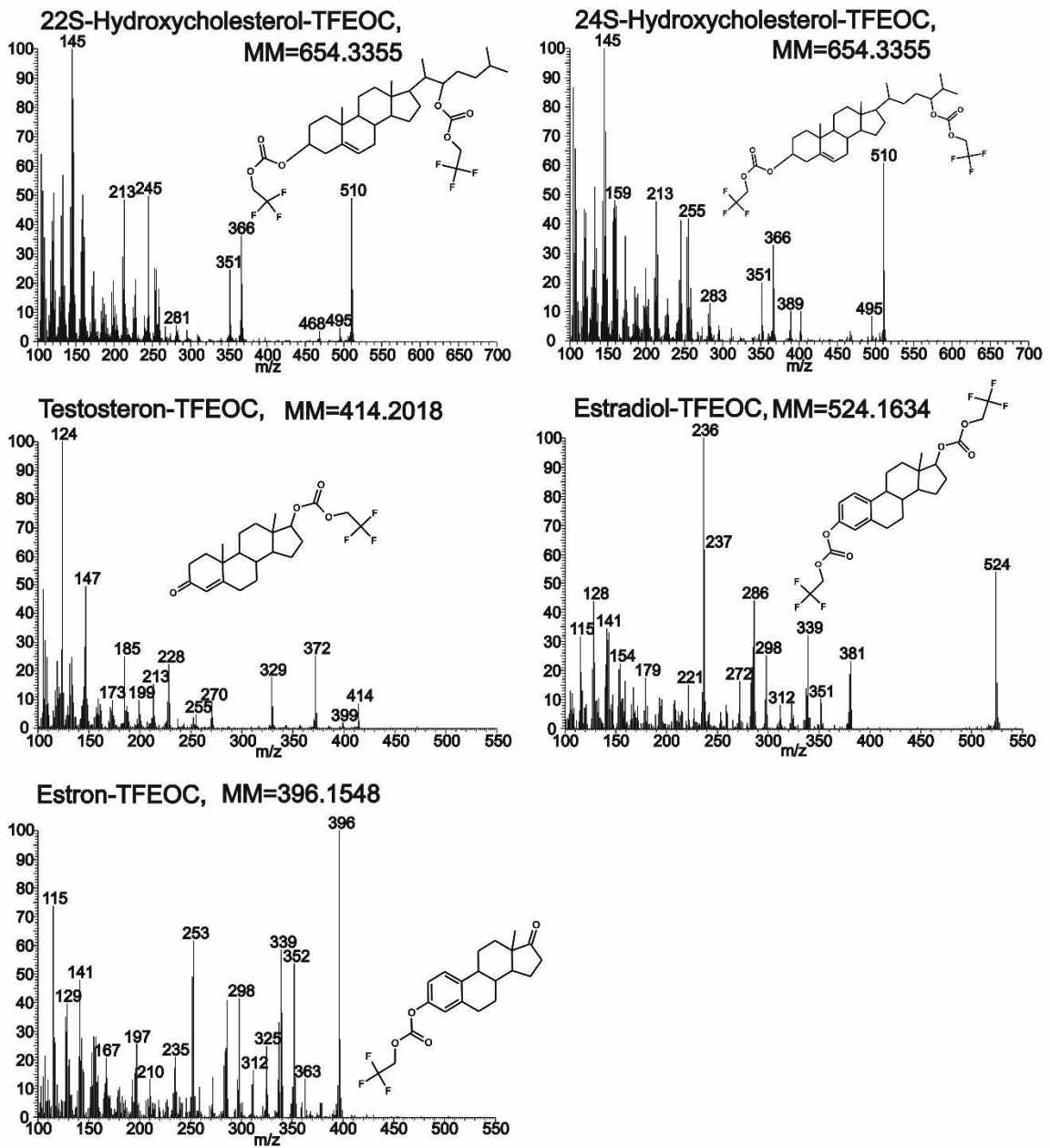




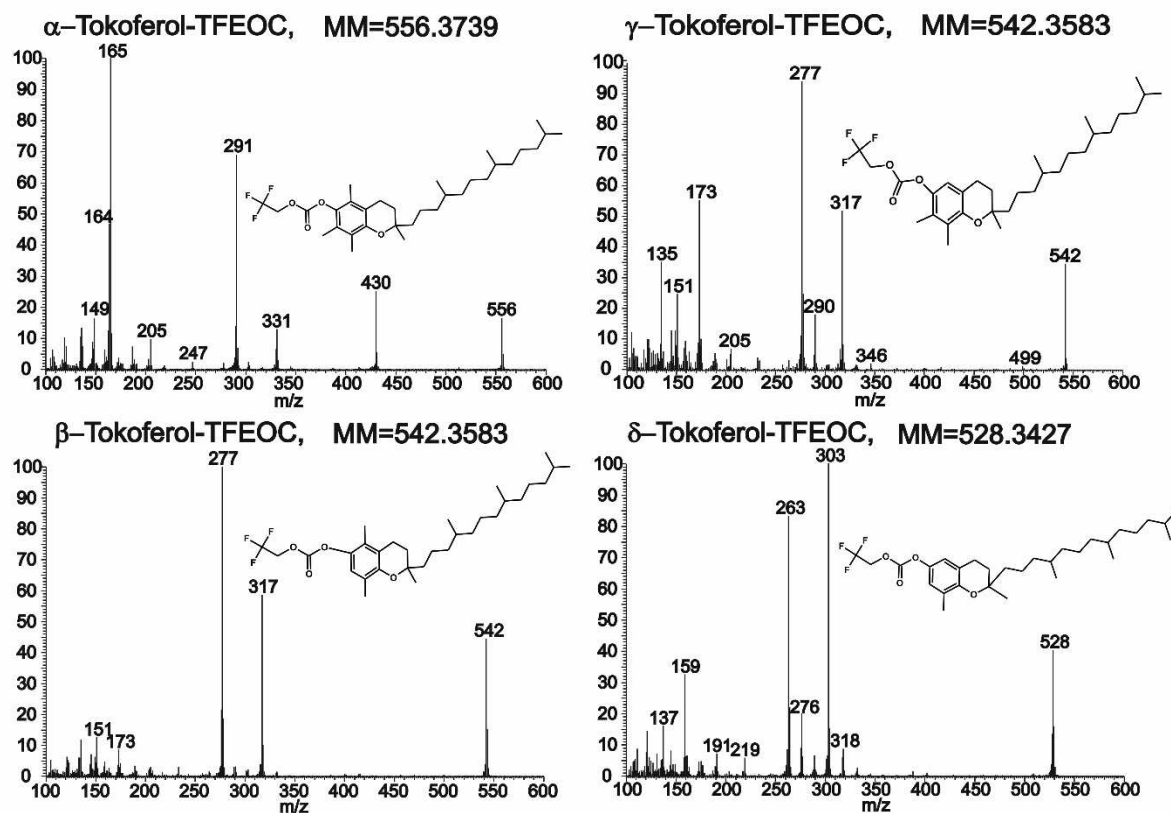
Obr. S.2: EI spektra zkoumaných sterolů, steroidů a tokoferolů po derivatizaci s TFECF.



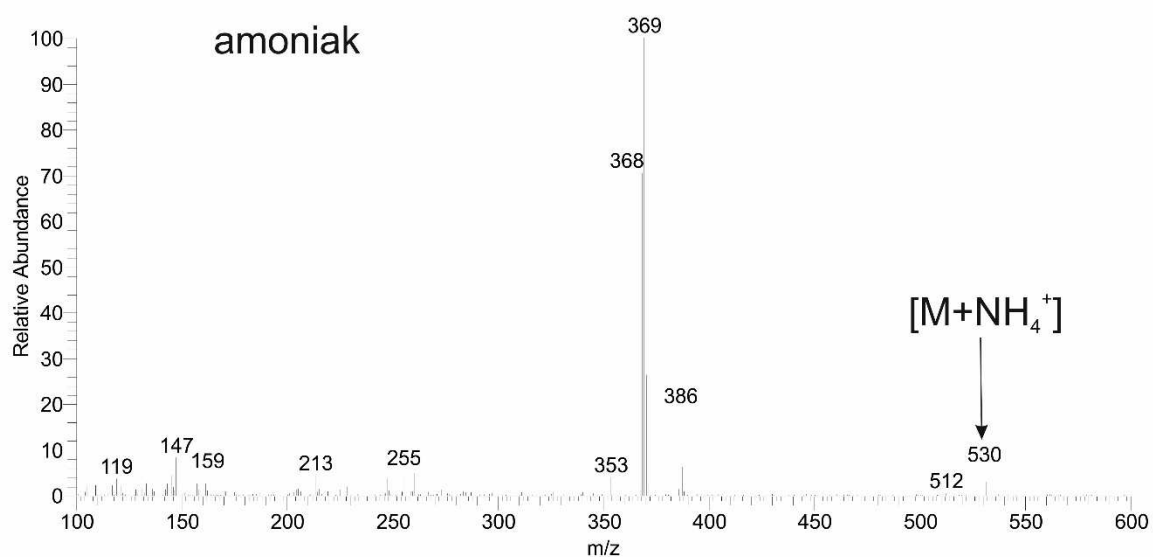
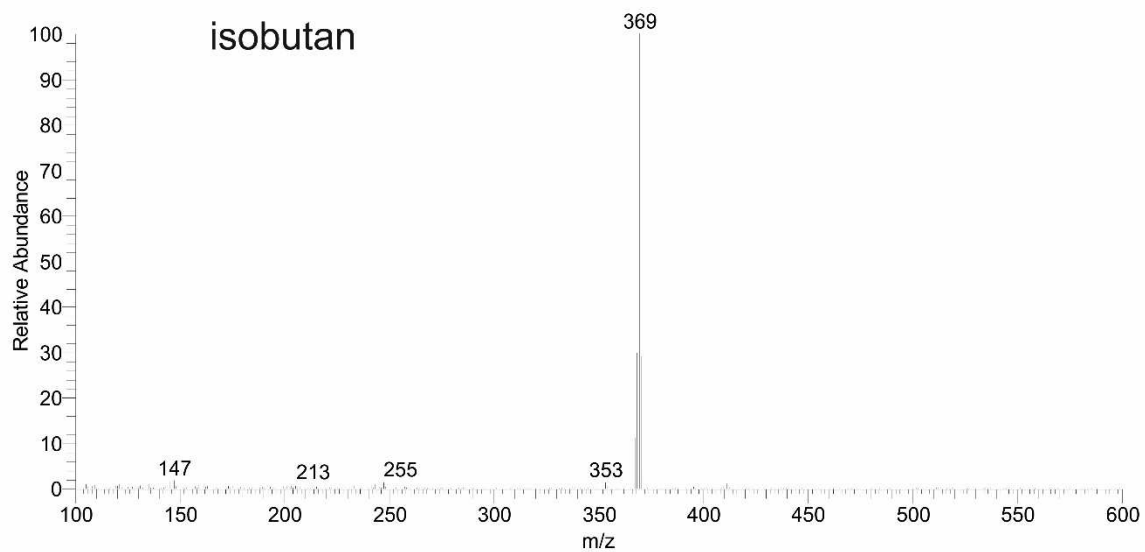
Obr. S.2 (pokračování): EI spektra zkoumaných sterolů, steroidů a tokoferolů po derivatizaci s TFEFC.



Obr. S.2 (pokračování): EI spektra zkoumaných sterolů, steroidů a tokoferolů po derivatizaci s TFECF.



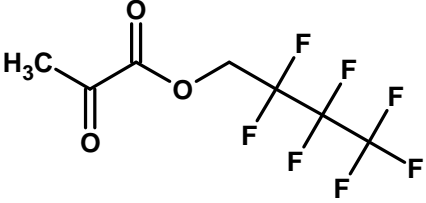
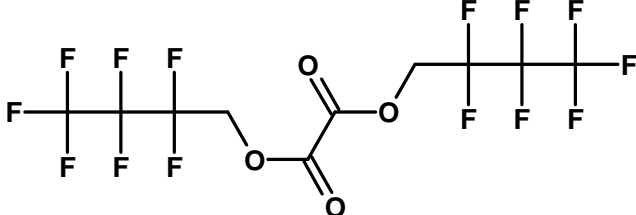
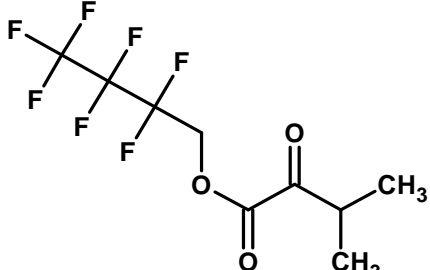
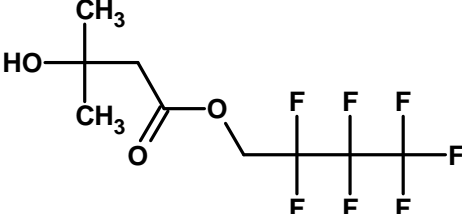
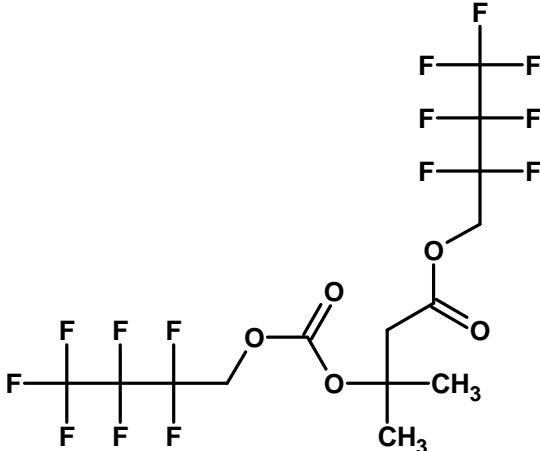
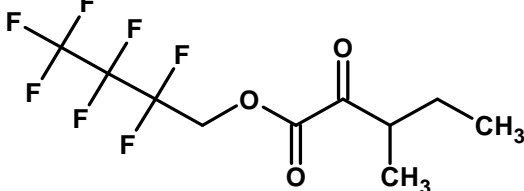
Obr. S.3: PICI spektra TFEOC derivátu cholesterolu, horní obrázek = ionizační plyn isobutan, dolní obrázek = ionizační plyn amoniak.

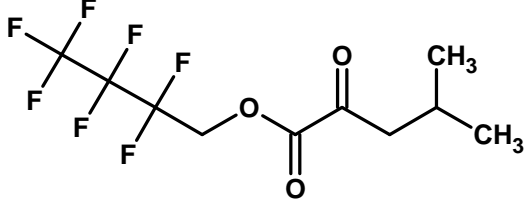
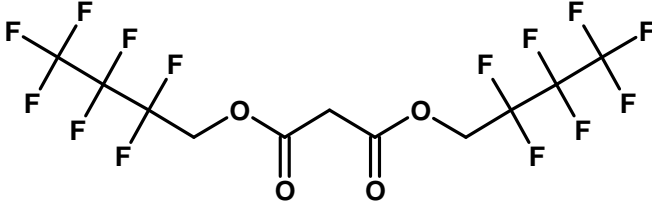
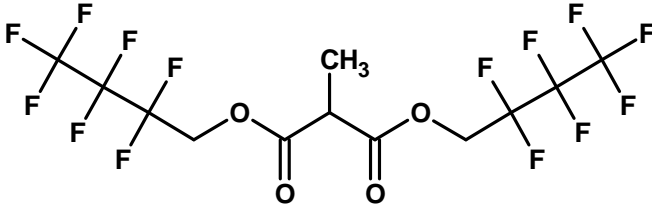
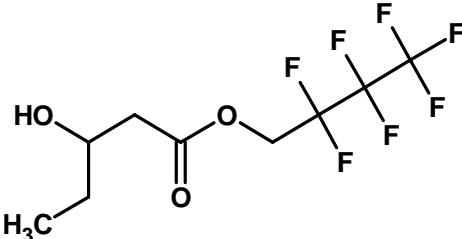
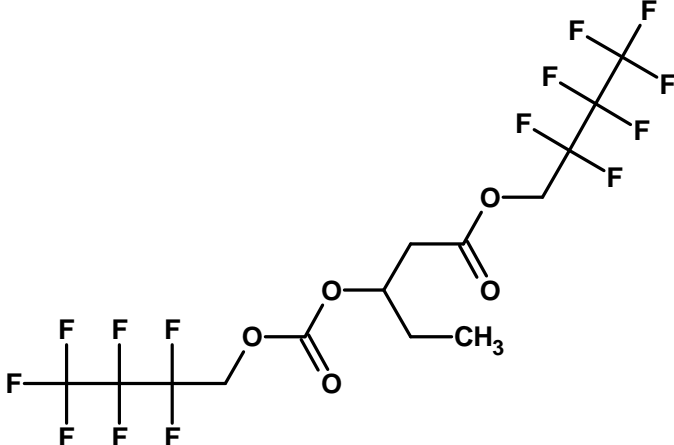
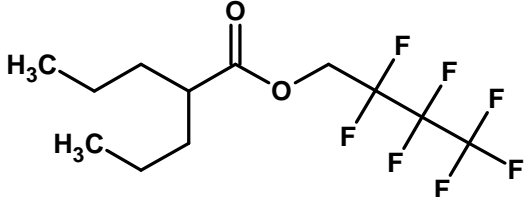


Tab. S.1: Preciznost, přesnost a výtěžnost stanovovaných sterolů a tokoferolů ve vzorcích kontrolního lidského séra po validaci metody.

Analyt	Koncentrační hladina ( $\mu\text{g/ml}$ )	Preciznost (%)		Přesnost (%)		Výtěžnost (%)
		within-run	between-run	within-run	between-run	
$\delta$ -Tokoferol	30	2,6	3,2	98	93	92
	3	5,2	4,6	104	112	90
	0,15	1,2	5,9	91	86	104
$\beta$ -Tokoferol	30	2,6	5,2	96	98	105
	3	3,8	2,5	98	96	108
	0,15	4,1	3,6	103	111	96
$\gamma$ -Tokoferol	30	1,2	5,2	91	89	105
	3	5,1	5,1	96	85	99
	0,15	1,9	4,9	82	86	102
$\alpha$ -Tokoferol	30	5,0	4,3	102	85	110
	3	14,7	0,2	94	86	96
	0,15	13,7	5,2	85	82	92
Dihydrocholesterol	10	11,0	11,1	115	83	94
	1	15,1	3,7	89	83	98
	0,05	19,5	6,6	86	89	97
Desmosterol	10	12,9	13,1	110	105	85
	1	14,2	10,8	104	109	86
	0,05	18,9	19,5	111	113	90
Lathosterol	10	5,3	12,1	95	86	96
	1	11,6	15,9	85	88	92
	0,05	2,2	7,5	86	91	91
Kampesterol	10	3,7	11,2	98	105	101
	1	0,9	5,8	86	98	108
	0,05	11,1	11,0	83	86	98
Stigmasterol	10	8,5	9,0	90	85	99
	1	3,6	4,8	85	90	101
	0,05	9,3	9,6	82	82	109
$\beta$ -Sitosterol	10	5,6	7,6	99	108	110
	1	4,6	12,6	98	87	98
	0,05	13,6	19,0	85	84	95

Tab. S.2: Navrhované struktury pro pozorované reakční produkty močových metabolitů po reakci s HFBCF.

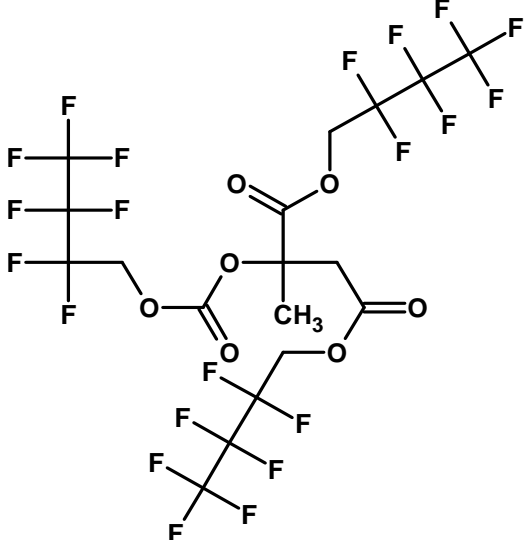
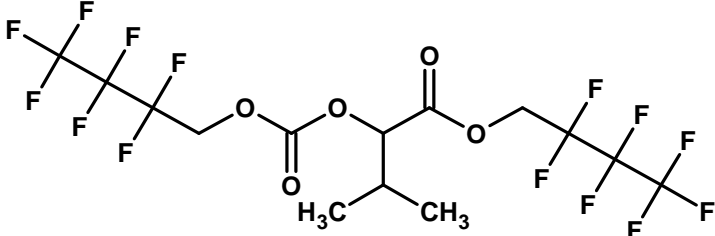
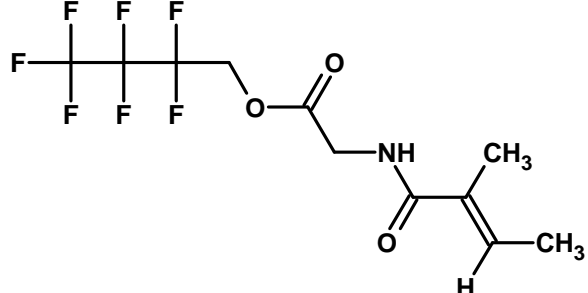
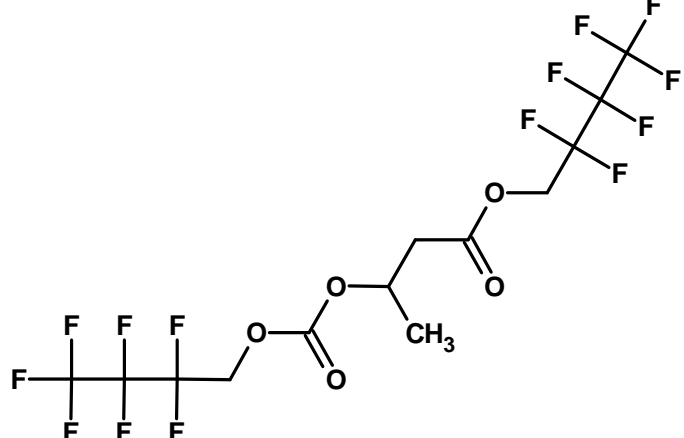
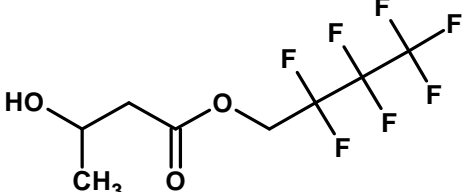
	<p>Číslo 1 K. pyrohroznová HMDB ID: HMDB00243 Molekulový vzorec: C7H5F7O3 Monoisotopická hmotnost = 270,012691 Da</p>
	<p>Číslo 2 K. šťavelová HMDB ID: HMDB02329 Molekulový vzorec: C10H4F14O4 Monoisotopická hmotnost = 453,988603 Da</p>
	<p>Číslo 3 K. 2-ketoisovalerová HMDB ID: HMDB00019 Molekulový vzorec: C9H9F7O3 Monoisotopická hmotnost = 298,043992 Da</p>
	<p>Číslo 4a K. 3-hydroxyisovalerová-1 (OH) HMDB ID: HMDB00754 Molekulový vzorec: C9H11F7O3 Monoisotopická hmotnost = 300,059642 Da</p>
	<p>Číslo 4b K. 3-hydroxyisovalerová-2 (OR, 65 %) Molekulový vzorec: C14H12F14O5 Monoisotopická hmotnost = 526,046118 Da</p>
	<p>Číslo 5 K. 2-keto-3-methylvalerová HMDB ID: HMDB00491 Molekulový vzorec: C10H11F7O3 Monoisotopická hmotnost = 312,059642 Da</p>

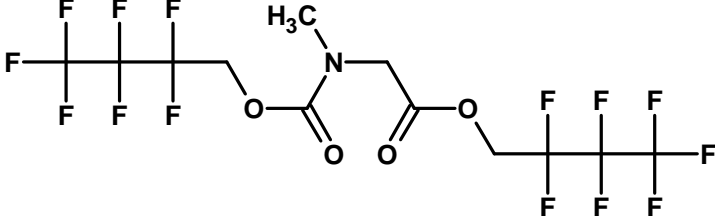
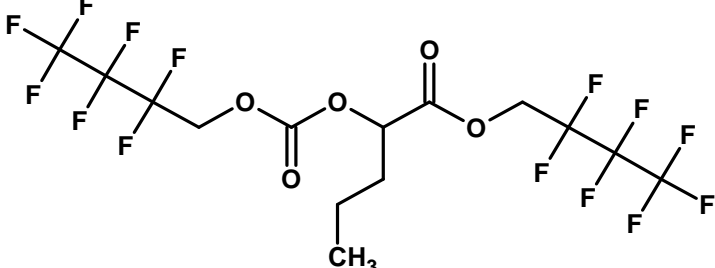
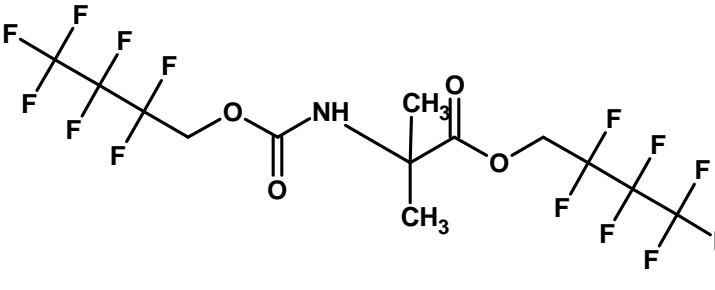
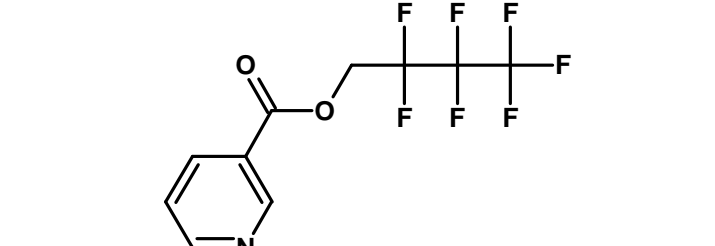
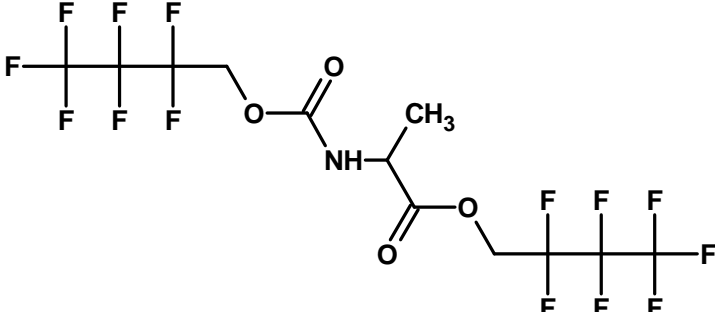
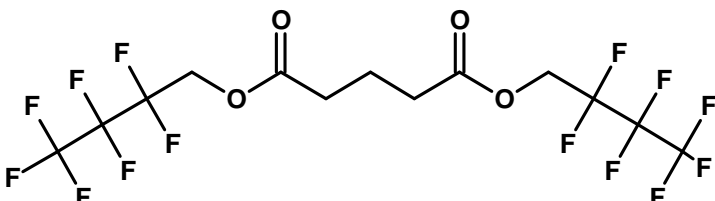
	<p>Číslo 6 K. 4-methyl-2-oxovalerová HMDB ID: HMDB00695 Molekulový vzorec: C10H11F7O3 Monoisotopická hmotnost = 312,059642 Da</p>
	<p>Číslo 7 K. malonová HMDB ID: HMDB00691 Molekulový vzorec: C11H6F14O4 Monoisotopická hmotnost = 468,004253 Da</p>
	<p>Číslo 8 K. methylmalonová HMDB ID: HMDB00202 Molekulový vzorec: C12H8F14O4 Monoisotopická hmotnost = 482,019904 Da</p>
	<p>Číslo 9a K. 3-hydroxyvalerová-1 (OH) HMDB ID: HMDB00531 Molekulový vzorec: C9H11F7O3 Monoisotopická hmotnost = 300,059642 Da</p>
	<p>Číslo 9b K. 3-hydroxyvalerová-2 (OR, 65 %) Molekulový vzorec: C14H12F14O5 Monoisotopická hmotnost = 526,046118 Da</p>
	<p>Číslo 10 K. 2-propylvalerová HMDB ID: HMDB01877 Molekulový vzorec: C12H17F7O2 Monoisotopická hmotnost = 326,111677 Da</p>

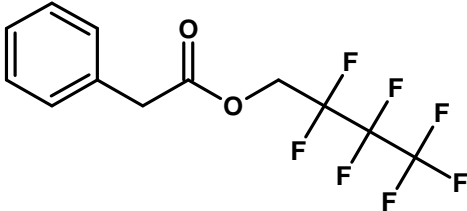
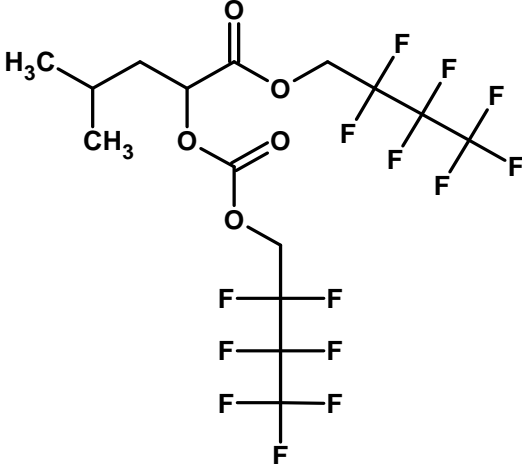
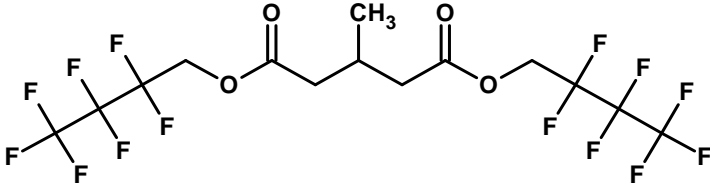
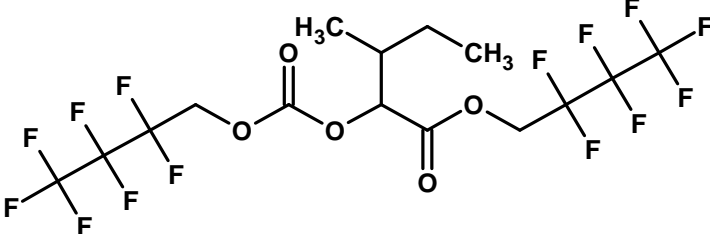
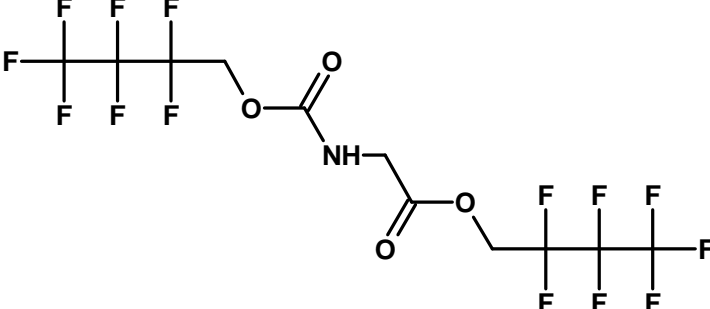
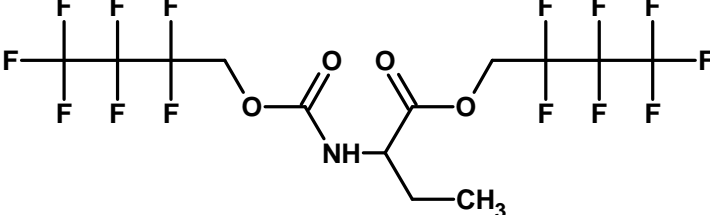
	<p>Číslo 11  K. 2-hydroxyisomáselná  HMDB ID: HMDB00729  Molekulový vzorec: C<sub>13</sub>H<sub>10</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost = 512,030468 Da</p>
	<p>Číslo 12  K. ethylmalonová  HMDB ID: HMDB00622  Molekulový vzorec: C<sub>13</sub>H<sub>10</sub>F<sub>14</sub>O<sub>4</sub>  Monoisotopická hmotnost = 496,0355 Da</p>
	<p>Číslo 13  K. mléčná  HMDB ID: HMDB00190  Molekulový vzorec: C<sub>12</sub>H<sub>8</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost = 498,014818 Da</p>
	<p>Číslo 14  K. glykolová  HMDB ID: HMDB00115  Molekulový vzorec: C<sub>11</sub>H<sub>6</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost = 483,999168 Da</p>
	<p>Číslo 15  K. fumarová  HMDB ID: HMDB00134  Molekulový vzorec: C<sub>12</sub>H<sub>6</sub>F<sub>14</sub>O<sub>4</sub>  Monoisotopická hmotnost = 480,004253 Da</p>

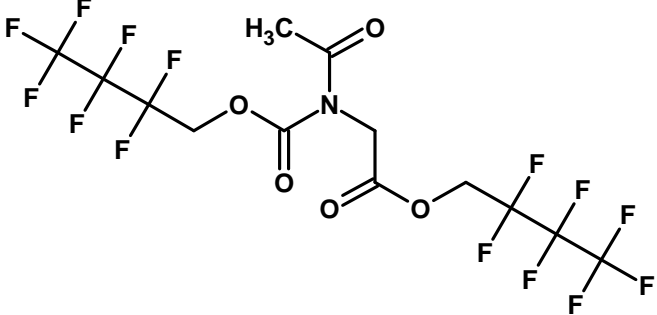
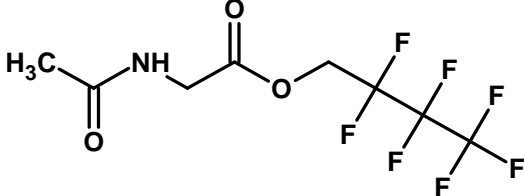
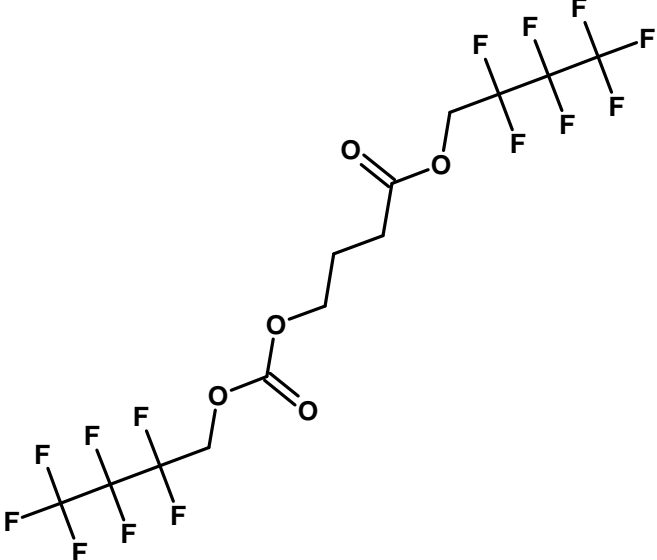
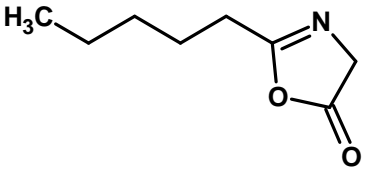
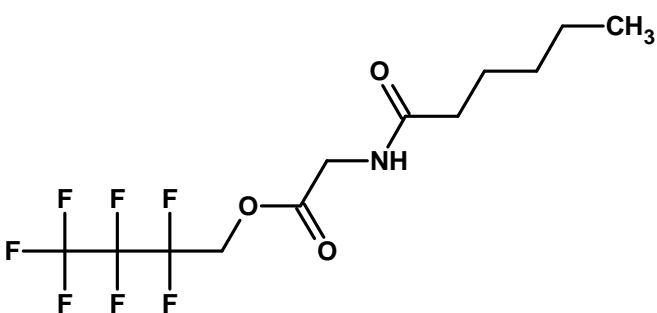


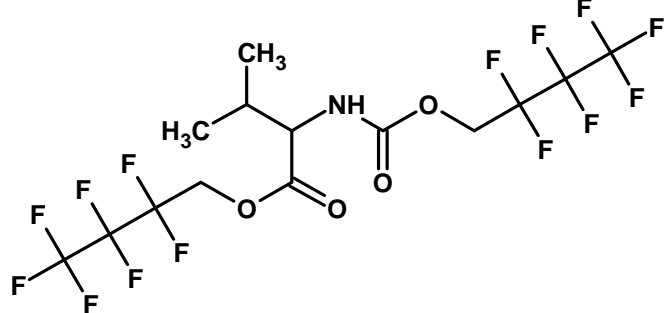
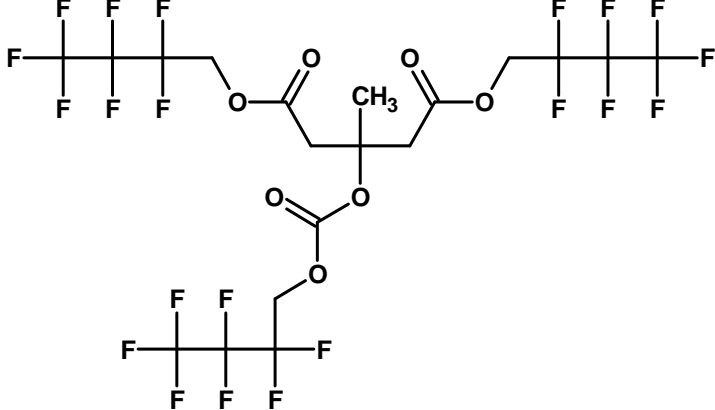
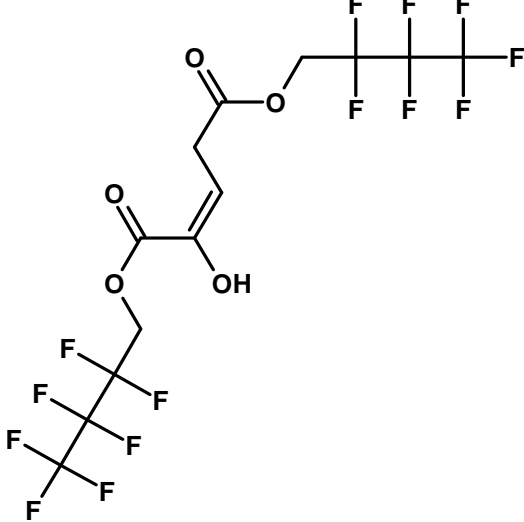
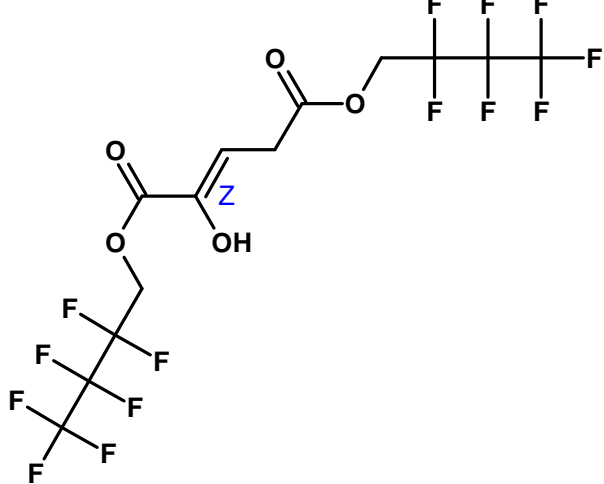
	<p>Číslo 16 K. citrakonová HMDB ID: HMDB00634 Molekulový vzorec: C13H8F14O4 Monoisotopická hmotnost = 494,019904 Da</p>
	<p>Číslo 17 K. jantarová HMDB ID: HMDB00254 Molekulový vzorec: C12H8F14O4 Monoisotopická hmotnost = 482,019904 Da</p>
	<p>Číslo 18 K. methyljantarová HMDB ID: HMDB01844 Molekulový vzorec: C13H10F14O4 Monoisotopická hmotnost = 496,035554 Da</p>
	<p>Číslo 19 K. 2-hydroxymáselná HMDB ID: HMDB00008 Molekulový vzorec: C13H10F14O5 Monoisotopická hmotnost = 512,030468 Da</p>
	<p>Číslo 20 K. benzoová HMDB ID: HMDB01870 Molekulový vzorec: C11H7F7O2 Monoisotopická hmotnost = 304,033427 Da</p>
	<p>Číslo 21a K. citramalová-1 (laktón) HMDB ID: HMDB00426 Molekulový vzorec: C9H7F7O4 Monoisotopická hmotnost = 312,023256 Da</p>

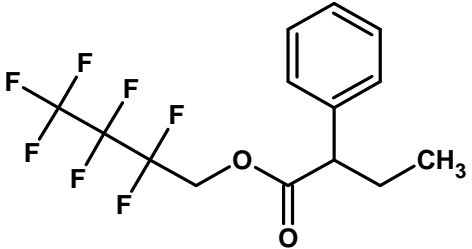
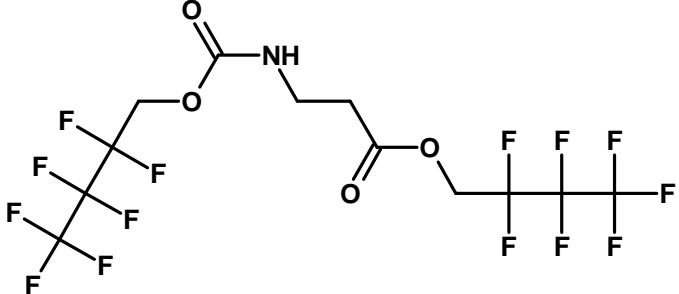
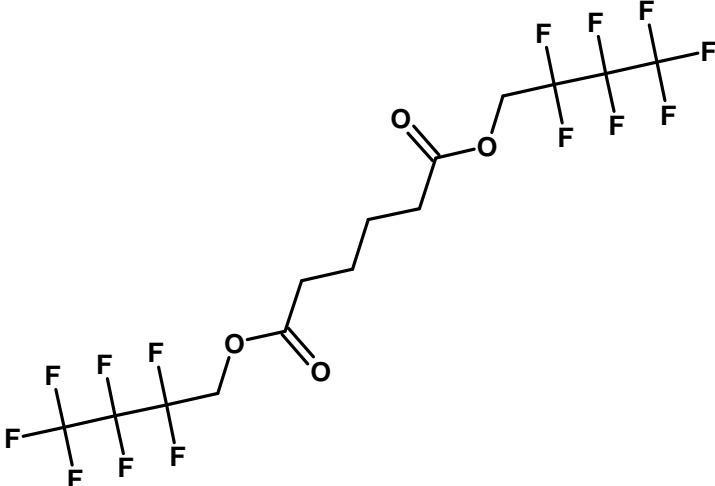
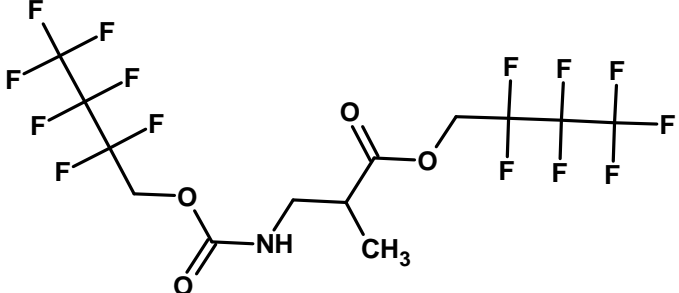
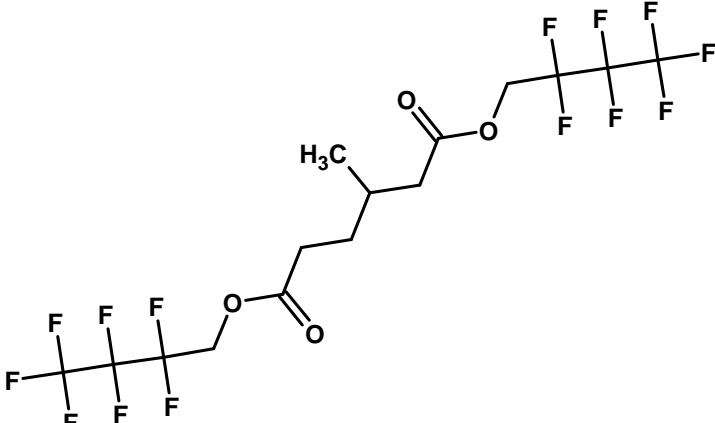
	<p>Číslo 21b  K. citramalová-2 (OR, 30 %)  HMDB ID: HMDB00426  Molekulový vzorec: C<sub>18</sub>H<sub>11</sub>F<sub>21</sub>O<sub>7</sub>  Monoisotopická hmotnost = 738,016945 Da</p>
	<p>Číslo 22  K. 2-hydroxyisovalerová  HMDB ID: HMDB00407  Molekulový vzorec: C<sub>14</sub>H<sub>12</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost = 526,046118 Da</p>
	<p>Číslo 23  Tiglylglycin  HMDB ID: HMDB00959  Molekulový vzorec: C<sub>11</sub>H<sub>12</sub>F<sub>7</sub>NO<sub>3</sub>  Monoisotopická hmotnost = 339,070541 Da</p>
	<p>Číslo 24a  K. 3-hydroxymáselná-2 (OR)  HMDB ID: HMDB00357  Molekulový vzorec: C<sub>13</sub>H<sub>10</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost = 512,030468 Da</p>
	<p>Číslo 24b  K. 3-hydroxymáselná-1 (OH, 65 %)  Molekulový vzorec: C<sub>8</sub>H<sub>9</sub>F<sub>7</sub>O<sub>3</sub>  Monoisotopická hmotnost = 286,043992 Da</p>

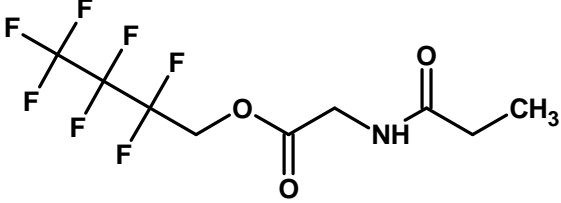
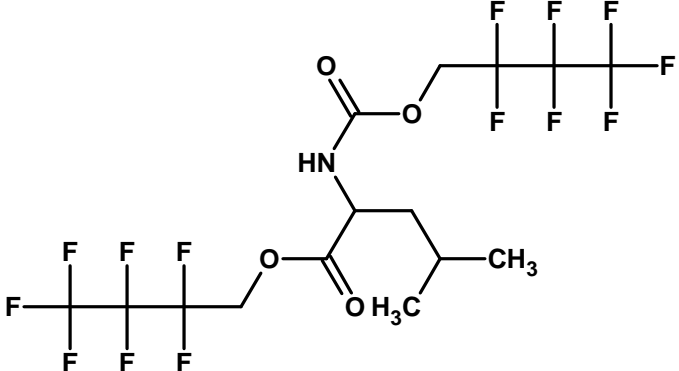
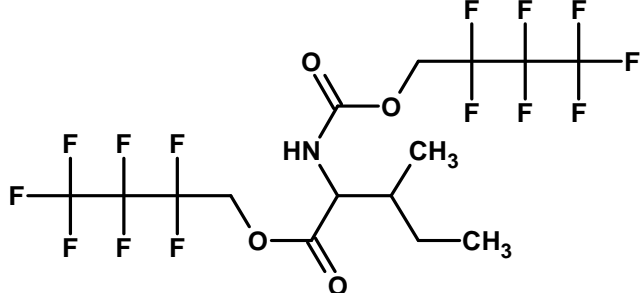
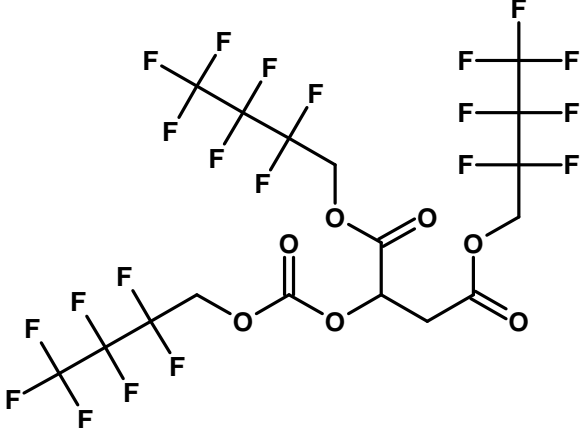
	<p>Číslo 25 Sarkosin HMDB ID: HMDB00271 Molekulový vzorec: C<sub>12</sub>H<sub>9</sub>F<sub>14</sub>NO<sub>4</sub> Monoisotopická hmotnost = 497,030803 Da</p>
	<p>Číslo 26 K. 2-hydroxyvalerová HMDB ID: HMDB01863 Molekulový vzorec: C<sub>14</sub>H<sub>12</sub>F<sub>14</sub>O<sub>5</sub> Monoisotopická hmotnost = 526,046118 Da</p>
	<p>Číslo 27 K. 2-aminoisomáselná HMDB ID: HMDB01906 Molekulový vzorec: C<sub>14</sub>H<sub>13</sub>F<sub>14</sub>NO<sub>4</sub> Monoisotopická hmotnost = 511,046453 Da</p>
	<p>Číslo 28 K. nikotinová HMDB ID: HMDB01488 Molekulový vzorec: C<sub>10</sub>H<sub>6</sub>F<sub>7</sub>NO<sub>2</sub> Monoisotopická hmotnost = 305,028676 Da</p>
	<p>Číslo 29 Alanin HMDB ID: HMDB00161 Molekulový vzorec: C<sub>12</sub>H<sub>9</sub>F<sub>14</sub>NO<sub>4</sub> Monoisotopická hmotnost = 497,030803 Da</p>
	<p>Číslo 30 K. glutarová HMDB ID: HMDB00661 Molekulový vzorec: C<sub>13</sub>H<sub>10</sub>F<sub>14</sub>O<sub>4</sub> Monoisotopická hmotnost = 496,035554 Da</p>

	<p>Číslo 31 K, fenylactová HMDB ID: HMDB00209 Molekulový vzorec: C12H9F7O2 Monoisotopická hmotnost = 318,049077 Da</p>
	<p>Číslo 32 K, 2-hydroxyisokapronová HMDB ID: HMDB00665 Molekulový vzorec: C15H14F14O5 Monoisotopická hmotnost = 540,061768 Da</p>
	<p>Číslo 33 K, 3-methylglutarová HMDB ID: HMDB00752 Molekulový vzorec: C14H12F14O4 Monoisotopická hmotnost = 510,051204 Da</p>
	<p>Číslo 34 K, 2-hydroxy-3-methylvalerová HMDB ID: HMDB00819 Molekulový vzorec: C15H14F14O5 Monoisotopická hmotnost = 540,061768 Da</p>
	<p>Číslo 35 Glycin HMDB ID: HMDB00123 Molekulový vzorec: C11H7F14NO4 Monoisotopická hmotnost = 483,015153 Da</p>
	<p>Číslo 36 K, 2-aminomáselná HMDB ID: HMDB00452 Molekulový vzorec: C13H11F14NO4 Monoisotopická hmotnost = 511,046453 Da</p>

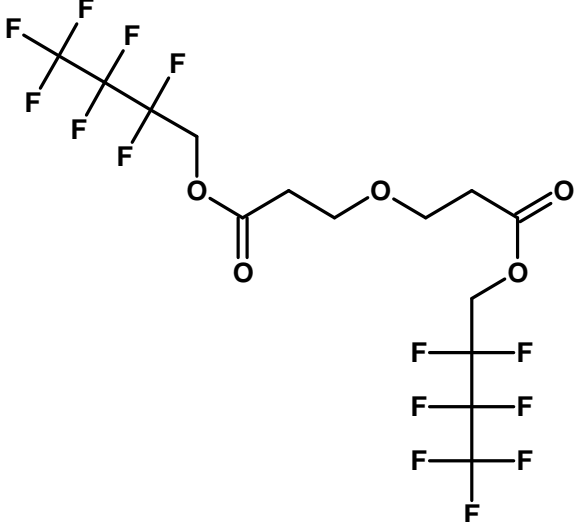
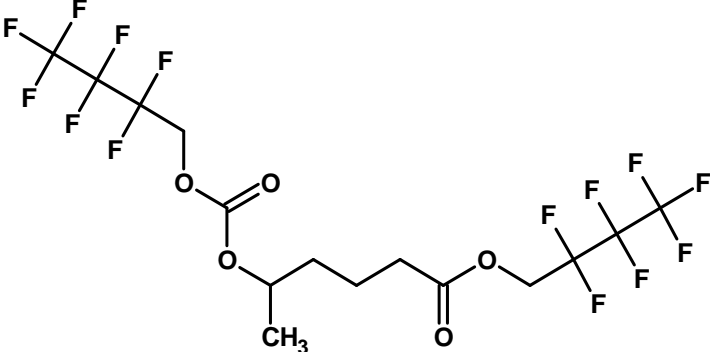
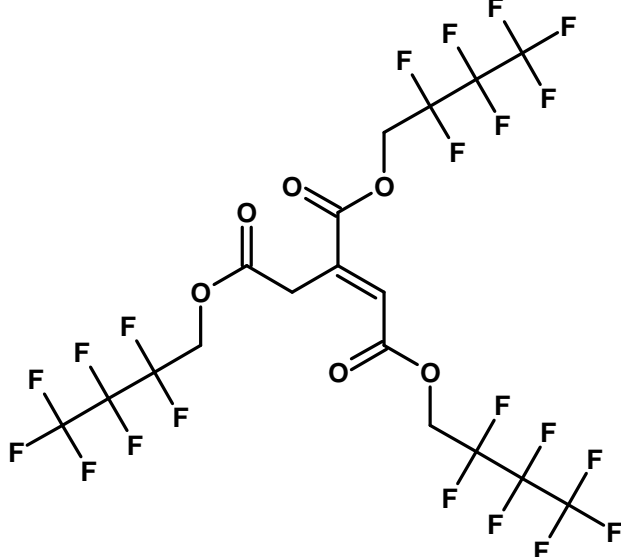
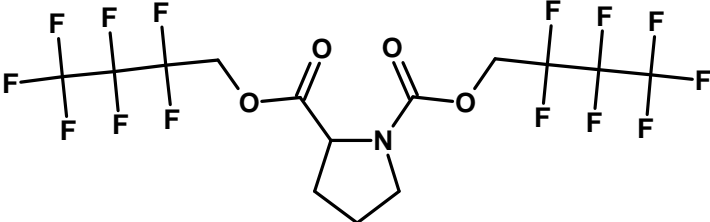
 <p>Chemical structure of N-Acetyl-2-(perfluoromethyl)glycine. It features a central nitrogen atom bonded to an acetyl group (H<sub>3</sub>C-C(=O)-), a perfluoromethyl group (-CH<sub>2</sub>-F<sub>7</sub>), and a perfluoroethyl ester group (-CH<sub>2</sub>-C(=O)-O-CH<sub>2</sub>-F<sub>5</sub>).</p>	<p>Číslo 37a  N-Acetylglycin-2  HMDB ID: HMDB00532  Molekulový vzorec: C<sub>13</sub>H<sub>9</sub>F<sub>14</sub>NO<sub>5</sub>  Monoisotopická hmotnost = 525,025717 Da</p>
 <p>Chemical structure of N-Acetyl-1-(perfluoroethyl)glycine. It consists of an acetyl group (H<sub>3</sub>C-C(=O)-) attached to the nitrogen of a glycine derivative, which is further substituted with a perfluoroethyl ester group (-CH<sub>2</sub>-C(=O)-O-CH<sub>2</sub>-F<sub>5</sub>).</p>	<p>Číslo 37b  N-Acetylglycin-1(NR, 65%)  Molekulový vzorec: C<sub>8</sub>H<sub>8</sub>F<sub>7</sub>NO<sub>3</sub>  Monoisotopická hmotnost = 299,039241 Da</p>
 <p>Chemical structure of 4-(perfluoromethyl)butanoic acid. It shows a four-carbon chain with a carboxylic acid group at one end and a perfluoromethyl group (-CH<sub>2</sub>-F<sub>7</sub>) at the other end.</p>	<p>Číslo 38  K. 4-hydroxymáselná  HMDB ID: HMDB00710  Molekulový vzorec: C<sub>13</sub>H<sub>10</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost = 512,030468 Da</p>
 <p>Chemical structure of 6-(perfluoromethyl)hexanamide. It features a six-carbon chain with an amide group (-NH<sub>2</sub>) at one end and a perfluoromethyl group (-CH<sub>2</sub>-F<sub>7</sub>) at the other end.</p>	<p>Číslo 39a  Hexanoylglycin-1 (cyklický)  Molekulový vzorec: C<sub>8</sub>H<sub>13</sub>NO<sub>2</sub>  Monoisotopická hmotnost = 155,094629 Da</p>
 <p>Chemical structure of N-(perfluoromethyl)hexanamide. It shows a six-carbon chain with an amide group (-NH-) at one end and a perfluoromethyl group (-CH<sub>2</sub>-F<sub>7</sub>) at the other end.</p>	<p>Číslo 39b  Hexanoylglycin-2 (65 %)  HMDB ID: HMDB00701  Molekulový vzorec: C<sub>12</sub>H<sub>16</sub>F<sub>7</sub>NO<sub>3</sub>  Monoisotopická hmotnost = 355,101841 Da</p>

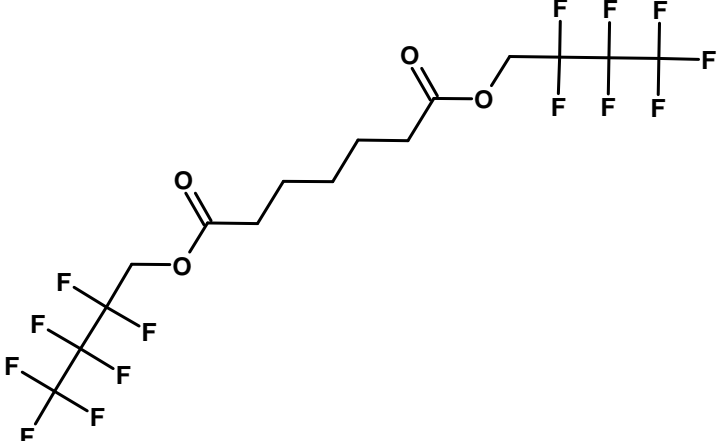
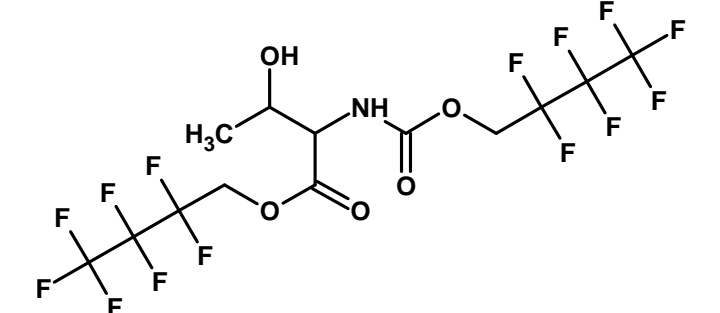
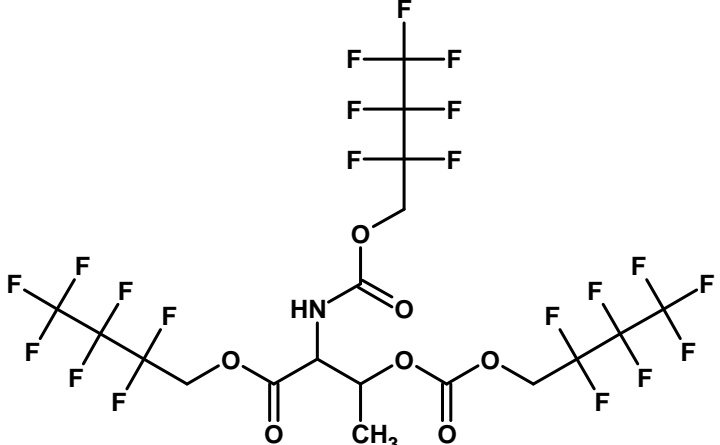
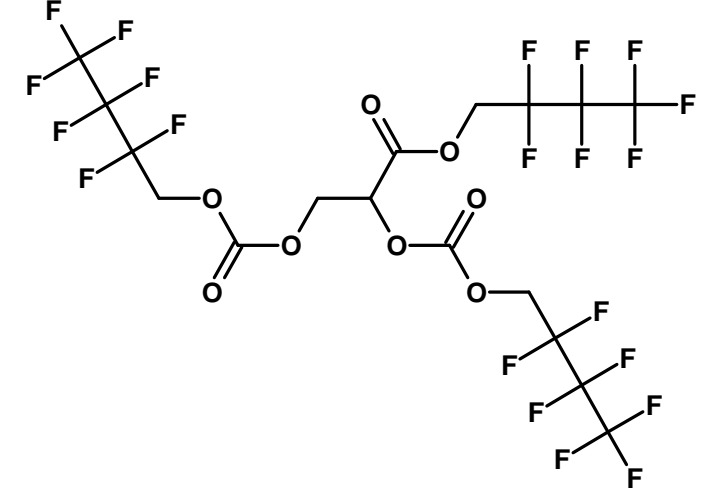
	<p>Číslo 40 Valin HMDB ID: HMDB00883 Molekulový vzorec: C14H13F14NO4 Monoisotopická hmotnost = 525,062103 Da</p>
	<p>Číslo 41 K. 3-hydroxy-3-methylglutarová HMDB ID: HMDB00355 Molekulový vzorec: C19H13F21O7 Monoisotopická hmotnost = 752,032595 Da</p>
	<p>Číslo 42a K. 2-ketoglutarová-1 (2E, 80 %) HMDB ID: HMDB00208 Molekulový vzorec: C13H8F14O5 Monoisotopická hmotnost = 510,014818 Da</p>
	<p>Číslo 42b K. 2-ketoglutarová-2 (2Z) HMDB ID: HMDB00208 Molekulový vzorec: C13H8F14O5 Monoisotopická hmotnost = 510,014818 Da</p>

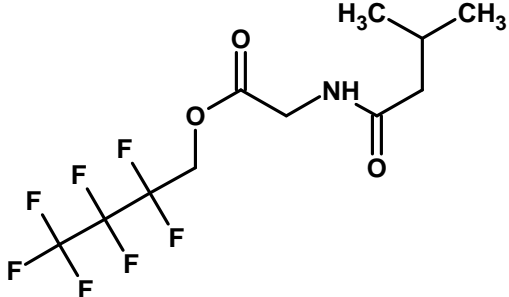
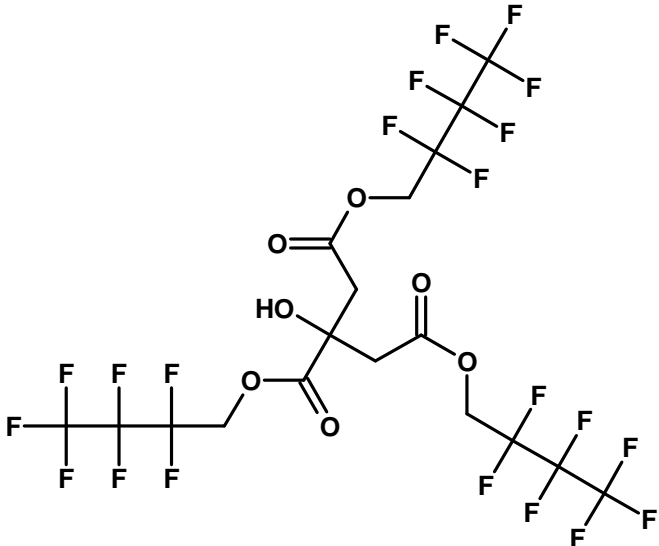
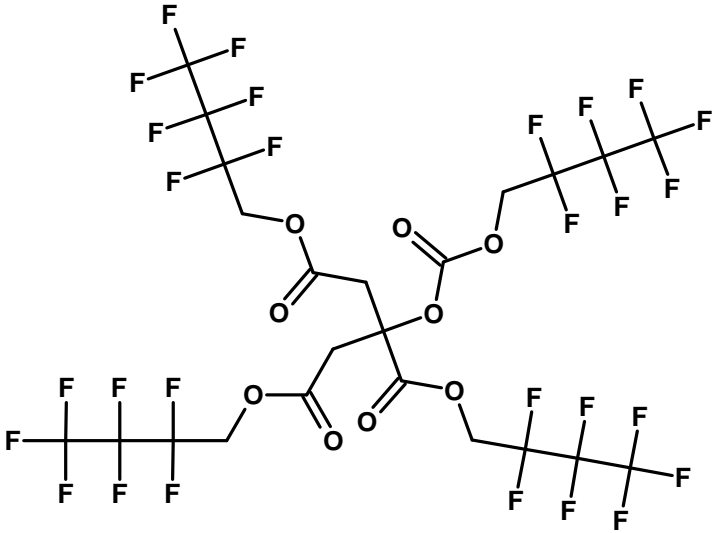
	<p>Číslo 43  K. 2-fenylmásečná – I.S.  Molekulový vzorec: C<sub>14</sub>H<sub>13</sub>F<sub>7</sub>O<sub>2</sub>  Monoisotopická hmotnosť =  346,080377 Da</p>
	<p>Číslo 44  3-Alanin  HMDB ID: HMDB00056  Molekulový vzorec: C<sub>12</sub>H<sub>9</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnosť =  497,030803 Da</p>
	<p>Číslo 45  K. adipová  HMDB ID: HMDB00448  Molekulový vzorec: C<sub>14</sub>H<sub>12</sub>F<sub>14</sub>O<sub>4</sub>  Monoisotopická hmotnosť =  510,051204 Da</p>
	<p>Číslo 46  K. 3-aminoisomásečná  HMDB ID: HMDB03911  Molekulový vzorec: C<sub>13</sub>H<sub>11</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnosť =  511,046453 Da</p>
	<p>Číslo 47  K. 3-methyladipová  HMDB ID: HMDB00555  Molekulový vzorec: C<sub>15</sub>H<sub>14</sub>F<sub>14</sub>O<sub>4</sub>  Monoisotopická hmotnosť =  524,066854 Da</p>

	<p>Číslo 48  Propionylglycin  HMDB ID: HMDB00783  Molekulový vzorec: C<sub>9</sub>H<sub>10</sub>F<sub>7</sub>NO<sub>3</sub>  Monoisotopická hmotnost =  313,054891 Da</p>
	<p>Číslo 49  Leucin  HMDB ID: HMDB00687  Molekulový vzorec: C<sub>15</sub>H<sub>15</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnost =  539,077753 Da</p>
	<p>Číslo 50  Isoleucin  HMDB ID: HMDB00172  Molekulový vzorec: C<sub>15</sub>H<sub>15</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnost =  539,077753 Da</p>
	<p>Číslo 51  K. jablečná  HMDB ID: HMDB00744  Molekulový vzorec: C<sub>17</sub>H<sub>9</sub>F<sub>21</sub>O<sub>7</sub>  Monoisotopická hmotnost =  724,001295 Da</p>

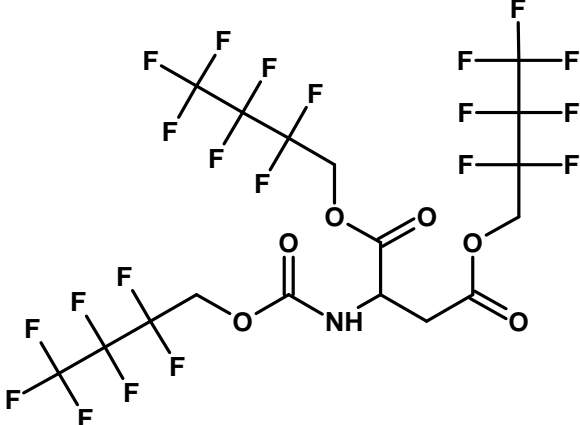
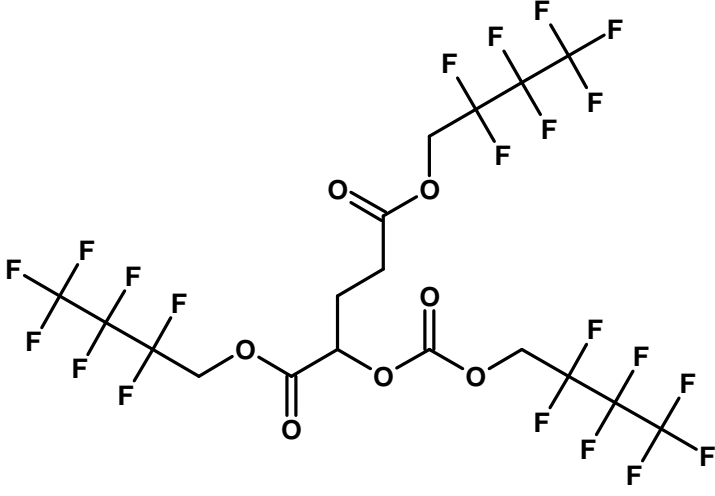
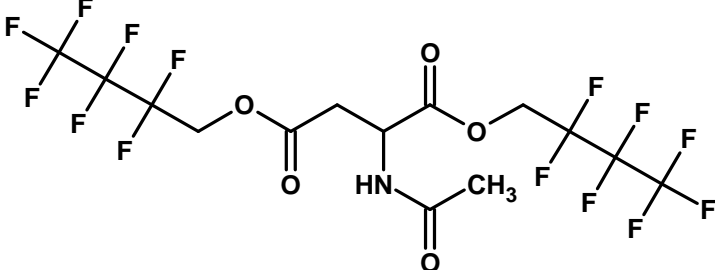
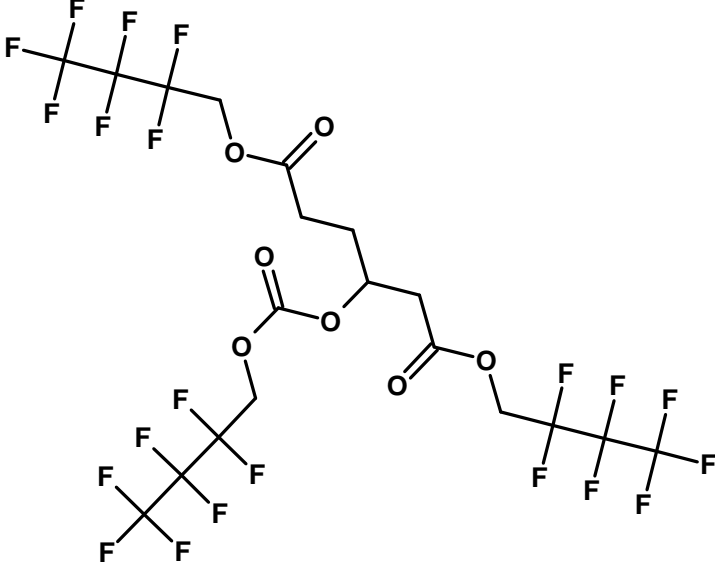


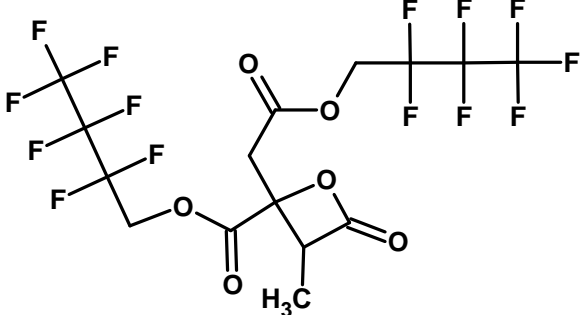
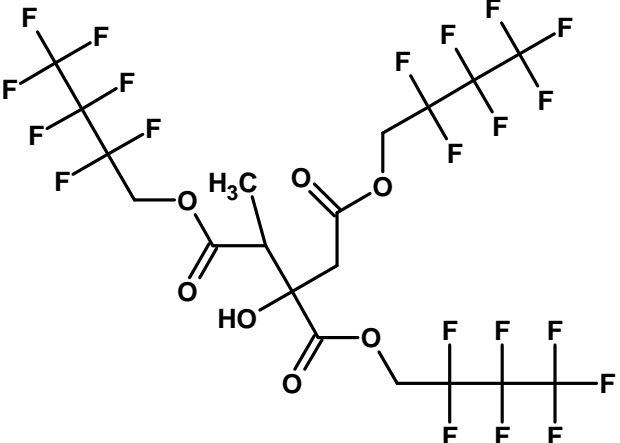
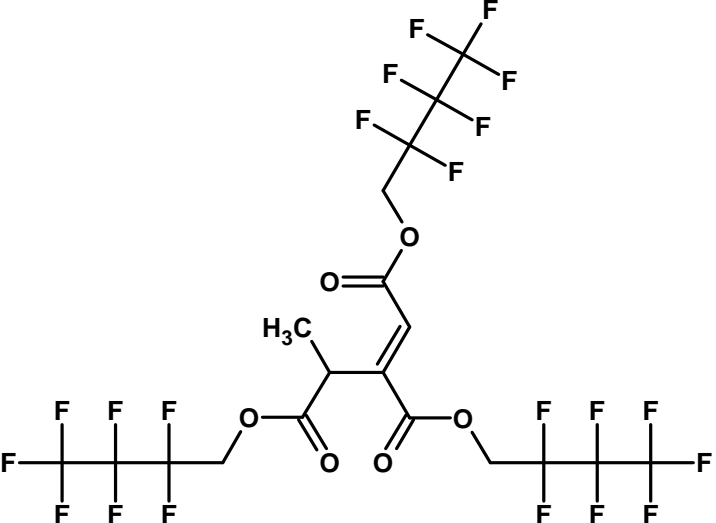
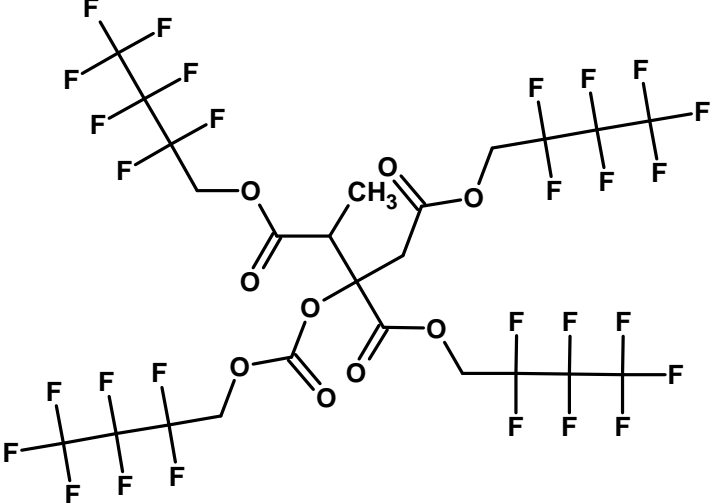
	<p>Číslo 52  K. 3-hydroxypropionová (dimer)  HMDB ID: HMDB00700  Molekulový vzorec: C<sub>14</sub>H<sub>12</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost =  526,046118 Da</p>
	<p>Číslo 53  K. 5-hydroxyhexanová  HMDB ID: HMDB00525  Molekulový vzorec: C<sub>15</sub>H<sub>14</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost =  540,061768 Da</p>
	<p>Číslo 54  K. <i>trans</i>-aconitová  HMDB ID: HMDB00958  Molekulový vzorec: C<sub>18</sub>H<sub>9</sub>F<sub>21</sub>O<sub>6</sub>  Monoisotopická hmotnost =  720,00638 Da</p>
	<p>Číslo 55  Prolin  HMDB ID: HMDB00162  Molekulový vzorec: C<sub>14</sub>H<sub>11</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnost =  523,046453 Da</p>

	<p>Číslo 56 K. pimelová HMDB ID: HMDB00857 Molekulový vzorec: C<sub>15</sub>H<sub>14</sub>F<sub>14</sub>O<sub>4</sub> Monoisotopická hmotnost = 524,066854 Da</p>
	<p>Číslo 57a Threonin-1 (OH) HMDB ID: HMDB00167 Molekulový vzorec: C<sub>13</sub>H<sub>11</sub>F<sub>14</sub>NO<sub>5</sub> Monoisotopická hmotnost = 527,041367 Da</p>
	<p>Číslo 57b Threonin-2 (OR, 40 %) HMDB ID: HMDB00167 Molekulový vzorec: C<sub>18</sub>H<sub>12</sub>F<sub>21</sub>NO<sub>7</sub> Monoisotopická hmotnost = 753,027844 Da</p>
	<p>Číslo 58 Glycerol (dihydroxy-propionat) HMDB ID: HMDB00139 Molekulový vzorec: C<sub>17</sub>H<sub>9</sub>F<sub>21</sub>O<sub>8</sub> Monoisotopická hmotnost = 739,996209 Da</p>

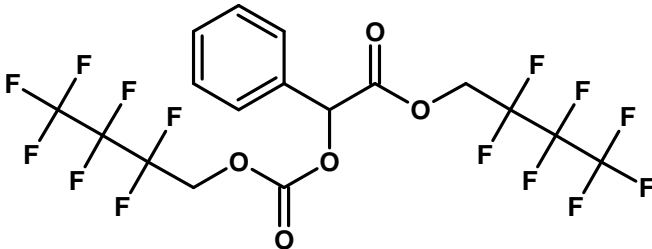
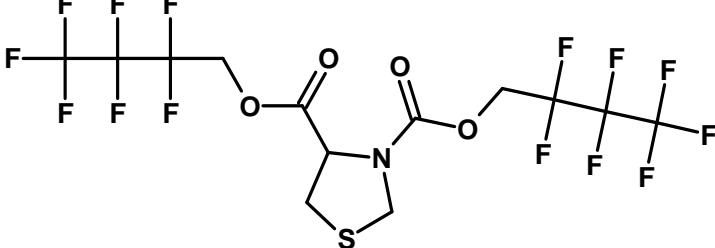
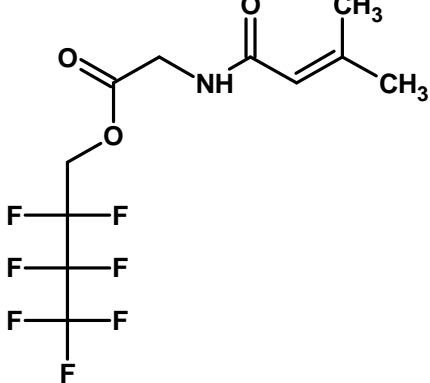
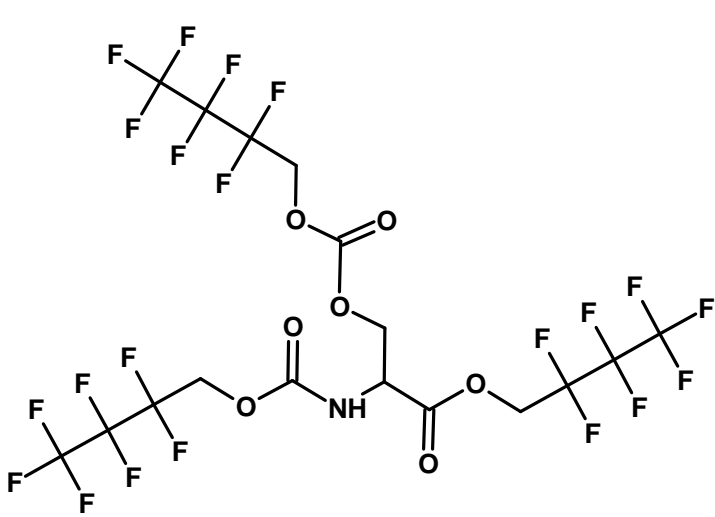
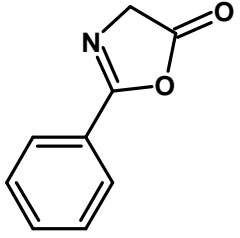
	<p>Číslo 59            Isovalerylglycin            HMDB ID: HMDB00678            Molekulový vzorec: C<sub>11</sub>H<sub>14</sub>F<sub>7</sub>NO<sub>3</sub>            Monoisotopická hmotnost =            341,086191 Da</p>
	<p>Číslo 60a            K. citronová-2 (OH)            HMDB ID: HMDB00094            Molekulový vzorec: C<sub>18</sub>H<sub>11</sub>F<sub>21</sub>O<sub>7</sub>            Monoisotopická hmotnost =            738,016945 Da</p>
	<p>Číslo 60b            K. citronová-3 (OR, 25 %)            Molekulový vzorec: C<sub>23</sub>H<sub>12</sub>F<sub>28</sub>O<sub>9</sub>            Monoisotopická hmotnost =            964,003422 Da</p>

<p>The structure shows a central cis-alkene core. One carbon of the double bond is part of a five-membered lactone ring. Two ester groups are attached to the alkene carbons. Each ester group consists of a carbonyl group bonded to a methylene group, which is further bonded to a pentafluoromethyl group (CF<sub>5</sub>).</p>	<p>Číslo 60c  K. <i>cis</i>-akonitová (2 %)  HMDB ID: HMDB00072  Molekulový vzorec: C<sub>18</sub>H<sub>9</sub>F<sub>21</sub>O<sub>6</sub>  Monoisotopická hmotnosť = 720,00638 Da</p>
<p>The structure features a central benzene ring. At the 1 and 3 positions of the ring, there are two phthalate groups. Each phthalate group consists of a carbonyl group bonded to a methylene group, which is further bonded to a pentafluoromethyl group (CF<sub>5</sub>).</p>	<p>Číslo 61  K. ftalová  HMDB ID: HMDB02107  Molekulový vzorec: C<sub>16</sub>H<sub>8</sub>F<sub>14</sub>O<sub>4</sub>  Monoisotopická hmotnosť = 530,019904 Da</p>
<p>The structure shows a central amide group (-NH-) bonded to a four-carbon alkyl chain. One end of the chain is attached to a carbonyl group, which is further bonded to a methylene group and a pentafluoromethyl group (CF<sub>5</sub>). The other end of the chain is attached to another carbonyl group, which is further bonded to a methylene group and a pentafluoromethyl group (CF<sub>5</sub>).</p>	<p>Číslo 62  K. 4-aminomáselná  HMDB ID: HMDB00650  Molekulový vzorec: C<sub>13</sub>H<sub>11</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnosť = 511,046453 Da</p>
<p>The structure shows a central ester group (-O-C(=O)-) bonded to a four-carbon alkyl chain. One end of the chain is attached to a pentafluoromethyl group (CF<sub>5</sub>). The other end of the chain is attached to a phenyl ring.</p>	<p>Číslo 63  K. 4-fenylmáselná – I.S.  Molekulový vzorec: C<sub>14</sub>H<sub>13</sub>F<sub>7</sub>O<sub>2</sub>  Monoisotopická hmotnosť = 346,080377 Da</p>

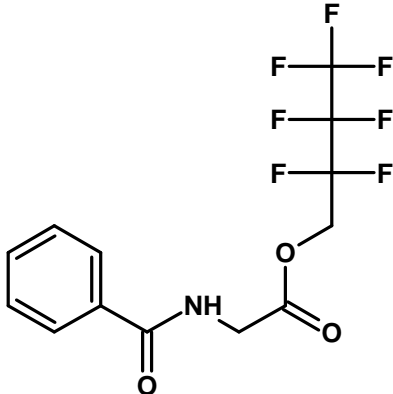
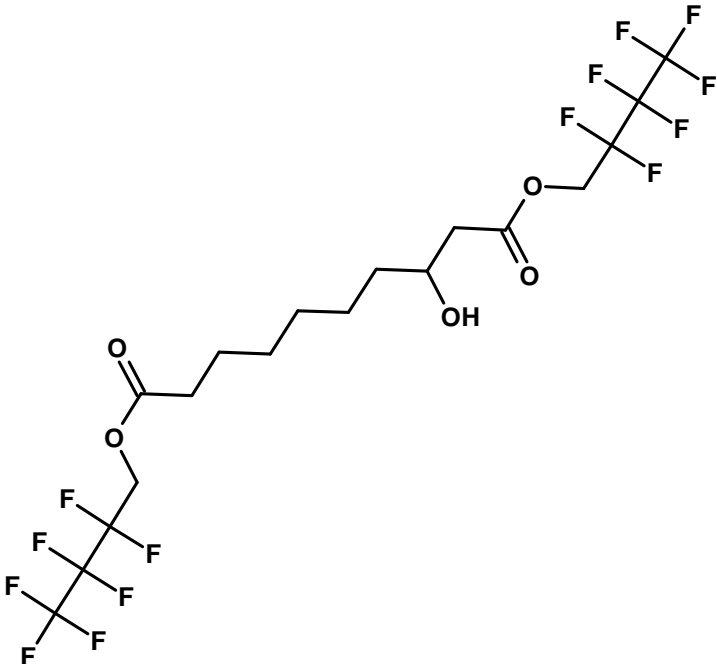
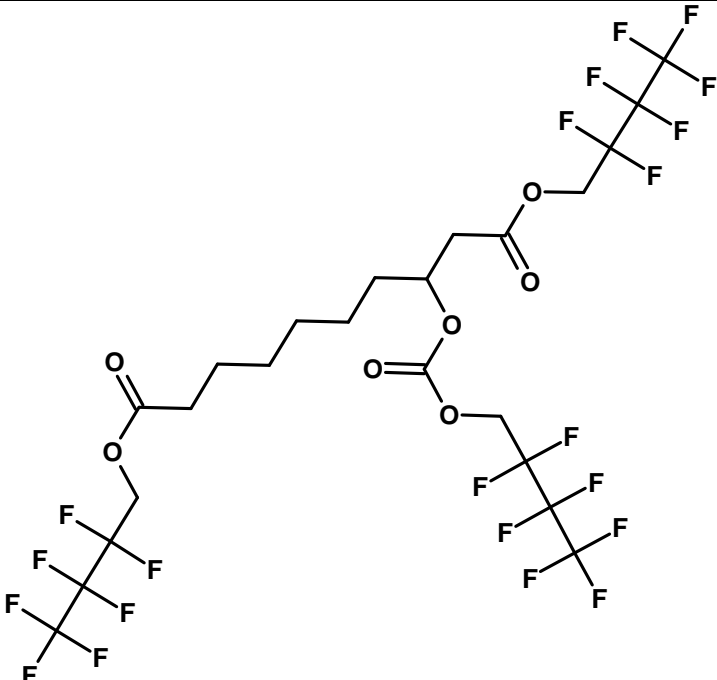
	<p>Číslo 64  K. asparagová  HMDB ID: HMDB00191  Molekulový vzorec: C<sub>17</sub>H<sub>10</sub>F<sub>21</sub>NO<sub>6</sub>  Monoisotopická hmotnost =  723,017279 Da</p>
	<p>Číslo 65  K. D-2-hydroxyglutarová  HMDB ID: HMDB00606  Molekulový vzorec: C<sub>18</sub>H<sub>11</sub>F<sub>21</sub>O<sub>7</sub>  Monoisotopická hmotnost =  738,016945 Da</p>
	<p>Číslo 66  K. N-acetylasparagová  HMDB ID: HMDB00812  Molekulový vzorec: C<sub>14</sub>H<sub>11</sub>F<sub>14</sub>NO<sub>5</sub>  Monoisotopická hmotnost =  539,041367 Da</p>
	<p>Číslo 67  K. 3-hydroxyadipová  HMDB ID: HMDB00345  Molekulový vzorec: C<sub>19</sub>H<sub>13</sub>F<sub>21</sub>O<sub>7</sub>  Monoisotopická hmotnost =  752,032595 Da</p>

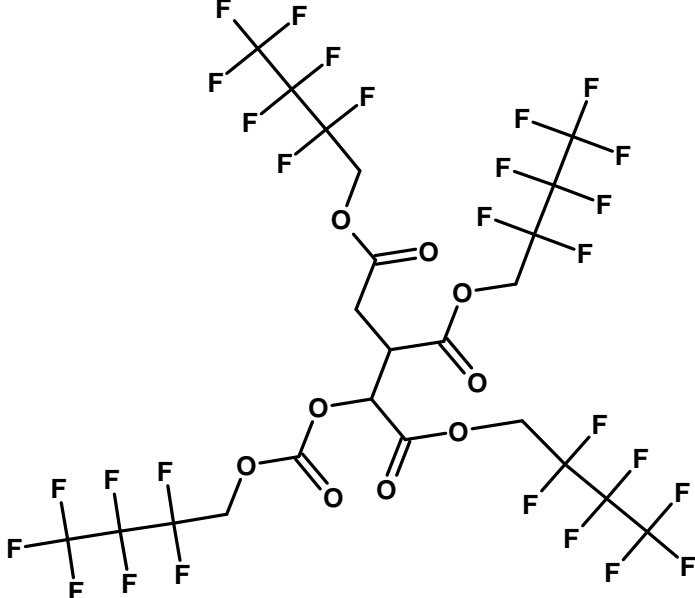
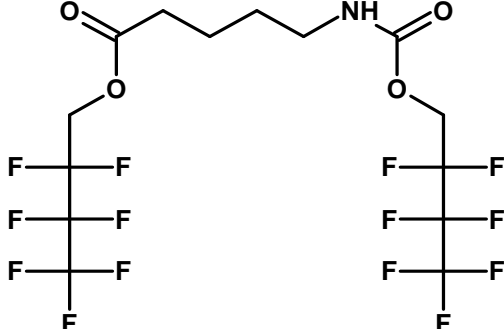
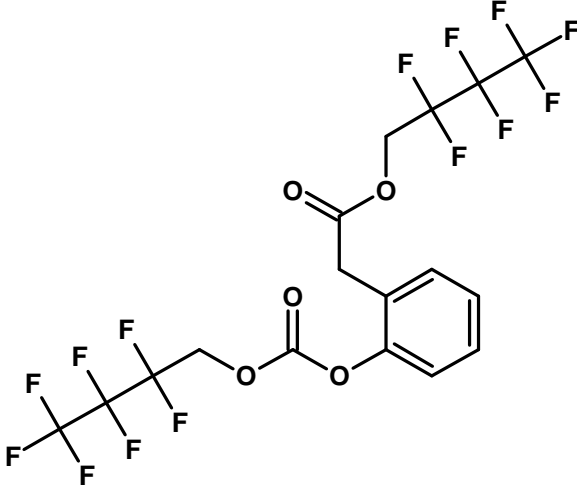
	<p>Číslo 68a  K. 2-methylcitronová (lakton)  HMDB ID: HMDB00379  Molekulový vzorec: C<sub>24</sub>H<sub>14</sub>F<sub>28</sub>O<sub>9</sub>  Monoisotopická hmotnost = 552,025383 Da</p>
	<p>Číslo 68b  K. 2-methylcitronová (triester, 60 %)  HMDB ID: HMDB00379  Molekulový vzorec: C<sub>24</sub>H<sub>14</sub>F<sub>28</sub>O<sub>9</sub>  Monoisotopická hmotnost = 752,032595 Da</p>
	<p>Číslo 68c  K. 2-methylcitronová (dehydrat., 20 %)  HMDB ID: HMDB00379  Molekulový vzorec: C<sub>24</sub>H<sub>14</sub>F<sub>28</sub>O<sub>9</sub>  Monoisotopická hmotnost = 734,02203 Da</p>
	<p>Číslo 68d  K. 2-methylcitronová (triester + OR, 10 %)  HMDB ID: HMDB00379  Molekulový vzorec: C<sub>24</sub>H<sub>14</sub>F<sub>28</sub>O<sub>9</sub>  Monoisotopická hmotnost = 978,019072 Da</p>

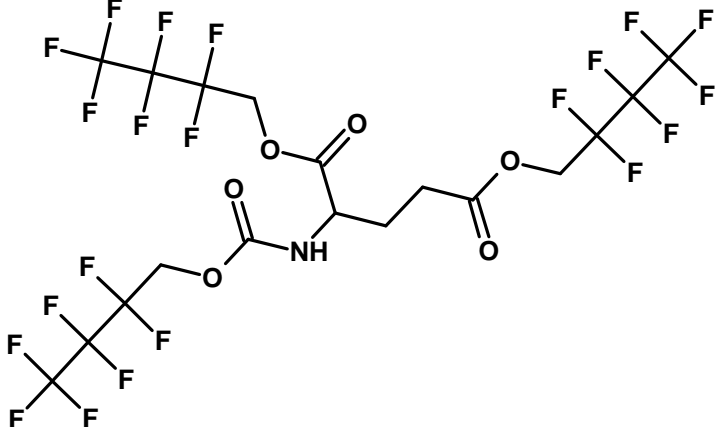
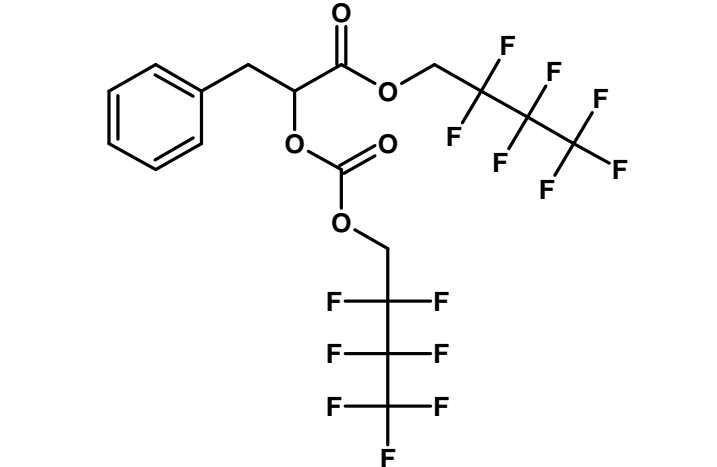
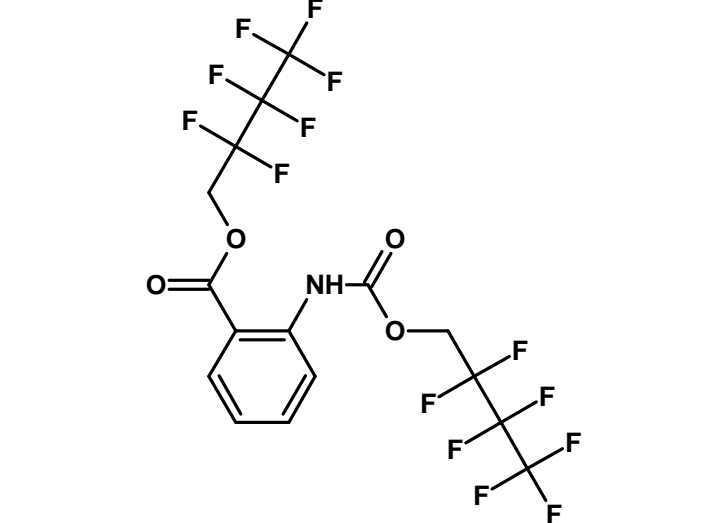
	<p>Číslo 69 K. pyroglutamová HMDB ID: HMDB00267 Molekulový vzorec: C<sub>14</sub>H<sub>9</sub>F<sub>14</sub>NO<sub>5</sub> Monoisotopická hmotnost = 537,025717 Da</p>
	<p>Číslo 70 Asparagin HMDB ID: HMDB00168 Molekulový vzorec: C<sub>13</sub>H<sub>8</sub>F<sub>14</sub>N<sub>2</sub>O<sub>4</sub> Monoisotopická hmotnost = 522,026052 Da</p>
	<p>Číslo 71 S-Methylcystein HMDB ID: HMDB02108 Molekulový vzorec: C<sub>13</sub>H<sub>11</sub>F<sub>14</sub>NO<sub>4</sub>S Monoisotopická hmotnost = 543,018523 Da</p>
	<p>Číslo 72 K. suberová HMDB ID: HMDB00893 Molekulový vzorec: C<sub>16</sub>H<sub>16</sub>F<sub>14</sub>O<sub>4</sub> Monoisotopická hmotnost = 538,082504 Da</p>
	<p>Číslo 73 K. salicylová HMDB ID: HMDB01895 Molekulový vzorec: C<sub>16</sub>H<sub>8</sub>F<sub>14</sub>O<sub>5</sub> Monoisotopická hmotnost = 546,014818 Da</p>

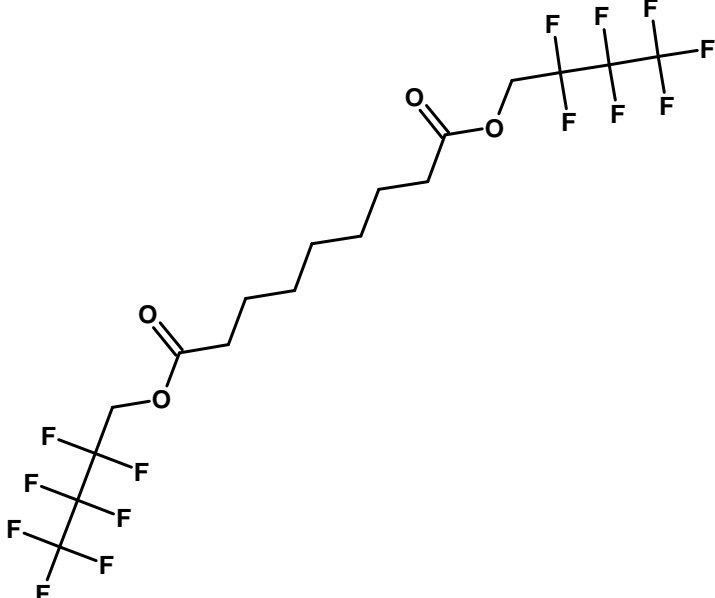
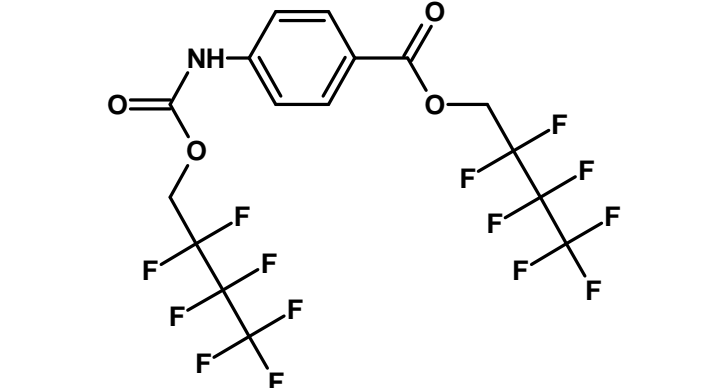
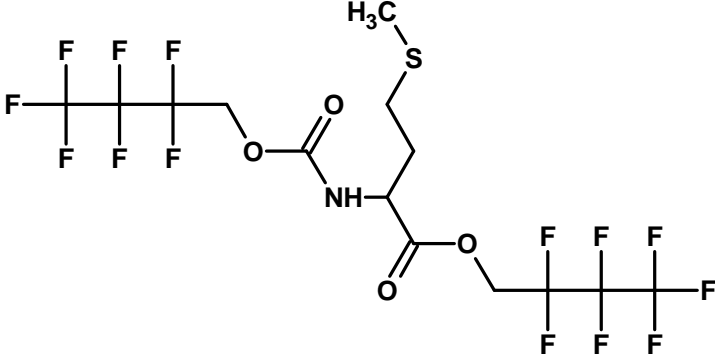
	<p>Číslo 74 K. mandlová HMDB ID: HMDB00703 Molekulový vzorec: C<sub>17</sub>H<sub>10</sub>F<sub>14</sub>O<sub>5</sub> Monoisotopická hmotnost = 560,030468 Da</p>
	<p>Číslo 75 Thioprolin HMDB ID: - Molekulový vzorec: C<sub>13</sub>H<sub>9</sub>F<sub>14</sub>NO<sub>4</sub>S Monoisotopická hmotnost = 541,002873 Da</p>
	<p>Číslo 76 Methylkrotonylglycin HMDB ID: HMDB00459 Molekulový vzorec: C<sub>11</sub>H<sub>12</sub>F<sub>7</sub>NO<sub>3</sub> Monoisotopická hmotnost = 339,070541 Da</p>
	<p>Číslo 77 Serin HMDB ID: HMDB00187 Molekulový vzorec: C<sub>17</sub>H<sub>10</sub>F<sub>21</sub>NO<sub>6</sub> Monoisotopická hmotnost = 723,0238 Da</p>
	<p>Číslo 78a K. hippurová-1 (cyklická) HMDB ID: HMDB00714 Molekulový vzorec: C<sub>9</sub>H<sub>7</sub>NO<sub>2</sub> Monoisotopická hmotnost = 161,047678 Da</p>

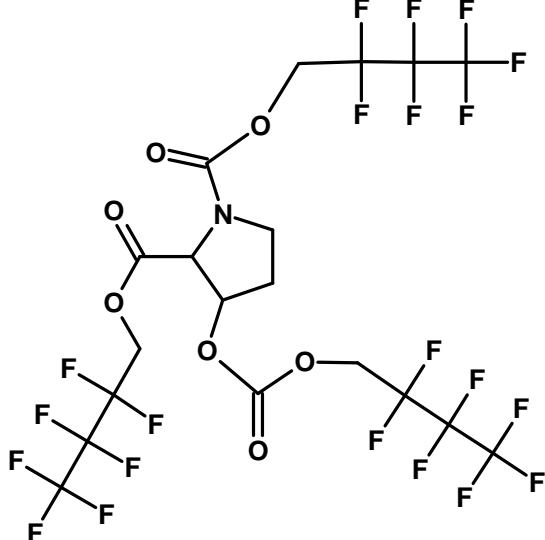
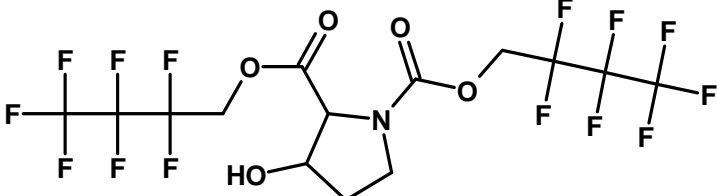
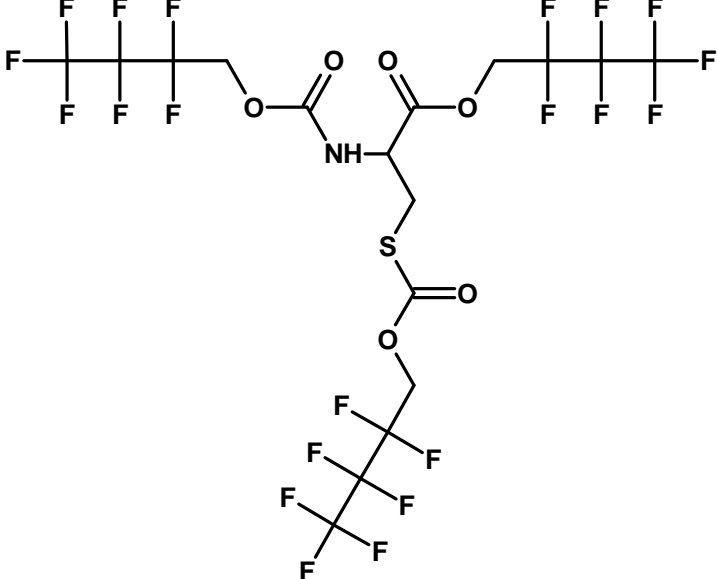
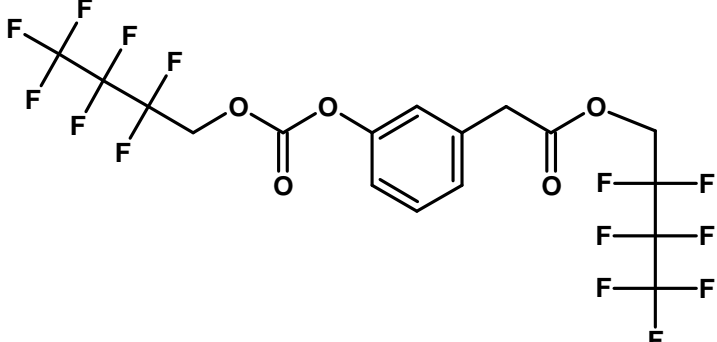


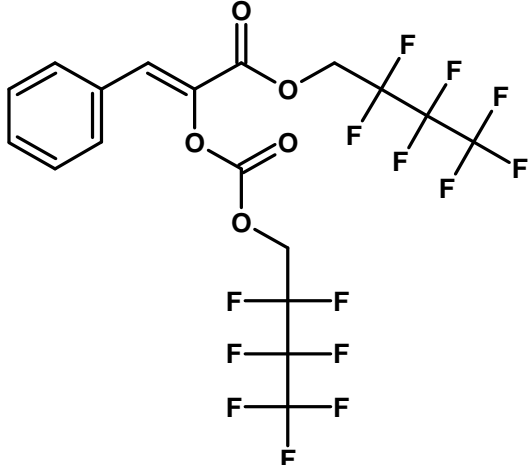
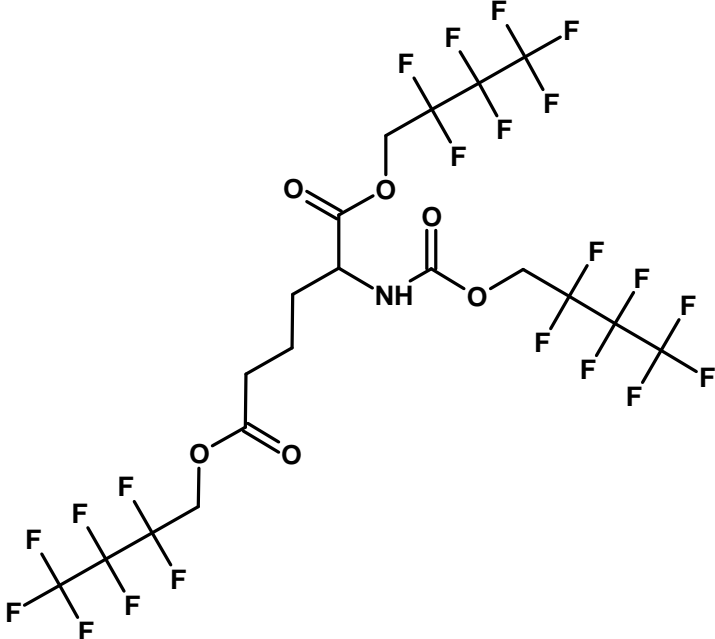
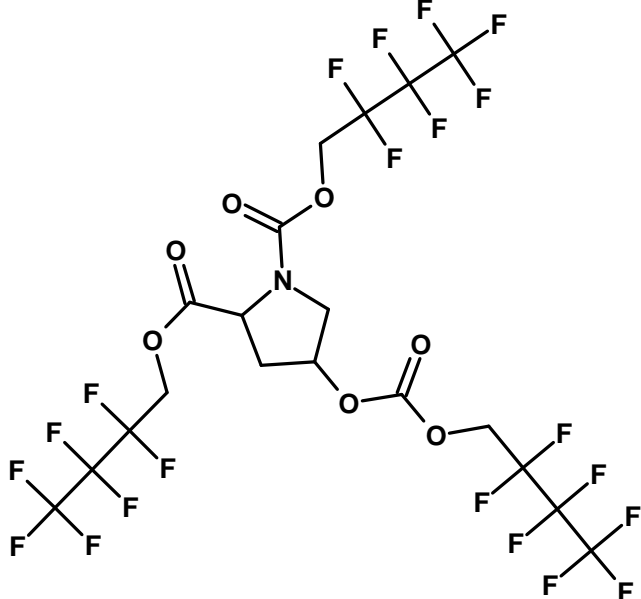
	<p>Číslo 78b  K. hippurová-2 (ester, 60 %)  HMDB ID: HMDB00714  Molekulový vzorec: C<sub>13</sub>H<sub>10</sub>F<sub>7</sub>NO<sub>3</sub>  Monoisotopická hmotnost = 361,054891 Da</p>
	<p>Číslo 79a  K. 3-hydroxysebaková-1 (OH)  HMDB ID: HMDB00350  Molekulový vzorec: C<sub>18</sub>H<sub>20</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost = 582,108719 Da</p>
	<p>Číslo 79b  K. 3-hydroxysebaková-2 (OR, 20 %)  HMDB ID: HMDB00350  Molekulový vzorec: C<sub>23</sub>H<sub>21</sub>F<sub>21</sub>O<sub>7</sub>  Monoisotopická hmotnost = 808,095195 Da</p>

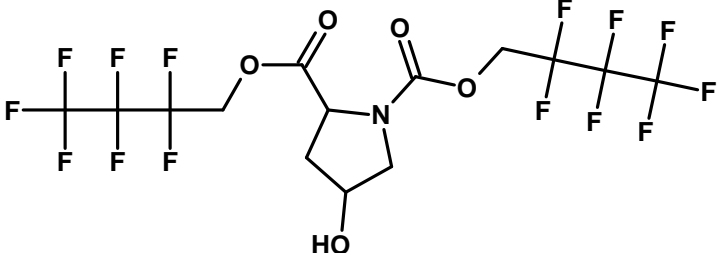
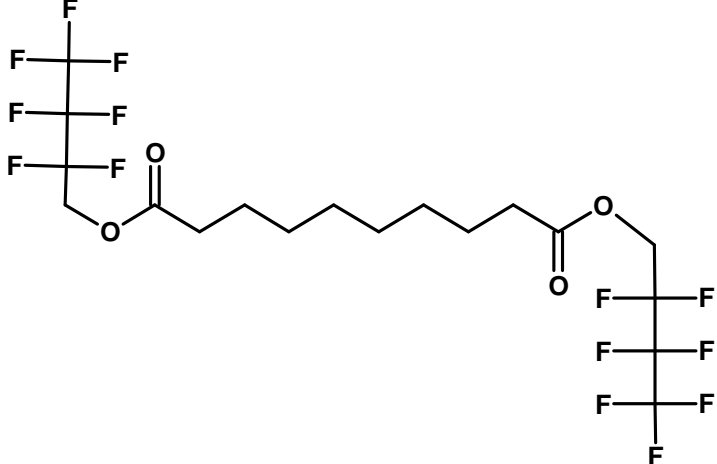
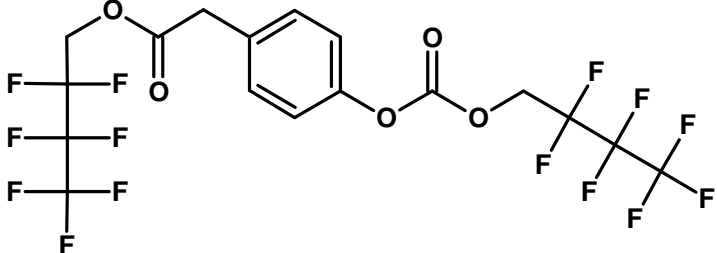
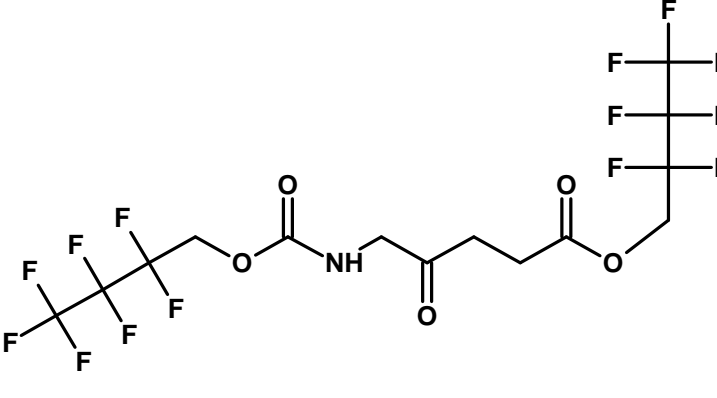
	<p>Číslo 80  K. isocitronová  HMDB ID: HMDB00193  Molekulový vzorec: C<sub>23</sub>H<sub>12</sub>F<sub>28</sub>O<sub>9</sub>  Monoisotopická hmotnosť =  964,003422 Da</p>
	<p>Číslo 81  K. 5-aminovalerová  HMDB ID: HMDB03355  Molekulový vzorec: C<sub>14</sub>H<sub>13</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnosť =  525,062103 Da</p>
	<p>Číslo 82  K. 2-hydroxyfenoctová  HMDB ID: HMDB00669  Molekulový vzorec: C<sub>17</sub>H<sub>10</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnosť =  560,030468 Da</p>

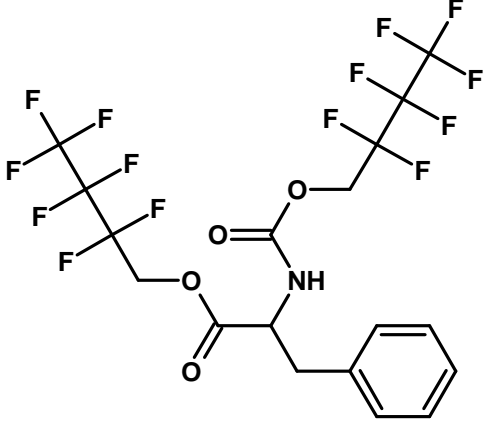
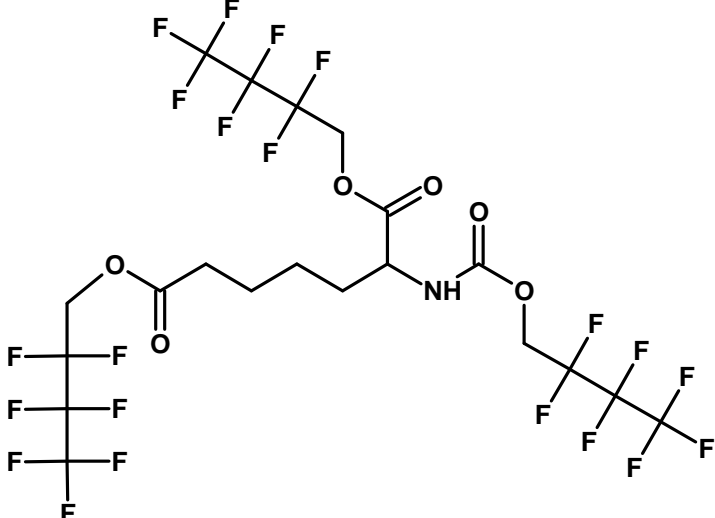
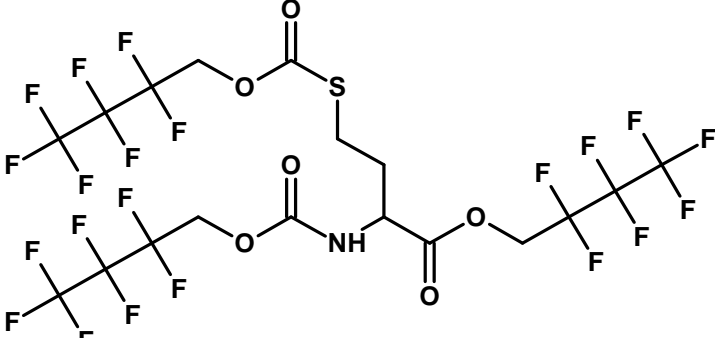
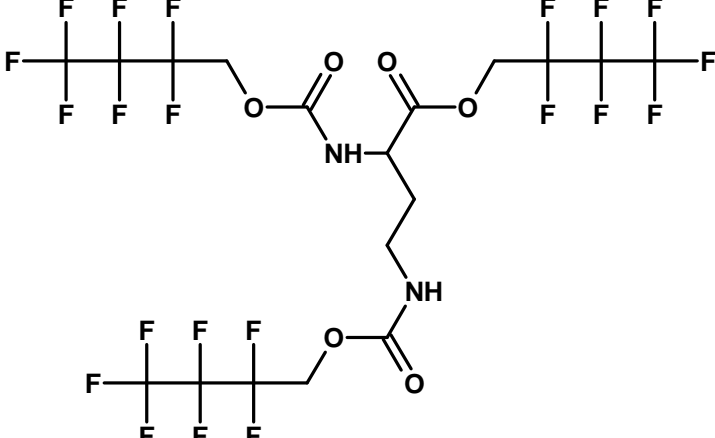
	<p>Číslo 83  K. glutamová  HMDB ID: HMDB00148  Molekulový vzorec: C<sub>18</sub>H<sub>12</sub>F<sub>21</sub>NO<sub>6</sub>  Monoisotopická hmotnosť =  737,25745 Da</p>
	<p>Číslo 84  K. 3-fenylmléčná  HMDB ID: HMDB00779  Molekulový vzorec: C<sub>18</sub>H<sub>12</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnosť =  574,046118 Da</p>
	<p>Číslo 85  K. antranilová  HMDB ID: HMDB01123  Molekulový vzorec: C<sub>16</sub>H<sub>9</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnosť =  545,030803 Da</p>

	<p>Číslo 86  K. azelaová  HMDB ID: HMDB00784  Molekulový vzorec: C17H18F14O4  Monoisotopická hmotnost =  552,098154 Da</p>
	<p>Číslo 87  K. 4-hydroxybenzoová  HMDB ID: HMDB00500  Molekulový vzorec: C16H8F14O5  Monoisotopická hmotnost =  546,014818 Da</p>
	<p>Číslo 88  Methionin  HMDB ID: HMDB00696  Molekulový vzorec:  C14H13F14NO4S  Monoisotopická hmotnost =  557,034173 Da</p>

	<p>Číslo 89a  3-hydroxyprolin-2 (OR)  HMDB ID: HMDB02113  Molekulový vzorec: C19H12F21NO7  Monoisotopická hmotnost =  765,027844 Da</p>
	<p>Číslo 89b  3-hydroxyprolin-1 (OH, 65 %)  HMDB ID: HMDB02113  Molekulový vzorec: C14H11F14NO5  Monoisotopická hmotnost =  539,041367 Da</p>
	<p>Číslo 90  Cystein  HMDB ID: HMDB00574  Molekulový vzorec:  C17H10F21NO6S  Monoisotopická hmotnost =  754,989349 Da</p>
	<p>Číslo 91  K. 3-hydroxyfenyloctová  HMDB ID: HMDB00440  Molekulový vzorec: C17H10F14O5  Monoisotopická hmotnost =  560,030468 Da</p>

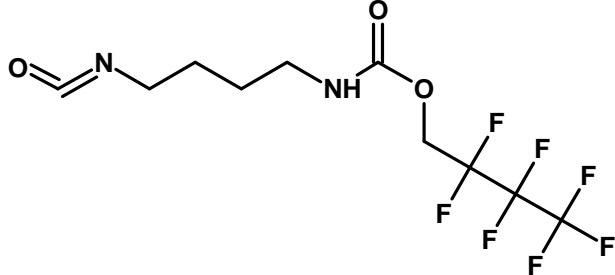
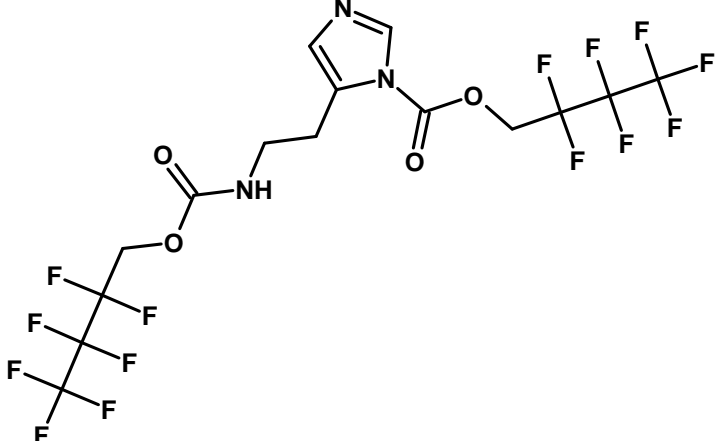
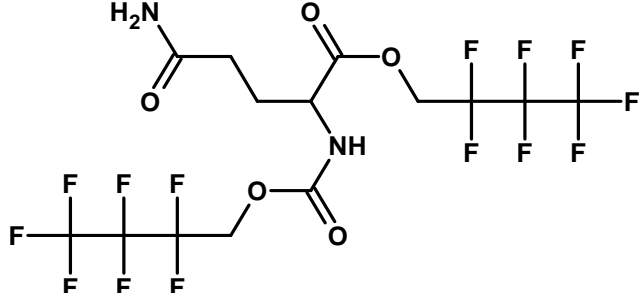
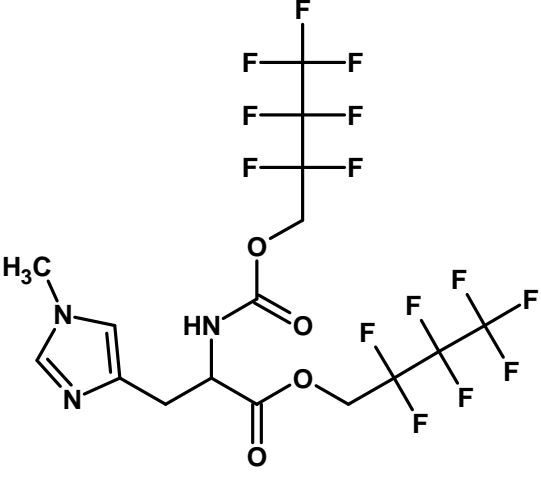
	<p>Číslo 92  K. fenyropyrohroznová  HMDB ID: HMDB12225  Molekulový vzorec: C<sub>18</sub>H<sub>10</sub>F<sub>7</sub>14O<sub>5</sub>  Monoisotopická hmotnosť =  572,030468 Da</p>
	<p>Číslo 93  K. 2-aminoadipová  HMDB ID: HMDB00510  Molekulový vzorec: C<sub>19</sub>H<sub>14</sub>F<sub>21</sub>NO<sub>6</sub>  Monoisotopická hmotnosť =  751,048579 Da</p>
	<p>Číslo 94a  4-hydroxyprolin-2  HMDB ID: HMDB00725  Molekulový vzorec: C<sub>19</sub>H<sub>12</sub>F<sub>21</sub>NO<sub>7</sub>  Monoisotopická hmotnosť =  765,027844 Da</p>

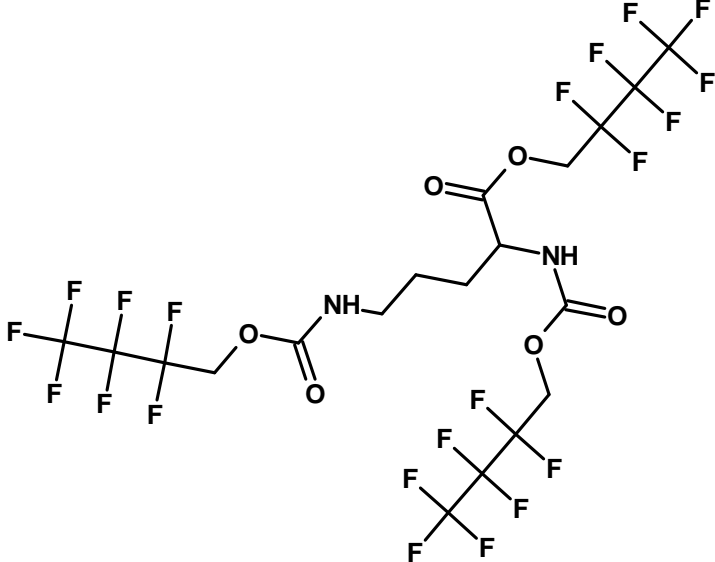
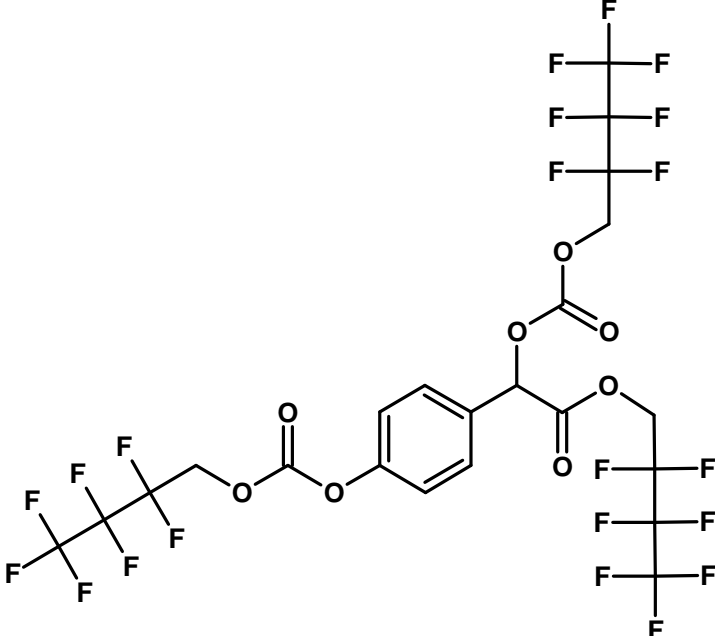
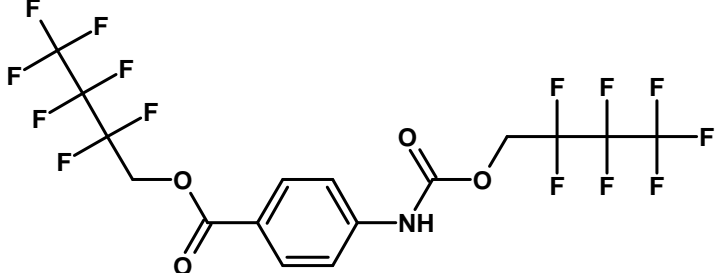
	<p>Číslo 94b  4-hydroxyprolin-1 (OH, 65 %)  HMDB ID: HMDB00725  Molekulový vzorec: C14H11F14NO5  Monoisotopická hmotnost =  539,041367 Da</p>
	<p>Číslo 95  K. sebaková  HMDB ID: HMDB00792  Molekulový vzorec: C18H20F14O4  Monoisotopická hmotnost =  566,113804 Da</p>
	<p>Číslo 96  K. 4-hydroxyfenoctová  HMDB ID: HMDB00020  Molekulový vzorec: C17H10F14O5  Monoisotopická hmotnost =  560,030468 Da</p>
	<p>Číslo 97  K. 5-aminolevulová  HMDB ID: HMDB01149  Molekulový vzorec: C14H11F14NO5  Monoisotopická hmotnost =  539,041367 Da</p>

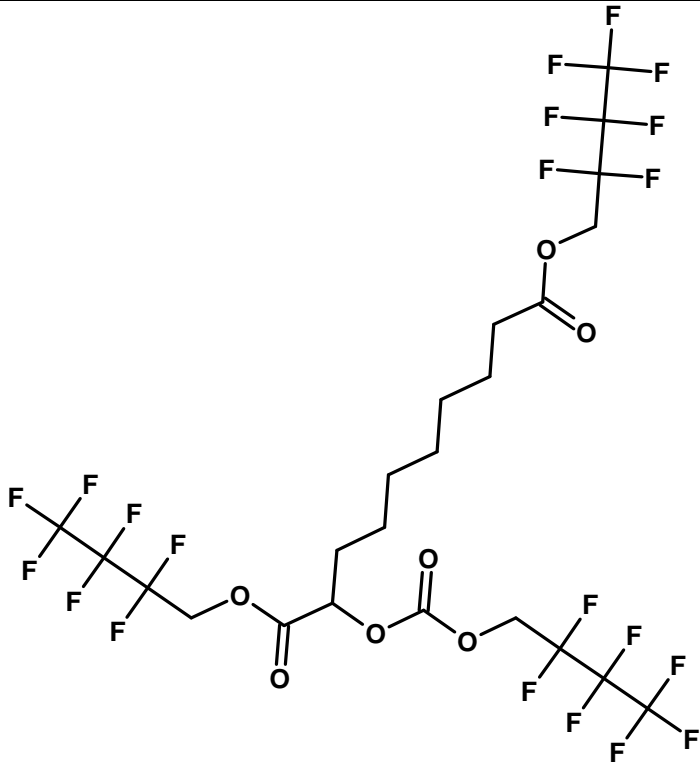
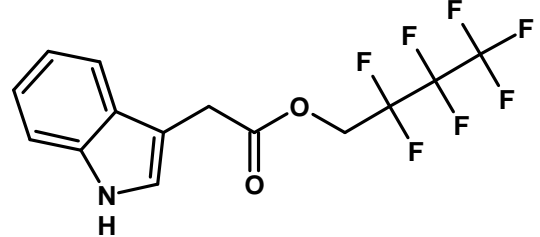
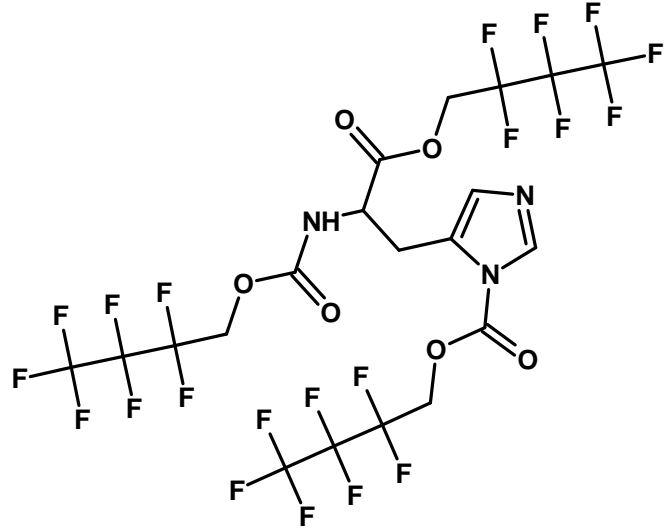
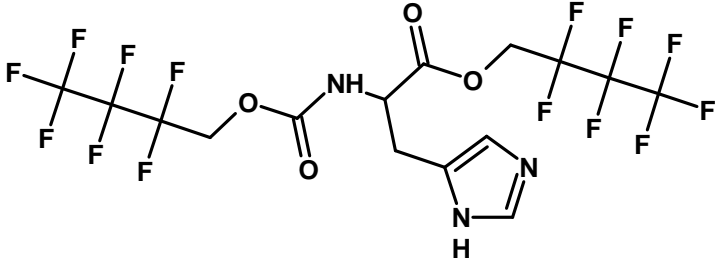
	<p>Číslo 98  Fenylylalanin  HMDB ID: HMDB00159  Molekulový vzorec: C<sub>18</sub>H<sub>13</sub>F<sub>14</sub>NO<sub>4</sub>  Monoisotopická hmotnost =  573,062103 Da</p>
	<p>Číslo 99  K. 2-aminopimelová  HMDB ID: HMDB34252  Molekulový vzorec: C<sub>20</sub>H<sub>16</sub>F<sub>21</sub>NO<sub>6</sub>  Monoisotopická hmotnost =  765,064229 Da</p>
	<p>Číslo 100  Homocystein  HMDB ID: HMDB00742  Molekulový vzorec: C<sub>18</sub>H<sub>12</sub>F<sub>21</sub>NO<sub>6</sub>  Monoisotopická hmotnost =  769,004999 Da</p>
	<p>Číslo 101  K. diaminomáselná  HMDB ID: HMDB06284  Molekulový vzorec:  C<sub>18</sub>H<sub>13</sub>F<sub>21</sub>N<sub>2</sub>O<sub>6</sub>  Monoisotopická hmotnost =  752,043828 Da</p>



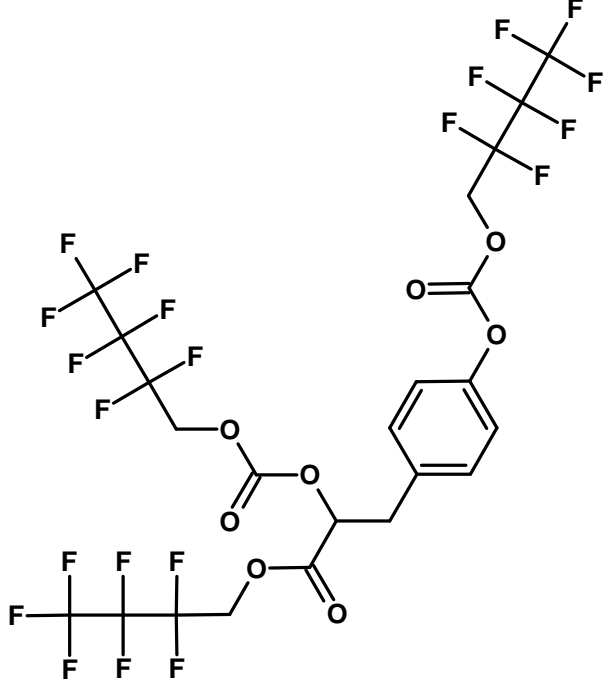
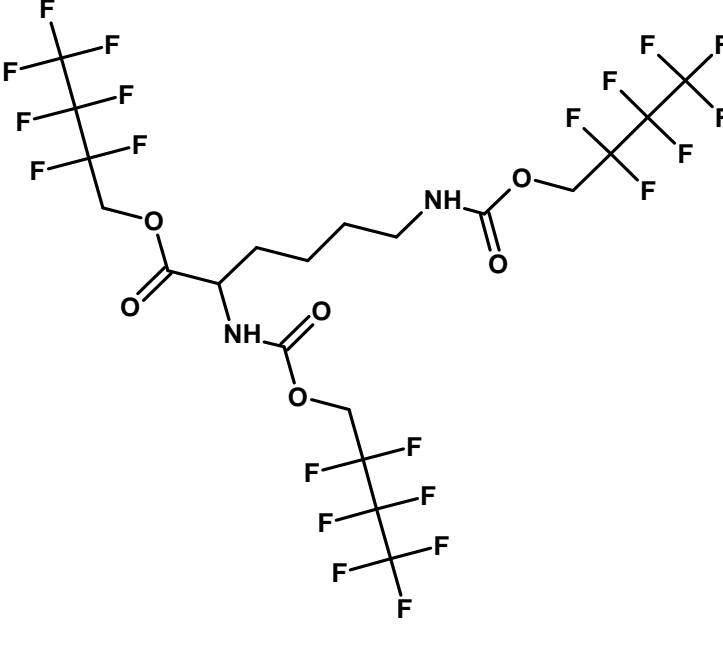
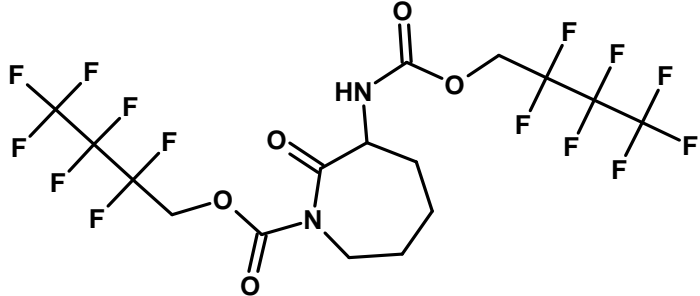
	<p>Číslo 102  <i>K.trans-urokanová</i>            HMDB ID: HMDB00301            Molekulový vzorec: C<sub>15</sub>H<sub>8</sub>F<sub>14</sub>N<sub>2</sub>O<sub>4</sub>            Monoisotopická hmotnost = 546,026052 Da</p>
	<p>Číslo 103  <i>Homofenylalanin – I.S.</i>            Molekulový vzorec: C<sub>19</sub>H<sub>15</sub>F<sub>14</sub>N<sub>2</sub>O<sub>4</sub>            Monoisotopická hmotnost = 587,077753 Da</p>
	<p>Číslo 104  <i>K. homovanilová</i>            HMDB ID: HMDB00118            Molekulový vzorec: C<sub>18</sub>H<sub>12</sub>F<sub>14</sub>O<sub>6</sub>            Monoisotopická hmotnost = 590,041033 Da</p>
	<p>Číslo 105a  <i>Putrescin-2</i>            HMDB ID: HMDB01414            Molekulový vzorec: C<sub>14</sub>H<sub>14</sub>F<sub>14</sub>N<sub>2</sub>O<sub>4</sub>            Monoisotopická hmotnost = 540,073002 Da</p>

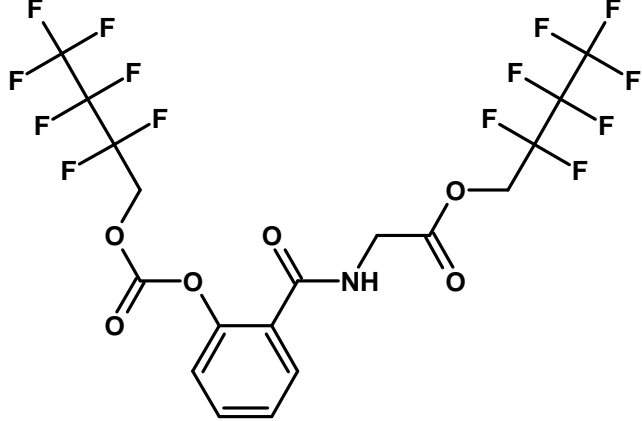
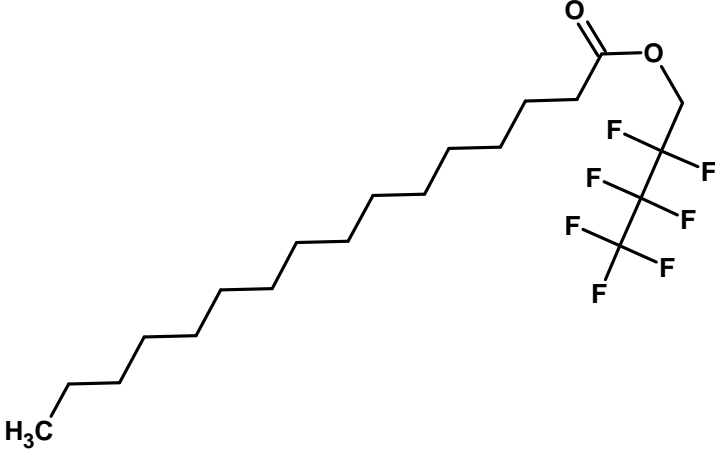
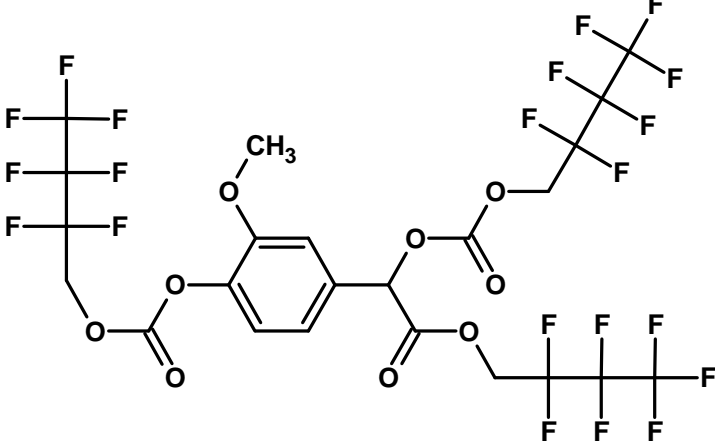
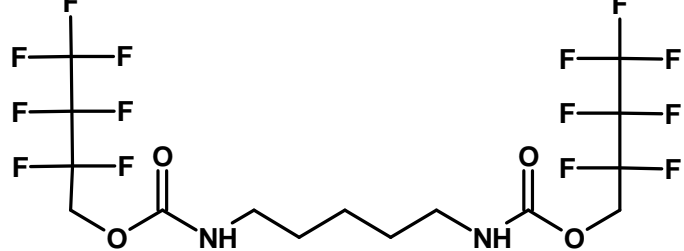
	<p>Číslo 105b Putrescin-1 (isokyanat, 75 %) HMDB ID: HMDB01414 Molekulový vzorec: C<sub>10</sub>H<sub>11</sub>F<sub>7</sub>N<sub>2</sub>O<sub>3</sub> Monoisotopická hmotnost = 340,06579 Da</p>
	<p>Číslo 106 Histamin HMDB ID: HMDB00870 Molekulový vzorec: C<sub>15</sub>H<sub>11</sub>F<sub>14</sub>N<sub>3</sub>O<sub>4</sub> Monoisotopická hmotnost = 563,052601 Da</p>
	<p>Číslo 107 Glutamin HMDB ID: HMDB00641 Molekulový vzorec: C<sub>14</sub>H<sub>12</sub>F<sub>14</sub>N<sub>2</sub>O<sub>5</sub> Monoisotopická hmotnost = 554,05227 Da</p>
	<p>Číslo 108 1-Methylhistidin HMDB ID: HMDB00479 Molekulový vzorec: C<sub>16</sub>H<sub>13</sub>F<sub>14</sub>N<sub>3</sub>O<sub>4</sub> Monoisotopická hmotnost = 577,068251 Da</p>

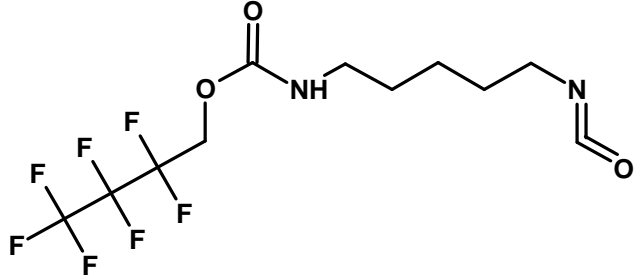
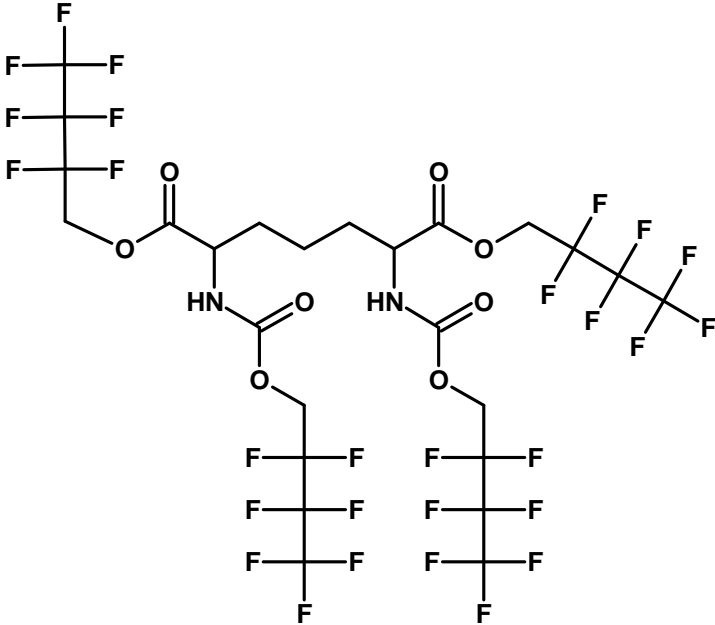
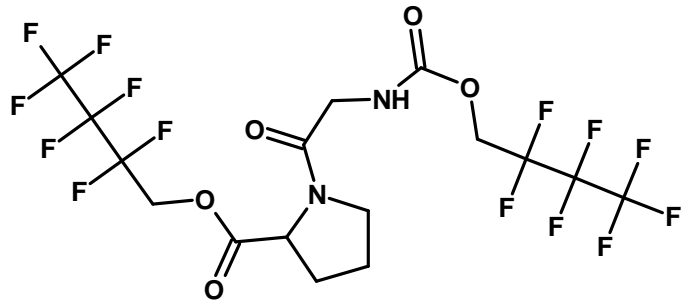
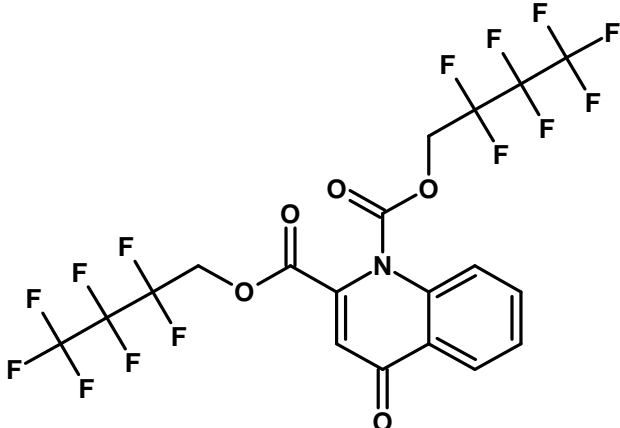
	<p>Číslo 109  Ornithin  HMDB ID: HMDB00214  Molekulový vzorec:  C19H15F21N2O6  Monoisotopická hmotnost =  766,059478 Da</p>
	<p>Číslo 110  K. 4-hydroxymandlová  HMDB ID: HMDB00822  Molekulový vzorec: C22H11F21O8  Monoisotopická hmotnost =  802,01186 Da</p>
	<p>Číslo 111  K. 4-aminobenzoová  HMDB ID: HMDB01392  Molekulový vzorec: C16H9F14NO4  Monoisotopická hmotnost =  545,030803 Da</p>

	<p>Číslo 112  K. 2-hydroxysebaková  HMDB ID: HMDB00424  Molekulový vzorec: C<sub>23</sub>H<sub>21</sub>F<sub>2</sub>O<sub>7</sub>  Monoisotopická hmotnosť = 808,095195 Da</p>
	<p>Číslo 113  K. indolactová  HMDB ID: HMDB00197  Molekulový vzorec: C<sub>14</sub>H<sub>10</sub>F<sub>7</sub>NO<sub>2</sub>  Monoisotopická hmotnosť = 357,059976 Da</p>
	<p>Číslo 114a  Histidin-2 (NR)  HMDB ID: HMDB00177  Molekulový vzorec: C<sub>20</sub>H<sub>12</sub>F<sub>21</sub>N<sub>3</sub>O<sub>6</sub>  Monoisotopická hmotnosť = 789,039077 Da</p>
	<p>Číslo 114b  Histidin-1 (NH, 15 %)  HMDB ID: HMDB00177  Molekulový vzorec: C<sub>15</sub>H<sub>11</sub>F<sub>14</sub>N<sub>3</sub>O<sub>4</sub>  Monoisotopická hmotnosť = 563,052601 Da</p>

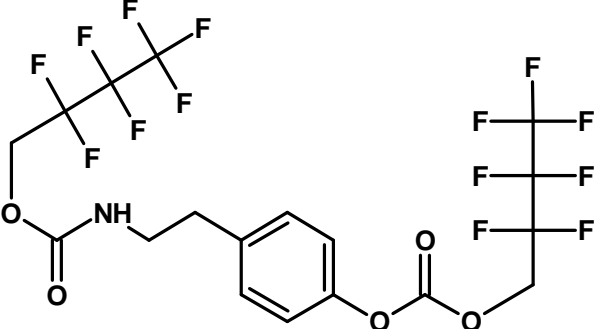
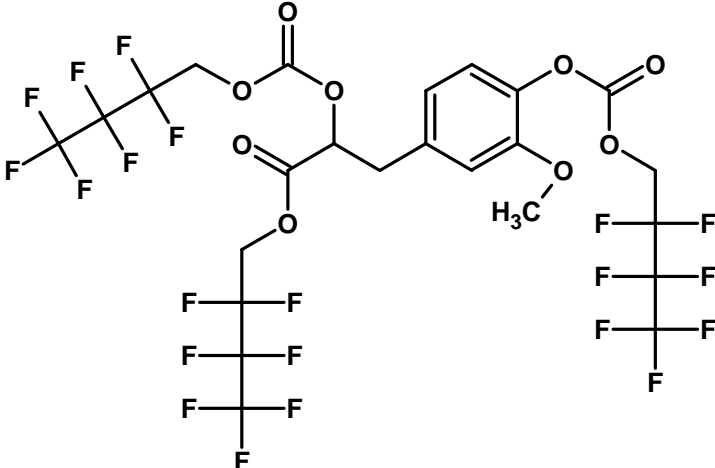
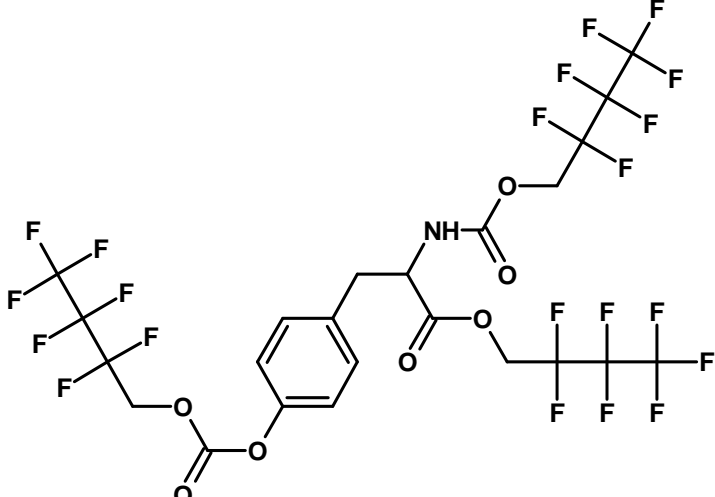
	<p>Číslo 115  K. 4-hydroxyskořicová  HMDB ID: HMDB02035  Molekulový vzorec: C<sub>18</sub>H<sub>10</sub>F<sub>14</sub>O<sub>5</sub>  Monoisotopická hmotnost =  572,030468 Da</p>
	<p>Číslo 116  3-Methylhistidin  HMDB ID: HMDB00479  Molekulový vzorec:  C<sub>16</sub>H<sub>13</sub>F<sub>14</sub>N<sub>3</sub>O<sub>4</sub>  Monoisotopická hmotnost =  577,068251 Da</p>
	<p>Číslo 117  K. 2-amino-4-(methylsulfonyl)  butanová  HMDB ID: –  Molekulový vzorec:  C<sub>14</sub>H<sub>13</sub>F<sub>14</sub>NO<sub>6</sub>S  Monoisotopická hmotnost =  589,024002 Da</p>

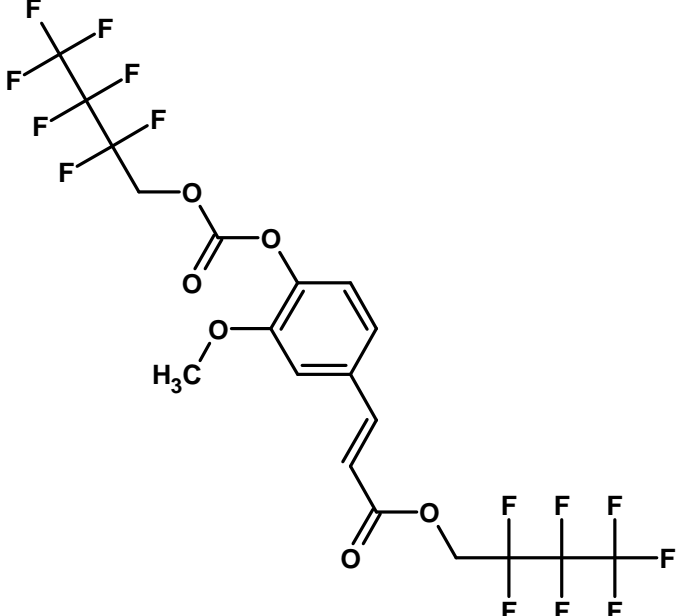
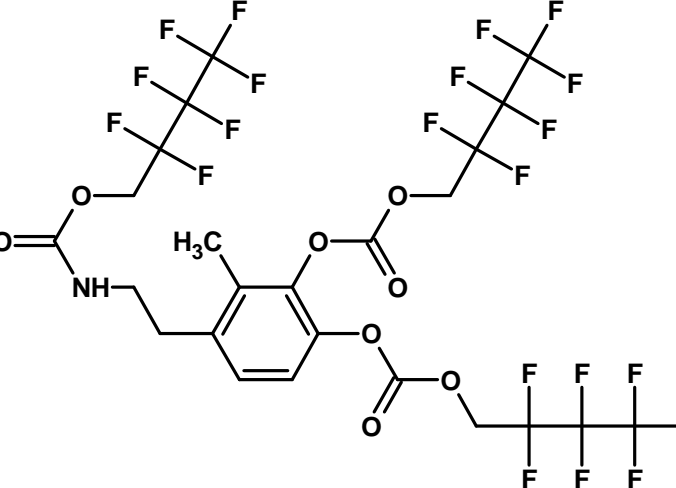
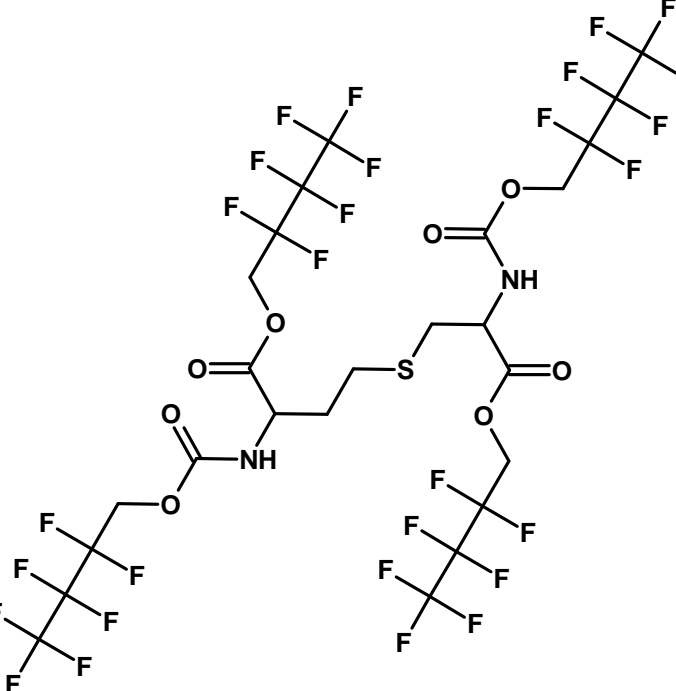
	<p>Číslo 118  K. 4-hydroxyfenylmléčná  HMDB ID: HMDB00755  Molekulový vzorec: C<sub>23</sub>H<sub>13</sub>F<sub>21</sub>O<sub>8</sub>  Monoisotopická hmotnosť = 816,02751 Da</p>
	<p>Číslo 119a  Lysin-2  HMDB ID: HMDB00182  Molekulový vzorec: C<sub>20</sub>H<sub>17</sub>F<sub>21</sub>N<sub>2</sub>O<sub>6</sub>  Monoisotopická hmotnosť = 780,075128 Da</p>
	<p>Číslo 119b  Lysin-1 (cyklická, 35 %)  HMDB ID: HMDB00182  Molekulový vzorec: C<sub>16</sub>H<sub>14</sub>F<sub>14</sub>N<sub>2</sub>O<sub>5</sub>  Monoisotopická hmotnosť = 580,067916 Da</p>

	<p>Číslo 120  K. 2-hydroxyhippurová  HMDB ID: HMDB00840  Molekulový vzorec: C<sub>18</sub>H<sub>11</sub>F<sub>14</sub>NO<sub>6</sub>  Monoisotopická hmotnost = 603,036282 Da</p>
	<p>Číslo 121  K. palmitová  HMDB ID: HMDB00220  MOLECULAR FORMULA: C<sub>20</sub>H<sub>33</sub>F<sub>7</sub>O<sub>2</sub>  Monoisotopická hmotnost = 438,236878 Da</p>
	<p>Číslo 122  K. vanilylmandlová  HMDB ID: HMDB00291  MOLECULAR FORMULA: C<sub>23</sub>H<sub>13</sub>F<sub>21</sub>O<sub>9</sub>  Monoisotopická hmotnost = 832,022424 Da</p>
	<p>Číslo 123a  Kadavarin-2  HMDB ID: HMDB02322  MOLECULAR FORMULA: C<sub>15</sub>H<sub>16</sub>F<sub>14</sub>N<sub>2</sub>O<sub>4</sub>  Monoisotopická hmotnost = 554,088652 Da</p>

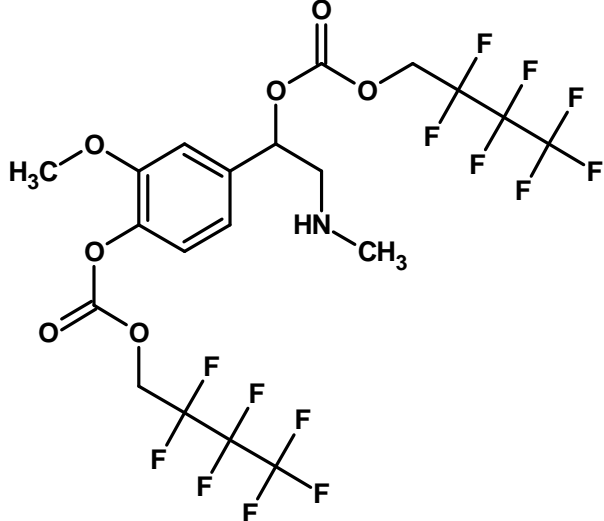
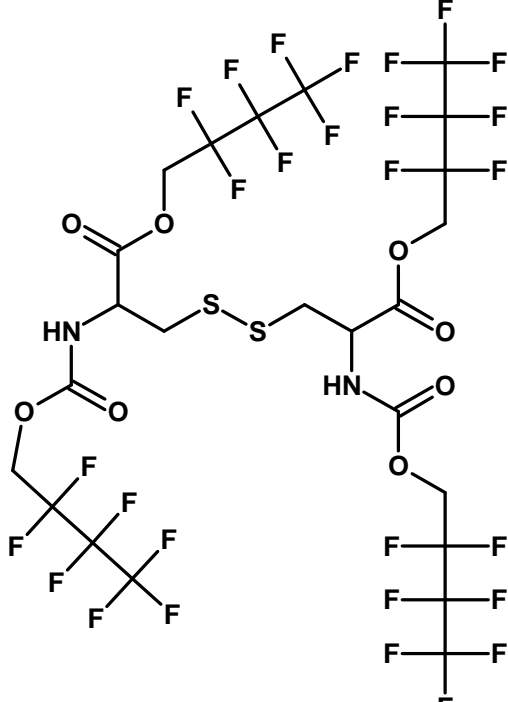
	<p>Číslo 123b Kadaverin-1 (isokyanát, 80 %) MOLECULAR FORMULA: C<sub>11</sub>H<sub>13</sub>F<sub>7</sub>N<sub>2</sub>O<sub>3</sub> Monoisotopická hmotnost = 354,08144 Da</p>
	<p>Číslo 124a K. 2,6-diaminopimelová (izom.) HMDB ID:HMDB01370 MOLECULAR FORMULA: C<sub>25</sub>H<sub>18</sub>F<sub>28</sub>N<sub>2</sub>O<sub>8</sub> Monoisotopická hmotnost = 1006,061605 Da</p>
	<p>Číslo 125 Glycylprolin HMDB ID: HMDB00721 MOLECULAR FORMULA: C<sub>16</sub>H<sub>14</sub>F<sub>14</sub>N<sub>2</sub>O<sub>5</sub> Monoisotopická hmotnost = 580,067916 Da</p>
	<p>Číslo 126 K. kynurenová HMDB ID: HMDB00913 MOLECULAR FORMULA: C<sub>19</sub>H<sub>19</sub>F<sub>14</sub>N<sub>1</sub>O<sub>5</sub> Monoisotopická hmotnost = 597,025717 Da</p>

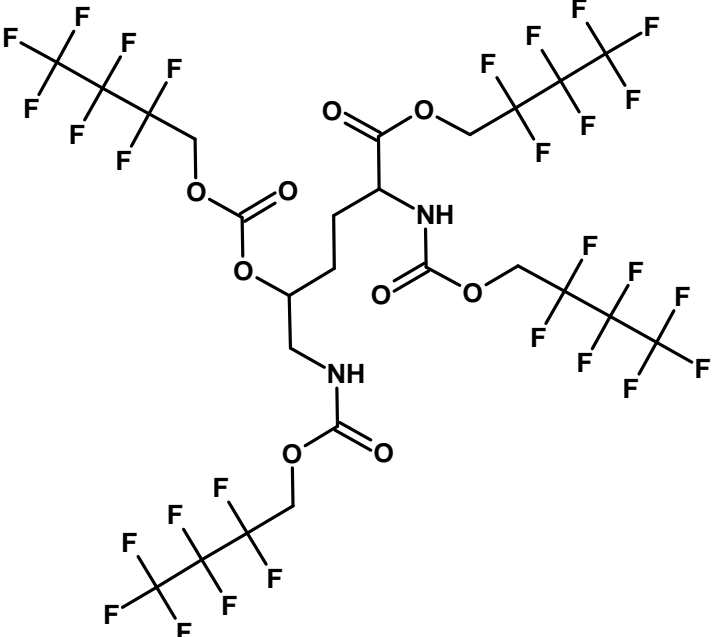
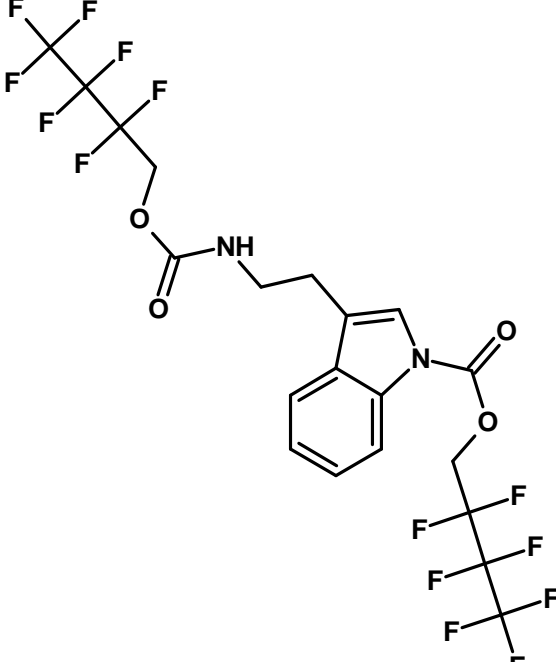


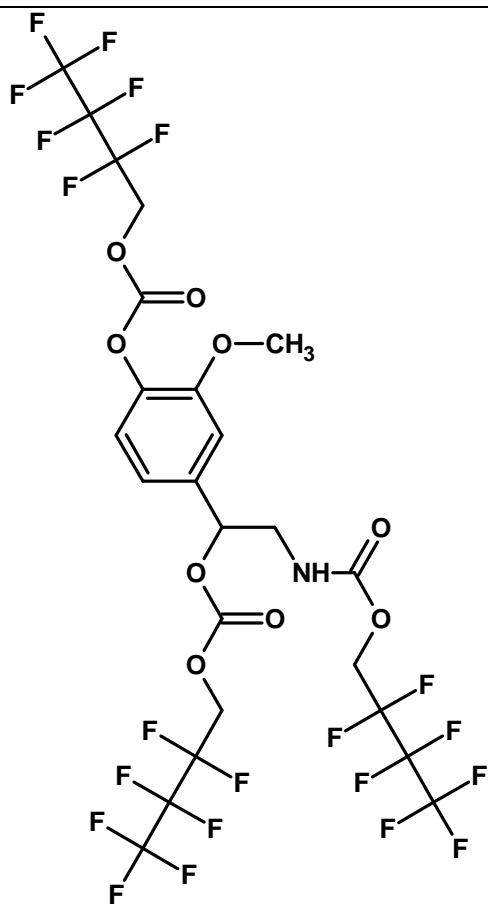
	<p>Číslo 127  Tyramin  HMDB ID: HMDB00306  MOLECULAR FORMULA:  C18H13F14NO5  Monoisotopická hmotnost =  589,057017 Da</p>
	<p>Číslo 128  K. 4-hydroxy-3-methoxyfenylmléčná  HMDB ID: –  MOLECULAR FORMULA:  C24H15F21O9  Monoisotopická hmotnost =  846,038074 Da</p>
	<p>Číslo 129  Tyrosin  HMDB ID: HMDB00158  MOLECULAR FORMULA:  C23H14F21NO7  Monoisotopická hmotnost =  815,043494 Da</p>

	<p>Číslo 130  K. 4-hydroxy-3-methoxyskořicová  HMDB ID: : HMDB00954  MOLECULAR FORMULA:  C19H12F14O6  Monoisotopická hmotnost =  602,041033 Da</p>
	<p>Číslo 131  3-Methoxytyramin  HMDB ID: HMDB00022  MOLECULAR FORMULA:  C24H16F21NO8  Monoisotopická hmotnost =  845,054059 Da</p>
	<p>Číslo 132  Cystathionin  HMDB ID: HMDB00099  MOLECULAR FORMULA:  C25H18F28N2O8S  Monoisotopická hmotnost =  1038,033675 Da</p>

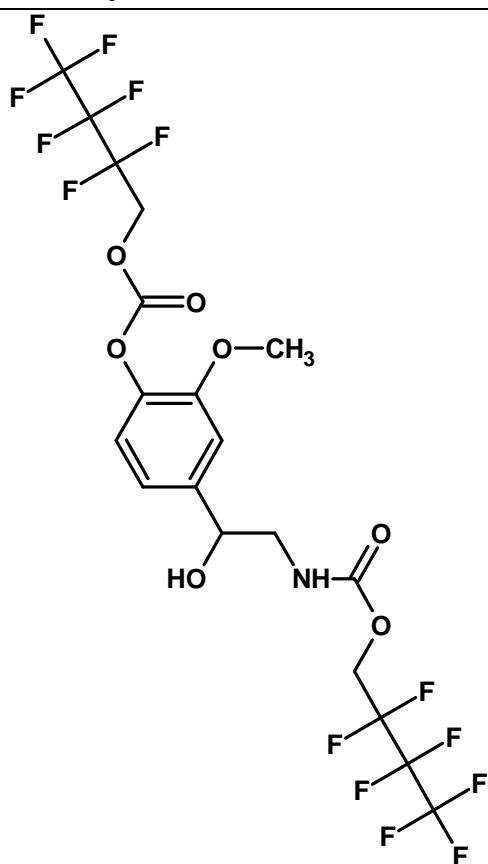
	<p>Číslo 133  Dopamin  HMDB ID: HMDB00073  MOLECULAR FORMULA:  C<sub>23</sub>H<sub>14</sub>F<sub>21</sub>NO<sub>8</sub>  Monoisotopická hmotnost =  831,038409 Da</p>
	<p>Číslo 134  K. stearová  HMDB ID: HMDB00827  MOLECULAR FORMULA:  C<sub>22</sub>H<sub>37</sub>F<sub>7</sub>O<sub>2</sub>  Monoisotopická hmotnost =  466,268178 Da</p>
	<p>Číslo 135  3,4-Dihydroxyfenylalanin  HMDB ID: HMDB00181  MOLECULAR FORMULA:  C<sub>28</sub>H<sub>15</sub>F<sub>28</sub>NO<sub>10</sub>  Monoisotopická hmotnost =  1057,024885 Da</p>

	<p>Číslo 136  Metanefrin  HMDB ID: HMDB04063  MOLECULAR FORMULA:  C<sub>20</sub>H<sub>17</sub>F<sub>14</sub>NO<sub>7</sub>  Monoisotopická hmotnost =  649,078147 Da</p>
	<p>Číslo 137  Cystin  HMDB ID: HMDB00192  MOLECULAR FORMULA:  C<sub>24</sub>H<sub>16</sub>F<sub>28</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>  Monoisotopická hmotnost =  1055,99009 Da</p>

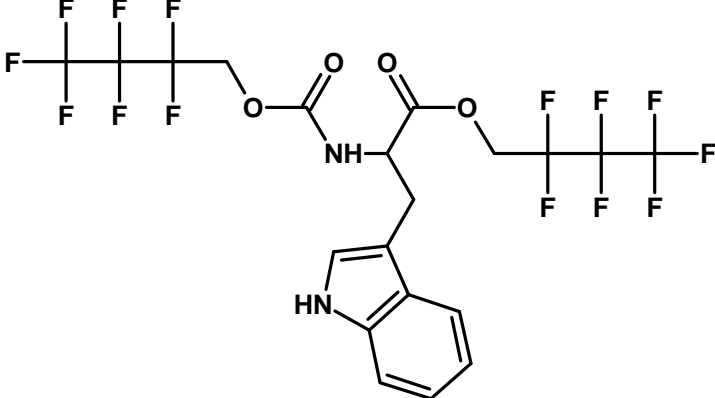
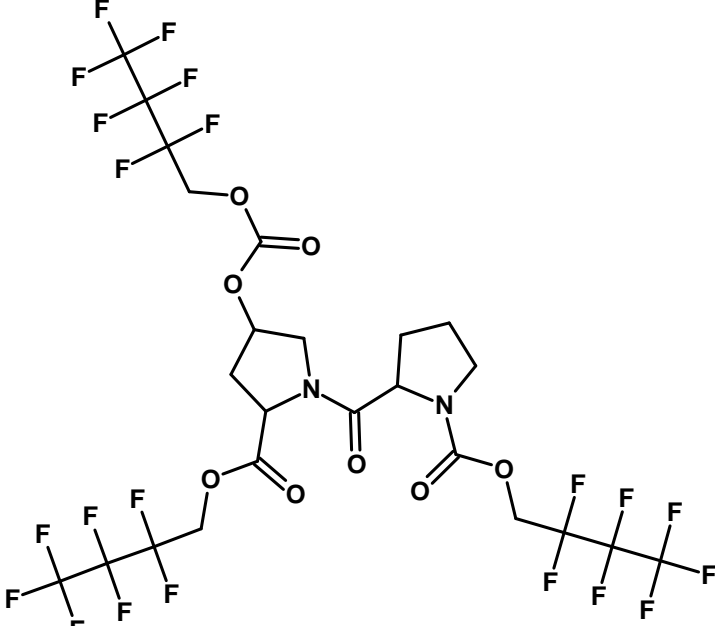
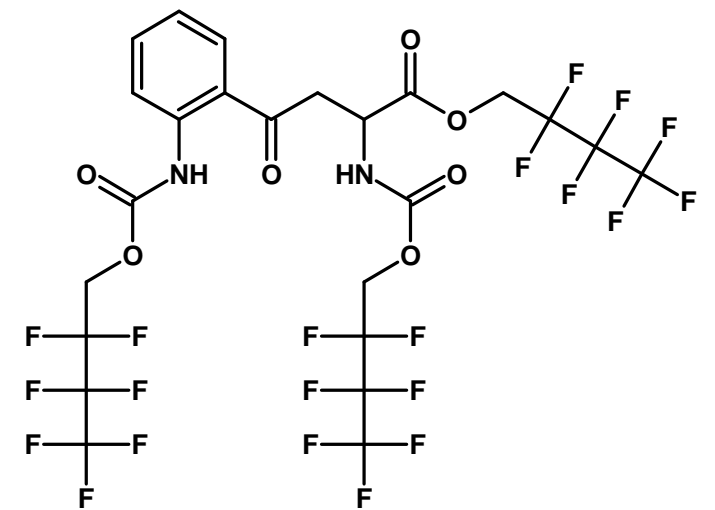
	<p>Číslo 138            5-Hydroxylysin            HMDB ID: HMDB00450            MOLECULAR FORMULA:            C<sub>25</sub>H<sub>18</sub>F<sub>28</sub>N<sub>2</sub>O<sub>9</sub>            Monoisotopická hmotnost =            1022,373710 Da</p>
	<p>Číslo 139            Tryptamin            HMDB ID: HMDB00303            MOLECULAR FORMULA:            C<sub>20</sub>H<sub>14</sub>F<sub>14</sub>N<sub>2</sub>O<sub>4</sub>            Monoisotopická hmotnost =            612,073002 Da</p>



Číslo 140a  
 Normetanefrin-1  
 HMDB ID: HMDB00819  
 MOLECULAR FORMULA:  
 C<sub>24</sub>H<sub>16</sub>F<sub>21</sub>NO<sub>9</sub>  
 Monoisotopická hmotnost =  
 861,048973 Da

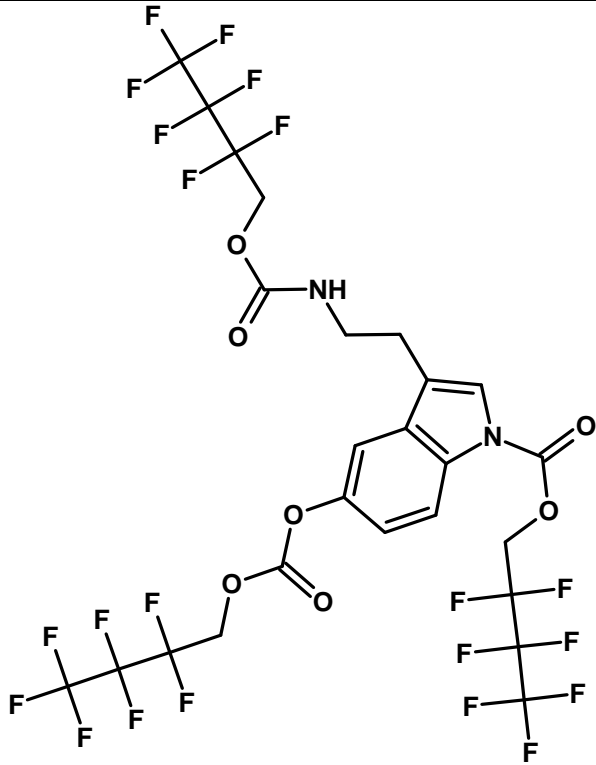
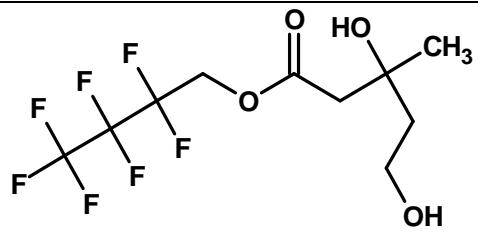
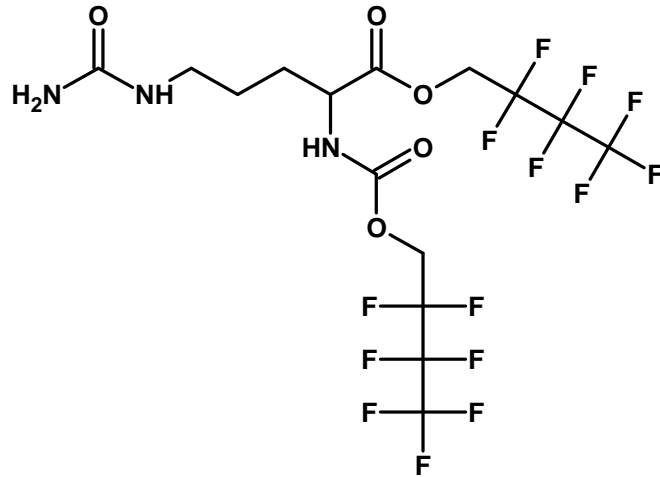


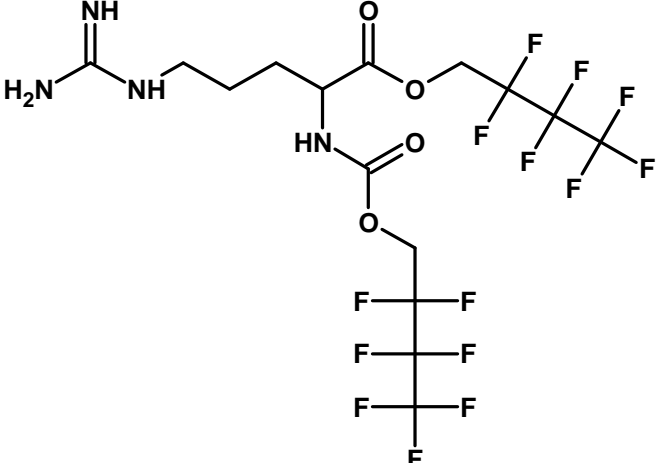
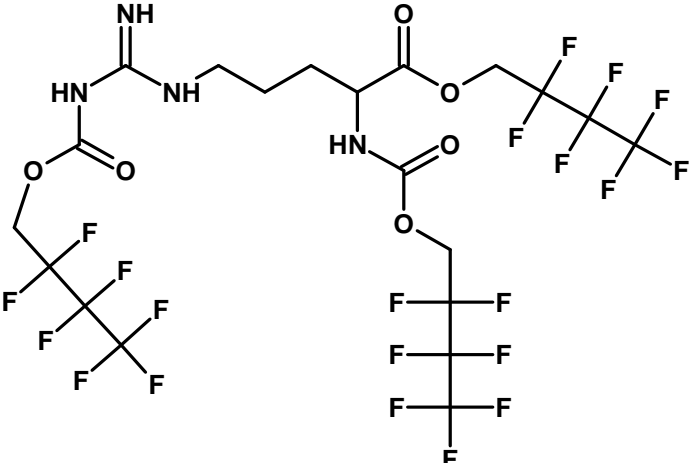
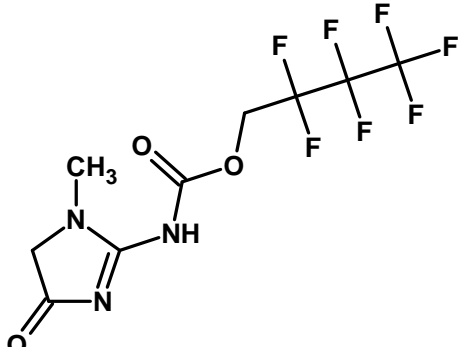
Číslo 140b  
 Normetanefrin-2 (10 %)  
 HMDB ID: HMDB00819  
 MOLECULAR FORMULA:  
 C<sub>19</sub>H<sub>15</sub>F<sub>14</sub>NO<sub>7</sub>  
 Monoisotopická hmotnost =  
 635,062497 Da

	<p>Číslo 141 Tryptofan HMDB ID: HMDB00929 MOLECULAR FORMULA: C<sub>20</sub>H<sub>14</sub>F<sub>14</sub>N<sub>2</sub>O<sub>4</sub> Monoisotopická hmotnost = 612,073002 Da</p>
	<p>Číslo 142 Prolylhydroxyprolin HMDB ID: HMDB06695 MOLECULAR FORMULA: C<sub>24</sub>H<sub>19</sub>F<sub>21</sub>N<sub>2</sub>O<sub>8</sub> Monoisotopická hmotnost = 862,080608 Da</p>
	<p>Číslo 143 Kynurenin HMDB ID: HMDB00684 MOLECULAR FORMULA: C<sub>24</sub>H<sub>15</sub>F<sub>21</sub>N<sub>2</sub>O<sub>7</sub> Monoisotopická hmotnost = 842,054393 Da</p>

	<p>Číslo 144  K. 5-hydroxyindolactová  HMDB ID: HMDB00763  MOLECULAR FORMULA:  C19H11F14NO5  Monoisotopická hmotnost =  599,041367 Da</p>
	<p>Číslo 145a  Homocystin  HMDB ID: HMDB00742  MOLECULAR FORMULA:  C26H20F28N2O8S2  Monoisotopická hmotnost =  1084,021395 Da</p>



	<p>Číslo 146            5-Hydroxytryptamin (Serotonin)            HMDB ID: HMDB00259            MOLECULAR FORMULA:            C<sub>25</sub>H<sub>15</sub>F<sub>21</sub>N<sub>2</sub>O<sub>7</sub>            Monoisotopická hmotnost =            854,054393 Da</p>
	<p>Číslo 147            K. mevalonová            HMDB ID: HMDB00227            MOLECULAR FORMULA:            C<sub>10</sub>H<sub>13</sub>F<sub>7</sub>O<sub>4</sub>            Monoisotopická hmotnost =            330,070206 Da</p>
	<p>Číslo 148            Citrulin            HMDB ID: : HMDB00904            MOLECULAR FORMULA:            C<sub>15</sub>H<sub>15</sub>F<sub>14</sub>N<sub>3</sub>O<sub>5</sub>            Monoisotopická hmotnost =            583,078815 Da</p>

	<p>Číslo 149a  Arginin-1  HMDB ID: HMDB00517  MOLECULAR FORMULA:  C15H16F14N4O4  Monoisotopická hmotnost = 582,0948  Da</p>
	<p>Číslo 149b  Arginin-2 (5 %)  HMDB ID: HMDB00517  MOLECULAR FORMULA:  C20H17F21N4O6  Monoisotopická hmotnost =  808,081276 Da</p>
	<p>Číslo 150  Kreatinin  HMDB ID: HMDB00562  MOLECULAR FORMULA:  C9H8F7N3O3  Monoisotopická hmotnost =  339,045389 Da</p>

## 8 Seznam publikací

### 8.1 Seznam publikací, prezentací a plakátových sdělení

#### 8.1.1 Publikace:

Řimnáčová L., Hušek P., Šimek P.: A new method for immediate derivatization of hydroxyl groups by fluoroalkyl chloroformates and its application for the determination of sterols and tocopherols in human serum and amniotic fluid by gas chromatography-mass spectrometry, *J.Chromatogr., A* **1339**, 154–167 (2014).

Koštál V, Urban T, Řimnáčová L, Berková P, Šimek P.: Seasonal changes in minor membrane phospholipid classes, sterols and tocopherols in overwintering insect, *Pyrrhocoris apterus*, *J. Insect Physiol.* **59**, 934–941 (2013).

Hušek P., Švagera Z., Hanzlíková D., Řimnáčová L., Zahradníčková H., Opekarová I., Šimek P.: Profiling of urinary amino-carboxylic metabolites by in-situ heptafluorobutyl chloroformate mediated sample preparation and gas chromatography-mass spectrometry, *J.Chromatogr., A* **1443**, 211–232 (2016).

Hušek P., Švagera Z., Hanzlíková D., Opekarová I., Řimnáčová L., Zahradníčková H., Šimek P.: GC-MS Metabolomic Profiling of Protic Metabolites Following Heptafluorobutyl Chloroformate Mediated Dispersive Liquid Liquid Microextraction, Theodoridis G., Gika H.G., Wilson I.D., eds., *Sample Preparation Protocol*, Springer, kapitola v knize, v tisku.

Řimnáčová L., Šimek P., Mráz J., Pejchal V., Moos M., Opekar S., Hušek P.: Simultaneous GC-MS profiling of acidic biomarkers of occupational exposure and endogenous metabolites in human urine, připraveno k odeslání do redakce Journal of Chromatography A.

#### 8.1.2 Prezentace:

Makuderová L., Šimek P., Mráz V., Stránský V., Čabala R.: Profiling of biomarkers of occupation exposure using extractive derivatization with chloroformates and GC-MS, Introduction, 5<sup>th</sup> ISC Modern Analytical Chemistry, Praha, ČR, 23.9.2009.

Makuderová L., Netland O., Ueland P.M., Šimek P.: Injektor jako kritické místo GC-MS analýzy metabolitů při výzkumu jednoduhlíkového metabolismu, Setkání uživatelů GC/MS Thermo Scientific Finnigan, Tichonice, ČR, 4. – 5.5. 2010.

Řimnáčová L., Berková P., Košťál V., Šimek P.: Comparative metabolomics of phospholipids, sterols and steroids in model insect and human body fluids, 3<sup>rd</sup> European Lipidomic Meeting, Pardubice, 2. – 4.6.2013.

### **8.1.3 Plakátová sdělení:**

Makuderová L., Šimek P., Hušek P., Mráz J.: Simultaneous GC-MS profiling of biomarkers of occupation exposure and acidic metabolites in urine, Metabolomics 2010, Amsterdam, Holandsko, 26.6. – 1.7.2010.

Řimnáčová L., Šimek P., Cimlová J., Zahradníčková H., Hušek P.: Alkyl chloroformates labelling strategies and their application in the identification and analysis of protic metabolites and xenometabolites, IMA 2011, Chania, Řecko, 18. – 22.9.2011.

Řimnáčová L., Šimek P., Mráz J., Šperlingová I.: The comparison of two analytical methods for determination of ethoxyacetic acid in human urine of ethoxyethanol-exposed people, 63. Zjazd chemikov, Vysoké Tatry, Slovensko, 5. – 9.9.2011.

Řimnáčová L., Šimek P., Mráz J.: Simultaneous screening of sixteen biomarkers of occupation exposure in urine, ISBM 2013, Londýn, UK, 2013.

## 8.2 Publikace 1:

Journal of Chromatography A, 1339 (2014) 154–167



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## A new method for immediate derivatization of hydroxyl groups by fluoroalkyl chloroformates and its application for the determination of sterols and tocopherols in human serum and amniotic fluid by gas chromatography–mass spectrometry



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#### ABSTRACT

A new method has been described for efficient derivatization of secondary alicyclic hydroxyl groups in steroids by alkyl chloroformates (RCFs). Cholesterol, an essential human sterol and a steroid precursor in eukaryotic cells, was used as a model for treatment with various RCFs in an aqueous and non-aqueous environment. While the cholesterol hydroxyl group did not react completely with any of the tested RCFs reagents in the former case, trifluoroethyl chloroformate (TFECF) or heptafluorobutyl chloroformate (HFBCF) fully converts cholesterol and related metabolites into the corresponding mixed carbonates under anhydrous conditions in seconds. The acylation reaction was combined with liquid–liquid microextraction (LLME) between isooctane and acetonitrile phase. The sample preparation requires just a stepwise addition of 50  $\mu$ l isooctane with 5  $\mu$ l of a pyridine catalyst, 100  $\mu$ l acetonitrile and 100  $\mu$ l isooctane with dissolved 5  $\mu$ l of the fluoroalkyl chloroformate reagent to a dried sample. The protocol developed in this study was successfully tested for GC–MS analysis of 12 important model steroids and four main tocopherols. Each analyte provided a single peak with excellent GC separation properties, well defined EI spectra containing diagnostic fragment ions suitable for their identification and quantitation. The new method was further validated for the determination of six diagnostic non-cholesterol sterols and four main tocopherols in human serum and in amniotic fluid. Satisfactory data were obtained in terms of calibration, quantitation limits (for sterols and tocopherols, 0.05 and 0.15  $\mu$ g/ml, respectively), within-run precision (0.9–19.5%) and between-run precision (0.2–19.0%), accuracy (82–115%) and recovery (90–110%). The validated method was successfully applied to GC–MS analysis of the analytes in woman sera and amniotic fluids and the results are well-comparable with those reported by other authors. The presented work demonstrates for the first time capability of the RCFs to derivatize alicyclic hydroxyls in steroids and tocopherols metabolites for GC–MS with excellent reaction rates, highest reaction yields, minor reagent consumption and easy conjunction with LLME methods.

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#### 1. Introduction

Organic analytes with a hydroxyl group are frequent subjects of gas chromatographic (GC) analysis. The active hydrogen of the hydroxyl can participate in a variety of intermolecular interactions that often impair gas–liquid/solid separation process. The undesirable adsorption phenomena may also occur via interactions of the analyte's hydroxyl with the GC injector and column. Furthermore, larger compounds such as steroids require high temperatures for their elution from GC columns and analytes with labile hydroxyls

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may tend to dehydration. The hydroxyl group is therefore preferably derivatized in order to increase the analyte volatility, stability and improve its separation and detection properties [1]. However, aliphatic and alicyclic hydroxyls in alcohols and steroids are only weakly acidic ( $pK_a$  15–18) and considerably resistant to conversion into derivatives with desired analytical properties.

Formation of silyl ethers by silylation with various trialkylsilyl reagents [2–4] and acylation with perfluorinated acyl anhydrides [5] have been most popular sample preparation methods in GC analysis of analytes possessing the OH group. Other derivatization methods, in particular alkylations were tested, e.g. methylation in 10% boron trifluoride methanol solution (BF<sub>3</sub>/MeOH) [6], derivatization with sulfur trioxide–pyridine complex [7], alkylation with *p*-nitrobenzyl chloride [8] and pentafluorobenzoyl bromide [9,10]. Nevertheless, sample preparation by using these reagents on analytical scale is often laborious, or it was used for specialized purposes, typically for sensitive negative ion electron capture detection [9,10]. The formation of alkyl and alkoxyalkyl ethers by alkylation reagents such as methyl iodide or dimethoxymethane and acylation of hydroxyl with alkyl chloroformates (RCFs) that provide carbonates, have been important methods for protecting OH groups in organic synthesis [11,12]. However, only the use of the latter RCF reagents has been increasingly popular in the current chemical analysis owing to their capability to react immediately with various protic groups in aqueous media [13].

The RCF reactions with protic groups are usually faster than the competing reagent hydrolysis and proceeds at the mixed organic–water interface where the RCFs rapidly convert amino groups to respective carbamates, esterify carboxyls and convert thiols and phenolic and activated hydroxyls into carbonates. Much less reactive aliphatic and alicyclic hydroxyl groups remain largely untouched in hydroxyalkyl amino acids (e.g. serine, threonine, 3-amino-2-hydroxybutyric acid) [14], 3- and 4-hydroxycarboxylic acids [15], while acylation of 2-hydroxycarboxylic acids is accompanied by formation of various side-products [16,17]. Nevertheless, small polyfunctional substances possessing aliphatic OH groups such as diethylene glycole or tartaric acid were successfully analyzed in salty, crude river waters and wines after the treatment with hexyl chloroformate decreasing thus the analyte polarity and volatility [18,19]. Introduction of chloroformates with fluorinated alkyls (FCFs), mostly trifluoroethyl (TFECF) [20,21], pentafluoropropyl (PFPCF) [22], heptafluorobutyl (HFBCF) [23–25] which are now commercially available and higher perfluoroalkyl chloroformates [26–28], considerably extended capabilities to analyze highly hydrophilic compounds directly in aqueous media. The FCFs are generally more reactive than their traditional alkyl analogs and provide highly volatile products easily amenable to GC analysis despite the rapidly increasing molecular weight of the arising derivatives.

Till now, the derivatization procedure with novel FCF reagents was mainly examined in conjunction with concurrent liquid–liquid microextraction (LLME) for GC analysis of small hydrophilic compounds possessing amino, carboxylic, thiol and phenolic groups [20–29]. However, biological samples such as human serum typically contain plenty of lipophilic substances possessing aliphatic and alicyclic hydroxyl groups that are readily extractable into the collected organic phase directly amenable to subsequent GC analysis [30]. Moreover, little is known about the reactivity of the alicyclic OH groups in abundant sterols and related steroids and other hydroxylated lipophilic substances toward the RCF reagents. Among these analytes, cholesterol (Chol) dominates as an essential membrane component of all eukaryotic cells and as a precursor of steroid hormones and bile acids which have various regulatory functions in numerous animal metabolic processes [31]. For instance, the knowledge of serum levels of non-cholesterol sterols can be highly useful because these sterols can be applied as

surrogate markers of cholesterol biosynthesis (mainly desmosterol, lathosterol, dihydrocholesterol) and absorption (campesterol,  $\beta$ -sitosterol, stigmaterol). The assessment of the non-cholesterol sterols is more affordable than the exact isotopic measurements of cholesterol synthesis and absorption [32–34].

In continuation of our research of the RCF mediated sample preparation methods, we therefore decided to investigate how various RCFs react with hydroxyls of metabolites playing a crucial role in steroid metabolism. Chol was selected as an initial model compound for the study of the OH conversion into a carbonate by action of various RCFs. By means of GC–MS, the non-derivatized Chol can easily be monitored on well deactivated GC columns and thus the reaction yields of the natural and treated Chol can easily be observed in a single GC chromatogram. The derivatization reaction of the cholesterol OH group was first examined by using the aqueous conditions optimized for the RCF treatment of amino and carboxylic acids in human body fluids [23–25] and in industrial waters [19,26,27]. Then the conversion of hydroxyl in Chol and other steroids with various RCFs was studied in non-aqueous media and conditions for the complete carbonate formation with FCFs were established. The testing set was further extended for several sterols, two hydroxysterols, the principal androgen steroid hormone testosterone, two crucial female estrogen hormones estrone, estradiol and four tocopherols, important fat soluble antioxidants from the vitamin E family. Tocopherols belong to potent fat-soluble antioxidants with protective effects against cardiovascular diseases and in the prevention of some types of cancer [35,36]. Reaction yields and further analytical properties of each RCF reaction product were carefully evaluated. Finally, the new sample preparation protocol was developed and validated for profiling of six diagnostic sterols and four tocopherols in human serum and amniotic fluid. The body fluids were collected from pregnant women in the last trimester and after the childbirth. The measured sterol and tocopherol levels were compared with the results reported earlier by other authors for serum and amniotic fluid.

## 2. Experimental

### 2.1. Chemicals

Cholesterol (Chol), dihydrocholesterol, 5- $\alpha$ -cholestane (I.S.),  $\alpha$ -tocopherol,  $\beta$ -tocopherol,  $\gamma$ -tocopherol,  $\delta$ -tocopherol, ethanol, isooctane, pyridine, hydrochloric acid, sodium carbonate, methyl chloroformate (MCF), ethyl chloroformate (ECF), isobutyl chloroformate (iBCF), hexyl chloroformate (HCF), 1-(trimethylsilyl)imidazole (TMSI), *N,N'*-dicyclohexylcarbodiimide (DCC) and cholesteryl palmitate were purchased from Sigma–Aldrich (Prague, Czech Republic). Lathosterol, campesterol, stigmaterol,  $\beta$ -sitosterol, desmosterol, testosterone, estradiol, estrone, 24S-hydroxycholesterol and 22S-hydroxycholesterol were from Steraloids (Newport, RI, USA). Potassium hydroxide and *n*-hexane from were from Merck (Prague, Czech Republic). Acetonitrile and methanol were obtained from Fisher Scientific (Pardubice, Czech Republic). TFECF and HFBCF were delivered by Genchem (Prague, Czech Republic).

### 2.2. Biological material

Serum samples and amniotic fluid samples used in this study were obtained from Dr. M. Hill (Institute of Endocrinology, Prague, Czech Republic) and stored at  $-80^\circ\text{C}$  until analysis. For the method development, serum obtained from five healthy volunteers was also used upon a written consent.



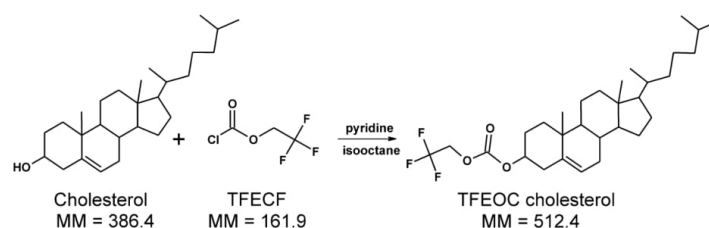


Fig. 1. Reaction scheme of cholesterol (Chol) with trifluoroethyl chloroformate (TFECF).

### 2.3. Laboratory equipment

Sample preparation was performed in micro reaction vessels with teflon lined screw cap closures (Supelco, PA, USA) which were used for saponification and derivatization. Special closures equipped with Restek mininert® precision sampling valves (Chromservis, Prague, Czech Republic) were used for sample preparation. The reagents were injected through a teflon-lined septum by means of a 100  $\mu$ l Hamilton syringe (Hamilton, Bonaduz, Switzerland) into the micro reaction vessels. A vortex mixer (50–2400 rpm) was supplied by P-Lab (Prague, Czech Republic). Autosampler vials and a 10  $\mu$ l autosampler syringe were from Thermo Fisher Scientific (Prague, Czech Republic). Drying of the reaction solvents (isooctane and acetonitrile) was accomplished by a molecular sieve, 10–20 mesh beads (Sigma–Aldrich, Prague, Czech Republic). For evaporation of samples to dryness, a Thermo Fisher Scientific Jouan RC 10.10 vacuum evaporator (Trigon Plus, Prague, Czech Republic) was used.

### 2.4. Stock and calibration solutions

Stock solutions of each examined steroid and tocopherol standard, cholesteryl palmitate and 5- $\alpha$ -cholestane used as an internal standard (I.S.) were prepared in ethanol at a concentration of 1 mg/ml. The calibration standards of sterols were prepared by a serial dilution in the concentration range between 0.25 and 50  $\mu$ g/ml at six levels (0.25, 0.5, 2.5, 5, 25 and 50  $\mu$ g/ml) in ethanol. Similarly, tocopherols calibration solutions were prepared by a

serial dilution within a 0.75–150  $\mu$ g/ml concentration range at six levels (0.75, 1.5, 7.5, 15, 75 and 150  $\mu$ g/ml) in ethanol. For spiking samples with the I.S., the 5- $\alpha$ -cholestane stock solution was diluted to the concentration 50  $\mu$ g/ml.

### 2.5. Method development

Chol (typically 3  $\mu$ g) was placed in a micro reaction vessel and the organic solvent evaporated under a mild stream of nitrogen. The residue was subjected to the reaction with a series of RCFs possessing the following alkyl and fluoroalkyl moieties: methyl (MCF), ethyl (ECF), isobutyl (iBCF), hexyl (HCF), trifluoroethyl (TFECF) and heptafluorobutyl (HFBCF). All manipulations with the RCFs and other organic chemicals were performed in a well ventilated fume hood. The derivatization procedure was combined with LLME which was carried out in both an aqueous and non-aqueous medium as follows.

#### 2.5.1. Alkyl chloroformate derivatization procedure in an aqueous medium

Chol (3  $\mu$ g) was covered with 100  $\mu$ l of water in a micro reaction vessel, briefly vortexed and then 10  $\mu$ l of pyridine, 30  $\mu$ l of an aliphatic alcohol (methanol, ethanol, hexanol or isobutyl alcohol), 12  $\mu$ l of RCF (MCF, ECF, HCF or iBCF) in 100  $\mu$ l of isooctane and 50  $\mu$ l of 1 mol/l hydrochloric acid were gradually added under vortexing. Finally, aliquot of the upper organic phase was aspirated into an autosampler vial and 1  $\mu$ l injected into a GC–MS instrument. For the derivatization of Chol with HCF, a sample preparation

Table 1

TIC EI response of the non-derivatized Chol and its alkyloxycarbonate (ROC) derivatives measured under uniform GC–MS conditions (full scan mode,  $m/z$  100–700 Da).

Analyte	Derivatization reagent	MM <sup>a</sup>	RT (min)	Chol (PA) <sup>b</sup>	Chol–ROC (PA)	Relative EI response Chol–ROC (%) <sup>c</sup>
<b>A. Sample preparation in an aqueous medium</b>						
Chol	None	386.4	4.23	16212150		
Chol–MOC <sup>d</sup>	MCF	444.4	4.78	15746879	1185249	7
Chol–EOC <sup>b</sup>	ECF	458.4	4.93	17052785	1894754	10
Chol–iBOC <sup>c</sup>	iBCF	486.4	5.28	19333588	805566	4
Chol–HOC <sup>d</sup>	HCF	514.4	5.81	20074728	1056565	5
Chol–HOC	HCF + DCC <sup>e</sup>	514.4	5.81	9842676	11099187	53
Chol–TFEOC <sup>e</sup>	TFECF	512.4	4.52	13746024	9552321	41
Chol–TFEOC	TFECF + DCC	512.4	4.52	1758047	23356924	93
Chol–HFBOC <sup>f</sup>	HFBCF	612.3	4.61	11416233	10538060	48
<b>B. Sample preparation under anhydrous conditions</b>						
Chol	None	386.4	4.23	18762260		
Chol–TMS	TMSI	458.4	4.45	n.d. <sup>k</sup>	21106631	100
Chol–MOC	MCF	444.4	4.78	14838147	6359205	30
Chol–EOC	ECF	458.4	4.93	14427267	4809089	25
Chol–iBOC	iBCF	486.4	5.28	12896723	6944388	35
Chol–HOC	HCF	514.4	5.81	11656353	10345633	47
Chol–HOC	HCF + DCC	514.4	5.81	3602825	17590266	83
Chol–TFEOC	TFECF	512.4	4.52	n.d. <sup>k</sup>	21477150	100
Chol–HFBOC	HFBCF	612.3	4.61	n.d. <sup>k</sup>	20847372	100

The carbonate derivatives: <sup>a</sup>MOC = methoxy-, <sup>b</sup>EOC = ethoxy-, <sup>c</sup>iBOC = isobutoxy-, <sup>d</sup>HOC = hexyloxy-, <sup>e</sup>TFEOC = trifluoroethoxy-, <sup>f</sup>HFBOC = heptafluorobutoxy  
<sup>a</sup>MM = monoisotopic mass, <sup>b</sup>PA = peak area, <sup>c</sup>DCC = N,N'-dicyclohexylcarbodiimide, <sup>d</sup>Relative EI response Chol–ROC(%) =  $\frac{\text{Chol-ROC(PA)}-100}{(\text{Chol(PA)}+\text{Chol-ROC(PA)})}$ , <sup>k</sup>n.d. = not detected.

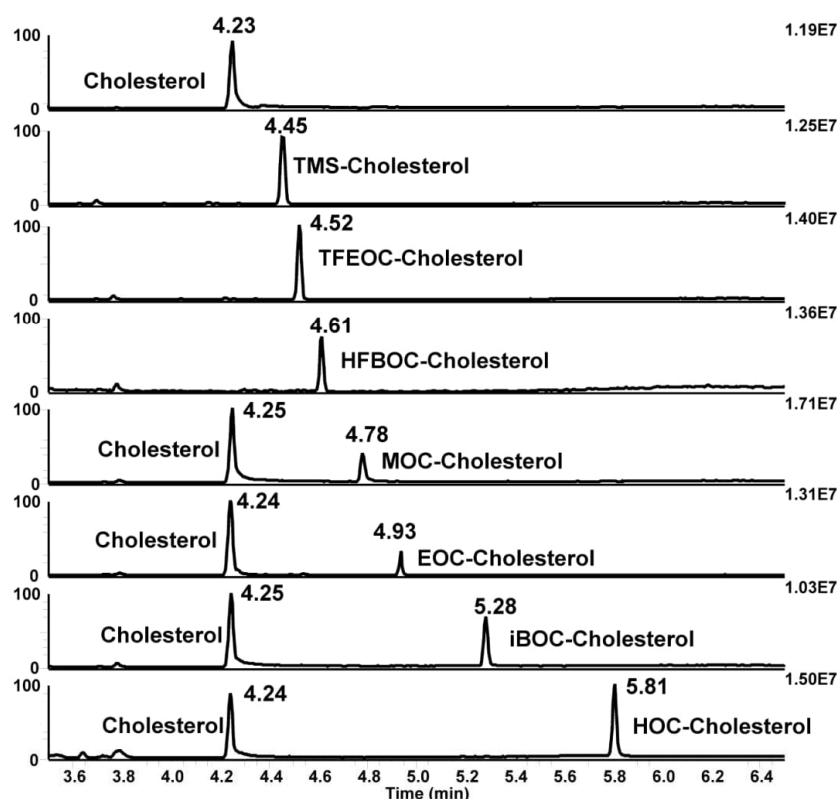


Fig. 2. The TIC GC–MS chromatograms of Chol and the derivatives under study; the non-derivatized cholesterol, its TMS-ether and the carbonates obtained by the reaction of Chol with various RCFs under non-aqueous conditions.

method of Vincenti et al. [19,26,27] was examined. A 10  $\mu$ l aliquot of a pyridine catalyst solution containing DCC (400 mg/ml) instead of simple pyridine was used.

For the derivatization with fluoroalkyl chloroformates, Chol residue was mixed with 150  $\mu$ l of 0.05 mol/l aqueous sodium carbonate in a micro reaction vessel and 50  $\mu$ l of an isooctane-TFECF (HFBCF) mixture (5:3, v/v) was added, vortexed for approximately 10 s. Then a second medium of 0.1 mol/l sodium carbonate with admixed pyridine (50  $\mu$ l, 5:1, v/v) was added, followed by vortexing for about 10 s. After adding 50  $\mu$ l of isooctane and vortexing for about 10 s, the upper organic phase was aspirated into an autosampler vial and a 1  $\mu$ l subjected to GC–MS analysis. For the Chol treatment with TFECF, the pyridine solution containing DCC (400 mg/ml) was also tested.

#### 2.5.2. Alkyl chloroformate derivatization procedure under anhydrous conditions

The Chol (3  $\mu$ g) was dried under mild stream of nitrogen in a micro reaction vessel and re-dissolved in 50  $\mu$ l of an isooctane–pyridine mixture (9:1, v/v). After vortexing, 100  $\mu$ l of acetonitrile was added. Subsequently, 100  $\mu$ l of isooctane–RCF medium containing an appropriate reagent: either MCF, ECF, iBCF, HCF, TFECF or HFBCF (each 95:5, v/v) was added, the content vortexed for about 20 s and two immiscible layers were obtained. The upper isooctane layer was aspirated into an autosampler vial and a 1  $\mu$ l aliquot was subjected to GC–MS analysis.

#### 2.5.3. Trimethylsilylation derivatization procedure

The properties of the new carbonate Chol derivatives were compared with the most common Chol trimethylsilyl ether derivative prepared by treatment of the same amount of Chol (3  $\mu$ g) with a TMSI reagent as described earlier [37,38]. The dry residue of the standard was dissolved in 100  $\mu$ l of dimethylformamide and treated with 30  $\mu$ l of TMSI. The sample was then heated at 80  $^{\circ}$ C for 30 min and cooled to room temperature. After the addition of 100  $\mu$ l of isooctane and vortexing, the arising immiscible phases were separated and the upper layer containing the TMS derivative was transferred into an autosampler vial and a 1  $\mu$ l aliquot measured by GC–MS.

#### 2.6. GC–MS analysis

For the characterization of all studied reaction products, GC–MS analysis was performed on a Trace Ultra GC gas chromatograph with a programmable temperature vaporizing (PTV) injector coupled directly to a quadrupole mass spectrometer Trace DSQ equipped with an electron ionization (EI) ion source (ThermoFisher Scientific, San Jose, USA). An Xcalibur software, version 2.1 (Thermo Fisher Scientific), was used for data acquisition and processing. A 1  $\mu$ l sample aliquot was injected in the splitless mode (a valve closed for 0.7 min) into a Restek 2 mm glass Siltek deactivated baffle liner (Restek, PA, USA). Autosampler syringes should be rinsed with propan-2-ol after injection. The injector temperature was set at



**Table 2**  
Analytical characteristics of the investigated TFEOC derivatives.

Analyte	RT (min)	MM <sup>a</sup> TFEOC der.	Quantification ion	Confirmation ion (1)	Confirmation ion (2)
Estrone	3.22	396.1	396	352	339
Testosterone	3.42	414.2	372	124	329
Estradiol	3.57	524.1	524	236	381
5- $\alpha$ -Cholestane (I.S.) <sup>b</sup>	3.72	372.4	217	357	372
$\delta$ -Tocopherol	4.01	528.3	263	528	303
$\beta$ -Tocopherol	4.15	542.4	277	542	317
$\gamma$ -Tocopherol	4.20	542.4	277	542	317
$\alpha$ -Tocopherol	4.32	556.4	165	556	291
Cholesterol	4.58	512.3	368	353	247
Dihydrocholesterol	4.60	514.4	499	514	359
Desmosterol	4.68	510.3	366	253	351
Lathosterol	4.70	512.4	399	512	497
Campesterol	4.78	526.4	382	213	145
Stigmasterol	4.83	538.4	394	255	351
22S-Hydroxycholesterol	4.83	654.3	510	366	495
$\beta$ -Sitosterol	4.96	540.4	396	381	147
24S-Hydroxycholesterol	5.03	654.3	510	366	495

Three  $m/z$  ions were found suitable for quantification of each analyte. However, two  $m/z$  ions (a quantification and confirmation (1) one with RT) were proved sufficient for reliable identification and method validation.

<sup>a</sup> MM = monoisotopic mass.

<sup>b</sup> Not derivatized internal standard.

280 °C. A DB-1HT, 15 m  $\times$  0.25 mm i.d., 0.1  $\mu$ m film thickness (Agilent Technologies, Santa Clara, CA, USA) capillary column was used for GC analysis. The oven was initially held at 180 °C for 1 min, raised at 30 °C/min to 330 °C and held for 2 min. Helium carrier gas flow rate was 1.1 ml/min, the ion source temperature was held at 250 °C and the transfer line at 300 °C. EI mode (70 eV) in a full scan mode (100–700 Da).

### 2.7. Method quantitation and validation

For the quantitation of selected steroid and tocopherol metabolites in biofluids, mixed trifluoroethyl carbonate derivatives (TFEOC) were employed for their GC–MS analysis on the same DB-1 HT column and under the identical instrumental conditions, but in the selected ion monitoring MS scan mode (SIM). For each analyte, a quantification and confirmation  $m/z$  ion was selected and detected in a 1.2 Da mass window and a 25 ms dwell time.

The total content of each free and bound analyte was determined after saponification of each sample with 2 M KOH in 90% ethanol as described in [34]. The efficiency of the saponification was verified by two methods:

- (i) The content of cholesteryl palmitate and the released Chol was analyzed in triplicate before and after the saponification procedure. A 1  $\mu$ l aliquot of cholesteryl palmitate standard

solution was mixed with 250  $\mu$ l of 2 M KOH in 90% ethanol and hydrolyzed in an oven at 60 °C for 120 min. The medium was then mixed with 500  $\mu$ l of hexane and a 300  $\mu$ l aliquot was transferred into a micro reaction vessel and evaporated to dryness by using a vacuum evaporator. The dry residue was derivatized by TFEFCF and measured by GC–MS as described below.

- (ii) A randomly selected serum and amniotic fluid ( $n=3$  of each) was saponified and the nonpolar components extracted from the aqueous phase into hexane as described above. The hexane phase was withdrawn, and evaporated to dryness under a mild stream of nitrogen. The residue was then dissolved in 300  $\mu$ l of methanol and abundance of triglycerides and diglycerides monitored on a RP-column Gemini C18, 110 A, 150 mm  $\times$  2 mm i. d., 3  $\mu$ m (Phenomenex, CA, USA) by positive electrospray mass spectrometry (+ESI MS) LC–MS method described elsewhere [39].

#### 2.7.1. Sample preparation

A 100  $\mu$ l aliquot of each biofluid was spiked with 10  $\mu$ l of the I.S. solution in a micro reaction vial. After vortexing, 250  $\mu$ l 2 M KOH in 90% ethanol was added, the mixture saponified at 60 °C in an oven for 120 min and extracted with 500  $\mu$ l of hexane. A 300  $\mu$ l hexane aliquot was evaporated to dryness by the vacuum evaporator. The dry residue was then treated with 50  $\mu$ l of isooctane/pyridine (9:1,

**Table 3**  
Calibration data obtained by GC–MS analysis of QC serum and QC amniotic fluid samples.

Analyte-TFEOC der.	RT (min)	Range of quantification ( $\mu$ g/ml)	Regression line serum	R2 serum	Regression line amniotic fluid	R2 amniotic fluid	LLOQ <sup>b</sup> ( $\mu$ g/ml)
5- $\alpha$ -Cholestane	3.69	5	Internal standard (I.S.)				
$\delta$ -Tocopherol	4.01	0.15–30	0.0075x – 0.0008	0.9907	n.d. <sup>a</sup>	n.d.	0.15
$\beta$ -Tocopherol	4.15	0.15–30	0.0163x – 0.0022	0.9986	n.d.	n.d.	0.15
$\gamma$ -Tocopherol	4.20	0.15–30	0.0220x + 0.0113	0.9531	n.d.	n.d.	0.15
$\alpha$ -Tocopherol	4.32	0.15–30	0.0141x – 0.0030	0.9796	n.d.	n.d.	0.15
Dihydrocholesterol	4.64	0.05–10	0.0216x + 0.0011	0.9983	0.0234x – 0.0027	0.9995	0.05
Desmosterol	4.66	0.05–10	0.0400x + 0.0409	0.9746	0.0364x – 0.0081	0.9959	0.05
Lathosterol	4.68	0.05–10	0.0644x + 0.0101	0.9992	0.0418x – 0.0030	0.9991	0.05
Campesterol	4.77	0.05–10	0.0932x + 0.0123	0.9994	0.1015x – 0.0152	0.9994	0.05
Stigmasterol	4.83	0.05–10	0.0472x – 0.0012	0.9998	0.0797x – 0.0058	0.9997	0.05
$\beta$ -Sitosterol	4.93	0.05–10	0.0352x + 0.0095	0.9978	0.1005x – 0.0117	0.9997	0.05

<sup>a</sup> n.d. = not determined.

<sup>b</sup> The LLOQ was the lowest point of the calibration curve and the same for serum and plasma.

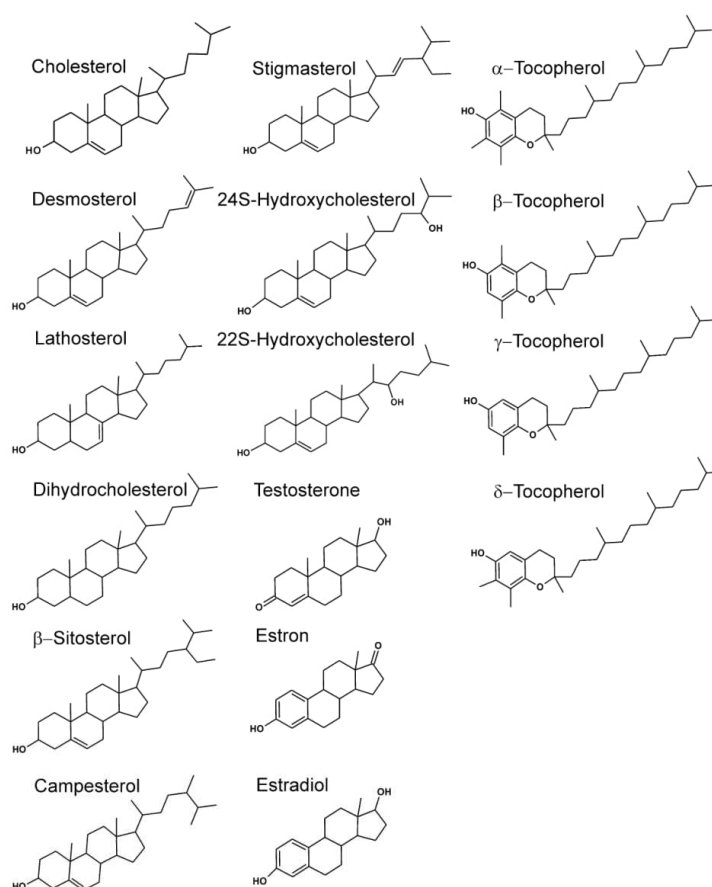


Fig. 3. The structures of sixteen analytes examined in this study.

v/v), vortexed for ca 10 s and 100  $\mu$ l of acetonitrile and 100  $\mu$ l of mixture of isooctane/TFEFCF (95:5, v/v) was gradually added and vortexed for further ca 20 s. Finally, a 50  $\mu$ l aliquot of upper layer was transferred into an autosampler vial and 1  $\mu$ l of the organic phase was subjected to GC–MS analysis.

#### 2.7.2. Calibration

Calibration curves were obtained by spiking the serum and amniotic fluid matrix with a 10  $\mu$ l I.S. solution and a 20  $\mu$ l calibration solution containing each sterol and tocopherol at six concentration levels. The final added concentrations in matrix were: 0.05, 0.1, 0.5, 1, 5, 10  $\mu$ g/ml and 0.15, 0.3, 1.5, 3, 15, 30  $\mu$ g/ml for sterols and tocopherols, respectively. The sample preparation and GC–MS analysis was then performed as described above. Peak area ratio of each analyte to the I.S. (5- $\alpha$ -cholestane, final serum concentration, 5  $\mu$ g/ml) was plotted against its concentration. Least square regression was used to determine slope, intercept and coefficient of determination ( $R^2$ ). The sera and amniotic fluids spiked with sterols and tocopherols at the respective 0.05, 1 and 10  $\mu$ g/ml and 0.15, 3 and 30  $\mu$ g/ml level were further used as quality control samples (QC) for validation of the developed method.

#### 2.7.3. Method validation

Calibration curves, lower limit of quantification (LLOQ), precision and accuracy, recovery and stability were assessed in accordance with generally accepted recommendations [40].

Lower limit of quantification (LLOQ) was determined as the lowest point of the calibration curve obtained by GC–MS analysis of the spiked matrix having precision less than 20% and accuracy within 80–120% ( $n = 5$ ).

Precision and accuracy were determined by analyzing the QC serum and amniotic fluid samples spiked with standards at the low, medium and high concentrations in single series (within-run assay) and over a 14-day period (between-run assay) ( $n = 5$ ).

Recovery was also estimated with the QC samples. The endogenous concentration of each sterol and tocopherol was first determined in the biofluids used as the QC samples and compared with the values measured in the same samples spiked with the analyte standards at three concentration levels. Recovery was calculated according to the formula: (measured concentration – endogenous concentration)/added concentration  $\times 100$ .

Stability of the TFEFCF derivatives was evaluated with the QC serum and amniotic fluid samples spiked with standards at the medium concentration level. Each sample was prepared in triplicate and stored after the sample preparation and GC–MS analysis

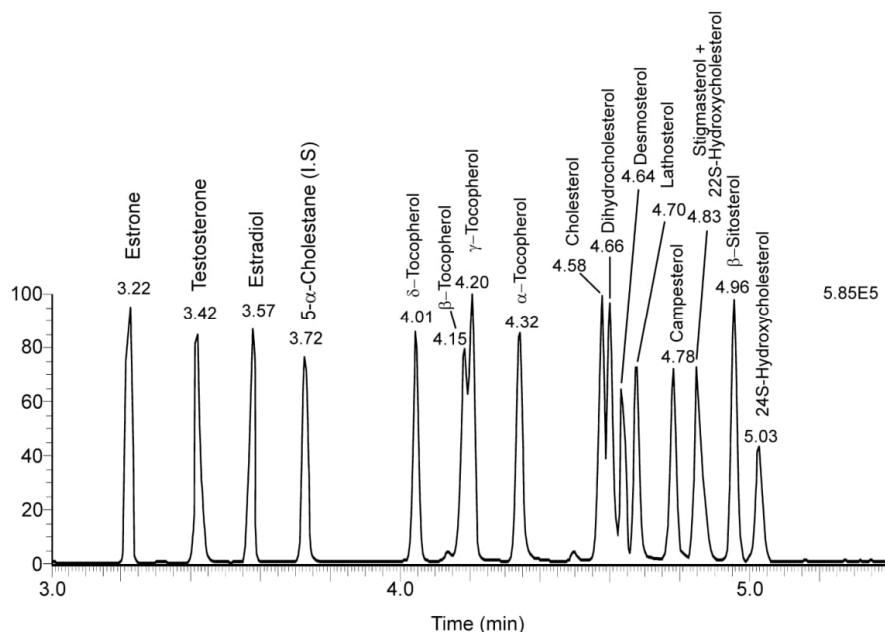


Fig. 4. TIC GC–MS separation of sixteen steroids, tocopherols as the TFEOC derivatives and 5- $\alpha$ -cholestane (I.S.) on a 15 m  $\times$  0.25 mm i.d.DB-1HT capillary column. Splitless injection, 20 ng of each analyte.

at room temperature and at 4 °C. The QC sample stability was measured by the validated method in regular intervals within 72 h. The analyte concentrations in the QC samples were compared with those obtained by the GC–MS analysis of the same samples prepared freshly on the day of analysis.

### 3. Results and discussion

#### 3.1. Reactivity of the cholesterol hydroxyl group toward alkyl chloroformates

For studying the reactions of the alicyclic hydroxyl with various RCFs on analytical scale, Chol was first tested as a pilot model compound. The reaction scheme for acylation of Chol with the TFEFCF reagent is shown in Fig. 1.

The reactivity of the OH group was first studied in aqueous medium. The reported derivatization conditions used earlier for the determination of polar metabolites in human body fluids [17,22–25,29] and in industrial waters [19,26] were tested. Chol was incubated with a series of chloroformates (from the MCF to FCF reagents) and the arising products extracted into organic phase and analyzed by GC–MS. The resulting products, their TIC GC–MS response and relative abundance are summarized in Table 1A.

From the relative EI response of the particular Chol carbonates (last column in Table 1A) it is evident that the secondary OH group of Chol remains in aqueous environment largely untouched and the yield of the corresponding carbonate depends clearly on the RCF's reactivity which is approximately increasing from hexyl/isobutyl over ECF, MCF to fluoroalkyl chloroformates. The effect of the DCC coupling reagent on the reaction yields was further tested in conjunction with HCF on the basis of the earlier observations of Vincenti et al. [27]. Interestingly, the yield of the Chol–HOC derivative increased upon the DCC action by an order of magnitude and

indicates an activation effect of DCC toward the carbonate formation. Comparable yields of the Chol derivatives in the aqueous medium, but without DCC, were obtained with FCFs which is in accordance with their observed higher reactivity [23,24]. Using DCC, the yield of the TFEOC Chol product exceeded 90% (calculated from the TIC response of the Chol and Chol–ROC peaks). Nevertheless, full conversion into the desired Chol–ROC product was not reached by any of the RCF reagents tested.

Since animal steroids are generally extractable from water into immiscible organic solvents, the reactivity of the Chol OH group was further studied under non-aqueous conditions in the next series of experiments. The obtained results are summarized in Table 1B.

While Chol was completely converted into the TMS-ether derivative at 80 °C for 30 min, the corresponding carbonates arising from acylation of Chol with RCFs were obtained with yields below 50%, except the trial when the carbonate formation was catalyzed by the DCC coupling reagent (83% yield), Table 1B. It is worth noting that the reaction yields rise with the decreasing chloroformate polarity, in reverse order than in aqueous environment, compare the reaction yields in Table 1A and B.

The smooth derivatization of the Chol hydroxyl was facilitated by action of both examined FCFs under the carefully maintained anhydrous conditions. The new derivatization procedure is very simple and involves an addition of a mixture of iso-octane with the FCF reagent to a dried sample. We tested a suitable ratio between the FCF reagent and the solvent dried over a molecular sieve in the range of 2–10% (v/v) and found the 5% aliquot of the commercially available TFEFCF (or HFBCF) in iso-octane as an optimum. The low reagent consumption, typically about 5  $\mu$ l per sample, makes the new sample preparation method very cost-effective. We initially used 100  $\mu$ l of 250 mM HCl in order to dissolve the undesired pyridine hydrochloride precipitate. However, addition of 100  $\mu$ l of dried acetonitrile prior to the derivatization step was found even



more efficient. Acetonitrile easily dissolves not only the white pyridine chloride salt but also is capable to retain extraneous interfering components while the nonpolar Chol-TFEOC derivative enters the upper isooctane layer.

The TIC GC-MS chromatograms of Chol and its derivatives, the non-derivatized Chol, its TMS-ether and the carbonates obtained by Chol reaction with various RCFs in non-aqueous environment are depicted in Fig. 2.

The chromatograms and EI responses of the Chol-carbonates which were measured by GC-MS under identical experimental conditions are shown in Fig. 2 and Table 1B, respectively. They indicate that among the tested carbonate derivatives of Chol, the fluoroalkyl carbonates exhibit advantageous analytical properties providing, symmetrical, sharp GC peaks and a very good EI response comparable to that obtained with the silylated Chol-TMS ether derivative.

### 3.2. Derivatization of other sterols, steroid hormones and tocopherols

Only the TFECF and HFBCF reagents enabled an immediate full conversion of Chol into the corresponding carbonate derivative. As the chromatographic and detection features of both Chol FCF derivatives were very similar, we further examined the TFECF reagent in derivatization of a larger range of animal and plant sterols, some important steroid hormones and tocopherols. The structures of all 16 investigated compounds are listed in Fig. 3. Under the sample preparation conditions developed for Chol, all examined analytes were smoothly converted by action of TFECF into the corresponding carbonates providing a single peak and defined EI mass spectra. The secondary hydroxyl groups, i.e. at position 3 (in sterols), 17 (in testosterone, and estradiol), at position 22 and 24 (in hydroxysterols) and in aromatic hydroxyls (estrogens,

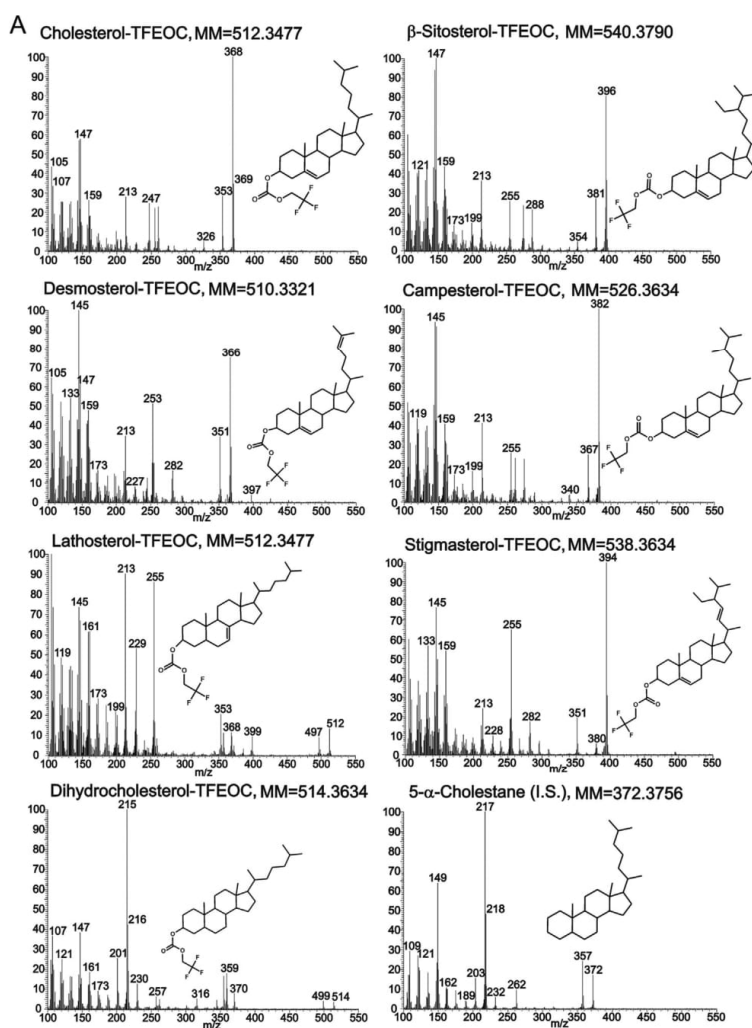


Fig. 5. (A) EI mass spectra of the TFEOC derivatives of Chol, six diagnostic non-cholesterol sterols and 5- $\alpha$ -cholestane (I.S.). (B) EI mass spectra of the TFEOC derivatives of two hydroxycholesterols and three steroid hormones. (C) EI mass spectra of the TFEOC derivatives of four tocopherols.

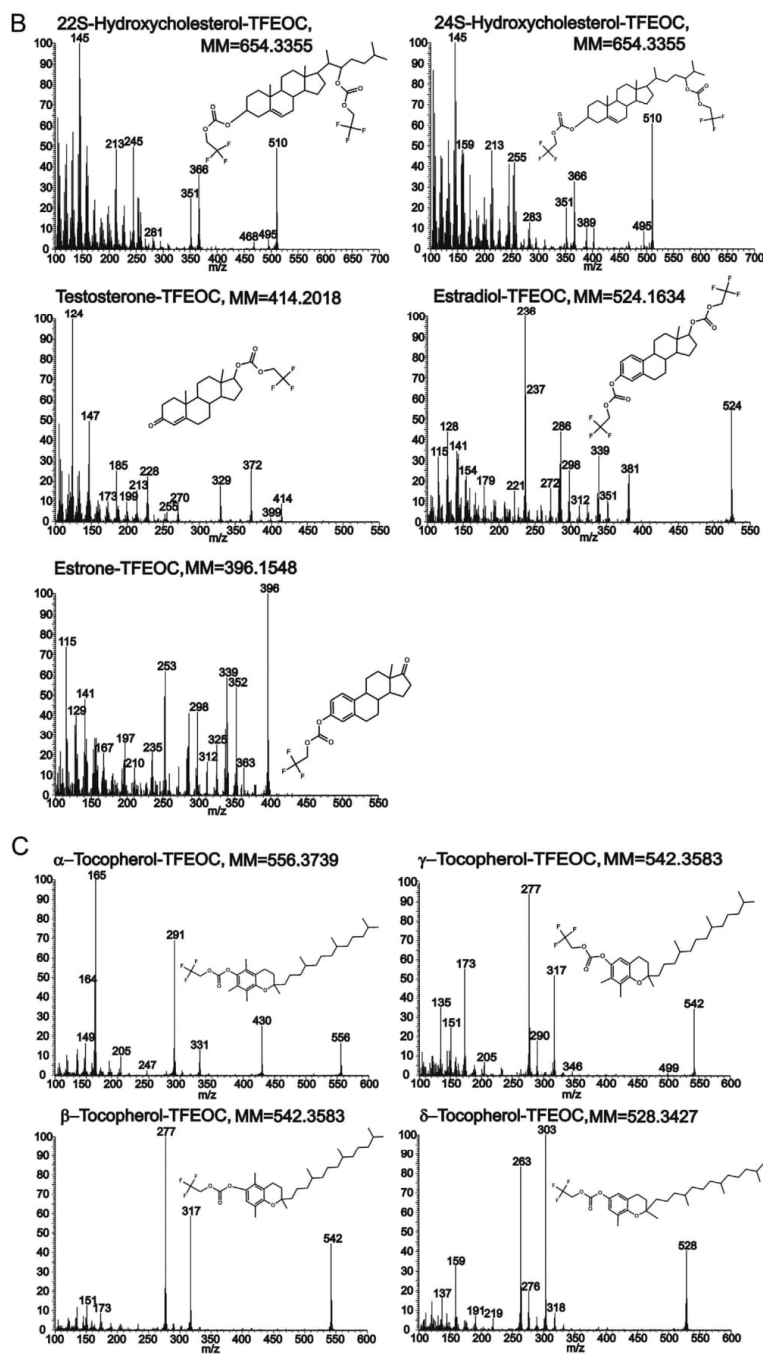
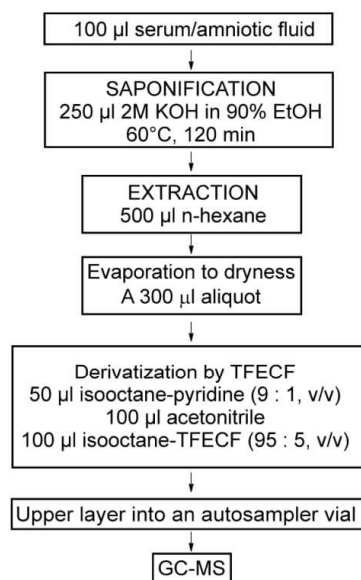


Fig. 5. (A) Continued .

**Table 4**  
Precision, accuracy and percent recovery of non-cholesterol sterols and tocopherols after GC–MS analysis of the QC woman serum samples.

Analyte	Concentration level (µg/ml)	Precision (%)		Accuracy (%)		Recovery (%)
		Within-run	Between-run	Within-run	Between-run	
δ-Tocopherol	30	2.6	3.2	98	93	92
	3	5.2	4.6	104	112	90
	0.15	1.2	5.9	91	86	104
β-Tocopherol	30	2.6	5.2	96	98	105
	3	3.8	2.5	98	96	108
	0.15	4.1	3.6	103	111	96
γ-Tocopherol	30	1.2	5.2	91	89	105
	3	5.1	5.1	96	85	99
	0.15	1.9	4.9	82	86	102
α-Tocopherol	30	5.0	4.3	102	85	110
	3	14.7	0.2	94	86	96
	0.15	13.7	5.2	85	82	92
Dihydrocholesterol	10	11.0	11.1	115	83	94
	1	15.1	3.7	89	83	98
	0.05	19.5	6.6	86	89	97
Desmosterol	10	12.9	13.1	110	105	85
	1	14.2	10.8	104	109	86
	0.05	18.9	19.5	111	113	90
Lathosterol	10	5.3	12.1	95	86	96
	1	11.6	15.9	85	88	92
	0.05	2.2	7.5	86	91	91
Campesterol	10	3.7	11.2	98	105	101
	1	0.9	5.8	86	98	108
	0.05	11.1	11.0	83	86	98
Stigmasterol	10	8.5	9.0	90	85	99
	1	3.6	4.8	85	90	101
	0.05	9.3	9.6	82	82	109
β-Sitosterol	10	5.6	7.6	99	108	110
	1	4.6	12.6	98	87	98
	0.05	13.6	19.0	85	84	95



**Fig. 6.** Optimized sample preparation workflow for the quantitative determination of six sterols and four tocopherols in human serum/amniotic fluid.

tocopherols) were also efficiently derivatized. Similarly, phenolic hydroxyl groups in tocopherol react smoothly with the RCF, while the keto groups in the tested testosterone and estrone structures remained untouched.

Separation and detection of all examined steroids and tocopherols carbonates prepared by the new method was easily achieved by GC–MS analysis on a nonpolar OV-1 type fused silica capillary column. TIC GC–MS separation of 16 TFEOC metabolites with the I. S. in nearly 5 min is depicted in Fig. 4.

Despite the relatively high molecular weight, the TFEOC derivatives were eluted at moderate temperature between 240 and 300 °C on the nonpolar GC column and approximately according to their increasing molecular masses. The basic analytical characteristics of the prepared TFEOC derivatives including the nominal molecular mass, retention data and diagnostic EI mass spectral fragments are summarized in Table 2.

### 3.3. Mass spectra of the TFEOC derivatives and evaluation of the diagnostic SIM ions

Replacing hydrogen in the hydroxyl by the TFEOC moiety increases molecular mass of the derivatization product by 126 daltons. The EI mass spectra of the studied steroids and tocopherols as the TFEOC derivatives are summarized in Fig. 5A–C.

The carbonate bond is generally weak and easily ruptures, here by a characteristic loss of neutral trifluoroethyl hydrogen carbonate species [TFEOCOOH,  $M^+ - 144$ ]. The fission process is driven by stabilization of the charge and radical on the cholesterol ring giving  $M^+ = 368.2$ . Molecular ion of sterols is therefore usually weak, Fig. 5A. Comparison of the sterol EI spectra indicates that the  $M^+$

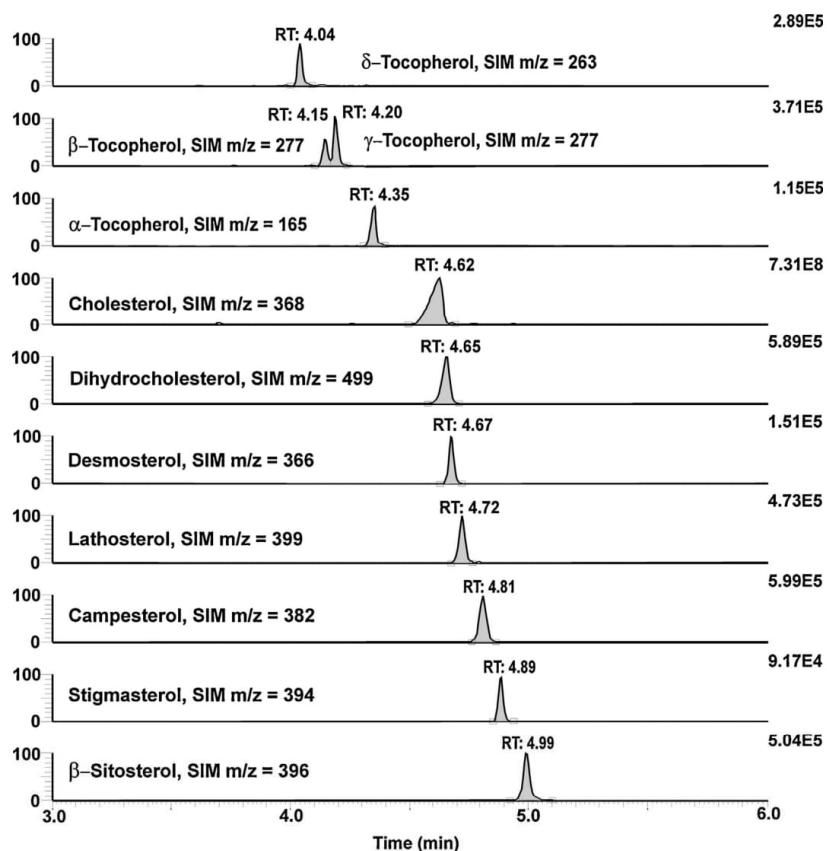


Fig. 7. Characteristic SIM GC-MS chromatograms of steroids and tocopherols detected in serum of a pregnant woman (sample Wb4).

stability and, thus intensity in the EI spectrum, is conducted by the stability of the steroid skeleton and is particularly influenced by the position of the double bond in the second ring. The presence of another OH in the Chol structure, at least in positions 22, 24 strengthens the TFEOCOOH loss giving a fragment ion  $m/z$  510 which is accompanied by the fission of a second TFEOCOOH moiety providing an ion  $m/z$  366 as documented in the EI spectra of estradiol and hydroxycholesterols, Fig. 5B.

The EI spectra of tocopherols contain a distinct molecular ion  $M^+$  because the TFEOC moiety is here stabilized by the present aromatic ring, Fig. 5C. Characteristic principal fragment ions  $M^+ - 225$  and  $M^+ - 224$  are formed through the non-aromatic portion of the chromanol ring both with and without hydrogen transfer on the long aliphatic chain, and via loss of the isoprenoid side chain  $M^+ - 265$  [41].

Potential diagnostic ions of each analyte suitable for the GC-MS SIM quantitation were carefully selected from the EI spectra obtained by GC-MS analysis of different human sera ( $n = 7$ ) after the application of the developed sample preparation protocol. Both the natural samples and the same sample aliquots spiked with standards at the medium QC level were examined for the evaluation of the quantification and confirmation SIM ions. The choice of the SIM ions largely depends on interferences arising from the serum matrix and from EI ionization of closely eluting structurally similar analytes, here particularly in the vicinity of the dominating Chol

peak. The quantification and confirmation SIM ions convenient for serum analysis were summarized in Table 2.

Assessment of the retention times with a tolerance window  $\pm 0.01$  min and two SIM ions were proved to be a reliable criterion for identification all measured analytes, although for dihydrocholesterol and lathosterol relatively less abundant ions  $m/z$  499, 512 and  $m/z$  399, 512 had to be used to avoid interferences with the coeluting cholesterol peak.

### 3.4. Sample preparation for the body fluid analysis

The new analytical procedure described in the present study was examined for the determination of 10 sterols and tocopherols in human serum and amniotic fluid. The complete workflow of the method is shown in Fig. 6 and involves a serum (amniotic fluid) saponification, extraction of sterols and tocopherols into hexane, solvent evaporation, derivatization by a TFEFCF-isooctane medium, acetonitrile-isooctane LLME, transfer of the upper layer into an autosampler vial and GC-MS analysis.

As the analytes exist in a free and bound form, each body fluid was subjected to an alkaline hydrolysis according to the procedure described in ref. [34]. The efficiency of the used saponification conditions was checked by hydrolysis of the cholesteryl palmitate standard and by GC-MS analysis of the released Chol before and after the hydrolysis step. Furthermore, the content of

**Table 5**  
Concentration levels (µg/ml) of non-cholesterol sterols and tocopherols in serum of ten healthy women before (Wb) and after (Wa) childbirth.

	Dihydrocholesterol	Desmosterol	Lathosterol	Campesterol	Stigmastanol	β-Sitosterol	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol
Wb1 <sup>a</sup>	32.38 ± 1.53	0.60 ± 0.01	7.44 ± 0.21	5.14 ± 1.09	1.73 ± 0.31	5.72 ± 0.09	9.90 ± 0.28	0.91 ± 0.10	0.44 ± 0.02	n.d. <sup>c</sup>
Wa1 <sup>b</sup>	2.64 ± 0.22	0.55 ± 0.01	5.42 ± 0.11	2.51 ± 0.03	0.06 ± 0.00	1.53 ± 0.06	3.19 ± 0.08	0.80 ± 0.07	0.07 ± 0.00	n.d.
Wb2	1.73 ± 0.41	0.85 ± 0.06	4.57 ± 0.35	4.85 ± 0.24	0.06 ± 0.01	2.85 ± 0.15	14.10 ± 0.11	0.92 ± 0.09	0.11 ± 0.01	n.d.
Wa2	0.70 ± 0.19	0.64 ± 0.06	2.12 ± 0.19	3.95 ± 0.14	0.07 ± 0.01	1.94 ± 0.05	2.52 ± 0.05	0.64 ± 0.02	n.d.	n.d.
Wb3	12.42 ± 1.49	0.81 ± 0.10	2.47 ± 0.20	2.08 ± 0.42	0.40 ± 0.10	1.85 ± 0.32	9.33 ± 0.31	1.18 ± 0.27	0.32 ± 0.02	n.d.
Wa3	0.48 ± 0.08	0.90 ± 0.05	1.57 ± 0.03	1.35 ± 0.07	0.04 ± 0.00	0.77 ± 0.07	7.18 ± 1.05	0.61 ± 0.09	n.d.	n.d.
Wb4	5.94 ± 0.02	0.26 ± 0.02	4.82 ± 0.01	6.93 ± 0.94	0.03 ± 0.00	4.85 ± 0.04	0.40 ± 0.02	0.52 ± 0.01	1.15 ± 0.21	0.72 ± 0.14
Wa4	0.94 ± 0.01	0.36 ± 0.01	0.82 ± 0.01	6.73 ± 0.23	0.24 ± 0.01	3.87 ± 0.13	0.24 ± 0.04	0.52 ± 0.01	1.23 ± 0.92	0.74 ± 0.02
Wb5	13.45 ± 2.71	0.65 ± 0.01	1.90 ± 0.45	1.26 ± 0.19	0.29 ± 0.08	0.78 ± 0.16	12.20 ± 1.10	0.66 ± 0.04	n.d.	n.d.
Wa5	4.33 ± 0.74	0.70 ± 0.03	0.08 ± 0.01	1.55 ± 0.16	0.05 ± 0.00	0.85 ± 0.01	1.22 ± 0.03	0.35 ± 0.03	n.d.	n.d.
Wb6	4.32 ± 0.62	10.50 ± 2.21	3.57 ± 0.32	3.64 ± 0.04	1.45 ± 0.14	3.72 ± 0.08	12.30 ± 0.41	1.50 ± 0.02	1.52 ± 0.61	0.66 ± 0.01
Wa6	2.57 ± 0.04	0.96 ± 0.11	1.81 ± 0.11	5.70 ± 0.21	0.82 ± 0.09	2.84 ± 0.11	6.19 ± 0.16	0.67 ± 0.01	1.09 ± 0.09	0.74 ± 0.03
Wb7	3.16 ± 0.18	0.46 ± 0.04	2.61 ± 0.08	3.59 ± 0.13	0.61 ± 0.02	2.70 ± 0.05	1.29 ± 0.50	0.26 ± 0.01	0.70 ± 0.07	0.67 ± 0.01
Wa7	1.04 ± 0.08	0.30 ± 0.01	0.84 ± 0.05	3.81 ± 0.02	0.68 ± 0.01	2.28 ± 0.25	1.42 ± 0.05	0.25 ± 0.03	0.68 ± 0.01	0.66 ± 0.00
Wb8	2.05 ± 0.17	0.71 ± 0.02	3.13 ± 0.12	4.66 ± 0.07	0.61 ± 0.03	2.64 ± 0.19	4.19 ± 0.08	0.38 ± 0.02	0.79 ± 0.05	0.71 ± 0.01
Wa8	1.67 ± 0.04	0.50 ± 0.01	1.30 ± 0.04	2.86 ± 0.12	0.56 ± 0.01	1.97 ± 0.03	3.00 ± 0.01	0.55 ± 0.06	0.98 ± 0.11	0.81 ± 0.00
Wb9	2.45 ± 0.03	0.56 ± 0.04	3.37 ± 0.31	4.20 ± 0.03	0.49 ± 0.05	2.56 ± 0.02	1.94 ± 0.05	0.29 ± 0.01	0.74 ± 0.02	0.67 ± 0.02
Wa9	1.98 ± 0.21	0.48 ± 0.01	2.16 ± 0.01	2.66 ± 0.11	0.34 ± 0.01	2.01 ± 0.09	5.04 ± 0.22	0.35 ± 0.02	0.85 ± 0.05	0.68 ± 0.10
Wb10	5.80 ± 0.18	0.56 ± 0.01	3.52 ± 0.20	2.63 ± 0.08	0.49 ± 0.05	4.12 ± 0.61	5.75 ± 0.11	0.80 ± 0.04	1.20 ± 0.18	0.64 ± 0.05
Wa10	3.91 ± 0.26	0.47 ± 0.02	1.32 ± 0.07	5.25 ± 0.20	0.62 ± 0.02	3.53 ± 0.07	2.00 ± 0.05	0.43 ± 0.01	0.86 ± 0.01	0.66 ± 0.08

Mean values (n = 3) ± SD.

<sup>a</sup> Wb1 = woman 1 before childbirth.

<sup>b</sup> Wa1 = woman 1 after childbirth.

<sup>c</sup> n.d. = not detected.

**Table 6**  
Measured and reference concentrations of total (free + esterified) non-cholesterol sterols and tocopherols.

Ref.	Dihydrocholesterol	Desmosterol	Lathosterol	Campesterol	Stigmastanol	β-Sitosterol	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol
<b>A. Human plasma or serum<sup>a</sup></b>										
This work	0.48 – 32.38	0.26 – 10.50	0.08 – 7.44	1.26 – 6.83	0.04 – 1.73	0.77 – 5.72	0.24 – 14.10	0.25 – 1.50	n.d. <sup>c</sup> – 1.52	n.d. – 0.81
[34] <sup>d</sup>	0.81 ± 0.11	0.81 ± 0.11	4.80 ± 0.27	3.05 ± 0.11	0.04 ± 0.01	2.36 ± 0.26				
[42] <sup>d</sup>	0.57	0.57	2.74	2.01	0.9 ± 0.7	3.53				
[44] <sup>e</sup>			2.4 ± 2.2	1.0 ± 1.0		1.6 ± 1.9				
[45] <sup>e</sup>		0.69	2.08	2.59		2.29				
[47] <sup>d</sup>	86.9 ± 4.1	27.5 ± 1.9	53.6 ± 7.3	194.1 ± 20.2	0.64 – 2.28	136.3 ± 9.8				
[48] <sup>d</sup>				1.96 – 12.27		1.01 – 6.07				
[49] <sup>d</sup>	3.12 ± 1.00	0.69 ± 0.27	6.12 ± 4.87	4.11 ± 1.66	0.004 – 0.25	3.46 ± 1.33				
[50] <sup>e</sup>		0.27 – 4.17	0.16 – 7.44	0.69 – 10.14		0.15 – 7.01				
[51] <sup>e</sup>							8.82 ± 3.39			
[52] <sup>e</sup>							7.73 ± 2.14			
[53] <sup>d,e</sup>							7.45 – 15.12			
<b>B. Amniotic fluid<sup>b</sup></b>										
This work	0.32 – 1.82	0.33 – 0.67	0.7 – 16.49	0.17 – 0.23	0.08 – 0.10	0.14 – 0.23	n.d.	n.d.	0.37 – 2.12	0.09 – 0.12
[54]		0.297 ± 0.032	0.331 ± 0.069			0.096 ± 0.016			n.d.	n.d.
[55]		0.163 ± 0.103	0.653 ± 0.069			0.040 ± 0.009				

<sup>a</sup> Mean value or range of the total sterol levels (µg/ml) in human plasma or serum.

<sup>b</sup> Mean value or range of total sterol levels (µg/ml) in amniotic fluid.

<sup>c</sup> n.d. = not detected.

<sup>d</sup> Concentration in serum.

<sup>e</sup> Concentration in plasma.



**Table 7**  
Concentration levels ( $\mu\text{g/ml}$ ) of non-cholesterol sterols and tocopherols in amniotic fluids obtained from five women in the last trimester.

	Dihydrocholesterol	Desmosterol	Lathosterol	Campesterol	Stigmasterol	$\beta$ -Sitosterol	$\alpha$ -Tocopherol	$\beta$ -Tocopherol	$\gamma$ -Tocopherol	$\delta$ -Tocopherol
W1 <sup>a</sup>	0.32 $\pm$ 0.01	0.38 $\pm$ 0.03	1.09 $\pm$ 0.17	0.19 $\pm$ 0.01	0.08 $\pm$ 0.00	0.15 $\pm$ 0.01	n.d. <sup>b</sup>	n.d.	n.d.	n.d.
W2	0.40 $\pm$ 0.08	0.36 $\pm$ 0.01	1.31 $\pm$ 0.15	0.17 $\pm$ 0.00	0.08 $\pm$ 0.00	0.14 $\pm$ 0.01	n.d.	n.d.	n.d.	n.d.
W3	1.82 $\pm$ 0.14	0.67 $\pm$ 0.02	16.49 $\pm$ 0.69	0.23 $\pm$ 0.00	0.10 $\pm$ 0.01	0.23 $\pm$ 0.00	n.d.	n.d.	n.d.	n.d.
W4	0.32 $\pm$ 0.03	0.33 $\pm$ 0.01	0.85 $\pm$ 0.04	0.17 $\pm$ 0.01	0.08 $\pm$ 0.00	0.16 $\pm$ 0.02	n.d.	n.d.	n.d.	n.d.
W5	0.29 $\pm$ 0.03	0.34 $\pm$ 0.02	0.70 $\pm$ 0.04	0.18 $\pm$ 0.01	0.09 $\pm$ 0.01	0.15 $\pm$ 0.01	n.d.	n.d.	n.d.	n.d.

Mean values ( $n=3$ )  $\pm$  SD.

<sup>a</sup> W1 = woman 1.

<sup>b</sup> n.d. = not detected.

triglycerides and diglycerides was measured in the biofluids after their saponification and extraction into the hexane phase (data not shown). Only traces of cholesteryl palmitate and glycerides were detected by the respective GC–MS and LC–MS analysis and confirmed that the saponification method releases analytes bound in the human biofluids with yields >98%. The TFEFCF derivatization was successfully coupled with the LLME step conducted between the immiscible isooctane and acetonitrile phase. As the extraction of the analytes is accompanied with plenty of polar interfering components commonly present in the complex biofluids, the combined derivatization LLME sample preparation ensures cleaner extracts and much lower contamination of the GC–MS system.

The saponification procedure was most time consuming step (about 130 min) and it is best carried out in sample batches. The derivatization coupled with the liquid extraction takes less than 10 min, a single GC–MS analytical run was accomplished within 8 min including the GC column equilibration. A representative SIM mass chromatogram of ten sterol and tocopherol analytes in a woman serum before childbirth is shown in Fig. 7.

All examined metabolites were clearly detected in sera and amniotic fluids subjected to the developed analytical protocol and their identity confirmed by their quantitation and confirmatory ions presented in Table 2.

### 3.5. Method quantitation and validation

The calibration data involving calibration curve parameters, the quantification range, the R square values, the LLOQ, are presented in Table 3.

Within-run precision ranged from 0.9% to 19.5% and the between-run values from 0.2% to 19.0%. Within-run accuracy was in the range 82–115% and the between-run accuracy between 82% and 112%. The criteria acceptable for precision and accuracy were fulfilled at each tested concentration level.

Mean recovery of sterols and tocopherols assayed in serum and amniotic fluid were between 90–110% and 92–110% for the medium concentration levels, respectively. The data indicate negligible matrix effects in view to the quantitation of sterols and tocopherols by the developed method.

The results obtained with QC serum samples are detailed in Table 4.

The TFEFCF derivatives and the analyte levels measured in woman serum and amniotic fluid were found stable. The concentration of the analytes varied in the range of  $\pm 12\%$  and thus was in the tolerance limit.

### 3.6. Real sample analysis

Analytical performance of the validated method was further evaluated by GC–MS of the sterol and tocopherol set in women sera and amniotic fluids. Total (free+esterified) sterol and tocopherol concentrations in sera of 10 women in last trimester, after the childbirth and in 5 amniotic fluids correspond well in most cases to those reported by other authors who applied other analytical methods [34,42–55]. The concentrations of cholesterol precursors, i.e. dihydrocholesterol and lathosterol were between 0.08 and 32.4  $\mu\text{g/ml}$  and found higher before childbirth in accordance with Nikkilä [47]. On the other hand, desmosterol and plant sterols fluctuated at approximately same concentration levels. Concentration of desmosterol and campesterol was between 0.3–10.5 and 1.4–6.9  $\mu\text{g/ml}$ , respectively. Concentration of  $\beta$ -sitosterol was ranged 0.7–5.7  $\mu\text{g/ml}$  and concentration of stigmasterol 0.07–1.7  $\mu\text{g/ml}$ , Table 5.

Only Nikkilä et al. [47] reported considerably different sterol serum levels an order of magnitude higher than others. The

abundance of  $\alpha$ -tocopherol was similar (in range 0.24–14.1  $\mu\text{g/ml}$ ) to that reported previously [51,52] and the levels of the other tocopherols were lower (0–1.52  $\mu\text{g/ml}$ ) in accordance with literature [53], Table 6A.

Concentration of Chol metabolites in amniotic fluid was found lower than those in serum; dihydrocholesterol, between 0.29 and 1.8  $\mu\text{g/ml}$ ; lathosterol 0.7 and 1.3  $\mu\text{g/ml}$ ; desmosterol in range 0.3–0.7  $\mu\text{g/ml}$  and campesterol about 0.2  $\mu\text{g/ml}$ , except a sample where high concentration of lathosterol (16.5  $\mu\text{g/ml}$ ) was detected, Table 7.

From the plant sterols,  $\beta$ -sitosterol and stigmasterol were detected at levels about 0.5  $\mu\text{g/ml}$  and 0.1  $\mu\text{g/ml}$ , respectively. The results are in accordance with other authors, Table 6B [54,55].

No tocopherols were detected in the measured amniotic fluids.

#### 4. Conclusions

Alkyl chloroformates exhibit considerable pyridine-catalyzed reactivity toward secondary alicyclic hydroxyl groups in both aqueous and non-aqueous environment. In particular, trifluoroethyl chloroformate and heptafluorobutyl chloroformate are powerful reagents that immediately convert the hydroxyl group of cholesterol and related metabolites into the corresponding mixed carbonates under anhydrous conditions. Each examined analyte provides a single product with very good GC separation properties, well-defined EI spectra containing diagnostic fragment ions suitable for their identification and quantitation. The GC–MS determination of six sterols and four tocopherols in a set of women sera and amniotic fluids provided data well-comparable with those reported earlier by other authors and proved the performance of the new method. The new derivatization procedure outperforms existing methods for immediate derivatization of hydroxyl groups, is advantageous for the low microliter reagent consumption and the stability of the carbonate products which is much higher than at commonly used trimethylsilyl derivatives. The derivatization can be easily combined with a LLME step, either in non-aqueous or aqueous conditions, and offers a convenient clean-up accessory, particularly for complex matrices. The reaction kinetics and phase transfer liquid–liquid equilibrium of these concurrent processes are very fast, the sample preparation procedure is very simple and does not require any specialized equipment. The new method has been successfully applied to GC–MS profiling of cholesterol, sitosterol, campesterol and two ( $\gamma$  and  $\delta$ ) tocopherol isomers in various tissues of the insect bug *Pyrrhocoris apterus* [56]. Studies extending the new approach to analysis of other hydroxylated compounds and to evaluation of various detection modes are in progress.

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## 8.3 Publikace 2

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### Seasonal changes in minor membrane phospholipid classes, sterols and tocopherols in overwintering insect, *Pyrrhocoris apterus*



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#### ABSTRACT

Ectotherm animals including insects are known to undergo seasonal restructuring of the cell membranes in order to keep their functionality and/or protect their structural integrity at low body temperatures. Studies on insects so far focused either on fatty acids or on composition of molecular species in major phospholipid classes. Here we extend the scope of analysis and bring results on seasonal changes in minor phospholipid classes, lysophospholipids (LPLs), free fatty acids, phytosterols and tocopherols in heteropteran insect, *Pyrrhocoris apterus*. We found that muscle tissue contains unusually high amounts of LPLs. Muscle and fat body tissues also contain high amounts of  $\beta$ -sitosterol and campesterol, two phytosterols derived from plant food, while only small amounts of cholesterol are present. In addition, two isomers ( $\gamma$  and  $\delta$ ) of tocopherol (vitamin E) are present in quantities comparable to, or even higher than phytosterols in both tissues. Distinct seasonal patterns of sterol and tocopherol concentrations were observed showing a minimum in reproductively active bugs in summer and a maximum in diapausing, cold-acclimated bugs in winter. Possible adaptive meanings of such changes are discussed including: preventing the unregulated transition of membrane lipids from functional liquid crystalline phase to non-functional gel phase; decreasing the rates of ion/solute leakage; silencing the activities of membrane bound enzymes and receptors; and counteracting the higher risk of oxidative damage to PUFA in winter membranes.

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#### 1. Introduction

Insects overwintering in temperate and polar zones evolved a whole array of acclimatization responses that prevent/counteract/repair damaging effects of low, often subzero body temperatures (reviewed in Denlinger and Lee, 2010). Biological membranes are considered as primary targets of cold injury in all organisms (Chapman, 1975; Quinn, 1985). All three basic types of cold injury are closely associated with biological membranes: (a) freezing of body fluids may cause either cell swelling beyond

the elastic limits of the membrane (intracellular freezing), or cellular dehydration, shrinkage, and membrane fusions (extracellular freezing); (b) *direct chilling* injury is almost synonymous with lipid bilayer phase transition, resulting in formation of phase separations, and loss of the membrane's barrier-function (Drobnis et al., 1993; Arav et al., 1996); and (c) the decline or loss of function in various membrane proteins, including the primary ion-pumping ATPases is one of the most important causes of the *indirect chilling* injury (Hochachka, 1986), resulting in gradual dissipation of membrane electrochemical potentials, followed by cell disintegration and death (Košťál et al., 2004).

Acute sensitivity of the lipidic bilayer's physical properties, *i.e.* phase and order (fluidity), to temperature is a cornerstone for mechanistic explanation of cold injury. Membrane integrity and functionality at low temperatures critically depend on bilayer's physical properties, and these, in turn, are dictated by the bilayer's chemical composition (Chapman, 1975; McElhaney, 1984; Hazel, 1995). Hence, compositional remodeling of lipidic bilayers linked to decrease of body temperature is a widespread adaptive and/or acclimatization response in many different poikilotherms including insects (Sinensky, 1974; Cossins and MacDonald, 1989; Hazel,

**Abbreviations:** PL, phospholipid; LPL, lysophospholipid; FFA, free fatty acids; (L)PE, (lyso)phosphatidylethanolamine; (L)PC, (lyso)phosphatidylcholine; (L)PG, (lyso)phosphatidylglycerol; (L)PS, (lyso)phosphatidylserine; (L)PI, (lyso)phosphatidylinositol; PCA, principal component analysis; GC–MS, gas chromatography–mass spectrometry; HPLC–MS, high performance liquid chromatography–mass spectrometry.

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1995; Crockett, 2008; van Dooremalen et al., 2009, 2011). The earlier studies on cold-related membrane restructuring in insects focused mainly on fatty acid composition in two major classes of phospholipids (PLs): phosphatidylethanolamines (PEs) and phosphatidylcholines (PCs) (reviewed in Košťál, 2010). Our knowledge on potential contribution of minor membrane lipid classes, such as phosphatidylglycerols (PGs), phosphatidylserines (PSs), phosphatidylinositols (PIs), lysophospholipids (LPLs), and free fatty acids (FFAs) is very limited. Just a single relevant observation exists for membrane sterols (Shreve et al., 2007). It shows that feeding the fruit fly (*Drosophila melanogaster*) larvae a cholesterol-augmented diet elevates cholesterol content in the adult flies' membranes 1.6-fold and is accompanied with significant increase of their cold-tolerance. Practically nothing is known on potential cold-related changes of membrane tocopherols. Sterols and tocopherols, however, are indispensable and ubiquitous architectural components of cell membranes (Wang and Quinn, 1999; Behmer and Nes, 2003; Atkinson et al., 2008). Besides their metabolic roles (sterols as precursors of steroid hormones; tocopherols as antioxidants), sterols and tocopherols play important structural functions in membranes. Sterols and tocopherols display the same general effect on membrane physical properties. They increase the order (decrease the fluidity) of the bilayers in the liquid-crystalline phase ( $L_{\alpha}$ ), while decreasing the order (increasing the fluidity) of the bilayers in the lamellar gel phase ( $L_{\beta}$ ) (Urano et al., 1988; Stillwell et al., 1996). Increasing the relative proportion of sterols and/or tocopherols in membrane lipids thus results in the decrease of the bilayer's specific transition temperature ( $T_m$ ) at which the "solid" phase  $L_{\beta}$  "melts" and forms a "fluid" phase  $L_{\alpha}$ . This way, high concentrations of sterols and tocopherols may counteract the ordering effects of seasonally low temperatures, and may serve as important adaptive trait by reducing the risk of membrane solidification, i.e. phase-transition into gel phase (Sørensen, 1993; Crockett and Hazel, 1995). The gel phase is considered to be non-functional on itself and, mainly, the transition to gel phase in restricted membrane portions is linked to direct chilling injury by formation of lipid phase separations between the gel and fluid phases resulting in rapid ion and solute leakage (Quinn, 1985). Indeed, artificially increasing the cholesterol:PL ratio in membranes proved to reduce the direct chilling injury associated with membrane phase separations and helped to increase the viability of cryopreserved biological materials (Purdy and Graham, 2004; Horvath and Seidel, 2006).

The main aim of this study was to fill the gap in our knowledge on insect seasonal membrane remodeling by supplementing new data on minor lipid classes such as PGs, PSs, PIs, LPLs, FFAs, sterols, and tocopherols. The linden bug, *Pyrrhocoris apterus* was used as a model species, since its overwintering biology has been intensively studied in the past (Hodek, 1968; Hodková and Hodek, 1997; Košťál and Šimek, 2000; Košťál et al., 2011), and a good knowledge exists on the seasonal remodeling of major phospholipid classes (PEs and PCs) in *P. apterus*' cell membranes in response to cold (Hodková et al., 1999, 2002; Tomčala et al., 2006). Here we show that the muscle tissue of adult linden bugs contains surprisingly high amounts of LPLs. In addition, muscle and fat body contain high amounts of  $\beta$ -sitosterol and campesterol, two phytosterols derived from their food (these bugs are seed eaters), while only small amounts of cholesterol are present. Two isomers ( $\gamma$  and  $\delta$ ) of tocopherol (vitamin E) are found in quantities comparable to, or even higher than phytosterols in both tissues. Distinct seasonal patterns of sterol and tocopherol concentrations are observed showing a minimum in reproductively active bugs in summer and a maximum in diapausing, cold-acclimated bugs in winter. The potential effects of relatively high winter concentrations of sterols and tocopherols on the structure, phase behavior and function of the linden bugs' cell membranes are discussed.

## 2. Materials and methods

### 2.1. Insects

Adults of the brachypterous wing-form of *P. apterus* (L.) (Heteroptera: Pyrrhocoridae) were collected by hand picking from the natural population occurring nearby České Budějovice (48°57' NW, 14°28' EL; Czech Republic) during 2011. The sampling dates and basic descriptions of the insects' developmental/reproductive state are provided in Table 1. The adults belonging to 2010/2011 – overwintering generation perished by the end of May and beginning of June. New (1st) generation of adults emerged during the second half of June and the adults of partial 2nd generation occurred by the end of summer and during autumn. The collected specimens were transported (ca. 10 min) in a temperature-insulated container to the Institute of Entomology and processed immediately, with the exception of a sample taken on 28 June 2011. This sample consisted of young reproducing adults that moulted from last (5th) instar nymphs that were collected in the field on 20 June 2011, were kept in outdoor cage (exposed to natural photoperiod and variations of temperature), and were supplied with water and dry seeds of the linden tree (*Tilia parviflora* Ehrh.) as food. This way, we ensured that all adults belong to the new (1st) generation.

In order to validate our analytical results, and to obtain comparative data, samples of other animal tissues were analyzed. Several species of insects were obtained from laboratory cultures that are maintained at the Institute of Entomology in České Budějovice. Muscle and fat body tissues were dissected from: locust (*Locusta migratoria*); cockroach (*Periplaneta americana*); wax moth (*Galleria mellonella*); and codling moth (*Cydia pomonella*). In addition, two mammalian species were analyzed: the human blood serum (obtained from healthy man, Hospital in České Budějovice); and the mouse (*Mus musculus*), from which the samples of hind femur muscle, liver and abdominal white fat were dissected. Finally, the contents of sterols, tocopherols and LPLs in the dry seeds of the linden tree (major diet of *P. apterus* in the field) were also analyzed.

### 2.2. Tissue dissection, extraction of total lipids, SPE

Thorax (containing mostly muscle tissue) and abdominal fat body were rapidly (3 min) dissected from *P. apterus* adults. Hemolymph, containing circulating cells and lipoproteins, was blotted from the tissues. Each sample contained tissues pooled from three animals and was prepared in four biological replications (two

**Table 1**  
Description of developmental/reproductive state of the adults of *P. apterus* taken for analysis.

Generation of insects	Sampling date <sup>a</sup>	Developmental/reproductive state
Overwintering	18 Jan	End of reproductive diapause/post-diapause quiescence
	10 Mar	Post-diapause quiescence
	20 May	Reproduction (mating pairs)
1st	20 Jun	Reproduction (mating pairs were collected in the field)
	28 Jun	Reproduction (mating pairs were reared in the outdoor cage)
1st & 2nd	17 Sep	Maintenance of reproductive diapause, warm-acclimated
	1 Nov	Maintenance/termination of reproductive diapause, cold-acclimation
	7 Dec	Termination of reproductive diapause, cold-acclimated

<sup>a</sup> All insects were collected during 2011.



replications with males and two replications with females). Since no statistically significant differences were later found between the sexes, the results for males and females were combined for statistical analysis. Details on the tissue dissection from other species are given in the Supplementary material (Table S2). Total lipids from all samples were extracted uniformly in ice-cold chloroform:methanol (2:1, v/v) solution using the slightly modified method of Folch et al. (1957) and Košťál and Šimek (1998). After the extraction, the solvents were evaporated under a stream of nitrogen, and the lipid extracts were stored at  $-80^{\circ}\text{C}$ .

For the GC–MS and HPLC–MS analysis, the lipid extracts were spiked with 2.5 nmol of  $^{13}\text{C}$  cholesterol (CIL, Andover, USA) and 10 nmol of 1,2-dihexadecanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) dissolved in isooctane and MeOH, respectively, both serving as internal standards. Each extract was then separated into individual lipid classes by dissolution and liquid–liquid extraction between 2 ml acetonitrile–water (80:20) and 1 ml hexane. A 1 ml aliquot of the lower aqueous acetonitrile phase containing phospholipids (PLs) was evaporated to dryness and diluted with 300  $\mu\text{l}$  of a mobile phase before the analysis. Non-polar lipids partitioned into the upper hexane phase were separated by solid phase extraction (SPE) on an aminopropyl silica Bond Elut LRC-NH<sub>2</sub> column NH<sub>2</sub> (Agilent Technologies, Santa Clara, CA, USA) into the particular lipid classes using a modified method by Kaluzny et al. (1985).

The following fractions were obtained: fr.1, steryl esters (eluent; hexane); fr.2, triacylglycerols (eluent; hexane:dichloromethane, 8:2); and fr.3, sterols and tocopherols plus diacylglycerols (eluent; hexane:ethylacetate, 7:3). After the SPE step, fractions were evaporated using Jouan Thermo Scientific RC 10.10 vacuum evaporator (Trigon Plus, Prague, Czech Republic) and stored at  $-80^{\circ}\text{C}$  until further analysis. All chemicals used for extraction and SPE were purchased from Sigma–Aldrich Co. (St. Louis, USA).

### 2.3. Analysis of phospholipids and free fatty acids

Total content of phospholipids in the PL fraction was measured using phosphorus assay (Rouser et al., 1970). The PL phosphorus was released by mineralization of the dry sample for 20 min at  $180^{\circ}\text{C}$  in 125  $\mu\text{l}$  of 70% perchloric acid. After adding distilled water (900  $\mu\text{l}$ ), 2.5% solution of ammonium molybdate tetrahydrate (125  $\mu\text{l}$ ), and 10% ascorbic acid (125  $\mu\text{l}$ ), the sample was heated to  $100^{\circ}\text{C}$  for 5 min during which the released inorganic phosphate complexes with ammonium molybdate. After cooling for 10 min in cold water bath and spinning, the absorbance of resulting blue solution was read at 820 nm and the amount of PL phosphorus was derived from the calibration curve obtained by measuring the range of monopotassium phosphate solutions.

Analysis of FFA, LPL, and PL molecular species was conducted in three selected sampling dates of *P. apterus* (collected on 20 Jun, 17 Sep, 7 Dec) using HPLC combined with electrospray ionization mass spectrometry (ESI-MS) as described in previous studies (Tomčala et al., 2006; Overgaard et al., 2008). An LTQ–XL mass spectrometer (Thermo Fisher Scientific) equipped with ESI, Accela 600 pump HPLC system, and Accela AS autosampler (Thermo Fisher Scientific, San Jose, CA, USA) was used. The stored dry PL fractions were dissolved in 300  $\mu\text{l}$  of methanol, and 5  $\mu\text{l}$  aliquots were injected into a Gemini C18 HPLC column (150  $\times$  2 mm ID, 3  $\mu\text{m}$  (Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) 10 mM ammonium acetate in methanol with ammonia (0.025%), (B) 10 mM ammonium acetate in water, and (C) isopropanol–MeOH 8:2. The gradient elution A:B:C was performed as follows: 0 min: 92:8:0, 7 min: 97:3:0, 12 min: 100:0:0, 19 min: 93:0:7, 20–23 min: 90:0:10, 24 min: 100:0:0 and for equilibration of column 26–45 min: 92:8:0 with applied flow rate of 250  $\mu\text{L min}^{-1}$ . The column temperature was maintained at  $35^{\circ}\text{C}$ .

The mass spectrometer was operated either in the positive or the negative ion detection mode at +3 kV or  $-2.5$  kV, respectively. Capillary temperature was  $200^{\circ}\text{C}$ , and nitrogen was used as both the sheath and the auxiliary gas. Eluting ions were detected with full scan mode from 200 to 1000 Da. For the collisionally induced MS2 fragmentations, ion isolation width was 2 Da and the microscan and maximum ion time was 50 ms for positive and 100 ms for negative mode; the normalized collision energy was 35%. Data were acquired and processed by means of Xcalibur 2.1 software (Thermo Fisher Scientific). The responses of analyzed phospholipids were corrected by comparison to the internal standard signals. The used common chemicals were purchased from Sigma–Aldrich Co. The lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

### 2.4. Analysis of sterols and tocopherols

Sterols and tocopherols in the fraction three were analyzed by GC–MS after their derivatization with a reaction medium (100  $\mu\text{l}$ ) containing 5  $\mu\text{l}$  of trifluoroethyl chloroformate (available from Genchem or Pragolab, Prague, Czech Republic), 10  $\mu\text{l}$  pyridine and 90  $\mu\text{l}$  isooctane. After the addition of 100  $\mu\text{l}$  of acetonitrile, 100  $\mu\text{l}$  isooctane and short vortexing, 1  $\mu\text{l}$  aliquot of the upper isooctane layer was injected into a GC–QMS mass spectrometer DSQ (Thermo Fisher Scientific) equipped with an electron ionization ion source (EI) and scanning EI mass spectra in the 100–700 Da mass range every 0.25 s. Helium carrier gas flow rate was 1.1 ml/min, the ion source temperature was held at  $250^{\circ}\text{C}$  and the transfer line at  $300^{\circ}\text{C}$ . Injection mode was splitless (valve closed for 0.75 min); the injector temperature was set at  $280^{\circ}\text{C}$ , the GC column was Agilent DB-1HT, 15 m  $\times$  0.25 mm i.d., 0.1  $\mu\text{m}$  film thickness (Agilent Technologies, Santa Clara, CA, USA). The GC oven was initially held at  $180^{\circ}\text{C}$  for 1 min, raised at  $30^{\circ}\text{C min}^{-1}$  to  $330^{\circ}\text{C}$  and held for 2 min. The data acquisition and processing was performed by Xcalibur software v. 2.0 (Thermo Fischer Scientific).

### 2.5. Data processing and statistical analysis

The relative amounts of sterols and tocopherols in tissues were calculated per phospholipid molecule and are expressed as mol% (moles of each compound per 100 moles of total PLs). The influence of season (sampling date) on the relative amounts of sterols and tocopherols in tissues was statistically analyzed using one-way ANOVA and the Bonferroni's multiple comparison tests were applied to find the differences among sampling dates using Prism v.4 (GraphPad Software, San Diego, CA, USA).

The results of FFA, LPL and PL molecular species analysis were expressed as relative proportion of each molecular species from the total of 100%. The complex association of lipidic compositional change with season was determined by principal component analysis (PCA) using Canoco v. 4.52 (Biometris-Plant Research International, Wageningen, The Netherlands).

## 3. Results

### 3.1. Free fatty acids, lysophospholipids and phospholipids

We identified six different FFAs, 13 species of LPLs, and 28 species of PLs in tissues of *P. apterus*. The complete list of detected compounds and their relative proportions are summarized in Table S1 and Fig. S1. None of the molecular species exceeded relative proportion of 17%. The overall compositions were similar in both tissues with few exceptions: linoleic acid (FFA 18:2) and two LPLs (LPE 18:2, LPC 18:2) were highly abundant in muscles where their relative proportions ranged around 7%, 10%, and 15%, respectively,

while these species were much less abundant in the samples prepared from fat (1.5%, 1%, and 2%, respectively). The palmityl linoleyl phosphatidylethanolamine (PE 16:0/18:2) was the molecular species showing the most significant change with seasonal time. Its relative proportion increased from 3.4% in June to 6.9% in December (thoracic muscles), and from 6.3% in June to 15.1% in December (fat body).

PEs and PCs were dominant lipid classes in both tissues. Their dominance, however, was much less pronounced in muscles than in fat, due to high relative proportions of FFAs and LPLs in muscles (Fig. 1). Slightly different patterns of seasonal change were identified in two tissues by statistical analysis using PCA. In the muscles, high concentrations of FFAs and LPLs correlated tightly with Sep sample. The Jun and Dec samples were not clearly associated with any lipid class, except for a weak association between Dec and high relative proportion of PEs (Fig. 1A). In the fat, there was a clear correlation between Jun sample and the high concentrations of FFAs and LPLs. In contrast, Dec sample associated with high relative proportions of PEs and PSs (Fig. 1B).

3.2. Sterols

Three different sterols, cholesterol, campesterol, and  $\beta$ -sitosterol, were identified in the tissues of *P. apterus*. The seasonal changes in relative amounts of sterols are shown in Fig. 2. Cholesterol contents were relatively low, ranging between 0.2 and 0.4 mol% in muscles, and between 0.5 and 0.8 mol% in fat. Two phytosterols were present at higher concentrations: campesterol, 1.2–2.5 mol% in muscles, and 1.5–4.6 mol% in fat; and  $\beta$ -sitosterol, 5.1–8.0 mol% in muscles, and 6.8–15.2 mol% in fat. While the concentrations of cholesterol were fairly constant during the year in both tissues, two phytosterols showed similar seasonal patterns with a broad minimum during spring and summer and a peak

during autumn/winter. The statistical analysis revealed that significant differences exist in total sterol concentration between the reproductive insects collected during May and June and the diapausing, cold-acclimated insects collected during November and December (min 6.6 vs. max 10.8 mol% in muscles; and 8.9 vs. 20.5 mol% in fat).

3.3. Tocopherols

Two isomers of tocopherol were found in the tissues of *P. apterus* (Fig. 3). The amounts of  $\delta$ -tocopherol ranged between 0.1–1.3 mol% in muscles, and 0.3–5.5 mol% in fat. The amounts of  $\gamma$ -tocopherol ranged between 3.0–8.6 mol% in muscles, and 9.1–31.3 mol% in fat. Both tocopherols showed a distinct seasonal pattern: the statistically significant minimum of total tocopherol concentration was found in the young adults of the 1st generation, while a peak occurred in diapausing, cold-acclimated insects (min 3.1 vs. max 9.9 mol% in muscles, and 9.4 vs. 36.8 mol% in fat).

3.4. Comparative data for other species

Analytical data collected for other insect species, mouse tissues, human blood serum, and seeds of linden tree are summarized in Table S2. They will be commented later, in Section 4.

4. Discussion

Complex lipidomic data on seasonal changes of relative proportions of 52 different species of membrane lipids in *P. apterus* are presented in this paper. Lipid classes that are not involved in membrane architecture, such as triacylglycerols, diacylglycerols, and sterols esterified to fatty acids, were not included in this study. We analyzed lipids in whole tissues (thoracic muscles, abdominal

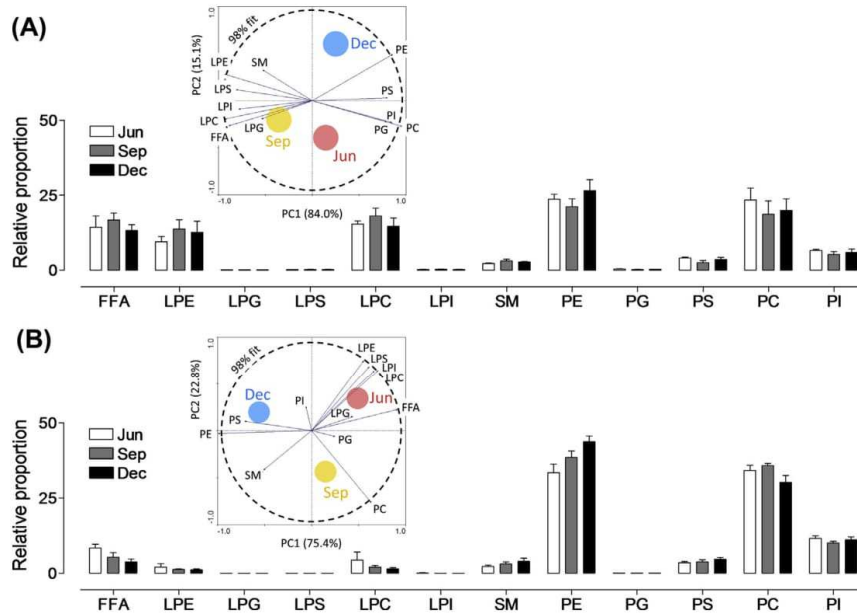
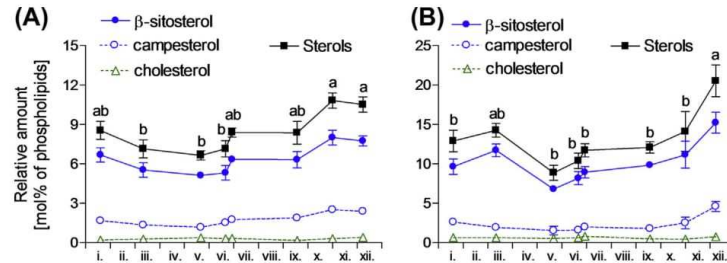
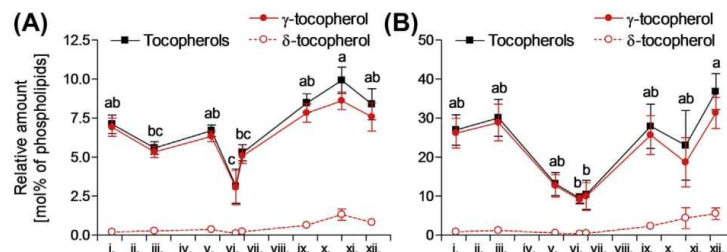


Fig. 1. Membrane lipid composition in thoracic muscles (A) and fat body (B) of the adult *P. apterus* collected in the field during June (Jun), September (Sep), and December (Dec) 2011. Relative proportions of major lipid classes (for abbreviations, see text) of a total 100% are shown. Each column is a mean of four independent samples (each sample contained tissues pooled from three individuals)  $\pm$  S.D. ( $n = 4$ ). The insets show results of PCA statistics on association between the treatments (colored circles representing the centroids of four replications for each season) and the relative proportions of major lipid classes (eigenvectors). The eigenvectors extending beyond the dashed correlation circle fit the model by more than 98%. Detailed results showing lipid molecular species are listed in Table S1 and depicted graphically in Fig. S1.





**Fig. 2.** Seasonal change of sterol composition in thoracic muscles (A) and fat body (B) of the adult *P. apterus* collected in the field during 2011 (x axis shows months coded using Roman numerals). Each point is a mean of four independent samples (each sample contained tissues pooled from three individuals)  $\pm$  S.D. ( $n = 4$ ). The influence of season on total sterol content (Sterols) was analyzed using one-way ANOVA and the Bonferroni's multiple comparison tests were applied to find the differences among treatments (sampling dates). Statistically different means are assigned different letters.



**Fig. 3.** Seasonal change of tocopherol composition in thoracic muscles (A) and fat body (B) of the adult *P. apterus* collected in the field during 2011 (x axis shows months coded using Roman numerals). Each point is a mean of four independent samples (each sample contained tissues pooled from three individuals)  $\pm$  S.D. ( $n = 4$ ). The influence of season on total tocopherol content (Tocopherols) was analyzed using one-way ANOVA and the Bonferroni's multiple comparison tests were applied to find the differences among treatments (sampling dates).

fat body) without isolating pure membrane fractions prior to analysis. Although this is a rather crude approach, similarly complex analysis is not currently available for any other insect species, at least not to our knowledge. Most of the earlier studies on insects focused on fatty acid composition in total polar lipid fraction obtained from the animal's whole body (reviewed in Košťál, 2010). All compounds analyzed in this study show spontaneous tendency to partition into lipid bilayers and monolayers in aqueous environments due to their amphipathic nature (Dowhan and Bogdanov, 2002). Thus, we believe that our results represent the pool of lipid compounds that form cell membranes (based on PL bilayers) and the envelopes of lipid droplets (based on PL monolayers).

In *P. apterus*, seasonal changes in composition of two major PL classes, PEs and PCs, were analyzed previously in our laboratory (Hodková et al., 2002; Tomčala et al., 2006). TLC analysis of lipid classes revealed that PEs and PCs typically represent more than 80% of all phospholipids (Hodková et al., 1999). When we restrict our choice of PEs and PCs to 14 major molecular species, similarly as in the previous studies, our new data would fully confirm that the winter increase of a single species, PE 16:0/18:2 is the most striking seasonal trend. Calculating with only 14 species of PEs and PCs as a total, the relative proportion of PE 16:0/18:2 would increase from 7.2% in Jun to 14.8% in Dec (muscles) or from 9.3% to 20.4% (fat). Calculating with all analyzed polar lipids (FFAs, LPLs and PLs), the winter increase of PE 16:0/18:2 still emerges as the most prominent change, its relative magnitude, however, becomes smaller (Fig. S1). It has been suggested (Hodková et al., 2002) that the adaptive meaning of specific pairing of palmitic and linoleic acyls in a single PE molecule may be related to the widening of the window between the temperatures of gel phase transition ( $T_m$ ) and hexagonal phase transition ( $T_h$ ), thus increasing the span of environmental temperatures at which the membrane remains

fluid and functional. However, the architecture, physicochemical properties and functionality of a particular membrane is influenced by all its components. Thus, it is important to consider the membrane composition in its complexity. In this study, we extend our earlier observations and report that *P. apterus* membranes contain surprisingly high concentrations of LPLs and FFAs (in muscle) and also high levels of phytosterols and tocopherols (in both tissues).

#### 4.1. Lysophospholipids

Free LPLs are found in all animal membranes in relatively low quantities of around 1 mol%. Albumine-bound LPLs and lipoprotein-associated LPLs circulate in vertebrate blood where they may represent 5–20% of all PLs (Croset et al., 2000). In vertebrates, LPL signaling is intensively studied as it is involved in numerous vital processes including embryogenesis, vascular development, oocyte survival, and immune cell trafficking (Skoura and Hla, 2009) and because the LPL concentrations increase in connection to specific pathologies including cancer, heart and brain strokes, and inflammatory reactions (Xiao et al., 2001; Adibhatla et al., 2006; Wang et al., 2010; Skoura and Hla, 2009). Membrane LPLs are mostly formed by hydrolysis of PLs by the enzyme phospholipase  $A_2$ , as part of the de-acylation/re-acylation cycle (or Lands' cycle; Lands, 1958) that controls the membrane's molecular species composition (Hishikawa et al., 2008).

Free LPLs represented as much as 25–32% of all PLs in *P. apterus* thoracic muscles. In addition, high relative proportions of FFAs (13–17%) were detected in muscles (Fig. 1A). Currently, we have no clear explanation for such high levels of LPLs and FFAs in *P. apterus* muscles. The brachypterous wing-morph of *P. apterus* possesses only rudimentary thoracic muscles that are not capable to support flight. Specific composition of their membranes may be related to

loss of their primary function. We verified that extending the time of dissection from ca. 1 min (on ice) to 5 min and 10 min (at room temperature) had no effect on the relative amounts of LPLs. Thus, the effect of non-regulated enzymatic degradation of PLs during dissection probably was not responsible for high LPL levels. The LPL levels were much lower in the fat body tissue of *P. apterus* and also in other animal tissues including flight muscles and non-flight muscles dissected from four other insect species (Table S2).

Free LPLs assume a molecular shape of inverted cone (large hydrophilic head group relative to small hydrophobic domain) which results in their tendency to self-associate in micellar structures with a small radius of curvature and hydrophobic core excluding water (Dowhan and Bogdanov, 2002). This detergent-like, micellar-forming effect can be useful for membrane architecture as it can stabilize convex surfaces with high curvature; similarly as PEs with unsaturated fatty acids can stabilize concave surfaces (Lundbaek and Andersen, 1994). It was shown that very high concentrations of LPLs (up to 72 mol%) can be incorporated into natural lipid membranes without destroying the bilayer structure (Van Meer et al., 1980). Inverted cones of LPLs closely associate with unsaturated PEs which assume normal conical shape and thus, the amphipatic balance of a complex of two molecules is restored (Mishima et al., 2004). Indeed, *P. apterus* membrane lipids are highly unsaturated in both, muscles and fat. Oleyl and linoleyl together represent more than 70% of all fatty acyls in both PEs and PCs (Hodková et al., 1999). We found small seasonal differences in the relative proportions of LPLs and FFAs in muscles and fat. The highest concentrations of LPLs and FFAs occurred in Sep in muscle or in Jun in fat (Fig. 1). The autumn maximum in thoracic muscles may be linked to the increased rate of tissue degradation and its conversion to fat deposits that are accumulated for incoming winter in the fat body. The spring/summer maximum in fat can be associated with high metabolic activity of reproductive animals.

#### 4.2. Phytosterols

Insects cannot synthesize sterols *de novo* and are absolutely dependent on dietary supply of sterols (Canavoso et al., 2001; Behmer and Nes, 2003). Fungi- and plant-eating insects encounter around 100 different sterol structures in their diet (Akihisa et al., 1991). Many phytophagous insects can dealkylate phytosterols and convert them to typical animal sterol-cholesterol (for numerous examples, see Table S2 and Behmer and Nes, 2003). Some insects, however, have lost dealkylation capacity in evolution, probably with their transition to animal diet (Behmer and Nes, 2003). Primitive heteropterans were probably predators, while plant feeding has arisen secondarily in them. That is probably why heteropterans cannot dealkylate phytosterols and must use them as architectural components of their membranes as well as precursors of their specific ecdysteroid hormones—makisterones (Svoboda, 1999; Behmer and Nes, 2003). The structural features of plant sterols, such as desaturation of tetracyclic nucleus and, mainly, the length, orientation, branching, and desaturation of the alkyl side chain dictates whether the plant sterol will serve as a good structural component of an animal membrane (Behmer and Nes, 2003). It has been shown that  $\beta$ -sitosterol and campesterol can functionally replace cholesterol in animal membranes. Similarly as cholesterol, these phytosterols prefer interactions with saturated PCs or SMs and exert condensing and ordering effects on them (Su et al., 2007; Hac-Wydro et al., 2009; Hodzic et al., 2008). Fruit fly, *D. melanogaster*, for instance, has fungal ergosterol as the predominant membrane sterol, which participates on formation of rafts similar to vertebrate cholesterol-ordered rafts (Rietveld et al., 1999).

We found relatively high contents of  $\beta$ -sitosterol and campesterol in *P. apterus* tissues (Fig. 2) and in the seeds of linden tree (Table S2) which is the major diet of *P. apterus*. In contrast, the contents of cholesterol were low (up to 0.8 mol%, Fig. 2). We verified our analytical method by measuring the free cholesterol content in human blood serum, which yielded a value perfectly fitting the range of reference values for healthy human (Table S2). *P. apterus* bugs are known to feed occasionally on invertebrate cadavers in the field, which may explain the presence of low quantities of cholesterol in their tissues. Higher relative amounts of sterols were systematically found in the fat than in the muscles. This is in accordance with the function of fat body as a storage center for lipid molecules, including sterols (Jouni et al., 2002; Arrese and Soulages, 2009). Free sterols are mostly present in the cell membranes but they also occur in the lipid monolayers forming the envelopes of lipid droplets. Large lipid droplets occur abundantly in fat body cells and the total lipid content varies widely between 1 and 10 mg per fat body depending on adult age (low in young, 1–3 d old individuals, higher in older animals), developmental mode (high in early diapause animals, decreases gradually during diapause development), and phase of reproduction cycle in females (high during early phases of egg production, low when mature eggs are present) (Šula et al., 1998). Considering such variation, it is difficult to assess what part of the observed winter increase in phytosterol content is caused simply by increasing the number of lipid droplets containing sterols in their envelopes and what part means the increase of relative proportion of sterols in cell membranes.

Increasing sterol content is known to have ordering, rigidifying effect on fluid membranes. Sterols together with saturated PCs and SMs are major constituents of highly ordered membrane domains rafts in both vertebrates and insects (Rietveld et al., 1999; Zhuang et al., 2002). Since the low winter temperatures exert the same ordering effect on membranes as the high content of sterols, it seems as counterintuitive to accumulate sterols for winter. The sterol molecules, however, disrupt tight packing of PLs and prevent the membrane transition from the functional, liquid crystalline phase to a non-functional, highly ordered gel phase (Chapman, 1975). Thus, the adaptive meaning of sterol accumulation for winter season may be similar as in the case of accumulation of other non-bilayer forming species such as unsaturated PEs, i.e. lowering the transition temperature  $T_m$  from the liquid crystalline to gel phase (Cossins, 1977; Crockett and Hazel, 1995; Hazel, 1995). Additionally, high concentrations of sterols in membrane, and subsequent increase in membrane order, result in decreasing the rate of ion leakage, decreasing the activities of membrane-bound enzymes (Haines, 2001), and decreasing the sensitivities of receptor molecules to their ligands. This effect is known as molecular freezing of the receptor molecule in the lipid matrix (Liu et al., 1994; Storlien et al., 1996; Clarke et al., 2000). Most insects overwinter in a state of diapause, which is characterized by developmental arrest, low activity and metabolic suppression (Košťál, 2006). Thus, it is possible that high winter sterol contents may contribute to diapause by decreasing the overall rates of membrane-bound processes and signaling.

#### 4.3. Tocopherols

Tocopherols (or vitamins E) are minor but ubiquitous constituents of cell membranes. Their concentrations in vertebrates typically range between 0.1 and 1.0 mol% (McMurchie and McIntosh, 1986; Wang and Quinn, 1999; Atkinson et al., 2008). Practically nothing is known about the content of tocopherols in insect membranes. Apart from their well recognized primary function as antioxidants acting to prevent free radical damage to unsaturated membrane lipids (Traber and Atkinson, 2007), the structural roles of tocopherols in membrane architecture are important (Quinn,



2007; Atkinson et al., 2008). Although the evidence is not yet conclusive, it seems that tocopherols preferentially incorporate into hyper-fluid PUFA-rich domains (Atkinson et al. 2010) and stabilize them in a role parallel to that fulfilled by cholesterol in highly ordered rafts. In addition, there is good evidence that tocopherols form complexes with LPLs or FFAs and counter their detergent-like, membrane-destabilizing effects, based on restoring the amphipatic balance in a complex (Erin et al., 1986; Quinn, 2007; Atkinson et al., 2008).

We observed unusually high concentrations of  $\gamma$  and  $\delta$  tocopherols in *P. apterus* tissues (Fig. 2). As in the case of phytosterols, the tocopherol concentrations were higher in fat than in muscles (see above). The maximum content was 8.6 mol% in muscle and it was as high as 31.3 mol% in fat. It is likely that high tocopherols in *P. apterus* tissues reflect high tocopherol content in the seeds of linden tree (Table S2). As in the case of phytosterols, accumulation of tocopherols during cold season was observed in both tissues.

The basic effect of tocopherols on membrane order is similar to that of cholesterol, it means that tocopherols order the liquid crystalline phase but disorder the gel phase (Stillwell et al., 1996). The stabilizing effect of tocopherols on liquid crystalline membranes is probably explained by their preferential association with unsaturated acyl chains, which reduces the fluidity of these structures (Atkinson et al., 2008). Similarly as in sterols, increasing proportions of tocopherols cause progressive broadening and decrease of  $T_m$  (Massey and Pownall, 1998; Wang and Quinn, 1999) and thus may counteract the unregulated transition to non-functional gel phase at low body temperatures during winter. Further, the interaction of tocopherols with PLs results in decreased permeability of membranes to ions and small polar solutes (Quinn, 2007), which may contribute to energy savings and conserving the electrochemical potentials during winter dormancy. Another adaptive explanation may be based on close co-localization of tocopherols with highly unsaturated membrane lipids in *P. apterus*, which concentrates tocopherols to the site where their anti-oxidant activity is most required. Oxidative damage to PUFA may occur repeatedly and unpredictably in overwintering insects such as *P. apterus*, where periods of very low metabolism of deep dormancy during cold spells are altered with periods of higher activity and oxygen reperfusion at warmer temperatures when the insects move, feed, drink and bask on tree trunks (Košťál and Šimek, 2000; Košťál et al., 2008).

Collectively, new results on minor phospholipid classes, lysophospholipids, free fatty acids, phytosterols and tocopherols in heteropteran insect, *P. apterus* significantly extend our understanding of seasonal changes in lipidomic composition of insect cell membranes. Functional assays are now required to test hypotheses on adaptive meaning of such changes. Lowering the transition temperature  $T_m$  and thus preventing the unregulated transition from functional liquid crystalline phase to non-functional gel phase during winter time is a theme unifying the winter accumulations of PEs (Hodková et al., 2002; Tomčala et al., 2006), phytosterols and vitamin E (this study). Small concentrations of phytosterols and vitamin E in membranes may also decrease the rates of ion/solute leakage, save energy required for ion pumping, and silence the activities of membrane bound enzymes and receptors. In addition, high levels of vitamin E may counteract the higher risk of oxidative damage to PUFA in winter membranes.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2013.06.008>.

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## Profiling of urinary amino-carboxylic metabolites by in-situ heptafluorobutyl chloroformate mediated sample preparation and gas chromatography–mass spectrometry



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GC–MS

### ABSTRACT

A novel 1,1,1,2,2,3,3-heptafluorobutyl chloroformate reagent (HFBCF) was examined for in-situ derivatization of amino-carboxylic metabolites in human urine. The arising reaction products exhibit greatly reduced polarity which facilitates combining the derivatization and liquid-liquid microextraction (LLME) from an aqueous urine into an isooctane phase and immediate gas chromatographic–mass spectrometric analysis (GC–MS). The sample preparation protocol is simple, proceeds without an alcohol excess and provides cleaner extracts than other urinary GC–MS based methods. Moreover, thiol metabolites bound in disulfide bonds can be released by reduction with tris(3-hydroxypropyl)phosphine (THP) prior to the developed derivatization and LLME step. In order to evaluate potential of the novel method for GC–MS metabolomics, reaction products of 153 urinary metabolites with HFBCF, particularly those possessing amino and carboxyl groups (56 amino acids and their conjugates, 84 organic acids, 9 biogenic amines, 4 other polar analytes) and two internal standards were investigated in detail by GC–MS and liquid chromatography–mass spectrometry (LC–MS). One hundred and twenty metabolites (78%) yielded a single product, 25 (16%) and 2 metabolites (2-methylcitrate, citrate) generated two and more derivatives. From the examined set, analytically applicable products of 5 metabolites were not detected; the derivatives of 3 metabolites were only suitable for LC–MS analysis. Electron ionization (EI) of the examined analytes contained characteristic, diagnostic ions enabling to distinguish related and isomeric structures. The new method was validated for 132 metabolites using two internal standards in artificial urine and with special attention to potential disease biomarker candidates. The developed sample preparation protocol was finally evaluated by means of a certified organic acid standard mixture in urine and by GC–MS analysis of 100 morning urines obtained from healthy patients (50 males and 50 females), where 112 physiological metabolites were quantified in a 25  $\mu$ L sample aliquot. The quantification data for the set were satisfactory, most metabolites were found within the range reported in the reference human metabolome (HMDB) database and literature. The reported results suggest that the described method has been a novel promising tool for targeted GC–MS based metabolomic analysis in urine.

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### 1. Introduction

Human urine is a complex biofluid of hundreds small polar metabolites that are excreted by kidney in a buffered aqueous medium containing high portions of inorganic salts, urea and other ionic organic species. The metabolite profile in urine

virtually reflects metabolic processes in cells and, consequently, health and disease status of organism. As the urine collection is non-invasive, analysis of urine has been a widely used diagnostic tool and new strategies expanding the metabolite coverage by means of separation-based technologies coupled to mass spectrometry (MS) are of a great interest for targeted metabolite analysis, metabolite profiling, metabolic fingerprinting and metabolomics [1–3].

Gas chromatography coupled to mass spectrometry (GC–MS) has been an efficient tool for comprehensive urinary metabolite analysis due to its high separation efficiency, sensitivity and

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robustness [4,5]. In comparison to the concurrent liquid chromatography (LC) or capillary electrophoresis (CE) coupled to atmospheric pressure ionization (API) MS detection, GC–MS benefits from lower matrix effects of co-eluting components and library searchable electron ionization (EI) mass spectra. On the other hand, the application range of GC–MS is limited to sufficiently volatile and thermally stable analytes so that the small polar metabolic entities of primary concern in urine such as amino and carboxylic acids have to be derivatized prior to GC analysis. As the urinary analytes usually contain multiple active hydrogen containing functional groups, their effective comprehensive analysis requires use of general-purpose reagents, preferably those that allow direct conversion of analytes in urine, *in situ*.

Many derivatization methods were explored in the past for treating as much as possible urinary metabolites. Among them, two-step oximation-silylation approach was the most widely used, typically using methoxylamine (MO) and a subsequent silylation with a trimethylsilyl (TMS) or tert-butyldimethylsilyl (TBDMS) reagent [6–10]. The oximation step prior silylation converts aldehyde and keto groups into oximes and inhibits thus multiple peak formation of sugars and cyclization and decarboxylation of ketocarboxylic acids [4,5]. The silylation procedure requires strictly anhydrous conditions and was mainly efficient for GC–MS analysis of O-silylated compounds, typically sugars [11,12] and steroids [13]. However, silylated protic sulfur and nitrogen functional groups are much less stable and prone to hydrolysis in the presence of water traces. The metabolites having NH<sub>2</sub> group often provide mono and double-silylated forms that continue to react in the reagent excess. Moreover, they are themselves very reactive donors of the silylation group and often decompose in GC injector port or even on the large specific surface area of a GC capillary column [14]. These phenomena undoubtedly affect the yield of each metabolite derivative to a different extent and can be a source of biases that complicate correction and standardization of the obtained metabolomic data and may eventually lead to misinterpretations [15–17]. Despite the extensive use of the oximation-silylation procedures in GC–MS metabolite profiling for more than 4 decades, the described practical problems stimulate looking for alternative sample preparation strategies.

Derivatization with alkyl chloroformates (RCFs) has been increasingly popular approach. Unlike silylation, the RCFs smoothly convert highly polar functional groups into the corresponding *N*-carbamate and (*S*, *O*)-carbonate carboxylic esters in the presence of the corresponding alcohol and pyridine catalyst. The reaction is quickly stopped by depletion of the reagent and the arising products are simultaneously extracted into an immiscible organic solvent layer which is directly amenable to GC–MS analysis [18,19].

In urinary analysis, the simple and cost-effective RCF mediated derivatization and concurrent liquid-liquid microextraction procedure was first applied to GC profiling of 44 organic acids [20] and branched chain carboxylic, keto, hydroxy and amino acids useful in diagnosis of maple syrup urine disease [21]. However, succinic and glutaric dicarboxylic acid and their analogues being important markers of impaired enzyme functions underwent undesired internal cyclizations to unstable anhydrides in the first studies. The facile esterification of a wide range of di- and tri-carboxylic acids with methyl and ethyl chloroformates (MCF, ECF), but not with less reactive RCFs of higher alkyls, was resolved by repeated addition of the reagent in presence of sodium hydroxide [22]. The improved sample preparation protocol enabled to increase metabolite coverage for simultaneous profiling of amino carboxylic species and has been successfully applied for GC–MS metabolomic analysis in various biological matrices [23–32].

The promising analytical features of the RCF reagents encouraged, by analogy with the historical development of perfluoroalkyl reagents (anhydrides or imidazoles), research of chloroformates

with fluorinated alkyls of a various chain length. Vincenti et al. [33–36] synthesized and employed long-chain fluoroalkyl chloroformates (FCFs) in GC–MS analysis of small very polar water disinfection by-products. A short chain trifluoroethyl chloroformate (TFECF) was prepared and examined to chiral separation of a set of amino acids by Abe et al. [37,38]. Analytical properties of RCFs with a pentafluoropropyl (PFPCF) or heptafluorobutyl (HFBCF) moiety were extensively studied by Hušek and co-workers [39–41]. Unlike the common RCFs, the FCF reagents are more reactive and capable to transform the target protic functional groups under pyridine catalysis without a presence of analogous alcohol. FCFs with five or seven fluorine atoms are stable and exhibit excellent volatility enabling enantiomeric separations of amino acids [40–42].

The HFBCF reagent was found highly efficient in nonchiral & chiral profiling of amino acids in human serum [43,44]. The sample preparation protocol comprised three simple steps; (i) release of bound thiols by a novel reducing agent 2,3-dimercaptopropane sulfonate (DMPS), (ii) protein precipitation by perchloric acid and (iii) direct treatment of the supernatant with HFBCF in iso-octane that simultaneously allows transfer of the nonpolar derivatization products into an immiscible organic phase. In this way, extraordinary clean extracts were obtained enabling plasma/serum GC–MS metabolite analysis in the full scan MS regime [43]. Moreover, amino acid derivatives with HFBCF exhibit excellent chiral separation properties on Chirasil-Val phases that outperform other RCF amino acid derivatives hitherto examined; more than 35 amino acid enantiomeric pairs were separated and analyzed by GC–MS in human serum, except the D,L enantiomers of arginine and cystine (not eluted) and proline (not separated), [44]. Furthermore, FCFs have been shown highly reactive towards alicyclic hydroxyl in steroids and tocopherols under anhydrous conditions [45].

The HFBCF-mediated sample preparation strategy undoubtedly indicates very good perspectives in GC–MS profiling of other acidic and multifunctional metabolites occurring in urine and which, to our knowledge, has not yet been investigated. However, for standardization of a novel, effective GC–MS metabolomics method, qualified experimental data sets must be acquired in order to guarantee comparability among the particular samples. This requires thorough knowledge of the properties not only of the obtained metabolite derivatives but also abundance of possible side products, their stability, possible metabolite inter-conversions and cross-reactions [15].

This study expands the current knowledge by development a new GC–MS method for urinary metabolomic analysis. To evaluate the new method, reaction products and analytical properties of more than 150 known urinary metabolites, particularly those possessing amino and carboxyl groups, were investigated. The developed sample preparation protocol involves an immediate conversion of disulfides into thiols by the tris(3-hydroxypropyl) phosphine (THP) reducing agent [46] followed by *in situ* treatment of urine with HFBCF under pyridine catalysis. The derivatives possessing highly non-polar HFB moieties are concurrently transferred from urine into an iso-octane immiscible layer by the employed liquid-liquid microextraction principle (LLME). The developed sample preparation protocol was evaluated by means of the analyte calibration in artificial urine, by determination of 19 diagnostic metabolites in a certified urine standard, and finally, by GC–MS analysis of 100 morning urines obtained from control adult subjects of both genders.

## 2. Experimental

### 2.1. Urine samples

Aliquots of the morning, second-void urine samples were gathered from adult clinical staff subjects in the Faculty Hospital Ostrava

who periodically attended preventive examinations. An informed consent was obtained from each subject and the participants claimed no suffering on any evident disease or metabolic disorder. The urinary creatinine concentration was measured in each sample aliquot by the colorimetric Jaffe method routinely used in the clinical laboratory. The specimens were supplied in closed plastic vials with known creatinine level and age of each participant, stored at 4 °C and processed within 3 days.

## 2.2. Chemicals, solutions, reaction media

Solution of protein amino acids (AAS-18) and other amino-carboxylic metabolites were obtained from Sigma-Aldrich (Prague, Czech Republic). 2-methylcitric acid (racemate) was purchased from C/D/N Isotopes (Quebec, Canada). Less common carboxylic acids 2-hydroxysebacic, 3-hydroxyadipic, 3-hydroxypropionic, 3-hydroxyvaleric and 5-hydroxyhexanoic acid, and glycine conjugates hexanoylglycine, methylcrotonylglycine and tiglylglycine were purchased from Dr. E. Brunet, Dept. Química Organica, University Autònoma de Madrid (Madrid, Spain). Urine samples with a certificated level of the diagnostic organic acid standards (product ORG-01, batch 2010.007) were obtained from SKB Winterswijk-MCA (Winterswijk, The Netherlands). The THP reducing agent and isooctane were purchased from Merck KGaA (Darmstadt, Germany). Heptafluorobutanol (HFBOH) was supplied by Fluorochem Ltd. (Derbyshire, UK), the HFBCF reagent was delivered by Genchem s.r.o. (Prague, Czech Republic). Other organic solvents and chemicals were from Sigma-Aldrich.

The artificial urine was prepared as reported earlier [47]. Stock solutions of the following chemicals were mixed in water to final 1% urea, 8 mmol L<sup>-1</sup> creatinine, 7 g L<sup>-1</sup> NaCl and 3 g L<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub>. A 0.5% aqueous solution of the THP reducing agent was used; a stock aqueous solution of THP was made 5% and kept in a freezer.

The organic reaction medium consisted of a mixture of isooctane-HFBO H-HFBCF in volume ratio of 15:1:4; when kept in refrigerator it remained stable for several months. The catalytic medium was a 4% (v/v) of pyridine in 1 mol L<sup>-1</sup> aqueous sodium hydroxide; this solution was prepared on monthly basis and kept in refrigerator.

## 2.3. Stock and working solutions

Stock solutions of the examined metabolites were prepared in 100 mmol L<sup>-1</sup> sodium bicarbonate and stored in freezer. However, the protein amino acids (AAS-18), homocystine and examined amines were dissolved in 50 mmol L<sup>-1</sup> HCl. For the identification of urinary metabolites and their reaction products arising after the HFBCF treatment, a series of 153 di- and tricarboxylic acids, aliphatic and aromatic acids, protein and non-protein amino acids, amines, 2-hydroxy and 3-hydroxy acids, glycine conjugates and some others was examined after diluting the stock solution in water or in the artificial urine to a 10 μmol L<sup>-1</sup> concentration.

Solutions of 4-phenylbutyric acid (4PB) and homophenylalanine (hF) used as I.S. were prepared in a 100 mmol L<sup>-1</sup> aqueous sodium bicarbonate; concentration of each I.S. was 200 μmol L<sup>-1</sup>.

Working solutions at varied concentrations were prepared by an appropriate dilution of each stock metabolite solution aliquot with the artificial urine solution.

## 2.4. Equipment

Glass culture tubes 6 × 50 mm (Kimble-Kontes, Vineland, NJ) were used for sample handling, 25 mm capillary pipette tips of gel-loading type from VWR International (Prague, Czech Republic) enabled suction of the organic phase after completing the coupled derivatization–extraction step. Adjustable Transferpettor

pipettes of 50–100 μL volumes with glass capillaries were supplied by Merck (Darmstadt, Germany) and were employed for dispensing the organic phase with the reagent. The reaction mixture was agitated by a REAX 1 vortex shaker from Heidolph (Schwabach, Germany). A portable Gilson centrifuge PMC-880 (6200 rpm) used for separation of immiscible layers was supplied by Labmark (Prague, Czech Republic).

## 2.5. Sample preparation procedure

In the 6 × 50 mm glass culture tube, 25 μL of urine was gradually mixed with 25 μL of the I.S. mixture, 25 μL of the 0.5% THP reducing agent and left to stand for 1 min (or longer). Next, 50 μL of the reactive organic medium and 25 μL of the catalytic medium were added and the content was vortexed for 3 s leaving the dispersed organic phase still cloudy. After second addition of 25 μL of the catalytic medium, the arising two-phase system was shaken for 5 s, until the dispersed milky organic phase turned clear. Finally, 50 μL of isooctane plus 25 μL of 1 mol L<sup>-1</sup> aqueous HCl was added, the mixture was vortexed for 3 s and the upper organic phase aspirated into an autosampler vial (alternatively after a brief centrifugation at 3000 rpm) and 1 μL injected into a GC–MS system.

For the study of the HFBCF treated metabolite reaction products, the same protocol was used except the human urine matrix which was replaced by the same volume of the artificial urine. If there was no expected product detected by GC–MS, the metabolite standard was treated again, the 1 mol L<sup>-1</sup> aqueous HCl addition step was omitted and the reaction products were searched, after evaporation of the isooctane phase and mobile phase dilution, by LC–MS analysis.

*Note.* Manipulation of the HFBCF reagent should be performed in a well-ventilated area (fume hood). After application, autosampler syringe should be rinsed with propan-2-ol followed by isooctane to prevent corrosion of the plunger.

## 2.6. Instrumental analysis

### 2.6.1. GC–MS

A 7890A gas chromatograph with 5975C mass spectrometric detector (Agilent Technologies, Santa Clara, CA) equipped with capillary flow technology and multi-mode injector (MMI) was employed for GC–MS analysis. A 30 m × 0.25 mm i.d., 0.25 μm film thickness ZB-XLB fused silica capillary column (Phenomenex, Torrance, CA) was operated at helium flow of 1.2 mL min<sup>-1</sup> and a temperature program from 60 °C at 5 °C min<sup>-1</sup> to 240 °C and then at 20 °C min<sup>-1</sup> to 320 °C (total analysis time of 38 min). The temperatures of the EI ion source, the quadrupole and the transfer line were 230, 150 and 250 °C, respectively; the electron ionization (EI) energy was 70 eV. Splitless injection of 1 μL at the column head pressure of 113 kPa (hold for 0.75 min) was carried out into a 4.0 mm ID Blue Sky™ cyclo double tapered gooseneck inlet liner (Restek, Bellefonte, PA); the MMI temperature was maintained at 220 °C. The EI mass spectra of the derivatized metabolites were studied by using a full-scan mass range mode of 40–1050 Da (scan speed 2.7 scans s<sup>-1</sup>).

For metabolite profiling and targeted quantification either full scan 53–900 Da (4.2 scans s<sup>-1</sup>) or selected ion monitoring (SIM) of the two appropriate diagnostic analyte fragment ions, q1 (quantification) and q2 (confirmation) were used. The SIM parameters: 35 time sequence groups, maximum 12 ions in each group, the electron multiplier voltage was set 1318 V. Data were acquired and processed using MSD ChemStation (version E.02, Agilent).

Structural elucidation of the HFBCF treated metabolites was further performed with the assistance of positive chemical ionization (PCI). A quadrupole mass spectrometer DSQ (Thermo Scientific, San Jose, CA, USA) equipped with a CI ion volume was employed.



The prepared HFBCF derivative of the investigated metabolite was injected into a Restek glass Siltek deactivated liner using the splitless mode; the injection volume, 1  $\mu\text{L}$ ; the split valve opened after 0.7 min. The injector temperature was set at 240 °C. The GC column was Agilent VF-5MS, 30 m  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness (HPST s.r.o., Prague, Czech Republic). The oven was initially held at 50 °C for 1.5 min, raised at 20 °C  $\text{min}^{-1}$  to 170 °C and at 30 °C  $\text{min}^{-1}$  to 300 °C and held for 1 min. Helium carrier gas flow rate was 1.1  $\text{mL min}^{-1}$ , the ion source temperature was held at 180 °C and the transfer line at 250 °C. PICI mass spectra were collected in the full scan regime (90–1150 Da, 2.5 scans  $\text{s}^{-1}$ ). Isobutane was used as the reagent gas at a flow rate of 1.5  $\text{mL min}^{-1}$ . The Thermo Scientific Xcalibur 2.0 software was used for the data acquisition and processing.

#### 2.6.2. LC–MS

Structure of some HFBCF derivatized reaction products was further investigated by LC–MS on an Accela 600 coupled to LTQ-XL mass spectrometer (Thermo Scientific, San Jose, CA, USA). A 150  $\times$  2.1 mm, 5  $\mu\text{m}$  Kinetex C18 column (Phenomenex, Torrance, USA) was used for separation of the detected components at 25 °C using methanol and water containing 5  $\text{mmol L}^{-1}$  ammonium formate (pH = 4.5) as the mobile phase. The initial methanol concentration of 30 vol% was linearly increased to 100% within 10 min and the composition kept at 100% for 3 min; flow-rate was set at 250  $\mu\text{L min}^{-1}$ ; injection volume, 5  $\mu\text{L}$ . Positive ion ESI mode detection was used with the following parameters: capillary temperature of 275 °C; vaporizer temperature of 150 °C; source voltage, 4 kV; capillary voltage 40 V; nitrogen served as a desolvation gas. The Xcalibur 2.0 software was used for the data processing.

#### 2.7. Method calibration and validation

Once the HFBCF metabolite reaction products were unequivocally elucidated, we consulted the literature with respect to the metabolite abundance, mainly the Human Metabolome Data Base (HMDB). From the examined set, 135 metabolites were further measured by the developed GC–MS method in the averaged urine sample obtained by mixing of 10 random male and 10 female urines without and after the addition of the corresponding metabolite standard at the approximately estimated medium level (L3) reported in Table 1.

For the calibration, the metabolite pool was divided into four groups and a five-point calibration plot was obtained from a triplicate GC–MS analysis of each metabolite in the artificial urine at a level L1, L2, L3, L4 and L5. The lower (L1 and L2) and higher calibration points (L4, L5) were 10 times, 2.5 times diluted, and 2.5 times and 10 times increased to the respective reference medium L3 concentration level. To cover the whole range of physiological concentrations inclusive a random occurrence of highly elevated levels of some metabolites, particularly those diagnostic for inherited metabolic disorders, an additional level L6 (25 times higher concentration to the reference L3 level) was calibrated for several biomarker candidates summarized in Table 2, i.e. 3-hydroxyisovalerate, methylmalonate, ethylmalonate, glycolate, fumarate, tiglylglycine, 3-hydroxybutyrate, 3-methylglutarate, 2-ketoglutarate, 4-hydroxybutyrate, hexanoylglycine, adipate, glycerate, 2-hydroxyglutarate, *N*-acetylaspartate, pyroglutamate and suberate.

The calibration curves were constructed by plotting the peak area ratios of each metabolite to a proper I.S. against its concentration in the artificial urine. The non-physiological amino acid homophenylalanine served as I.S. for determination of amino acids and biogenic amines, while acidic metabolites were normalized to 4-phenylbutyrate, refer for the analytes in Table 1, No. 103 and 63, respectively. Urinary metabolite concentration levels were

deduced from the regression calibration curves obtained by the linear regression of the L1–L5 (L6) calibration points.

The limit of detection (LOD) was determined by a serial dilution of each derivatized metabolite in the artificial urine as the concentration at which a signal/noise (S/N) ratio of 3:1 was achieved. The lower limit of quantification (LLOQ) was estimated as the lowest L1 point of the calibration curve (obtained with the RSD = < 20%, Table 1), because concentration levels of the determined metabolites in urine samples were rarely found below this level.

Method precision and accuracy for each analyte were determined by GC–MS of the separate metabolite mixture in the artificial urine at low (L1), medium (L3) and high (L5) level; precision was evaluated from the relative standard deviation (RSD, %) of the three replicates. Accuracy was calculated by direct comparison of mean measured level of each spiked analyte with the expected concentration. The analytical protocol was further evaluated by measuring a certified set of 19 analytes in the reference urine (ORG-01). The given concentration values were compared with those obtained by the developed analytical protocol and validation criteria of the method precision, accuracy and stability were evaluated.

The method recovery was assessed by means of the averaged urine ( $n = 10$  of each gender) spiked with the metabolite set at the medium level (L3) and further by addition of a known amount of each measured metabolite ( $5 \times$  the medium level) into the SKB certified reference urine. The recovery was calculated according to the formula: (measured concentration in the spiked urine – endogenous concentration)/added concentration  $\times 100$ .

Statistical calculations were performed with the NCSS 2007 program, version 07.1.21.

### 3. Results and discussion

#### 3.1. Reaction products of metabolites with the HFBCF reagent

Most methods for GC–MS metabolomic analysis have only described a main reaction product of each metabolite formed during the essential derivatization step. However, standardization of a truly metabolomics method requires detailed knowledge of pitfalls associated with formation of side-products or a possible conversion of one metabolite to another product or metabolite present in the sample. To establish a convenient metabolite set for the targeted GC–MS metabolomics analysis, we first prepared and aimed to identify the reaction products of more than 150 urinary metabolites. The list of the examined metabolites is compiled in Table 1.

The metabolite set was obtained by reviewing current literature and metabolite databases, with respect to the capability of RCFs to block protic functional groups and the current GC technology to separate the volatile metabolite derivatives. As a result, metabolites possessing mainly amino and carboxyl groups were included in the list which contained 56 amino acids and their conjugates, 84 organic acids, 9 biogenic amines, 4 other diagnostic urinary metabolites including 2-propylvalerate (No. 10, valproic acid, an antiepileptic drug), phthalate (No. 61, a contaminant) and also two internal standards (4-phenylbutyrate, No. 63; homophenylalanine, No. 103).

Reaction products of each analyte (typically 1–10 nmol) were studied in an aqueous medium and artificial urine and their 70 eV EI and isobutane PICI mass spectra were recorded. If the analyte reaction products were detected poorly or not all, the organic phase was carefully evaporated and re-dissolved in an LC mobile phase and positive/negative ES spectra recorded by LC–MS analysis. The proposed structures for the all examined and detected metabolite derivatives treated by the HFBCF reagent are summarized in the supplementary Table S1.

**Table 1**  
The list of urinary metabolite standards treated with the HFBCF reagent. Basic analytical characteristics of the reaction products, the diagnostic ions, calibration data. Mean values, n = 3.

No.	Metabolite and its products	GC RT (min)	MM	Diagnostic ions		R <sup>2</sup>	LOD μmol L <sup>-1</sup>	L3 μmol L <sup>-1</sup>	Precision (RSD%)			Accuracy (%)			Recovery (%)	
				m/z <sup>a1</sup>	m/z <sup>a2</sup>				L1	L3	L5	L1	L3	L5		
1	Pyruvate <sup>c</sup>	n.d.	2700	43												
2	Oxalate	5.38	454.0	113	183	0.99	0.750	200	14.3	8.2	3.7	95	115	115	115	
3	2-Ketoglutarate	5.81	298.0	71	113	1.00	0.067	20	3.4	2.7	1.6	112	107	114	87	
24b	3-OH-Valerate-1 (OH, 65%)	6.37	286.0	71	242	0.99	0.187	100	3.7	2.5	1.2	106	94	99	101	
4a	3-OH-Valerate-1 (OH)	6.46	300.1	59	85	1.00	0.125	100	6.0	3.0	1.2	91	90	109	114	
5	2-Keto-3-methylvalerate	7.34	312.1	57	85	1.00	0.081	20	1.8	1.6	1.0	100	97	103	95	
6	2-Ketocaproate	7.43	312.1	85	57	1.00	0.047	20	2.1	1.7	1.7	108	97	100	89	
7	Malonate	7.51	468.0	269	183	0.99	0.041	40	2.4	7.1	5.4	85	87	105	108	
8	Methylmalonate	7.54	482.0	283	438	0.99	0.013	40	5.2	1.9	2.4	92	91	100	116	
9a	3-OH-Valerate-1 (OH)	8.21	300.1	71	271	1.00	0.250	40	2.4	1.8	0.9	101	93	82	103	
10	2-Propylvalerate	8.61	326.1	255	284	1.00	0.015	40	4.5	2.4	2.8	98	105	112	103	
11	2-OH-Isobutyrate	8.74	512.0	241	285	1.00	0.025	40	7.7	8.0	5.6	84	96	101	106	
12	Ethylmalonate	8.78	496.0	297	468	0.99	0.033	40	2.9	4.9	3.8	95	90	100	96	
13	Lactate	9.14	498.0	271	255	1.00	0.013	100	7.9	3.7	1.1	94	100	103	98	
14	Glycolate	9.35	484.0	285	213	1.00	0.174	40	3.1	2.1	1.7	99	95	98	102	
15	Fumarate	9.53	480.0	281	253	0.99	0.090	40	8.3	7.0	7.8	101	86	111	87	
16	Citraconate	10.29	494.0	295	267	1.00	0.060	40	6.8	2.5	5.6	114	105	100	83	
17	Succinate	10.32	482.0	283	55	1.00	0.005	40	7.2	3.9	5.4	92	100	106	116	
18	Methylsuccinate	10.38	496.0	297	268	1.00	0.005	40	5.0	3.8	8.5	99	95	105	94	
19	2-OH-Butyrate	10.49	512.0	285	241	1.00	0.009	20	2.8	2.1	1.0	100	97	100	88	
20	Benzoate	10.74	304.0	105	304	1.00	0.096	40	5.2	8.7	3.4	104	115	95	101	
21a	Citramalate-1 (lactone)	10.8	312.0	85	285	0.98	0.158	100	3.1	4.4	5.3	100	94	105	110	
22	2-OH-3-methylbutyrate (isovalerate)	11.16	526.0	55	299	1.00	0.018	20	3.1	2.5	1.8	99	105	105	86	
23	Tiglylglycine	11.27	339.1	83	55	0.96	0.127	40	4.9	3.7	3.9	106	99	102	80	
24a	3-OH-Butyrate-2 (OR)	11.93	512.0	268	69	1.00	0.155	100	4.3	3.0	2.2	104	96	99	115	
25	Sarcosine	12.07	497.0	270	226	1.00	0.048	20	5.2	3.9	4.5	106	104	103	101	
26	2-OH-Valerate	12.12	526.0	55	299	1.00	0.049	20	5.6	1.6	1.2	100	97	99	98	
27	2-Aminoisobutyrate	12.14	525.1	284	241	0.95	0.637	40	9.4	12.6	7.1	99	110	120	106	
28	Nicotinate	12.17	305.0	106	78	0.99	0.170	40	6.2	4.7	5.7	100	109	97	92	
4b	3-OH-Isovalerate-2 (OR, 45%)	12.29	526.0	241	425	0.96	0.333	100	5.7	4.6	5.9	94	105	114	98	
29	Alanine	12.35	497.0	270	70	0.99	0.025	40	5.0	3.5	4.7	86	91	113	115	
30	Glutarate	12.51	496.0	227	297	1.00	0.086	40	1.7	2.4	8.2	97	92	105	107	
31	Phenylacetate	12.65	318.0	91	318	1.00	0.031	20	6.9	1.7	1.6	102	100	101	114	
32	2-OH-Isocaproate	12.79	540.1	296	113	1.00	0.055	20	5.6	1.3	1.0	90	93	103	101	
37b	N-Acetylglycine-1 (NR, 65%)	12.84	299.0	256	113	212										
33	3-Methylglutarate	12.93	510.1	311	282	69	1.00	0.038	40	7.6	3.3	3.1	99	93	105	108
34	2-OH-3-Methylvalerate	13.06	540.1	284	484	69	0.99	0.059	20	3.3	2.8	2.8	98	108	107	100
9b	3-OH-Valerate-2 (OR, 65%)	13.44	526.0	83	411	113	1.00	0.850	40	4.7	2.5	3.6	98	98	109	93
35	Glycine	13.56	483.0	256	212	56	0.99	0.113	200	7.5	3.3	4.8	85	97	113	113
36	2-Aminobutyrate	13.61	511.0	284	84	113	0.98	0.098	20	3.2	2.4	3.2	96	111	123	98
42a	2-Ketoglutarate-1	13.66	510.0	283	55	113	0.98	0.364	200	7.1	4.1	1.3	97	102	103	103
37a	N-Acetylglycine-2 (NH)	13.88	525.0	256	483	113	0.94	2.550	200	4.6	8.7	4.1	114	100	77	95
38	4-OH-Butyrate	14.05	512.0	227	269	113	0.99	0.167	100	2.8	4.5	5.9	111	94	100	104
35a	Hexanoylglycine-1 (cyclic)	14.07	155.1	99	71	55	0.99	0.861	20	1.6	3.2	4.1	105	82	78	93
40	Valine	14.1	525.1	298	283	98	0.99	0.031	40	1.5	1.7	3.7	100	107	116	98
41	3-OH-3-Methylglutarate	14.37	752.0	85	285	69	0.99	0.148	100	4.8	1.9	7.7	91	80	108	90
42b	2-Ketoglutarate-2 (80%)	14.6	510.0	283	284	55	0.99	0.250	200	6.4	2.3	1.7	127	115	84	116
43	2-Phenylbutyrate <sup>c</sup>	14.71	346.1	91	346	119										
44	3-Alanine	14.91	497.0	270	113	226										
45	Adipate	14.95	510.1	282	311	55	1.00	0.081	40	2.3	3.1	4.0	93	97	107	108
46	3-Aminoisobutyrate	15.02	511.0	256	112	113	0.99	0.105	100	5.2	4.2	6.6	96	98	101	103
21b	Citramalate-2 (OR, 30%)	15.37	738.0	295	425	113	1.00	0.128	100	7.4	6.5	8.3	82	88	104	97

Table 1 (Continued)

No.	Metabolite and its products	GC RT (min)	MM	Diagnostic ions		R <sup>2</sup>	LOD $\mu\text{mol L}^{-1}$	L3 $\mu\text{mol L}^{-1}$	Precision (RSD%)			Accuracy (%)			Recovery (%)	
				$m/z^1$	$m/z^2$				L1	L3	L5	L1	L3	L5		
47	3-Methyladipate	15.53	524.1	325	55	1.00	0.225	20	6.2	3.2	1.5	89	91	105	107	
48	Propionylglycine	15.55	313.1	57	56	0.93	0.520	100	12.1	7.2	4.3	102	98	106	111	
49	Leucine	15.84	539.1	312	270	256	0.98	0.084	40	2.6	2.2	2.9	100	102	109	100
50	Isoleucine	15.93	539.1	283	312	256	1.00	0.083	40	7.5	3.0	2.5	88	99	108	92
51	Malate	16.04	724.0	281	253	113	1.00	0.027	40	0.8	3.4	5.4	115	106	92	83
52	3-OH-Propionate (dimer)	16.06	526.0	255	298	55	1.00	0.150	100	2.0	2.6	2.1	95	92	100	96
53	5-O-Hexanoate	16.67	540.1	227	113	69	0.99	0.175	100	3.6	4.3	6.5	98	94	113	113
60c	cis-Aconitate (2%)	16.78	720.0	321	492	100	0.98	0.781	200	6.8	4.5	1.7	100	115	137	106
54	trans-Aconitate	16.78	720.0	321	492	100	0.98	0.781	200	6.8	4.5	1.7	100	115	137	106
55	Proline	16.84	523.0	296	297	69	1.00	0.026	40	4.1	4.5	4.5	99	104	106	115
56	Phenylate	17.09	524.1	296	325	69	1.00	0.023	20	4.7	2.5	2.3	92	92	103	101
105b	Putrescine-1 <sup>+</sup> (isocyanate, 15%)	17.38	340.1	236	212	295	1.00	0.023	20	4.7	2.5	2.3	92	92	103	101
57a	Threonine-1 (OH)	17.42	527.0	100	283	56	0.99	0.480	100	1.8	5.2	5.4	105	88	93	112
58	Glycerate (2,3-diOH-propionate)	17.68	740.0	113	497	183	0.99	0.079	40	5.8	6.3	1.4	93	89	78	111
59	Isovaleryl-glycine	17.77	341.1	85	525	57	0.98	0.270	40	5.9	11.7	9.2	109	95	87	98
60a	Citrate-2 (OH)	17.79	738.0	311	269	111	1.00	0.065	400	2.4	7.8	1.8	97	116	123	103
61	Phthalate	17.79	530.0	331	332	149	0.99	0.026	20	8.6	3.7	6.9	95	117	102	107
62	4-Aminobutyrate <sup>+</sup>	17.84	511.0	112	256	69	0.99	0.026	20	8.6	3.7	6.9	95	117	102	107
68b	2-Methylcitrate-1 (triester, 60%)	17.9	752.0	325	283	256	1.00	0.026	20	8.6	3.7	6.9	95	117	102	107
63	4-Phenylbutyrate (L.S.)	17.95	346.1	104	147	91	1.00	0.026	20	8.6	3.7	6.9	95	117	102	107
68c	2-Methylcitrate-2 (dehydrat., 20%)	17.99	734.0	325	535	551	1.00	0.026	20	8.6	3.7	6.9	95	117	102	107
68d	2-Methylcitrate-3 (triester+OR, 10%)	18.13	978.0	325	308	280	1.00	0.026	20	8.6	3.7	6.9	95	117	102	107
64	Aspartate	18.3	723.0	254	496	296	0.98	0.102	40	4.3	5.2	2.8	100	126	116	104
65	D-2-OH-Glutarate	18.34	738.0	283	239	296	0.98	0.110	100	2.8	7.1	2.1	100	91	103	97
66	N-Acetyl-aspartate	18.38	539.0	270	312	296	1.00	0.158	200	3.0	5.2	3.7	101	82	84	105
67	3-OH-Adipate	18.41	752.0	85	127	240	0.99	0.336	100	1.2	4.4	1.9	109	101	91	101
68a	2-Methylcitrate-4 (lactone)	18.46	552.0	152	334	153	1.00	0.102	40	8.8	7.6	3.4	102	84	102	100
57b	Threonine-2 (OR, 40%)	18.75	753.0	282	227	113	1.00	0.484	100	8.8	5.0	1.6	93	80	114	102
69	Pyroglutamate	18.9	537.0	310	84	282	0.98	0.226	200	2.5	6.8	1.9	100	88	100	103
70	Asparagine	19.1	522.0	295	95	113	0.99	0.236	100	6.4	2.6	3.9	100	108	113	94
71	S-Methylcysteine	19.13	543.0	61	300	116	1.00	0.057	20	2.9	2.8	3.1	98	104	108	87
72	Suberate	19.2	538.1	339	138	69	1.00	0.028	20	4.3	2.5	2.0	93	94	103	101
60b	Citrate-3 <sup>+</sup> (OR, 25%)	19.31	964.0	321	521	113	1.00	0.046	20	3.2	2.9	4.5	98	109	102	95
73	Salicylate	19.41	546.0	120	303	347	1.00	0.047	20	2.8	1.5	1.2	101	100	101	117
74	Mandelate	19.45	560.0	289	333	105	1.00	0.008	20	2.1	3.6	1.4	107	106	94	98
75	Thioproline	19.46	541.0	314	287	86	1.00	0.008	20	2.1	3.6	1.4	107	106	94	98
76	Methylcrotonylglycine	19.54	339.1	83	82	55	1.00	0.066	40	4.0	11.7	4.7	108	98	82	91
77	Serine	19.6	739.0	268	295	51	1.00	0.132	100	5.2	5.3	4.8	89	82	115	121
123b	Cadaverine-1 <sup>+</sup> (isocyanate, 12%)	19.84	354.1	256	113	69	0.95	2.500	400	3.2	11.2	5.4	122	96	83	90
78a	Hippurate-1 (cyclic, 60%)	19.86	161.0	105	161	77	0.99	0.441	20	2.7	2.5	3.0	117	80	98	94
79a	3-OH-Sebacate-1 (OH)	20.01	582.1	71	271	69	0.99	0.900	100	9.1	6.4	4.8	84	93	96	91
80	Isocitrate	20.18	964.0	465	321	113	1.00	0.436	40	5.0	3.3	3.9	103	90	99	107
81	5-Aminovalerate	20.41	525.1	256	269	113	1.00	0.159	20	6.3	2.3	1.2	96	103	110	93
82	2-OH-Phenylacetate	20.52	560.0	91	333	133	0.98	0.429	40	8.8	4.2	5.4	80	89	108	110
83	Glutamate	20.82	737.0	310	282	82	0.98	0.429	40	8.8	4.2	5.4	80	89	108	110
84	3-Phenylactate	20.92	574.0	330	131	91	1.00	0.063	20	2.4	1.6	1.2	104	99	99	109
39b	Hexanoylglycine-2 (65%)	20.93	355.1	99	256	71	0.99	0.200	20	3.5	7.4	7.1	117	90	93	94
89b	3-OH-Proline-1 (OH, 65%)	21.14	539.0	312	129	256	0.97	0.240	40	2.4	16.2	5.2	114	89	94	96
85	Anthranilate(2-aminobenzoate) <sup>+</sup>	21.21	545.0	146	345	90	1.00	0.078	20	4.6	2.3	2.2	93	92	103	103
86	Azelaate	21.23	552.1	353	152	55	1.00	0.096	20	2.1	2.3	1.3	107	93	89	115
87	4-OH-Benzate	21.41	546.0	303	347	113	0.99	0.429	40	7.3	2.2	3.8	84	95	114	99
88	Methionine	21.65	557.0	61	357	113	0.99	0.110	20	10.8	7.6	3.4	85	14	0	87
94b	4-OH-Proline-1 (OH, 65%)	21.83	539.0	312	294	68	1.00	0.156	20	6.9	5.5	2.8	104	97	97	104
89a	3-OH-Proline-2 (OR)	21.9	765.0	521	538	294	1.00	0.350	100	6.2	2.7	2.8	103	116	114	114
90	Cysteine (total) <sup>+</sup>	22.22	755.0	328	285	113	1.00	0.350	100	6.2	2.7	2.8	103	116	114	114



Table 1 (Continued)

No.	Metabolite and its products	GC RT (min)	MM	Diagnostic ions		R <sup>2</sup>	LOD $\mu\text{mol L}^{-1}$	L3 $\mu\text{mol L}^{-1}$	Precision (RSD%)					Accuracy (%)			Recovery (%)
				$m/z^1$	$m/z^2$				L1	L3	L5	L1	L3	L5			
91	3-OH-Phenylacetate	22.61	560.0	333	277	0.99	0.023	20	7.3	2.8	1.7	81	83	106	105		
79b	3-OH-Sebacate-2 (OR, 20%)	22.67	808.1	110	153	0.94	0.290	40	7.7	5.2	3.9	80	78	87	87		
92	Phenylpyruvate	22.84	572.0	118	329	0.99	0.168	40	2.8	2.0	5.3	97	78	86	92		
93	2-Aminoadipate	22.88	751.0	124	282	1.00	0.123	40	3.2	2.1	3.6	104	98	94	118		
94a	4-OH-Proline-2 (OR)	23.12	765.0	294	521	1.00	0.016	20	7.0	5.9	2.5	80	95	99	99		
95	Sebacate	23.16	566.1	98	367	1.00	0.021	40	2.8	1.7	1.8	89	100	98	103		
96	4-OH-Phenylacetate	23.31	566.0	289	333	1.00	0.010	40	1.3	1.9	1.4	102	101	99	115		
97	5-Aminolevulinat	23.36	535.0	283	256	0.99	0.066	40	7.1	3.0	4.5	93	88	104	99		
98	Phenylalanine	23.39	573.1	91	330	1.00	0.016	40	5.9	2.6	1.9	89	104	107	107		
99	2-Aminopimelate	24.6	765.1	338	138	0.97	0.062	40	4.1	4.9	1.6	99	92	115	104		
100	Homocysteine (total) <sup>f</sup>	24.68	769.0	282	342	0.95	0.118	40	7.4	3.2	18.3	101	95	58	101		
101	2,4-Diaminobutyrate	24.74	752.0	282	256	0.96	0.220	40	5.5	2.6	3.5	99	81	107	95		
102	trans-Urocanate	25.39	546.0	347	546	0.99	0.026	20	6.0	4.2	3.0	90	104	119	114		
78b	Hippurate-2 (ester)	25.85	361.1	105	134	0.98	0.237	400	6.9	15.9	11.6	115	112	110	94		
103	Homophenylalanine (L,S)	25.98	587.1	91	283	1.00	0.039	20	2.8	3.8	1.4	101	96	99	111		
104	Homovanillate	26.07	590.0	107	590	1.00	0.039	20	2.8	3.8	1.4	101	96	99	111		
105a	Putrescine-2 <sup>e</sup>	26.08	540.1	256	296	212											
106	Histamine	26.48	563.1	308	320	113			8.3	6.1	3.5	80	91	116	97		
107	Glutamine	26.64	554.1	84	282	327			7.9	3.2	3.8	91	78	117	90		
114b	Histidine-1 <sup>c</sup> (NH, 15%)	27.14	563.1	307	362	113											
115b	Lysine-1 <sup>c</sup> (Cyclic, 35%)	27.18	580.1	153	310	353											
108	1-Methylhistidine	27.2	577.1	95	350	150			2.3	5.3	15.1	105	88	85	102		
109	Ornithine	27.25	766.1	296	256	69			6.7	2.2	2.6	99	102	103	94		
110	4-OH-Mandelate	27.25	802.0	575	347	113			1.3	4.3	2.8	123	93	83	115		
111	4-Aminobenzoate	27.29	545.0	146	345	346			7.4	9.8	5.7	98	113	59	101		
112	2-OH-Sebacate	27.34	808.1	95	381	255			2.6	1.7	1.7	102	91	97	94		
113	Indolacetate	27.61	357.1	130	357	131			2.8	2.6	1.2	100	97	102	87		
114a	Histidine-2 (NR)	27.9	789.0	307	362	113			4.9	2.7	2.3	99	117	112	96		
115	4-O H-Cinnamate	27.98	572.0	329	572	373			2.3	0.6	2.5	105	98	99	109		
116	3-Methylhistidine	28.38	577.1	95	150	96			1.4	6.5	14.9	101	90	48	92		
117	Methionine sulfone	28.51	589.0	282	82	69			6.7	7.9	6.3	94	65	122	88		
118	4-OH-Phenylacetate	28.54	816.0	572	345	113			2.0	1.6	1.5	108	96	96	112		
119a	Lysine-2 (N/M-R)	28.79	780.1	310	256	153			5.7	4.2	1.6	99	103	109	108		
120	2-O H-Hippurate	28.82	603.0	120	403	176			3.4	9.1	6.6	97	104	111	100		
121	Palmitate	28.91	438.2	255	438	57			8.5	7.1	2.8	94	114	105	113		
122	Vanillylmandelate	28.92	832.0	832	377	113			5.1	4.0	1.8	91	91	103	109		
123a	Cadavarine-2 <sup>e</sup>	28.98	554.1	256	310	212											
124a	2,6-Diaminopimelate	29.45	1006.1	308	536	310											
124b	(isomers)	29.53															
125	Glycylproline	29.83	580.1	70	153	296			1.4	3.6	2.2	116	106	86	105		
126	Kynurenate	29.99	597.0	371	354	372			0.4	1.8	2.8	123	107	94	82		
127	Tyramine	30.06	589.1	346	333	289			4.3	3.6	1.1	93	99	92	99		
128	4-OH-3-Methoxyphenylacetate	30.38	846.0	375	561	319			1.8	1.0	1.9	111	96	92	111		
129	Tyrosine	30.58	815.0	333	289	113			2.8	3.8	1.6	90	97	106	116		
130	4-OH-3-Methoxycinnamate	30.64	602.0	602	375	147			6.9	1.2	2.8	88	86	103	94		
131	3-Methoxytyramine	32.36	845.1	319	376	419			3.0	2.8	1.8	99	106	105	121		

Table 1 (Continued)

No.	Metabolite and its products	GC RT (min)	MM	Diagnostic ions		R <sup>2</sup>	LOD $\mu\text{mol L}^{-1}$	L3 $\mu\text{mol L}^{-1}$	Precision (RSD%)			Accuracy (%)			Recovery (%)		
				m/z <sup>d1</sup>	m/z <sup>d2</sup>				L1	L3	L5	L1	L3	L5	L1	L3	L5
132	Cystathionine	32.42	1038.0	328	282	0.95	0.083	40	6.2	4.1	4.5	97	160	145	92		
133	Dopamine <sup>e</sup>	33.38	831.0	256	531	1.00	0.005	20	4.22	5.53	2.67	94	110	106	110		
134	Stearate	32.5	466.3	255	466	0.93	0.028	40		11.2	4.22	80	62	131	97		
135	3,4-dihydroxyphenylalanine	32.63	1057.0	149	388	1.00											
136	Metanephrine <sup>e</sup>	33.34	649.1	270	113	0.99											
137	Cystine	33.43	1056.0	496	295	1.00											
138b	5-OH-Lysine (isomers)	34.12	1022.1	269	256	0.99	0.645	40	9.76	1.25	5.21	107	118	113	92		
138a		34.16															
139	Tryptamine	34.18	612.1	130	386	1.00	0.050	20	5.56	3.94	3.72	98	105	121			
140a	Normetanephrine-1 <sup>e</sup>	34.41	861.1	561	418	1.00											
141	Tryptophan	34.44	612.1	130	131	1.00	0.013	40	3.46	1.11	0.79	97	90	107	111		
142	Ptylhydroxyproline	34.5	862.1	296	297	0.96	0.091	100	9.4	6.2	5.35	107	118	109	100		
143	Kynurenine	35.07	842.1	146	372	1.00	0.063	40	2.44	4.03	3.84	92	96	109	95		
144	5-OH-Indoleacetate	35.16	599.0	372	599	1.00	0.039	20	2.25	4.26	0.67	106	98	104	97		
145a	Homocystine-1 (isomer)	36.63	1084.0	314	510	1.00											
145b	Homocystine-2 (isomer)	36.69	1084.0	310	314	1.00											
140b	Normetanephrine-2 <sup>e</sup> (10%)	38.37	635.1	435	379	1.00											
146	5-OH-Tryptamine (Serotonin) <sup>f</sup>	38.8	854.1	372	373	1.00											
147	Mevalonate <sup>e</sup>	n.d.	330.1	331													
148	Citrulline <sup>e</sup>	n.d.	583.1	584													
149a	Arginine-1 <sup>e</sup>	n.d.	582.1	583													
149b	Arginine-2 <sup>e</sup> (5%)	n.d.	808.1	809													
150	Creatinine <sup>e</sup>	n.d.	339.1	340													
151	Urea <sup>g,h</sup>	n.d.	60.1														
152	Uric acid <sup>g,h</sup>	n.d.	168.1														
153	3,4-Dihydroxyphenylacetic acid <sup>g,h</sup>	n.d.															
154	3,4-Dihydroxymandelic acid <sup>g,h</sup>	n.d.															
155	Oxaloacetic acid <sup>g,h</sup>	n.d.															

MM = a molecular mass of each observed metabolite derivative; m/z<sup>d1</sup> = diagnostic ions in the EI spectrum of each metabolite.

R<sup>2</sup> = coefficient of determination; LOD = limit of detection.

a = main reaction product of the metabolite with HFBCF; its peak area EI or ESI response = 100%; b, c, d = minor reaction products.

g1, g2 = diagnostic (quantitation and qualifier) fragment ions used for the quantitative GC-SIM-MS analysis.

<sup>e</sup> = reaction products detected only by HPLC-MS as [M+H]<sup>+</sup> ions; <sup>g,h</sup> = metabolites not treated or decomposed by the HFBCF reagent.

<sup>f</sup> = uncalibrated.

<sup>g</sup> = added as cystine or homocystine, but reduced by THP to the respective total cysteine or total homocystine.

**Table 2**

Mean concentration values with 95% confidence intervals for the assessment of the method accuracy, precision and recovery of 19 organic acids by means of the certified reference urine (SKB, ORG-01).

No.	Metabolite	SKB certified urine std.		GC-MS mean value (n = 16)		Accuracy (%)		Precision (%)		Recovery (%) (n = 5)
		Reference ( $\mu\text{mol L}^{-1}$ )	95% CI	Mean ( $\mu\text{mol L}^{-1}$ )	95% CI	Within-run (n = 16)	Between-run (n = 10)	Within-run (n = 16)	Between-run (n = 10)	
42a	2-Ketoglutarate	395	350–440	406	384–427	103	102	3.1	5.8	120
33	3-Methylglutarate	70	66–73	69	66–73	99	93	3.5	6.4	99
24a	3-OH-Butyrate	1192	984–1400	1224	1153–1281	93	101	3.7	4.0	115
4a	3-OH-Isovalerate	184	162–206	199	187–212	98	95	3.8	4.5	118
38	4-OH-Butyrate	87	66–107	104	97–114	102	104	5.4	7.7	97
45	Adipate	217	200–234	230	215–245	101	98	3.6	7.2	119
65	D-2-OH-Glutarate	187	173–201	200	190–210	97	101	3.1	3.1	100
12	Ethylmalonate	99	93–104	96	88–105	97	100	5.5	3.6	117
15	Fumarate	63	57–68	65	59–72	104	96	6.1	8.1	93
30	Glutarate	101	95–107	96	92–101	95	92	3.1	8.7	99
58	Glycerate	461	391–531	501	461–527	99	98	3.9	2.1	102
14	Glycolate	194	175–213	203	187–219	105	95	4.9	3.5	97
39	Hexanoylglycine	32	28–36	37	34–40	96	107	5.4	5.8	97
8	Methylmalonate	1473	1343–1603	1501	1432–1569	102	94	2.7	3.5	116
66	N-Acetylaspartate	3266	2647–3885	3792	3588–3964	106	102	3.3	7.0	101
69	Pyroglutamate	2414	1832–2996	2538	2405–2756	90	88	4.3	6.8	103
95	Sebacate	214	189–239	230	216–243	103	105	3.7	4.1	96
72	Suberate	216	198–234	215	202–225	99	97	3.4	3.8	92
23	Tiglylglycine	89	79–100	85	82–88	95	90	2.6	6.2	81

In accordance with our previous experience [47], majority of the tested metabolites provided a distinct single peak (120 analytes, 78%) in the TIC GC-MS chromatogram.

The HFB ester of pyruvate (Table 1, analyte No.1) was also detected, but its diagnostic fragment ion  $m/z$  43 ion was overlapped by the EI signal of the solvent peak on the used GC column. A relative TIC EI response of the minor detected side-products that arise particularly from the not completely derivatized functional groups, was mostly <4% compared to the peak of the expected major derivative. The less reactive OH groups in 3-hydroxy carboxylic acids (Table 1, metabolites No. 4, 9, 24, 79), hydroxyamino acids (threonine, No. 57; hydroxyprolines, No. 89, 94) and histidine (No. 114) remain partly untouched and thus typically two products, designated with a suffix a, b were observed, refer to Tables 1 and 3.

Functional groups of cystine (No. 137) and homocystine (No. 145) are completely derivatized by the HFBCF reagent. However, the THP reduction step of these sulfur amino acids that possess a disulfide bond converts them completely to the respective cysteine and homocysteine [39], [43], and thus the total content of cysteine-cystine and homocysteine-homocystine metabolites is measured.

Biogenic amines, putrescine (No.105a), cadaverine (No.123a) provided, in addition to the assumed carbamate derivatives, the respective minor isocyanate-carbamate side-products (Table 3, No. 105b, 123b). Two isomeric peaks were further formed in the case of 2-ketoglutarate (No. 42a, b), lysine (No. 119a, b) and at some acylated amino acid metabolites, typically at *N*-acetylglycine (No. 37), *N*-hexanoylglycine (No. 39), and hippurate (No. 78) that show tendency to dehydration and cyclization reactions (with a water loss).

The reaction route is apparently more complex in polyfunctional organic acids (No. 21, 60, 68). For instance, the HFBCF treatment of citrate which possesses three sterically hindered carboxyls and an OH group, results in formation of three products; one of them arises by rearrangement to *cis*-aconitate (No. 60c) in a small extent. Glutamine (No. 107) which is not stable in solutions [48] provided minor products of pyroglutamate (No. 69) and glutamate in the HFBCF reaction medium. Consequently, an option of the metabolite conversion into another one should always be taken into account, particularly, when data sets of two metabolite stages have to be compared.

The last studied group represents metabolites (Table 1, No. 147–155) which were not found suitable to GC-MS analysis. Meval-

onate (No. 147) did not provide a satisfactory GC peak. As it was proved by LC-MS analysis, only the carboxyl was esterified, while the 3-OH and 5-OH groups were not acylated. Similarly, the amino-carboxylic functional groups of citrulline (No. 148) and arginine (No. 149) were smoothly derivatized with HFBCF; however, the respective guanidine and ureido groups remained nearly untouched. LC-MS analysis not only proved the arising double-derivatized products (data not shown) but also revealed high efficiency and selectivity of the ESI process for detection of both amino acids and also for histidines (No. 108, 114, 116) and creatinine (No. 150). It is interesting to note that LC-MS analysis showed that the guanidino group is treated to some extent (No. 149b). Nevertheless, the corresponding minor Arg product remains strongly polar and/or thermally labile and thus not detectable by GC-MS.

With exception of the above metabolites (No. 148–150 and 108, 114, 116) and partly lysine (No. 119), the strongly nonpolar and electron withdrawing properties of the HFB group have detrimental effect on the positive ion ESI ionization efficiency of the other metabolites examined making them useless for positive ESI detection. LC-MS analysis further proved that urea (No. 151) and uric acid (No. 152) highly abundant in urine, are not treated with HFBCF and thus remain in the aqueous phase after the LLME. The absence of urea and other highly polar metabolites and salts in the organic extraction phase brings a distinct plus over traditional silylation methods where urea is derivatized and its high content may overlap other co-eluting metabolites. The urea interference must therefore be strictly checked and often eliminated by prior treatment with urinase before silylation [49]. Some tested metabolites, particularly those sensitive to oxidation, afforded no analyzable reactions products by GC-MS or LC-MS under the examined reaction conditions.

Among them we found particularly labile dihydroxyphenyl carboxylic acids (metabolites No. 153–154), i.e. 3,4-dihydroxyphenylacetic and 3,4-dihydroxymandelic acid and further oxaloacetate. The last metabolite is known to decompose rapidly in aqueous solutions, particularly under catalysis of inorganic cations [50]. Finally, biogenic amines metanephrine (No. 136), normetanephrine (No. 140) exhibited rather irreproducible reaction yields, are present in urine at very low levels and therefore were not subject to further study.

**Table 3**  
Excreted metabolite levels ( $\mu\text{mol mmol}^{-1}$  creatinine) in the second void urine of the control healthy women (F, n = 50) and men (M, n = 50) expressed in medians and excretion ranges (1st–3rd quartile) plus reference metabolite levels in adult control and diseased subjects (mainly the HMDB reference data). Statistical significance of the gender difference, the Mann-Whitney test; \*p < 0.05, \*\*p < 0.01.

No.	Urinary metabolite	HMDB ID	Measured concentrations ( $\mu\text{mol mmol}^{-1}$ creatinine)			HMDB reference creatinine)			Associated adult disorders, diseases or a source	Reported values ( $\mu\text{mol mmol}^{-1}$ creatinine)
			F		M		Range			
			Median	Range	Median	Range	Range	Range		
108	1- Methylhistidine	00001	14	10–17	13	11–16	1.3–87.6	Diabetes mellitus	0–43	
93	2- Amino adipate	00510	2.5	1.7–4.2	3.2	1.9–4.3	2.5–16.4	Obesity 2-Aminoadipic aciduria 2-Ketoadipic acidemia	0.8–21 13000 9.8–99	
36	2- Amino butyrate	00452	0.71	0.46–1.1	0.62	0.52–0.71	0.04–4.4	Bone metastases	0–48	
5	2- Keto-3- methylvalerate	00491	0.77	0.55–0.95	0.69	0.53–0.88	0.11–1.42	Maple syrup urine disease	174–244	
6	2- ketoisocaproate	00695	0.65	0.48–0.86	0.62	0.48–0.72	0.02–0.45	Maple syrup urine disease	300–800	
3	2- ketoisovalerate	00019	0.92	0.78–1.2	0.96	0.79–1.1	0.01–1.1	Maple syrup urine disease	2–12	
68a	2- Methyl citrate	00379	1.9*	1.4–2.9	1.7*	1.2–1.8	0.05–1.2	Biotinidase deficiency	50–152	
42a	2- Ketoglutarate (2-KC)	00208	16**	12–20	9**	7–11	2–17	2-KG dehydrogenase deficiency	>25	
19	2- OH- Butyrate	00008	0.44	0.31–0.69	0.36	0.26–0.53	0–4.9	Pyruvate dehydrogenase deficiency	0–8	
120	2- O H- Hippurate	00840	0.68	0.59–0.87	0.79	0.61–0.87	1.7–5.9	Aspirine mtb	850–3600	
11	2- OH- Iso butyrate	00729	6.0	4.4–7.4	6.1	4.4–7.6	0–2	Lung cancer	0–76	
22	2- OH- 3- Methyl butyrate	00407	0.35	0.21–0.48	0.34	0.23–0.42	0.9–11.4	Isovaleric acidemia	0–23	
82	2- OH- Phenylacetate	00669	0.53*	0.38–0.72	0.43*	0.36–0.53	0.40–2.2	Maple syrup urine disease		
112	2- OH- Sebucate	00424	0.53*	0.39–0.67	0.42*	0.37–0.55	1.4–156	Phenylketonuria		
44	3- Alanine	00056	0.79	0.58–1.1	0.89	0.57–1.4	0.70–10.5	Peroxisomal disorders		
46	3- Amino isobutyrate	03911	6.7	3.6–13	6.4	4.3–8.5	1.3–2.0	Dihydroxyrimidine dehydrogenase and GABA transaminase deficiency		
131	3- Methoxytyramine	00022	0.40	0.31–0.55	0.38	0.29–0.54	0.40–2.2	Pyrimidine mtb		
47	3- Methyl adipate	00555	1.1	0.72–1.5	1.1	0.92–1.4	0.70–10.5	Tyrosine, dopamine mtb	3.0–8.5	
33	3- Methyl glutarate	00752	0.93**	0.75–1.2	0.66**	0.54–0.80	1.3–2.0	Refsum's disease	5–10	
116	3- Methyl histidine	00479	13	5.8–50	14	5.4–35	2.8–65.6	3-Methylglutaconic aciduria (TYPE 1)	14–1000	
41	3- OH- 3- Methyl glutarate	00355	3.6**	2.6–4.5	2.7**	2.4–3.6	1.1–5.2	3-OH-3-Methylglutaryl-CoA Lyase Deficiency	7–25	
67	3- OH- Adipate	00345	4.3	3.1–6.0	4.6	4.2–5.7	0.1–4	Obesity	5–18	
24a	3- OH- Butyrate	00357	1.8	1.4–2.7	1.7	1.4–2.2	0.1–4	Diabetes	12–20	
4a	3- OH- Isovalerate	00754	4.9	4.3–5.7	5.5	4.5–6.3	0.1–4	3-Hydroxy-3-Methylglutaryl-CoA lyase deficiency	200–11000	
91	3- OH- Phenylacetate	00440	1.5	1.0–2.4	1.4	0.81–2.0	0.1–4	3-Hydroxydicarboxylic aciduria	4.9–21	
52	3- OH- Propionate	00700	4.0	2.4–6.6	3.6	2.3–5.3	0.1–17.2	Ketosis	50–500	
9a	3- OH- Valerate	00531	1.1	0.87–1.4	1.1	0.94–1.2	1.4–15	Diabetes Mellitus, type 1	20–2000	
84	3- Phenyl lactate	00779	0.35	0.23–0.49	0.27	0.23–0.39	0–2.0	Biotinidase deficiency	20–500	
								Rutin mtb		
								Propionic acidemia		
								Biotinidase deficiency		
								Methylmalonic/proprionic acidemia		
								Phenylketonuria		

Table 3 (Continued)

No.	Urinary metabolite	HMDB ID	Measured concentrations ( $\mu\text{mol mmol}^{-1}$ creatinine)				HMDB reference creatinine ( $\mu\text{mol mmol}^{-1}$ )	Associated adult disorders, diseases or a source	Reported values ( $\mu\text{mol mmol}^{-1}$ creatinine)
			F		M				
			Median	Range	Median	Range			
130	4-OH-3-Methoxycinnamate	00954	1.0	0.80–1.3	0.92	0.78–0.98	0.18–1.2		
128	4-OH-3-Methoxyphenylacetate	00913	1.0**	0.83–1.2	0.85**	0.75–0.93	0–0.19	Polyphenol mtb	
87	4-OH-Benzoate	00500	0.55	0.39–0.75	0.46	0.36–0.57	1.3–3.6	Catecholamine mtb	
38	4-OH-Butyrate	00710	2.1	1.8–2.9	1.8	1.6–2.0	0.32–10.8	Catechin mtb	
115	4-O-H-Cinnamate	02035	0.43	0.31–0.57	0.37	0.29–0.45		Glutaric aciduria II	
110	4-OH-Mandelate	00822	1.2	0.91–1.7	1.1	0.98–1.5		Polyphenol mtb	
96	4-OH-Phenylacetate	00020	8.6	5.8–12	7.1	5.2–12	2.4–9.7	Catecholamine mtb	
118	4-OH-Phenylacetate	00755	0.82	0.55–1.2	0.66	0.51–0.94	0.2–3.9	Preterm infants	
54a	4-OH-Proline	00725	0.41	0.26–0.64	0.35	0.26–0.45	0.59–2.1	Phenylketonuria	
97	5-Aminolevulinate	01149	1.2*	1.1–1.4	1.1*	1.1–1.2	0.7–4.4	Collagen catabolism	
81	5-Aminovalerate	03355	1.1	1.0–1.2	1.1	0.98–1.2	0.1–2	Porphyria synthesis pwy	
53	5-O-H-Hexanoate	00525	1.8**	1.1–2.9	1.4**	0.86–1.8	0.80–5.7	Lysine mtb	
144	5-OH-Indoleacetate	00763	1.7*	1.5–2.0	1.6*	1.4–1.7	0–7.2	Medium chain acyl CoA	
138ab	5-OH-Lysine	00450	0.98	0.78–1.2	0.95	0.82–1.2	0.11–3.2	defydrogenase deficiency	
45	Adipate	00448	1.2*	0.84–1.9	0.95*	0.72–1.2	1–13	Serotonine mtb	
29	Alanine	00161	13	9–19	14	10–20	7.2–47.1	Lung cancer	
70	Asparagine	00168	3.4	2.1–5.5	2.9	2.5–3.7	4.6–18	Lung cancer	
64	Aspartate	00191	0.61	0.43–0.82	0.59	0.44–0.68	1.9–26.8	Nonessential amino acid mtb	
86	Aselate	00784	0.77	0.50–1.2	0.63	0.45–0.81	1.2–3.2	Nonessential amino acid mtb	
20	Benzoate	01870	0.51	0.35–0.63	0.61	0.50–0.75	1.2–10.5	Dicarboxylic acid mtb	
60c	cis-Aconitate	00072	78	48–103	71	58–85	2.7–95	Breast cancer	
54	trans-Aconitate	00958	0.58**	0.35–0.84	0.41**	0.33–0.51	1.8–25	Lung cancer	
16	Citraconate	00634	3.3*	2.5–4.5	2.8*	2.5–3.4	0.90–2.1	Lung cancer	
21b	Citramalate	00426	181	135–271	166	137–210	1.0–4.8	Citric acid mtb	
60a	Citrate	00094	9.2*	5.9–15	11*	9.6–16	47–484	Pyruvate and acetate mtb	
150	Creatinine <sup>1)</sup>	00562	9.2*	5.9–15	11*	9.6–16	4.1–25	Propionic acidemia	
132	Cystathionine	00099	1.4	0.94–2.3	1.1	0.80–1.6	0.40–20.7	Tyrosinemia I	
90	Cysteine (total) <sup>2)</sup>	00574	25*	22–27	22*	19–27	23.1–134.5	Maple syrup urine disease	
65	D-2-OH-Glutarate	00606	1.7**	1.5–2.1	1.4**	1.0–1.8	3–79.9	Maple syrup urine disease	
12	Ethylmalonate	00622	2.5*	1.9–3.1	2.0*	1.5–2.6	0.90–4.0	Phenylketonuria, Tyrosinemia I	
15	Fumarate	00134	1.1*	0.80–1.8	0.9*	0.75–1.2	0.2–1.9	Cystathioninuria	
83	Glutamate	00148	1.8*	1.2–2.3	1.4*	1.1–1.7	0.33–18	Alzheimer's disease	
107	Glutamine	00541	26	17–37	24	18–34	9.0–77.9	Cystinuria	
30	Glutarate	00661	1.4**	0.96–1.8	1.1**	0.87–1.4	0.50–3.6	Glutaric Aciduria II	
35	Glycine	00123	61	35–95	49	34–83	24–126	2-Hydroxyglutaric aciduria	
								Malonyl CoA decarboxylase deficiency	
								Fumaric Aciduria	
								Lung cancer	
								Nonessential amino acid mtb	
								Aminoaciduria	
								Glutaric aciduria I	
								Propionic acidemia	
								Lung cancer	



Table 3 (Continued)

No.	Urinary metabolite	HMDB ID	Measured concentrations ( $\mu\text{mol mmol}^{-1}$ creatinine)						HMDB reference creatinine ( $\mu\text{mol mmol}^{-1}$ )	Associated adult disorders, diseases or a source	Reported values ( $\mu\text{mol mmol}^{-1}$ creatinine)
			F			M					
			Median	Range	Median	Range	Median	Range			
58	Glycerate	00139	1.7**	1.5–2.1	1.4**	1.0–1.8	0.20–31	Primary hyperoxaluria II	1.2–1450		
14	Glycolate	00115	35	23–53	33	26–47	2.9–78	Primary hyperoxaluria I	11–103		
125	Glycolproline	00721	1.9*	1.4–2.8	1.6*	1.1–2.0	0–4.7	Prolidase deficiency	510–672		
39a	Hexanoylglycine		<LOQ		<LOQ		0–2	Medium chain acyl CoA dehydrogenase deficiency			
78b	Hippurate	00714	166**	96–398	100**	64–145	19–622	Phenylketonuria	45–55		
Maple syrup urine disease											
114a	Histidine	00177	67	45–100	88	66–106	21–118	Histidinuria	339–3054		
Aminoaciduria											
100	Homocysteine (total) <sup>3)</sup>	00742	1.1	0.91–1.4	1.0	0.86–1.2	0.48–3.2	Cystinuria			
Cardiovascular disease											
104	Homovanillate	00118	2.3	2.0–2.5	2.0	1.7–2.7	0.91–8.9	Catecholamine mtb.			
113	Indolacetate	00197	2.0	1.5–2.8	1.9	1.5–2.6	0.61–5.4	Irritable bowel syndrome	2.5–6.8		
Appendicitis											
80	Isocitrate	00193	43*	30–57	33*	27–43	19.4–119	TCA cycle	0.01–0.13		
50	Isoleucine	00172	1.1*	0.78–1.4	0.92*	0.71–1.1	0.50–2.7	Autosomal dominant polycystic kidney disease			
Alzheimer's disease											
126	Kynurenate	00715	0.89**	0.73–1.1	0.69**	0.62–0.86	0.66–4.2	Tryptophan mtb			
143	Kynurenine	00684	1.0*	0.63–1.6	0.78*	0.71–0.95	0.08–2.8	Tryptophan mtb			
13	Lactate	00190	7.0	3.6–11	5.3	3.9–9.8	0.9–44.6	Diabetes Mellitus, type 1	23–424		
49	Leucine	00687	2.0	1.4–2.3	1.8	1.4–2.3	1.6–6.0	Alzheimer's disease	0.8–1.1		
119a	Lysine	00182	7.6	5.2–16	11	5.6–15	3.7–51.3	Carbamoyl phosphate synth. deficiency	500–5000		
Hyperidibasic aminoaciduria I											
51	Malate	00744	0.67**	0.57–0.81	0.52**	0.41–0.65	0–1.1	TCA cycle	40–440		
7	Malonate	00691	1.1	0.8–1.5	0.9	0.8–1.1	1.2–3.5	Malonyl CoA decarboxylase deficiency			
74	Mandelate	00703	0.39*	0.27–0.48	0.28*	0.23–0.40	0.05–1.7	Occupational exposure to styrene	121–127		
88	Methionine	00696	1.0	0.75–1.4	0.86	0.75–1.1	0.51–2.5	Essential amino acid mtb.			
117	Methionine sulfone		0.77*	0.52–1.1	0.60*	0.46–0.83		Methionine oxidation	400–1000		
76	Methylcrotonylglycine	00459	1.4*	0.9–2.0	1.1*	0.8–1.2	0–2.0	3-Methyl-Crotonyl-Glycinuria			
Biotinidase deficiency											
8	Methylmalonate	00202	2.3*	1.7–2.8	1.7*	1.5–2.4	0.50–3.6	Transcobalamin deficiency	150–500		
Malonyl CoA decarboxylase deficiency											
18	Methylsuccinate	01844	0.95**	0.69–1.2	0.71**	0.53–0.85	0.7–9.9	Refsum's disease	1.3–2.5		

Table 3 (Continued)

No.	Urinary metabolite	HMDB ID	Measured concentrations ( $\mu\text{mol mmol}^{-1}$ creatinine)			HMDB reference creatinine ( $\mu\text{mol mmol}^{-1}$ )	Associated adult disorders, diseases or a source	Reported values ( $\mu\text{mol mmol}^{-1}$ creatinine)
			F	M	Range			
	Ethylmalonic encephalopathy							
66	20-60	00812	4.3*	3.2**	2.6–3.9	1.3–7.0	1240–2504	
109	N-Acetylaspartate	00214	1.9	1.7	1.2–2.3	1.2–2.2	70–2500	
	Cystinuria							
2	Ornithine	02329	8.8	8.8	7.7–10	3.9–14	90–350	
121	200–500	00220	0.82*	0.57*	0.45–0.73	0.3–24.3	Primary Hyperoxaluria I	
31	Oxalate	00209	0.32	0.29	0.20–0.41	0.30–4.16	Fatty acid mtb	
96	Palmitate	00159	3.6	3.6	3.1–5.0	2.8–11	Kidney disease, uremia	
58	Phenylacetate	00857	0.83*	0.74*	0.61–1.2	0.50–4	Essential amino acid mtb.	
55	Phenylalanine	00162	0.59	0.53	0.41–0.66	0.10–2.5	Fatty acid catabolism	
	Proline						Autosomal dominant polycystic kidney disease	
142	Prolylhydroxyproline	06695	6.7	6.1	4.5–7.7	2–26 <sup>4)</sup>	Bone collagen catabolism	
69	Pyroglutamate	00267	25*	22*	17–25	4.5–32.6	5-Oxoprolinase Deficiency	
25	Sarcosine	00271	0.28	0.28	0.25–0.31	0.20–9.4	Sarcosinemia	
95	Sebacate	00792	<LLOQ	<LLOQ		0.18–5.0	Carnitine-acylcarnitine translocase deficiency	
77	Serine	00187	25	23	18–34	10–53	ADPKD	
71	S-Methylcysteine	02108	0.51	0.46	0.35–0.63	0.038–0.56 <sup>5)</sup>	Methyl transfer metabolism	
134	Stearate	00827	0.58*	0.41*	0.35–0.50	0.07–7.7	Fatty acid mtb	
72	Sulberate	00893	0.72*	0.54*	0.44–0.76	0.5–2.9	Glutaric Aciduria II	
17	Succinate	00254	1.7	1.5*	1.4–1.7	0.30–33	TCA cycle	
75	Thioproline		0.50	0.34	0.26–0.46		Formaldehyde, acetaldehyde mtb	
57a	Threonine	00167	9.1**	14**	11–20	4–29	Alzheimer's disease	
23	Tiglylglycine	00959	1.4**	1.0**	0.78–1.2	0.1–6.3	Beta-Ketothiolase deficiency	
141	Tryptophan	00929	5.0	5.9	4.4–6.9	1.3–11.1	Cachexia	
127	Tyramine	00306	0.52	0.44	0.33–0.60	0.20–0.78	Alzheimer's disease	
129	Tyrosine	00158	6.6*	8.7*	6.1–11.1	2.5–23.2	Tyrosine mtb	
102	Urocanate-trans	00301	1.1*	0.95*	0.68–1.2	0–0.78	Cachexia	
40	Valine	00883	2.7	2.4	1.9–2.9	1.1–12.4	Alzheimer's disease	
	Vanillylmandelate	00291	1.8**	1.4**	1.1–1.7	0.64–2.8	Diabetes mellitus, type 1 Hypervalinemia Catecholamine mtb	

1) Creatinine concentration as determined by the Jaffe method and expressed in  $\mu\text{mol/L}$ .  
 2) Total cysteine and 3) total homocysteine obtained after the reduction of the disulfide bonds by the THP reagents.  
 4) From ref. [47]. 5) Adapted from ref. [57]. <LLOQ = below the lower limit of quantitation = level 1.  
 ADPKD = autosomal dominant polycystic kidney disease, TCA = tricarboxylate cycle, MtB = metabolism, pwy = pathway.

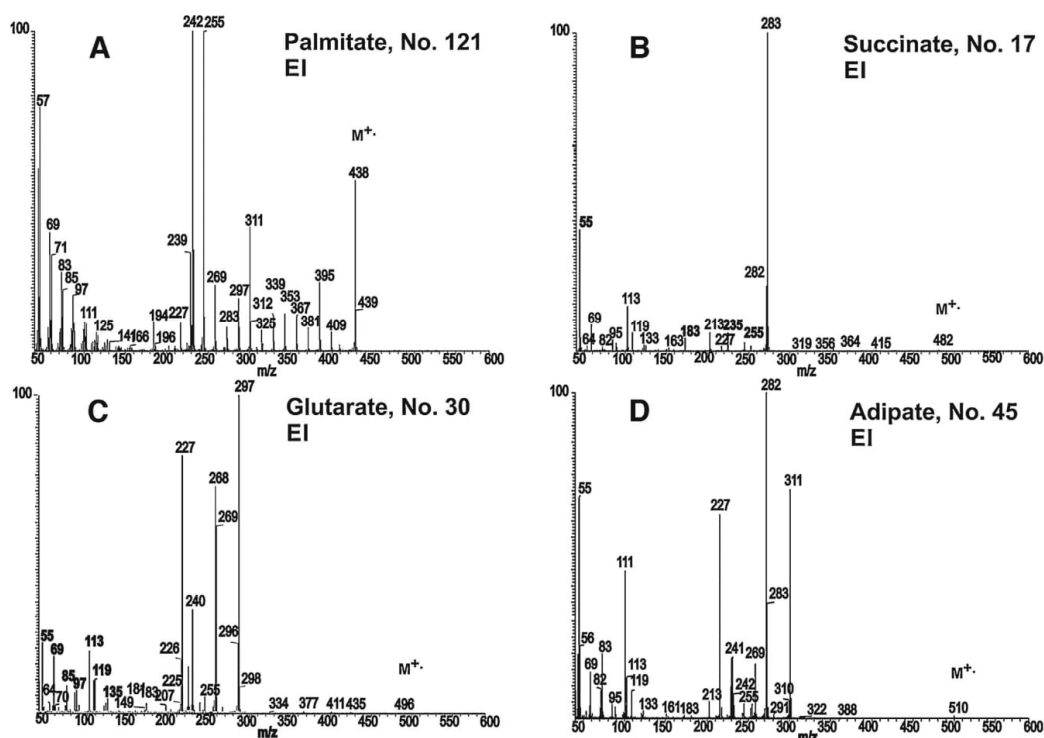


Fig. 1. Electron ionization mass spectra of the HFBCF treated metabolites: (A) palmitate (No. 121); (B) succinate (No. 17); (C) glutarate (No. 30), (D) adipate (No. 45).

### 3.2. Mass spectra of the HFBCF derivatives

The HFBCF derivatization of protic groups yields the relevant HFB esters and carbonates, carbamates and thus increases molecular mass of analytes by 182 and 226 daltons, respectively. Majority of the examined urinary metabolites (Table 1, metabolites No. 1–146) provided well defined EI spectra with a weak molecular ion in most cases, but characteristic fragment ions. Nevertheless, isobutane PICI ionization gently ionized all the HFBCF treated derivatives providing a distinct [M+H]<sup>+</sup> molecular ion and enabled thus confirmation of the molecular mass and structural features. While diagnostic EI ions of the HFBCF treated serum amino acids and some steroids, sterols and tocopherols have been reported in the past [43–45], EI spectra of urinary metabolites, particularly of carboxylic acids, have not been described yet.

Palmitate (Table 1 and the supplementary Table S1, metabolite No. 121) represents a simple example of a metabolite with a carboxyl group. The EI spectrum of the palmitate HFB ester (Fig. 1A) resembles the EI spectrum of the common palmitate methyl ester which is available in commercial mass spectral databases. The main unimolecular M<sup>+</sup> decomposition route of m/z 438 proceeds via reaction initiation on the carbonyl group [51] by known reactions;  $\beta$ -cleavage with  $\gamma$ -hydrogen McLafferty rearrangement yielding a highly characteristic fragment ion m/z 242 (analogous to methyl ester m/z 74 ion fragment). The strongly electron withdrawing HFB moiety evidently increases abundance of the molecular ion (M<sup>+</sup>, m/z 438) and an  $\alpha$ -cleavage product which gives characteristic (M-199)<sup>+</sup>, i.e. m/z 239, an OCH<sub>2</sub>CF<sub>2</sub>CF<sub>2</sub>CF<sub>3</sub> radical loss. Further decomposition of the arising alkyl moiety produces characteristic m/z 55/57, 69/71, 83/85, 97/99, 111/113, 125/127,

139/141C<sub>n</sub>H<sub>2n-1</sub>/C<sub>n</sub>H<sub>n+1</sub> ion series. The HFBCO(CH<sub>2</sub>)<sub>n</sub><sup>+</sup> ion series arising from the alkyl radical loss, i.e. m/z 255, 269, 283, 297, 311, 325, 339, 353, 367, 381, 395 and 409 exhibit a similar abundance periodicity as the EI spectrum of methyl palmitate in the NIST 2.0 mass spectral database. On the other hand, the COOCH<sub>2</sub>CF<sub>2</sub>CF<sub>2</sub>CF<sub>3</sub> loss, i.e. m/z 211 (M-227)<sup>+</sup> remains very small. The EI spectrum further contains fragment ions corresponding to the pieces of the HFB moiety; m/z 69, CF<sub>3</sub><sup>+</sup>; m/z 119, CF<sub>3</sub>CF<sub>2</sub><sup>+</sup>; m/z 183, CF<sub>3</sub>CF<sub>2</sub>CF<sub>2</sub>CH<sub>2</sub><sup>+</sup>; m/z 227, CF<sub>3</sub>CF<sub>2</sub>CF<sub>2</sub>CH<sub>2</sub>OCO<sup>+</sup>. Finally, loss of hydrogen fluoride (M-HF)<sup>+</sup> is sometimes observable from M<sup>+</sup> and some fragment ions.

Introduction of a second HFB ester group in the structure of dicarboxylic acids significantly changes appearance of their EI spectrum. Molecular ions are small and, with the exception of oxalate (No. 2), where m/z 183 (CF<sub>3</sub>CF<sub>2</sub>CF<sub>2</sub>CH<sub>2</sub><sup>+</sup>) ion from the fluoroalkyl chain dominates,  $\alpha$ -cleavage of the CF<sub>3</sub>CF<sub>2</sub>CF<sub>2</sub>O<sup>•</sup> radical providing (M-199)<sup>+</sup> dominates at dicarboxylates. EI spectra of C4–C6 dicarboxylates (No. 17, 30, 45) are depicted in Fig. 1B–D. An increasing number of methylene units facilitates hydrogen rearrangements with the saturated oxygen atom of the ester group. The concurrent CO loss results in formation of characteristic doublets of (M-227)<sup>+</sup> and (M-228)<sup>+</sup>, giving rise to characteristic m/z 269, 283 (M-OOCCH<sub>2</sub>CF<sub>2</sub>CF<sub>2</sub>CF<sub>3</sub>)<sup>+</sup> and m/z 268, 282 (M-HOOCCH<sub>2</sub>CF<sub>2</sub>CF<sub>2</sub>CF<sub>3</sub>)<sup>+</sup>, Fig. 1C and 1D. Furthermore, the increasing chain length increases probability of  $\beta$  and  $\gamma$ -cleavages resulting in the corresponding (M-(CH<sub>2</sub>)<sub>1-2</sub>COOCH<sub>2</sub>CF<sub>2</sub>CF<sub>2</sub>CF<sub>3</sub>)<sup>+</sup> losses without or with a hydrogen rearrangement; as indicate m/z 255, 240 and m/z 269, 255 in Fig. 1C and D, respectively.

Influence of a more labile carbonate group in 2-, 3-, 4-hydroxycarboxylic acid derivatives (No. 19, 24a, 38) on the appearance of the EI spectrum is documented in Fig. 2A–C. While



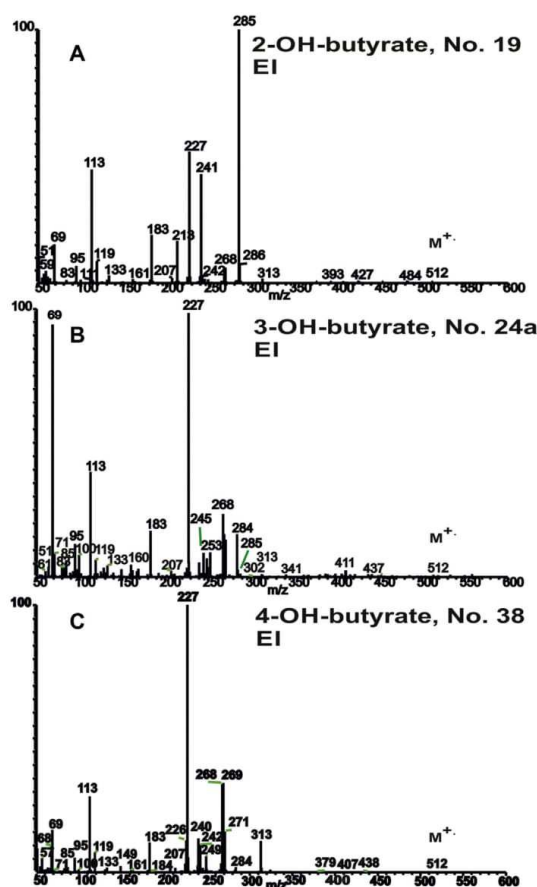


Fig. 2. Electron ionization mass spectra of the HFBCF treated metabolites: (A) 2-OH-butyrate (No. 19); (B) 3-OH-butyrate (No. 24a); (C) 4-OH-butyrate (No. 38).

proximity of the 2-OH group to carboxyl in the 2-OH-butyrate initiates the concurrent  $(M-OCH_2CF_2CF_2CF_3)^+$  providing the main peak  $m/z$  285 peak,  $(M-227)^+$ , Fig. 2A, a more distant position of the OH group from the carboxyl makes the ionization process less efficient and  $m/z$  227 ion dominates in the EI spectrum, Fig. 2B and C. Moreover, a characteristic loss of a neutral heptafluorobutyl hydrogen carbonate species  $m/z$  268,  $(CF_3CF_2CF_2CH_2OCOOH, M^+-244)$  reported earlier at steroid derivatives [45] indicates the presence of a carbonate functional group in the analyte structure.

Some hydroxycarboxylic acids can be labile in aqueous solutions and tend to oxidation or polymerization reactions with HFBCF. An example is 3-OH-propionate (No. 52), whose dominant product was detected as a dimer having molecular mass  $m/z$  527, which was proved by PICI MS (Fig. 3A1–A2). It is probably formed by dehydration of the 3-OH group to acrylate which may react with another 3-OH group to the proposed dimer structure (Table S1, analyte No. 52).

Ketocarboxylic acids represent another important group of urinary metabolites. The HFBCF derivatization leaves the 2-keto group mostly untouched. The keto group substantially influences appearance of the ketocarboxylate EI mass spectrum because the  $\alpha$ -cleavage and positive charge retention on the carbonyl part

deliver the dominant acylium fragments  $m/z$  43, 57, 71, 85 etc., as exemplified by the  $m/z$  71 peak in Fig. 3B for the derivatized 2-ketoisovalerate (No. 3) and documented in Tables 1 and S1 (metabolites No. 1, 3, 5, 6). Nevertheless, the high HFBCF reactivity may affect keto-enol equilibrium in more complex structures and either of the forms can occur. Hence, an important metabolite 2-ketoglutarate (No. 42a, 42b) provided 2 isomeric structures with nearly identical EI mass spectra and the identical mass  $m/z$  510 in the PICI MS spectra, Fig. 3, C1–C2. Although MS is not capable to estimate their exact structure, formation of the two Z, E isomers may be presumed.

The developed method enabled GC–MS analysis of simpler biogenic amines such as putrescine (No.105), cadaverine (No.123) or tyramine (No.127), Fig. 4A–C. The presence of an amino group bearing the heptafluorobutoxycarbonyl moiety initiates an extensive  $\alpha$ -cleavage providing  $m/z$  256 ion  $(CF_3CF_2CF_2CH_2OCONH^+ = CH_2)$  which is accompanied by the  $m/z$  212 ion ( $CO_2$  loss), Fig. 4A, B. The fission of the  $CF_3CF_2CF_2CH_2O(H)$  moiety is less abundant; it gives rise to a doublet  $m/z$  341, 340 and is followed by the  $(H)CONH_2$  loss ( $m/z$  296), Fig. 4A. The presence of a hydroxyl/phenolic group in biogenic amines yields the corresponding carbonates and their EI spectrum becomes more complex. The expected consecutive cleav-

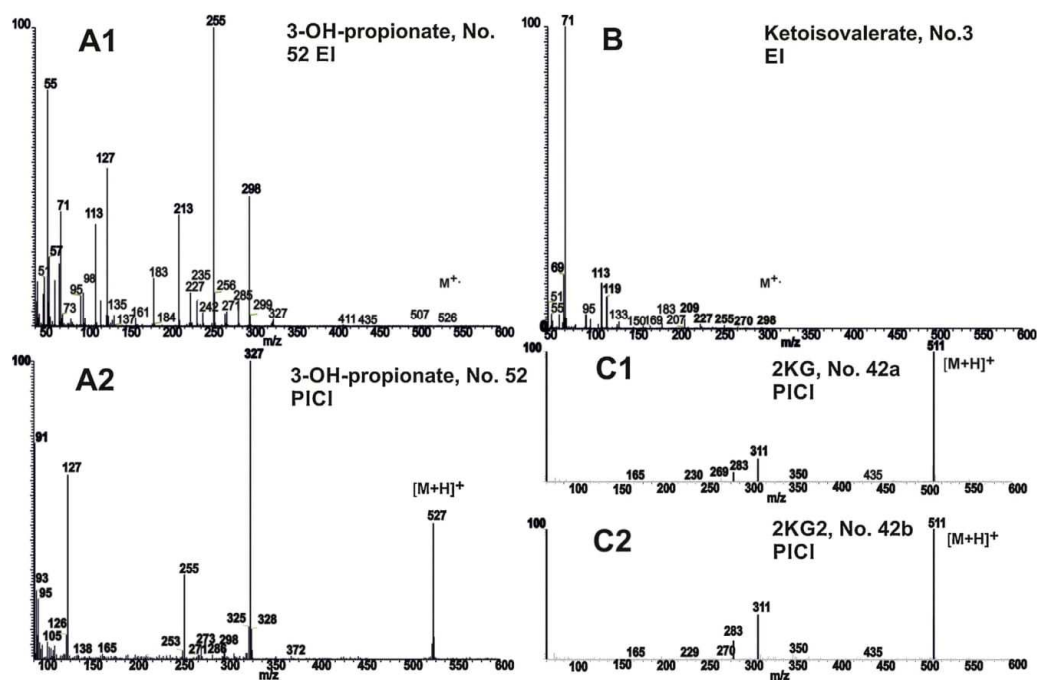


Fig. 3. Mass spectra of the HFBCF treated metabolites: (A1) 3-OH-propionate (No. 52), EI spectrum; (A2) 3-OH-propionate (No. 52), PICI spectrum; (B) ketoisovalerate (No. 3), EI; (C1) 2-ketoglutarate-1 (No. 38), EI; (C2) 2-ketoglutarate-1 (No. 38), PICI spectrum.

age of the  $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{O}(\text{H})$  moiety and a neutral  $\text{CO}_2$  species gives rise to a respective  $m/z$  390 and the dominant  $m/z$  346 ion ( $\text{M}-243$ )<sup>+</sup> in the EI spectrum of tyramine derivative (No. 127), Fig. 4B, while the diagnostic amine ions  $m/z$  256, 212 arising from the typical  $\alpha$ -cleavage and  $\text{CO}_2$  neutral loss, become less abundant. The aromatic group is also an efficient competitor for the charge retention. This indicates the distinct  $m/z$  107 peak characteristic for alkyl phenols and the  $m/z$  333 peak, which can be formally deduced as a loss of the  $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{OCONHCH}_2$  radical accompanied by a  $\text{CO}_2$  elimination ( $m/z$  289), Fig. 4B.

Amino acids typically possess an amino group in the position 2-, 3-, 4- to carboxyl and both functional groups are smoothly converted to the derivatives providing diagnostic ions in their EI spectra [43,44]. Ubukata et al. [52] investigated in detail the EI fragmentation pathways of ethoxycarbonyl-HFB esters of alanine, glycine and their  $^{13}\text{C}$ -labeled analogues. The high resolution and tandem MS experiments revealed that the unimolecular fragmentation of the RCF derivatized 2-amino acids proceeds via an uncommon rearrangement initiated by a loss of the alkoxy radical from the carbamate moiety which is coupled with the CO elimination from the HFB ester group. The plausible fragmentation mechanism proceeds via a concurrent rearrangement of the  $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{O}$ - moiety from ester to the  $-\text{CONHCH}_2\text{R}^+$  2-amino acid residue giving rise to a novel carbamate bond. As a result, the abundant  $m/z$  284 peak is observed in the EI spectra of the derivatized 2-aminobutyrate (No. 36, 27), Fig. 5A, 5B. EI spectra of 5 isomeric structures of aminobutyric acid shown in Fig. 5A–E indicate the capability of the HFBCF derivatives for the identification of structurally close metabolites. A methylene unit between the carboxyl and amino group in the 3-aminobutyrate structures, Fig. 5C and D, enables for amines highly characteristic  $\alpha$ -cleavage giving a principal fragment  $m/z$  270 ( $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{OCONHCH}_2\text{CH}_2$ )<sup>+</sup>

and  $m/z$  256 ( $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{OCONHCH}_2$ )<sup>+</sup> in the EI spectra of the respective 3-aminobutyrate (No. 46) and 3-aminobutyrate derivative. As 3-aminobutyrate is not present in urine, it was not originally included in the metabolite set and the EI spectrum of its HFBCF derivative was recorded additionally for comparison, Fig. 5C. The  $\alpha$ -cleavage giving  $m/z$  256 remains a prominent fragmentation pathway in 4-aminobutyrate (No. 62). A weak  $m/z$  312 peak indicates a  $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{O}$  radical cleavage which is evidently coupled with hydrogen rearrangement and thus the  $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{OH}$ - loss giving rise to the most abundant  $m/z$  112 peak in the EI spectrum, Fig. 5D. Further methylene unit also opens a  $\beta$ -cleavage pathway to the intensive  $m/z$  270 peak.

Inspection of the EI spectra of the derivatized-aminobutyrate isomers shown in Fig. 5A–E reveals further fragment ions indicative for their different geometrical structure. Thus, the ethyl radical cleavage gives fragments  $m/z$  482 ( $\text{M}-29$ )<sup>+</sup> and  $m/z$  283 (a  $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{OH}$  cleavage accompanied by an ethyl loss) in the 2-aminobutyrate (No. 36) EI spectrum, Fig. 5A, while a loss of methyl in the 2-aminoisobutyrate derivative (No. 27) is less evident, Fig. 5B. Here the fragment ions indicating the methyl loss might be  $m/z$  496, ( $\text{M}-15$ )<sup>+</sup> or  $m/z$  296 (a  $\text{CF}_3\text{CF}_2\text{CF}_2\text{CH}_2\text{OH}$  cleavage accompanied by a methyl loss), however, they are weaker than the ethyl radical cleavages. The remarkably different EI spectra of the 2, 3 and 4-aminobutyrate underline capability of the HFBCF derivatization for the structural investigation of isomeric structures. Moreover, the reported EI and isobutane PICI mass spectra of the HFBCF treated metabolites indicate that the developed methodology can be a valuable tool in the structural analysis of many small protic metabolites by GC–MS.

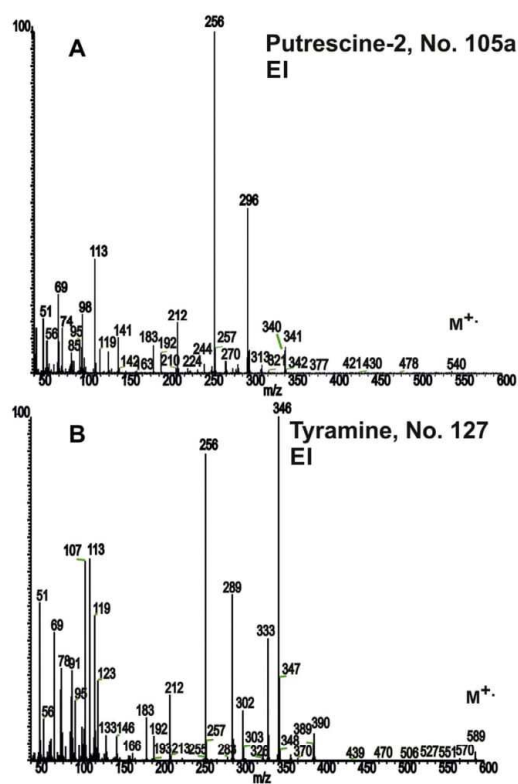


Fig. 4. Electron ionization mass spectra of the HFBCF treated metabolites: (A) putrescine (No. 105a); (B) Tyramine (No. 127).

### 3.3. Sample preparation

The complete sample preparation workflow of the new GC–MS method for urinary metabolite profiling is shown in Fig. 6. The protocol involves in-situ release of bound thiols by the THP reduction of disulfide bonds (step 2–3), pH adjustment (step 4), derivatization of the protic metabolites by the HFBCF–isooctane and NaOH–pyridine organic and catalytic media (step 5–7), aqueous–isooctane LLME (step 7), transfer of the residual pyridine into lower phase by acidification (step 8) and upper organic layer into an autosampler vial (step 9) and final GC–MS analysis. The powerful reducing efficiency of THP releases thiol metabolites such as cysteine and homocysteine bound in disulfide bonds in urine in a minute. Moreover, the reagent's reaction products do not enter the organic phase during the sample preparation process [46]. If the reduction step 3 is not required, it can be omitted without any other protocol change. The in-situ HFBCF mediated sample treatment is efficient, fast and is coupled with the LLME process which in fact generates three phases: (i) the upper isooctane organic phase containing the derivatized metabolites, (ii) the lower aqueous urinary phase bearing excess of the pyridine salt, ionic substances and (iii) carbon dioxide (CO<sub>2</sub>) which is formed by decomposition of the HFBCF reagent during the reaction course. The solubility of CO<sub>2</sub> in isooctane is one order magnitude greater than in water [53]. Nevertheless, the arising CO<sub>2</sub> has been well dissolved in the whole organic–aqueous environment in form of small droplets observed as a compact dispersed milky phase. The evolving CO<sub>2</sub> droplets enhance the effective surface area of both the immiscible organic and aqueous phase and the final equilibrium is reached quickly

in 5 s. The described sample preparation protocol in Fig. 6 can therefore be classified as a dispersive liquid–liquid microextraction method capable to transfer the treated protic metabolites in the upper organic phase. After the reaction is ceased, the CO<sub>2</sub> gas phase can be easily removed from the sample extract after further acidification by vortexing and/or brief microcentrifugation. Interestingly, the repeated addition of the pyridine catalyst in an alkali medium (steps 6–7) facilitates the subsequent reaction of not yet reacted functional groups increasing thus the yields of many derivatized metabolites. The sample preparation workflow is simple; it requires only 25 µL urine sample and successive pipetting of a particular medium, either in a 25 or 50 µL aliquot in each step for GC–MS analysis of major diagnostic amino–carboxylic metabolites. The developed protocol is thus faster, much less laborious and capable to cover wider range of urinary metabolites than the earlier proposed alkyl chloroformate mediated solid–phase microextraction [54] and dispersive LLME methods [55].

### 3.4. Method calibration and validation

For quantification, a set of 132 analytes was selected and their analytical properties obtained from GC separation and EI MS detection were evaluated by GC–MS analysis of the prepared standards in the artificial urine. The tested metabolites were divided into two standard mixtures containing each metabolite at the medium (L3) level. A TIC GC–MS record of this comprehensive set of metabolites (Table 1, No. 2–146) is depicted in Fig. 7. Using the temperature program, separation and detection of all the metabolites was accomplished in 38 min.

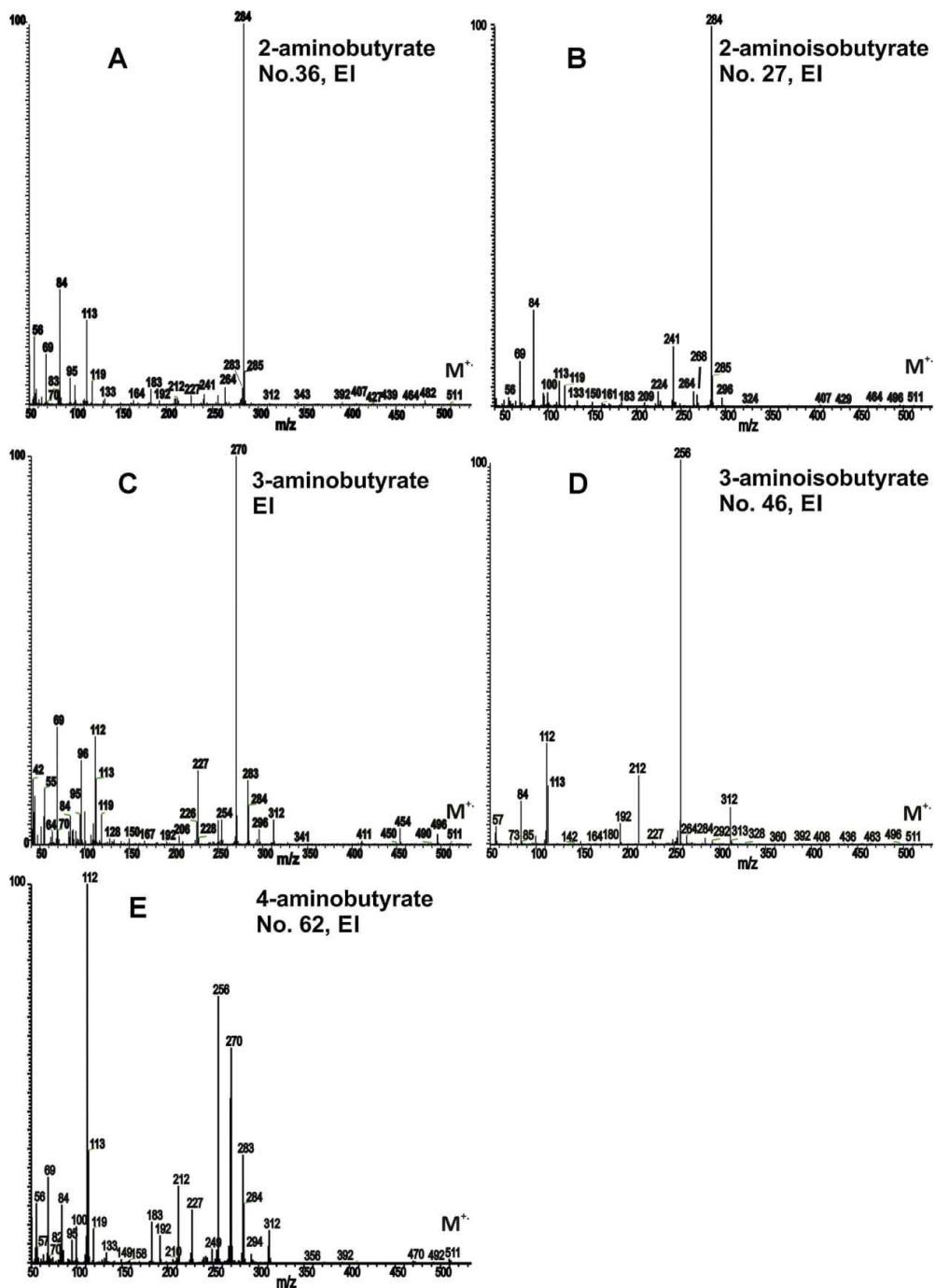
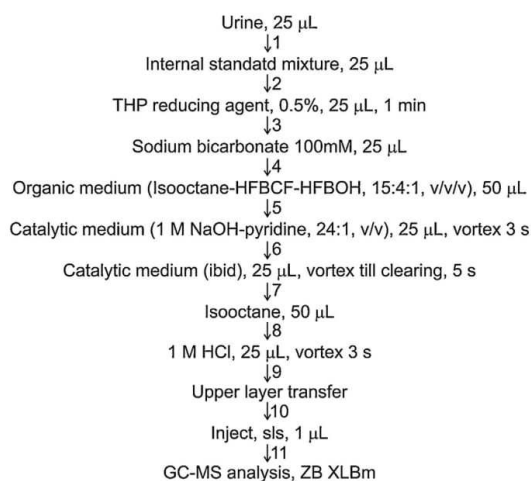


Fig. 5. Electron ionization mass spectra of the HFBCF treated aminobutyrate isomers: (A) 2-aminobutyrate (No. 36); (B) 2-aminoisobutyrate (No. 27); (C) 3-aminobutyrate; (D) 3-aminoisobutyrate (No. 46); (E) 4-aminobutyrate (No. 62).





**Fig. 6.** Flow chart diagram of the novel sample preparation protocol for the GC-MS profiling of protic metabolites in urine.

Calibration curves were mostly generated within three orders of magnitude around the estimated medium physiological level (L3) because the urinary metabolite levels rarely fluctuate outside this range. The calibration data including the calibration curve parameters, the R square values, the LOD, the LLOQ (i.e. the level L1) are shown in Table 1. The use of an appropriate internal standard structurally similar to every analyte has been a widely accepted practice in the MS based analysis to reduce measurement uncertainties associated with the sample preparation and instrumental analysis. However, with the set of more than 100 metabolites this approach is technically hardly feasible. To ascertain acceptable calibration linearity we used two internal standards; 4-phenylbutyric acid (No. 63) and homophenylalanine (No. 103) for quantification of most organic acids and amino-carboxylic acids, respectively. The option to measure metabolite levels in a narrower concentration interval is a practical approach in metabolite analysis because the R square-values rapidly decrease with the increasing calibration range when a relevant I.S. or their set labeled with stable isotopes is not available [56].

Here the R-square values were acceptable in most cases ( $R^2 > 0.97$ ) except of amino acids possessing *N*-acyl, such as tiglylglycine (No.23), *N*-acetylglycine-2 (No. 37a), propionylglycine (No. 48) and hippurate-1 (No. 78a), basic amino acids lysine (No. 119a), cystathionine (No. 132), 2,6-diaminopimelate (No. 124a, 124b), prolyldihydroxyproline (No. 142) and dihydroxyphenylalanine (No. 135) and 4-OH-mandelate (No. 110). Conversion of citrate (No. 60a) into a small portion (~2%) of *cis*-aconitate (No. 60c) may impair quantification of *cis* and *trans* aconitate (No. 54) isomers because these isomers are not separated on the used XLB phenylmethylsiloxane phase and thus, their total level can only be determined.

Using the SIM mode measurement on a single quadrupole mass spectrometer, the LOD values were below  $0.2 \mu\text{mol L}^{-1}$  for majority of metabolites, except oxalate (No. 2), some amino acids (No. 27, No. 39a, No. 57, No. 59) and the co-eluting aconitate isomers (No. 60c + 54).

The method precision and accuracy was satisfactory for most metabolites ranging between 0.8–18% and 81–123%, respectively. Mean recovery of the metabolite set assayed in the averaged urine pool by adding an average metabolite concentration L3 (Table 1) was also acceptable with values between 82 and 121% and indicates

that matrix effects do not impair the quantitative determination at least around the normal urine metabolite levels.

Analytical performance of the developed method was further evaluated by GC-SIM-MS analysis of 20 diagnostic organic acids in the certified urine standard (ORG1) delivered with the declared analyte concentrations. The results of the study involving the metabolite mean value, 95% confidence intervals, the method accuracy, precision and recovery are summarized in Table 2. Except mevalonate, whose analysis failed on the used GC column (see Section 3.1), all the determined organic acids were in a very good agreement with the given values. After measuring the batch of the SKB standard urine sample aliquots ( $n = 16$ ), the residue of the certified urine was stored 15–30 days in a refrigerator at  $-20^\circ\text{C}$  and then the between-run experiments were conducted. The acceptable method accuracy and precision data indicate good stability of the organic acids in the stored urine. The decomposition of the tiglylglycine derivative (No. 23) was an exception, most probably owing to the presence of the labile 2-methylbutenyl moiety in the structure. The GC-MS quantification of this analyte must therefore be accomplished as soon as possible after the sample preparation step. Finally, the satisfactory recovery data obtained after spiking the SKB certified urine with the organic acids standards further indicate negligible matrix effects of the described method.

### 3.5. Quantification of metabolites in human urine

A typical picture obtained by the full scan GC-MS analysis of urine metabolites in the pooled urine (a random mixture of healthy 10 male and 10 female urine samples) is shown in Fig. 8. Absence of any remarkable interference in the full scan TIC GC-MS record of the isooctane sample extract indicates capability of the developed method to ascertain comprehensive metabolite profiling in human urine by the described method.

The new analytical method was finally examined for the metabolite GC-SIM-MS quantification in a cohort of second-void urine samples of 50 female and 50 male healthy subjects. The average age of the gathered female and male subjects was 42 years (18–84 years) and 46 years (24–86 years), respectively. The list of the measured metabolites is shown in Table 3. The median values were used instead of the mean because the measured urinary metabolite concentrations do not exhibit normal distribution in the examined data set. The measured concentration of each metabolite was expressed in  $\mu\text{mol mmol}^{-1}$  creatinine and their respective range as a 25th–75th percentile variation.

The average creatinine (No. 150) concentration measured by the Jaffe UV spectrophotometric method in the urine sample set showed the median value 9.2 (5.9–15) and 11.0 (9.6–16)  $\text{mmol L}^{-1}$  for adult females and males, respectively, and as expected, the gender difference was statistically significant ( $p < 0.05$ ).

Finally, the metabolite concentrations measured by the new developed method in the examined urine set were compared with literature data, particularly with those amenable in the HMDB database. The HMDB metabocard entry code, metabolite concentration ranges obtained by GC-MS analysis of female and male urines samples and typical reported reference concentration range are collected in Table 3. Closer inspection of the data set in Table 3 indicates that the work-up of a 25  $\mu\text{L}$  urine sample aliquot allowed direct quantification of 112 metabolites which were detected in approximately 3 orders of the concentration magnitude, in the range  $0.2\text{--}400 \mu\text{mol mmol}^{-1}$  creatinine. Twenty calibrated metabolites were not detected in most examined female and male urine samples, evidently due to their low abundance and limited capability of the used single quadrupole MS to detect the analytes below the  $0.2 \mu\text{mol mmol}^{-1}$  creatinine level. Nevertheless, a metabolic disease may increase concentration of a certain metabolite in urine and then the metabolite can be quantified. This

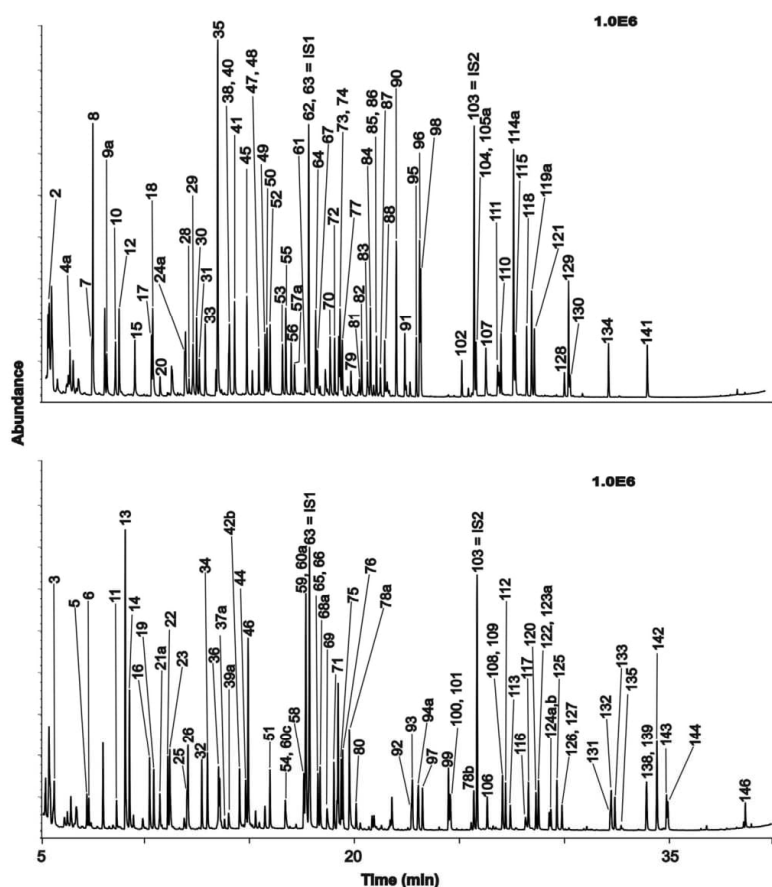


Fig. 7. Full scan TIC chromatogram of the comprehensive set of the urinary metabolite standards (Table 1, No. 2–146), each measured at the equimolar medium L3 level with the internal standards (IS1 and IS2) in the artificial urine. The metabolites were divided into 2 groups; the first group contains mainly protein amino acids, mono-, di-, tri- carboxylic, 3-hydroxycarboxylic and aromatic acid; the other involves non-protein amino acids, keto- and 2-hydroxycarboxylic acids and glycine conjugates. For the reported metabolites, refer to Table 1.

feature was corroborated by GC–MS analysis of hexanoylglycine (No. 39a) and sebacate (No. 95) in the SKB certified urine, where elevated levels of the metabolite diagnostic for inherited metabolic diseases were determined, Table 2 and Table 3.

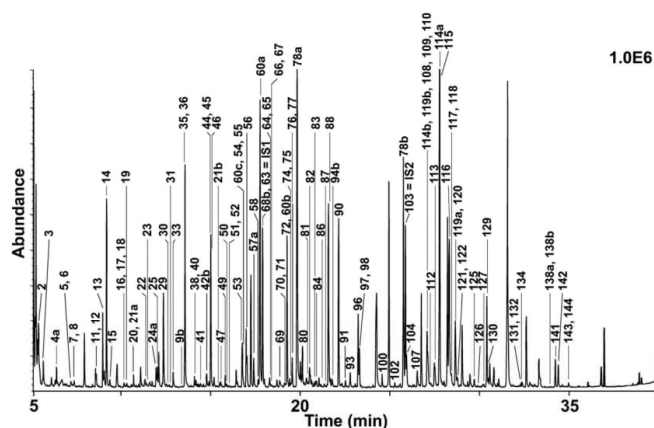
Three particular metabolites, i.e. hippurate (No. 78), citrate (No. 60) and histidine (No. 114) were detected above  $100 \mu\text{mol mmol}^{-1}$  creatinine. The excess of the HFBCF reagent ( $10 \mu\text{L}$ , i.e.  $60 \mu\text{mol}$ ) in the organic medium facilitates the derivatization of metabolites at highest levels in the  $25 \mu\text{L}$  worked-up urine volume. Moreover, the most abundant urine components such as urea, inorganic salts, creatinine, creatine, uric acid react with the reagent in a negligible extent and remain in the aqueous phase without deteriorating the assay. The levels of the detected metabolites were mostly within an order of magnitude in the examined urine sample set of healthy objects, justifying thus the use of the calibration within the narrower concentration range for the targeted metabolite profiling.

Vast majority of the urinary metabolites were also in a very good accordance with the concentration range reported in the HMDB database and relevant literature, refer to Table 3. The observed metabolite levels exhibited a rather smaller individual variability

and the highest observed values were often at lower levels than those reported in the HMDB. The individual variability was higher than the gender difference in most cases. Nevertheless, the Mann–Whitney U-test revealed a statistically significant difference ( $p < 0.01^{**}$ ) in the profiles of 16 urinary metabolites between the measured gender groups, Table 3.

Three metabolites were measured either as a sum of the isomers or the metabolite plus its precursor, refer to the respective aconitate (No. 54a + 54b), the total cysteine (No. 90 + 137) and total homocysteine (No. 100 + 145) content in Table 1 and Table 3. Two metabolites, thioproline (No. 75) and methionine sulfone (No. 117) were clearly detected in all urine samples, although they are not in the HMDB database and their formation during the sample preparation was carefully checked and thus excluded. Thioproline is known to be excreted in human urine by formaldehyde and acetaldehyde metabolism and was earlier reported at low but not reported concentrations [58]. Methionine sulfone is a suspected product of the methionine oxidation, but its origin remains uncertain. As we carefully checked that the metabolite does not arise during the sample work-up, it may be excreted from the human body or may arise





**Fig. 8.** Full scan TIC record obtained by comprehensive GC–MS analysis of urinary metabolite profiles in a random mixture of healthy 10 male and female urine samples. The new described method enables determination of 112 metabolites in a 25  $\mu$ L urine sample aliquot. IS1 and IS2 = internal standards. The analyzed metabolites are reported in Table 3.

from the oxidation of methionine in urine by microbial contamination or urine aging during the sample storage. The abundance of this metabolite in urine therefore requires further study.

#### 4. Conclusions and future perspectives

In this study, a novel GC–MS method based on the combined 1,1,1,2,2,3,3-heptafluorobutyl chloroformate derivatization and liquid-liquid microextraction was designed, tested and evaluated for metabolomic profiling of protic, mostly amino- carboxylic metabolites in urine. A 25  $\mu$ L sample aliquot was efficient for the direct single quadrupole GC–MS quantification of 112 metabolites in human urine collected from adult healthy persons as well as for the determination of 19 metabolites relevant to the inherited metabolic diseases at their increased level in the certified urine. We aimed to evaluate critical points of the GC–MS metabolomic analysis and revealed that the method provides well predictable derivatives possessing excellent volatility, separation properties and EI mass spectra being very useful in elucidation of structural isomers and unknown structures. The study also showed limitations of the described method, consisting in the incapability to derivatize analytes possessing the guanidino- and ureido-functional group, polyhydroxycarboxylic acids with sterically hindered OH-groups, sugars and labile, often to oxidation susceptible metabolites. On the other hand, for analytes possessing at least a single non-derivatized polar functional group, the LLME equilibrium is largely shifted towards the aqueous phase and thus the nonpolar iso-octane phase contains much less interferences arising from the urine matrix than any other comparable GC–MS based method. The results of this study suggest that the proposed method may become an efficient, complementary tool in GC–MS metabolomic analysis. Moreover, its application potential may further be increased by automation of the sample preparation procedure, advanced metabolite quantification by means of the  $^{13}$ C-labeled standards, examination of chemical ionization MS techniques, expansion of the described methodology to a wider range of urinary metabolites and to other complex biological matrices, with the option of comprehensive chiral amino acid analysis [44].

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2016.03.019>.

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## 8.5 Publikace 4

### **GC-MS Metabolomic Profiling of Protic Metabolites Following Heptafluorobutyl Chloroformate Mediated Dispersive Liquid Liquid Microextraction Sample Preparation Protocol**

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#### **Running Head:**

**GC-MS Metabolomic Profiling of Protic Metabolites**

#### **Abstract**

A simple analytical workflow is described for gas chromatographic-mass spectrometric (GC-MS) based metabolomic profiling of protic metabolites, particularly amino-carboxylic species in biological matrices. The sample preparation is carried out directly in aqueous samples and uses simultaneous in-situ heptafluorobutyl chloroformate (HFBCF) derivatization and dispersive liquid liquid microextraction (DLLME), followed by GC-MS analysis in single ion monitoring (SIM) mode. The protocol involves 10 simple pipetting steps and provides quantitative analysis of 132 metabolites by using two internal standards. A commentary of each analytical step and explaining notes are provided with particular attention to GC-MS analysis of 112 physiological metabolites in human urine.

#### **Keywords**

Metabolomic profiling; GC-MS; Dispersive liquid liquid microextraction; Chloroformate derivatization; Urine; Quantitative analysis

### **1. Introduction**

Comprehensive metabolomic analysis of small protic metabolites possessing amino-, carboxy-, thio- or hydroxy- groups in complex biological matrices has been a demanding task because of the frequent occurrence of structurally close and isomeric structures that are difficult to separate and detect by at present prevailing liquid chromatographic-mass spectrometric (LC-MS) techniques. As a result, GC-MS combined with an efficient sample preparation strategy involving metabolite derivatization (as a prerequisite), has still been a popular, cost-effective tool in the analysis of amino and organic acids and other protic metabolites that play important biochemical roles in central metabolism. Current GC-MS based metabolomics relies mainly on two derivatization strategies: (i) oximation with silylation and (ii) reaction with alkyl chloroformates. The former approach requires strictly anhydrous condition for silylation and has proved useful for profiling of polyhydroxylic metabolites such as sugars (1,2), steroids (3),

sterols and tocopherols (4), but much less effective for metabolites bearing protic nitrogen functional groups (5-7). The latter approach has been complementary and even more attractive; it can be applied in situ in a complex biological matrix, liquid-liquid microextraction proceeds simultaneously under pyridine catalysis and, importantly, with simultaneous carbon dioxide evolution (8-11). The arising CO<sub>2</sub> is partly dissolved in the whole sample medium; it enhances the effective surface area between the immiscible organic and aqueous phase and renders thus a powerful dispersive phase enabling the system to reach the final equilibrium in 5 second (4,12).

Fluoroalkyl chloroformates (FCFs) possess some advanced features over the traditionally used alkyl chloroformates (RCFs) providing highly volatile and much less polar derivatives extractable into nonpolar hydrocarbon solvents. The reaction scheme for reaction of heptafluorobutyl chloroformate (HFBCF) with threonine, an amino acid possessing 3 protic functional groups, is shown in **Fig. 1**.

The reaction product is formed directly in aqueous environment with high yields in less than 5 seconds. In this way, extraordinary clean protic metabolite extracts have been obtained from complex biological matrices and have been successfully applied to metabolite profiling of amino acids and steroids in human serum (4,13) or amino-carboxylic metabolites in human urine (12). Moreover, the clean extracts enabled chiral GC-MS analysis of 35 amino acid enantiomeric pairs in human serum (14).

The workflow of the GC-MS based metabolomic analysis is depicted in **Fig. 2**.

Here, we describe a HFBCF based GC-MS profiling method for quantification of protic metabolites possessing amino-, carboxy-, activated hydroxy- and thiol functional groups (12). The elaborated sample preparation protocol is simple, fast and follows a procedure described in detail for urinary analysis in reference (12) which should be consulted whenever necessary for getting more comprehensive knowledge. It involves gradual pipetting of uniform small volumes of a sample and necessary liquid media in 10 steps: (1) a sample, (2) an internal standard solution, (3) a reducing medium, (4) a pH adjustment, (5) an organic reaction medium containing the HFBCF reagent, (6-7) a repeated addition of a catalytic medium with pyridine, (8) an organic extraction medium, (9) an acidification medium, (10) an upper extraction phase transfer into a GC autosampler vial, see **Fig. 3**, and finally, the sample extract injection into a GC-MS spectrometer.

Although the protocol was primarily developed for metabolomic GC-MS analysis of protic metabolites in urine normalized to creatinine (12), the procedure can directly be applied to any aqueous biological material with low protein content. If the content of biopolymers is high (> 2mg/ml), then a prior precipitation step must be adapted to the described workflow (*see Note 1*). The protocol may be modified for some applications, for instance the reducing step 3 can be omitted without any change, if the reduction of disulfide bonds is not required.

## 2. Materials

## 2.1 Samples

Samples containing no or little protein and cell residues (urine, cell culture media) or cell and tissue extracts.

## 2.2 Chemicals, Solutions, Reaction Media

1. Chemicals of analytical grade should be used. All solutions, except those containing the HFBCF reagent, should be prepared in distilled deionized water (DI water,  $< 1.5 \mu\text{S}\cdot\text{cm}^{-1}$ ,  $25^\circ\text{C}$ ) and stored at  $4^\circ\text{C}$  (unless otherwise indicated).
2. Tris(3-hydroxypropyl)phosphine reducing agent (THP, 80%, Merck): Prepare a 5 % stock solution by transferring 0.625 ml of the THP liquid to a 10 ml volumetric flask and adjust by DI water up to 10 ml. Prepare a 0.5 % working solution by dilution 1:9 in a 10 ml volumetric flask (*see Note 2*).
3. 100 mM  $\text{NaHCO}_3$  (99,998% purity, Alfa Aesar) solution: Dissolve 840 mg in 100 mL of DI water.
4. 1 M NaOH (99.99 % purity, Alfa Aesar) solution: Dissolve 4 g in 100 mL of DI water.
5. Heptafluorobutanol (HFBOH) (99 % purity, Pragolab, Prague, Czech Republic).
6. Heptafluorobutyl chloroformate (HFBCF, 98 %, Pragolab), (*see Note 3*).
7. Solvents: Isooctane (2,2,4-trimethylpentane, 99.5%), pyridine (p.a., 99.0%), isopropanol (2-propanol, 99.5% purity) (all Sigma-Aldrich).
8. The organic reaction medium: prepare isooctane, HFBCF and HFBOH in a volume ratio 15:4:1 (v/v/v) in a Teflon-capped, well-tightened 4-mL glass vial. Store in a refrigerator, where the mixture remains stable for several months.
9. The catalytic medium: mix 1 M NaOH with pyridine in a volume ratio of 24:1 (v/v).
10. The artificial urine solution: prepare the following chemicals in DI water to final 10 g/L urea, 1 g/L creatinine, 7 g/L NaCl and 3 g/L  $\text{K}_2\text{SO}_4$  (12).
11. The certified urine standard (product ORG-01) containing diagnostic organic acids (ERNDIM Foundation (<http://cms.erndimqa.nl/>)). For the analyte concentrations, refer to the website. Order the latest available batch.

### 1.1. 2.3 Analytical Standards, Stock Solutions

1. 1. Internal standard solution (IS): 4-phenylbutyric acid (4PB, Sigma-Aldrich, MW = 164.2 g/mol); homo-phenylalanine (hF, Sigma-Aldrich, MW = 179.2 g/mol). Prepare a stock solution in 100 mM  $\text{NaHCO}_3$  with a final concentration of 200  $\mu\text{mol/L}$ , i.e. 5 nmol in 25  $\mu\text{L}$  of the applied internal standard solution.
2. 2. Protein amino acid (AA) standard solution (Sigma-Aldrich, P/N AAS-18) in 0.1 M HCl containing alanine, glycine, valine, leucine, isoleucine, threonine, serine, proline, aspartic acid, methionine, glutamic acid, phenylalanine, lysine, histidine, tyrosine and cystine at a concentration of 2.5 and 1.25 mmol/L, respectively. Alternatively, the protein AA mixture can be prepared from stock solutions of particular AAs in 0.1 M HCl. For the complete metabolite list refer to **Table 1**.
3. 3. Non-protein amino acid, biogenic amine (Sigma-Aldrich) standard solutions: prepare a stock solution of each metabolite in 100 mM HCl with a final concentration of 100  $\mu\text{mol/L}$ , **Table 1**. Store at  $4^\circ\text{C}$ .

4. Organic acid standards (Sigma-Aldrich); less common carboxylic acids 2-hydroxysebacic, 3-hydroxyadipic, 3-hydroxypropionic, 3-hydroxyvaleric and 5-hydroxyhexanoic acid, and glycine conjugates hexanoylglycine, methylcrotonylglycine and tiglylglycine can be purchased from Dr. E. Brunet, Dept. Organic Chemistry, University Autonoma de Madrid (Madrid, Spain), <http://www.uam.es/gruposinv/lumila/list.pdf>; a racemate of 2-methyl citric acid (90%, C/D/N Isotopes, P/N X-4176).

5. The organic acid stock solutions data are summarized in **Table 1** (*see Note 4*).

#### 2.4 GC-MS instrumentation

1. Agilent 7890A GC system equipped with G4513A autosampler (Agilent), multi-mode injector (MMI), equipped with a 10  $\mu$ l syringe (CTC Analytics, P/N PAL3-SYH-207807).
2. Sky® 4 mm I.D. cyclo double taper inlet liner (Restek, P/N 23310).
3. ZB-XLB type, 30 m x 0.25 mm ID, 0.25  $\mu$ m film thickness (Phenomenex, P/N 7HG-G019-11).
4. Single quadrupole mass triple-axis detector (5975 MSD Inert XL, Agilent) equipped with an inert EI ion source.
5. Autosampler 2 ml vials (12 x 32 mm) with 9mm PP open hole caps (Labicom, P/N 5310F-09) and 0.040" PTFE/silicone/PTFE Septa (Labicom, P/N 604060-09).
6. Inert conical glass insert, 200  $\mu$ l volume, (Chromacol, P/N 02-MTV).

#### 2.5 Additional equipment

1. The sample preparation glass culture tubes 6x50 mm, material: sodium-potassium silicate
2. glass (Merci, P/N Z1632000605010), or borosilicate glass (Kimble-Kontes, P/N 73500-650).
3. Common screw cap teflon-lined 2 and 4 ml amber vials for the reagent solutions.
4. An adjustable 50 and 100- $\mu$ l Transferpettor pipette with a glass capillary (Brand, P/N 701868 and 701873) for manipulation with the reagents and their mixtures in isooctane. The pipette tips with 25 mm capillary (gel-loading type, VWR Int.) for aspirating the upper organic phase in the 6x50 mm vial.
5. Alternatively, a common pipette (10-100  $\mu$ l) such as a Biohit Prolin® mechanical pipette (Sartorius, P/N 720050) equipped with an Optifit tip 200 (P/N 4059.9002) can be used.
6. A common vortex for sample mixing and a minicentrifuge such as MySpin 6 (Thermo Scientific, 2000 x g) for a complementary separation of immiscible layers in sample vials.
7. A commercial assay kit for creatinine analysis. For instance a creatinine kit (Dialab, P/N D95595).
8. A common spectrophotometer capable of measuring creatinine at 490-510 nm in urine, such as Specord® Plus (Jena Analytik), by an appropriate kit (*see 2.5.7.*) used for clinical applications.

### 3. Methods

Use appropriate laboratory wear, glasses and other personal protective equipment is recommended; follow standard laboratory precautions and local guidelines, especially waste disposal regulations. Use a functional fume hood for the sample work-up.

#### 3.1 Sampling and storage, normalization of samples

1. Serious attention should be paid to sample collection, transport and storage, because any omission may result in false results. For urine, collection of the morning second-void samples is most common practice and the easiest sampling method.
2. For urine analysis, store freshly collected samples at 4 °C within 2 hours. For longer than 48 h storage, freeze the samples and keep at -20 °C (*see Note 5*).
3. If urine is a subject of study, measure creatinine concentration in each collected urine aliquot used the analysis data for normalization of each determined metabolite (*see Note 6*).

#### 3.2 Preparation of calibration solutions

1. Prepare a calibration mixture following the procedure described in (12) by adding an appropriate volume of each stock solution standard, **Table 1**, in a 10 volumetric flask. Adjust to a final volume by the artificial urine solution.
2. The concentration of each individual analyte denotes its average level (here, level 3 = L3) measured in a pooled urine sample of normal morning urine, **Table 1** (12). Prepare the lower (L1 and L2) and higher (L4, L5) calibration points; i.e. 10 times, 2.5 times diluted and 2.5 times and 10 times increased to the respective medium L3 level. For the metabolites with highest abundance prepare level L6 (25 times higher than L3).
3. Distribute the calibration solution into appropriate aliquots before freezing.
4. Prepare an appropriated pooled sample by mixing an equal small volume of all samples included in the study for the verification of the average EI MS response for each target metabolite and for the quality control (QC) analysis.

#### 3.3 Sample preparation protocol

1. Transfer 25 µL of aqueous sample into a 6x50 mm culture tube.
2. Spike the sample with the 25 µl of the internal standard solution.
3. Add 25 µL 0.5% THP reducing solution, mix the content gently for 1-2 s and left to stand for 1 min.
4. Adjust pH to ca 9 with 25 µL 100mM NaHCO<sub>3</sub> solution and vortex gently.
5. Add 50 µL of the organic reaction medium (isooctane, HFBCF and HFBOH, 15:4:1, v/v/v) (*see Note 7*).
6. Add 25 µL of the catalytic medium (1 M NaOH-pyridine, 24:1, v/v) and vortex the content for ca 3 s leaving the organic phase milky.
7. Add a second portion (25 µL) of the catalytic medium and shake the content for 5 s until the milky phase becomes clarified.
8. Add 50 µL of the isooctane extraction medium, mix for about 1-2 s.

9. Add of 25  $\mu\text{L}$  of 1M aqueous HCl and vortex briefly; if the phases are not well separated, a minicentrifuge may be a convenient option (*see Note 8*).
10. Aspirate 70-80  $\mu\text{L}$  of the upper organic phase into a vial insert (150-200  $\mu\text{L}$  volume).
11. Inject a sample extract aliquot (1  $\mu\text{L}$ ) by using a pulsed splitless injection into a GC injector, start the GC-MS acquisition (*see Note 9*).

### 3.4 GC-MS Analysis

1. The instrument GC-MS conditions are summarized in **Table 2**.
2. First, analyze the standard mixtures to check the separation performance, retention times, the analyte peak shape and acquisition of the employed fragment ions in the obtained EI spectra, **Table 1**.
3. Using a single quadrupole MS analyzer, single-ion monitoring (SIM) mode is commonly used for quantification. Use the characteristic  $m/z$  ions (a quantifier and a qualifier) listed in **Table 1**. The SIM scanning should be arranged into convenient time sequence groups (windows) associating closely co-eluting analytes  $m/z$  ions enabling thus an appropriate SIM dwell time for each detected analyte (12).
4. Prepare an appropriate analysis sequence of a sample series consisting of repeated blank, standard, the QCs (*see 3.2.2.*), calibration and real sample extracts. Measure regularly the blanks, QC samples and standards (at least every 10 sample runs). Analyze samples in a random order to avoid a systematic error.
5. Change the GC injector liner approximately after 150 samples depending on the sample matrix. Before use, condition each new liner by running the following sequence: solvent blank, standard mixtures, the pooled QC sample extract (twice) and solvent blank (twice).

### 3.5 Data Analysis

1. Peak area for quantifier and qualifier ion of each metabolite are integrated. Their ratio is calculated to test for potential interferences.
2. The peak area of each quantifier is normalized by the peak area of the corresponding internal standard: amino acids and biogenic amines against homophenylalanine, compound No. 75; organic acids against 4-phenylbutyric acid, compound No. 37, **Table 1** (12).
3. Use appropriate vendor data processing software for data calibration and metabolite quantification.
4. Check metabolite responses in the QC samples measured regularly throughout the whole sample set. If the analyte's relative response to the IS fluctuates with RSD > 30%, then even a semi-quantitative measurement of such metabolite is difficult and it should be excluded from the metabolomic study (*see Note 10*).
5. Once the metabolite levels have been determined and met predefined acceptance criteria, normalize appropriately the measured metabolite concentrations. For urine recalculate the metabolite levels to creatinine or other suitable reference factor. Export the analytical data matrix into a Microsoft Excel<sup>®</sup> spreadsheet or other format suitable to explore for further chemometric analysis.
6. Use an appropriate statistical software to recognize differences among the studied metabolite sample sets. The calculation of p-values by means of a t-test helps to determine significance of

the obtained results. The data set can be conveniently examined graphically by means of box plots that display patterns of quantitative data and thus facilitate interpretation of observed metabolite changes in the studied organism.

#### 4. Notes

1. For serum/plasma, (lipo)proteins must be precipitated by a suitable medium prior to the application of this protocol. The work-up requires first an internal solution addition (step 2), which may be followed by the THP reduction of disulfide bonds (step 3). However, the protein precipitation must precede the step 4, pH adjustment; it can be carried out by perchloric acid (13), trichloroacetic acid or an organic solvent (15,16). Note that the selected precipitation conditions for plasma or serum may affect the obtained metabolite profile (15) and thus the described protocol must always be adapted and validated to a particular demand.

2. The 5% stock THP solution should be kept in a freezer for a year; the 0.5% working solution in a refrigerator for a month.

3. The 2,2,3,3,4,4,4-heptafluorobutyl chloroformate (HFBCF) reagent is a liquid with a boiling point 105 - 107 °C and density 1.6 g/cm<sup>3</sup> (14). The reagent must be stored in tightly closed Teflon-lined cap glass vials at 4 °C and thus is stable for at least 24 months.

WARNING! Manipulation with HFBCF must be performed in a well-ventilated area (fume-hood).

4. A list of metabolites covered by the protocol is summarized in **Table 1**. For the analytical purposes, the compounds can be sorted into six groups, i.e. protein amino acids, non-protein amino acids, dicarboxylic acids, hydroxycarboxylic acids, organic (aromatic) acids—and metabolites sensitive to storage conditions. The last group comprised oxoacids, lactate, 5-hydroxyindol- and indolacetate, 3- and 4-hydroxyphenylacetate, 4-hydroxymandelate, 4-hydroxyphenyllactate, kynurenate, vanillylmandelate, kynurenine, glycyproline, prolylhydroxyproline and 3-methylcrotonylglycine; the stock and working solutions of this group were kept in freezer at - 20 °C.

5. Urine like other important biological matrices is a metabolite- rich mixture. Be careful and always take into account properties of each metabolite of interest in the studied matrix. Check carefully the metabolite stability by sample measurement within a convenient time period before making final conclusions from the measured data.

6. In metabolomics, normalization of samples is an important practice (17). For urinary analysis, creatinine is the commonly used reference that indicates well the urine concentration (18). Urine of healthy women and men typically contains around 5-16 mmol/L of creatinine (12), (19). If its concentration is highly increased (more than 3-4 times), the sample should be diluted by DI water before analysis maintaining thus urine composition closer the average creatinine abundance.

7. The used HFBC reagent volume (10 µl, 60 µmol) is efficient for work-up urine volumes below 50 µl. In contrast with classical alkyl chloroformates, the corresponding heptafluorobutyl alcohol is not necessary in the reaction medium. Nevertheless, its small 5 % aliquot facilitates esterification of polycarboxylic acids such as citrate.

8. The acidification step substantially further decreases the pyridine catalyst content in the arising upper organic layer and thus contamination of the GC-MS system. Consequently, the liner change follows typically after 120-150 samples, less frequently than in earlier methods (20).

9. If the prepared organic sample extracts are not measured immediately, they can be stored in tightly closed Teflon-lined autosampler vials up to 2 weeks at -20 °C. A slow degradation of some metabolites was observed, in particular kynurenate, tiglylglycine, 3-hydroxybutyrate, fumarate and prolylhydroxyproline, histidine, 1-methyl- and 3-methylhistidine and isocitrate.

10. Note that demands on metabolomic analysis do always not conform strict guidelines requested for instance by guidelines in drug analysis and data showing a higher uncertainty may be useful in the study. Moreover, metabolite levels observed between two studied models rarely change by more than 1 order of magnitude, and thus narrower calibration ranges can be used throughout a metabolomic study with respect to the amount estimated in the pooled QC sample.

### Acknowledgments

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Figures Captions

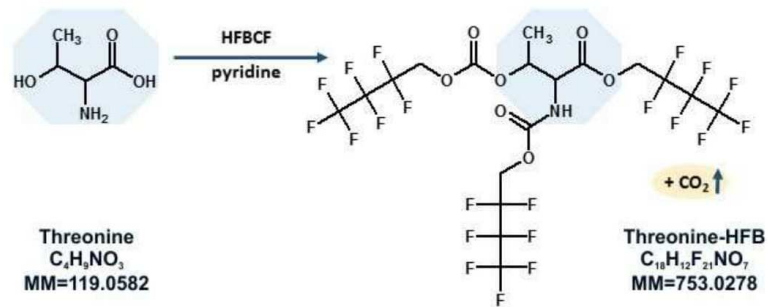


Fig. 1

Reaction scheme for the threonine protic functional groups with the HFBCF reagent. The carboxyl group yields a HFB ester, the amino group a corresponding HFB carbamate, while hydroxyl is transformed into a HFB carbonate. MM= monoisotopic mass.

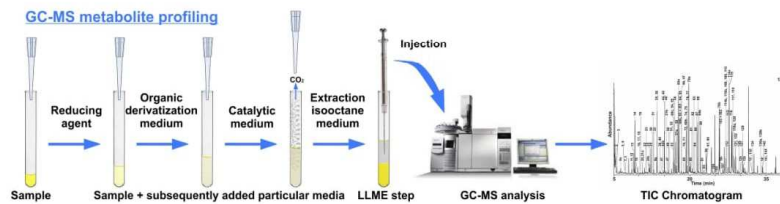
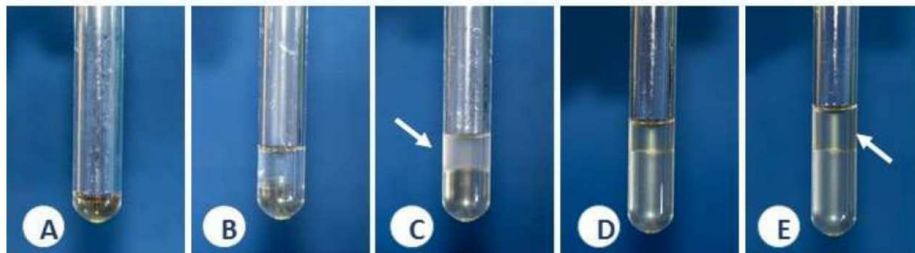


Fig. 2

The workflow for the GC-MS metabolomic analysis of protic metabolites in aqueous biological matrices.



**Fig. 3**

A view on a 6x50 mm culture tube containing an aqueous sample (here urine) or an aqueous sample extract and gradually added media during the sample preparation process: (A) an aqueous sample; (B) an arising two-phase system after performing the steps 2-5, before the reaction initiation; (C) a turbid upper phase after the first addition of the catalytic medium, step 6; occasionally visible CO<sub>2</sub> bubbles can appear; (D) the organic upper phase is clarified after the step 7, which indicates that the reaction was completed; (E) increasing the sample extract volume and its acidification in steps 8-9 enables an easy organic upper phase into an autosampler vial in step 10, and final GC-MS analysis.

### Table Captions

#### Table 1

The list of protic metabolites determined by the described GC-MS protocol in human urine. The traditional name, the metabolite product after the HFBCF derivatization, retention data, monoisotopic mass of the arising HFBCF derivatized product, diagnostic SIM ions, the used internal standard, the observed medium metabolite concentration in urine (12).

#### Table 2

GC-EI-SIM-MS operating conditions

Table 1.

No.	Traditional Name	The profiled metabolites as the HFBCF derivatives	RT (min)	MM product	Diagnostic ions		Internal Std <sup>b</sup>	L3 c [μM] <sup>1a</sup>	Stock solvent	Stock Solution c [mM]	Metabolite Database Coding			No. (12) <sup>a</sup>
					m/z <sup>1</sup>	m/z <sup>2</sup>					HMDB	PubChem	KEGG	
1	1-Methylhistidine	1-Methylhistidine	27.2	577.1	95	350	hF	40	0.1 M HCl	10	HMDB00001	92105	C01152	108
2	2,4-Diaminobutyric acid	2,4-Diaminobutyrate	24.74	752.0	282	256	hF	40	DI water	10	HMDB02362	470		101
3	2-Aminobutyric acid	2-Aminobutyrate	13.61	511.0	284	84	hF	20	DI water	10	HMDB00452	80283	C02356	36
4	2-Aminoheptanedioic acid	2-Aminopimelate	24.6	765.1	338	138	hF	40	DI water	10	HMDB34252	101122		99
5	2-Aminoisobutyric acid	2-Aminoisobutyrate	12.14	525.1	284	241	hF	40	DI water	50	HMDB01906	6119	C03665	27
6	2-Hydroxy-3-methylbutyric acid	2-Hydroxy-3-methylbutyrate (isovalerate)	11.16	526.0	55	299	PB	20	DI water	10	HMDB00407	99823		22
7	2-Hydroxy-3-methylpentanoic acid	2-Hydroxy-3-methylvalerate	13.06	540.1	284	484	PB	20	DI water	10	HMDB00317	10796774		34
8	2-Hydroxybutyric acid	2-Hydroxybutyrate	10.49	512.0	285	241	PB	20	DI water	10	HMDB00008	11266	C05984	19
9	2-Hydroxydecanedioic acid	2-Hydroxysebacate	27.34	808.1	95	381	PB	20	acetonitrile	10	HMDB00424	128458		112
10	2-Hydroxyglutaric acid	2-Hydroxyglutarate	18.34	738.0	283	239	PB	100	DI water	100	HMDB02307	439340	C00894	65
11	2-Hydroxyisobutyric acid	2-Hydroxyisobutyrate	8.74	512.0	241	285	PB	40	DI water	10	HMDB00729	11671		11
12	2-Hydroxyphenylacetic acid	2-Hydroxyphenylacetate	20.52	560.0	91	333	PB	20	DI water	50	HMDB00669	11970	C05852	82
13	2-Hydroxyvaleric acid	2-Hydroxy-valerate	12.12	526.0	55	299	PB	20	DI water	10	HMDB01863	98009		26
14	2-Ketoisovaleric acid	2-Ketoisovalerate	5.81	298.0	71	113	PB	20	DI water	10	HMDB00019	49	C00141	3
15	2-Methylcitric acid	2-Methylcitrate-4 (lactone)	18.46	552.0	152	334	PB	40	DI water	10	HMDB00379	515		68a
16	3-Aminoisobutanoic acid	3-Aminoisobutyrate	15.02	511.0	256	112	hF	100	0.1 M HCl	100	HMDB03911	64956	C05145	46
17	3-Hydroxyadipic acid	3-Hydroxyadipate	18.41	752.0	85	127	PB	100	0.1 M HCl	50	HMDB00345	151913		67
18	3-Hydroxybutyric acid	3-Hydroxybutyrate-2	11.93	512.0	268	69	PB	100	DI water	10	HMDB00357	441	C01089	24a
19	3-Hydroxyisovaleric acid	3-Hydroxyisovalerate-1 (OH)	6.46	300.1	59	85	PB	100	DI water	10	HMDB00754	69362		4a
20	3-Hydroxymethylglutaric acid	3-Hydroxy-3-methylglutarate	14.37	752.0	85	285	PB	100	DI water	10	HMDB00355	1662	C03761	41
21	3-Hydroxyphenylacetic acid	3-Hydroxyphenylacetate	22.61	560.0	333	277	PB	20	0.1 M HCl	100	HMDB00440	12122	C05593	91
22	3-Hydroxy-proline	3-Hydroxyproline-2	21.9	765.0	521	538	hF	20	0.1 M HCl	10	HMDB02113	11137200	C04397	89a
23	3-Hydroxysebacic acid	3-Hydroxysebacate-1 (OH)	20.01	582.1	71	271	PB	20	DI water	10	HMDB00350	3017884		79a
24	3-Hydroxyvaleric acid	3-Hydroxyvalerate-1 (OH)	8.21	300.1	71	271	PB	40	DI water	10	HMDB00531	107802		9a
25	3-Methoxytyramine	3-Methoxytyramine	32.36	845.1	319	376	hF	20	0.1 M HCl	10	HMDB00022	1669	C05587	131
26	3-Methyl-2-oxovaleric acid	2-Keto-3-methylvalerate	7.34	312.1	57	85	PB	20	DI water	10	HMDB00491	47	C03465	5
27	3-Methyladipic acid	3-Methyladipate	15.53	524.1	325	55	PB	20	DI water	50	HMDB00555	6999745		47
28	3-Methylcrotonylglycine	Methylcrotonylglycine	19.54	339.1	83	82	hF	40	DI water	10	HMDB00459	169485		76
29	3-Methylhistidine	3-Methylhistidine	28.38	577.1	95	150	hF	40	0.1 M HCl	10	HMDB00479	64969	C01152	116
30	4-Aminobenzoic acid	4-Aminobenzoate	27.29	545.0	146	345	hF	20	ethanol	10	HMDB01392	978	C00568	111
31	4-Hydroxybenzoic acid	4-Hydroxybenzoate	21.41	546.0	303	347	PB	20	ethanol	100	HMDB00500	135	C00156	87
32	4-Hydroxybutyric acid	4-Hydroxybutyrate	14.05	512.0	227	269	PB	100	DI water	100	HMDB00710	10413	C00989	38
33	4-Hydroxycinnamic acid	4-Hydroxycinnamate	27.98	572.0	329	572	PB	20	DI water	10	HMDB02035	637542	C00811	115
34	4-Hydroxymandelic acid	4-Hydroxymandelate	27.25	802.0	575	347	PB	20	DI water	10	HMDB00822	328	C11527	110
35	4-Hydroxyphenylacetic acid	4-Hydroxyphenylacetate	23.31	560.0	289	333	PB	40	0.1 M HCl	100	HMDB00020	127	C00642	96
36	4-Hydroxy-proline	4-Hydroxyproline-2	23.12	765.0	294	521	hF	20	0.1 M HCl	50	HMDB06055	69248	C01015	94a
37	4-Phenylbutyric acid (4PB, I.S.)	4-Phenylbutyrate	17.95	346.1	104	147			0.1 M NaHCO <sub>3</sub>	100				63
38	5-Aminolevulinic acid	5-Aminolevulinate	23.36	539.0	283	256	hF	40	0.1 M HCl	10	HMDB01149	137	C00430	97
39	5-Aminopentanoic acid	5-Aminovalerate	20.41	525.1	256	269	hF	40	0.1 M HCl	10	HMDB03355	138	C00431	81
40	5-Hydroxyhexanoic acid	5-Hydroxyhexanoate	16.67	540.1	227	113	PB	100	DI water	10	HMDB00525	170748		53
41	5-Hydroxyindoleacetic acid	5-Hydroxyindoleacetate	35.16	599.0	372	599	hF	20	DI water	10	HMDB00763	1826	C05635	144
42	5-Hydroxylysine	5-Hydroxylysine (isomers)	34.12	1022.1	269	256	hF	40	0.1 M HCl	50	HMDB00450	3032849	C16741	138b
43	Acetylglycine	N-Acetylglycine-2 (NH)	13.88	525.0	256	483	hF	200	DI water	10	HMDB00532	10972		37a
44	Aconitic acid (trans)	Aconitate	16.78	720.0	321	492	PB	200	DI water	100	HMDB00958	444212	C02341	54
45	Adipic acid	Adipate	14.95	510.1	282	311	PB	40	ethanol	100	HMDB00448	196	C06104	45
46	Alanine	Alanine	12.35	497.0	270	70	hF	40	0.1 M HCl	100	HMDB00161	5950	C00041	29
47	Amino adipic acid	2-Amino adipate	22.88	751.0	124	282	hF	40	0.1 M HCl	100	HMDB00510	469	C00956	93
48	Asparagine	Asparagine	19.1	522.0	295	95	hF	100	0.1 M HCl	100	HMDB00168	6267	C00152	70
49	Aspartic acid	Aspartate	18.3	723.0	254	496	hF	40	0.1 M HCl	100	HMDB00191	5960	C00049	64
50	Azelaic acid	Azelaate	21.23	552.1	353	152	PB	20	ethanol	10	HMDB00784	2266	C08261	86
51	Benzoic acid	Benzoate	10.74	304.0	105	304	PB	40	ethanol	100	HMDB01870	243	C00180	20
52	Beta-Alanine	3-Alanine	14.91	497.0	270	113	hF	40	0.1 M HCl	10	HMDB00056	239	C00099	44
53	Citraconic acid	Citraconate	10.29	494.0	295	267	PB	40	DI water	10	HMDB00634	643798	C02226	16
54	Citramalic acid	Citramalate-1 (lactone)	10.8	312.0	85	285	PB	100	DI water	10	HMDB00426	1081	C00815	21a
55	Citric acid	Citrate-2 (OH)	17.79	738.0	311	269	PB	400	DI water	100	HMDB00094	311	C00158	60a

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56	Cystathionine	Cystathionine	32.42	1038.0	328	282	hF	40	0.1 M HCl	100	HMDB00099	439258	C02291	132
57	Cysteine	Cysteine (total)**	22.22	755.0	328	285	hF	100	0.1 M HCl	100	HMDB00574	5862	C00097	90
58	Diaminopimelic acid	2,6-Diaminopimelate	29.45	1006.1	308	536	hF	40	DI water	10	HMDB01370	439283	C00666	124a
59	DOPA	3,4-Dihydroxyphenylalanine	32.63	1057.0	149	388	hF	40	0.1 M HCl	10	HMDB00609	836	C00355	135
60	Ethylmalonic acid	Ethylmalonate	8.78	496.0	297	468	PB	40	DI water	10	HMDB00622	11756		12
61	Ferulic acid ( <i>trans</i> )	4-Hydroxy-3-methoxycinnamate	30.64	602.0	602	375	PB	20	ethanol	100	HMDB00954	445858	C01494	130
62	Fumaric acid	Fumarate	9.53	480.0	281	253	PB	40	DI water	10	HMDB00134	444972	C00122	15
63	Glutamic acid	Glutamate	20.82	737.0	310	282	hF	40	0.1 M HCl	10	HMDB03339	23327	C00217	83
64	Glutamine	Glutamine	26.64	554.1	84	282	hF	100	DI water	100	HMDB00641	5961	C00064	107
65	Glutaric acid	Glutarate	12.51	496.0	227	297	PB	40	DI water	10	HMDB00661	743	C00489	30
66	Glyceric acid	Glycerate (2,3-Dihydroxypropionate)	17.68	740.0	113	497	PB	40	DI water	10	HMDB00139	439194	C00258	58
67	Glycine	Glycine	13.56	483.0	256	212	hF	200	DI water	100	HMDB00123	750	C00037	35
68	Glycolic acid	Glycolate	9.35	484.0	285	213	PB	40	DI water	100	HMDB00115	757	C00160	14
69	Glycylproline	Glycylproline	29.83	580.1	70	153	hF	40	0.1 M HCl	10	HMDB00721	79101		125
70	Hexanoylglycine	Hexanoylglycine-1 (cyclic)	14.07	155.1	99	71	hF	20	DI water	10	HMDB00701	99463		39a
71	Hippuric acid	Hippurate-1 (cyclic, 60%)	19.86	161.0	105	161	hF	400	ethanol	100	HMDB00714	464	C01586	78a
72	Histamine	Histamine	26.48	563.1	308	320	hF	40	0.1 M HCl	10	HMDB00870	774	C00388	106
73	Histidine	Histidine-2 (NR)	27.9	789.0	307	362	hF	200	0.1 M HCl	100	HMDB00177	6274	C00135	114a
74	Homocysteine	Homocysteine (total)**	24.68	769.0	282	342	hF	40	MeCN	10	HMDB00742	778	C05330	100
75	Homophenylalanine (HF, L.S.)	Homophenylalanine	25.98	587.1	91	283			0.1M NaHCO3	100				103
76	Homovanillic acid	Homovanillate	26.07	590.0	107	590	PB	20	0.1 M HCl	50	HMDB00118	1738	C05582	104
77	Hydroxisocaproic acid	2-Hydroxisocaproate	12.79	540.1	296	113	PB	20	DI water	10	HMDB00746	83697		32
78	Hydroxyphenyllactatic acid	4-Hydroxy-Phenyllactate	28.54	816.0	572	345	PB	20	DI water	10	HMDB00755	9378	C03672	118
79	Hydroxypropionic acid	3-Hydroxypropionate (dimer)	16.06	526.0	255	298	PB	100	DI water	10	HMDB00700	68152	C01013	52
80	Indolacetate	Indolacetate	27.61	357.1	130	357	hF	20	MeCN	10				113
81	Isoctic acid	Isocitrate	20.18	964.0	465	321	PB	100	DI water	100	HMDB00193	1198	C00311	80
82	Isoleucine	Isoleucine	15.93	539.1	283	312	hF	40	0.1 M HCl	10	HMDB00172	6306	C00407	50
83	Isovalerylglycine	Isovalerylglycine	17.77	341.1	85	525	hF	40	DI water	10	HMDB00678	546304		59
84	Ketoleucine	2-Ketoisocaproate	7.43	312.1	85	57	PB	20	DI water	10	HMDB00695	70	C00233	6
85	Kynurenic acid	Kynurenate	29.99	597.0	371	354	hF	40	NaHCO3	10	HMDB00715	3845	C01717	126
86	Kynurenine	Kynurenine	35.07	842.1	146	372	hF	40	0.1 M HCl	10	HMDB00684	161166	C00328	143
87	Lactic acid	Lactate	9.14	498.0	271	255	PB	100	DI water	100	HMDB00190	107689	C00186	13
88	Leucine	Leucine	15.84	539.1	312	270	hF	40	0.1 M HCl	50	HMDB00687	6106	C00123	49
89	Lysine	Lysine-2 (N,N-R)	28.79	780.1	310	256	hF	100	0.1 M HCl	100	HMDB00182	5962	C00047	119a
90	Malic acid	Malate	16.04	724.0	281	253	PB	40	DI water	10	HMDB31518	92824	C00497	51
91	Malonic acid	Malonate	7.51	468.0	269	407	PB	40	ethanol	10	HMDB00691	867	C00383	7
92	Mandelic acid	Mandelate	19.45	560.0	289	333	PB	20	DI water	10	HMDB00703	439616	C01984	74
93	Methionine	Methionine	21.65	557.0	61	357	hF	40	0.1 M HCl	10	HMDB00696	6137	C00073	88
94	Methionine sulfone	Methionine sulfone	28.51	589.0	282	82	hF	40	0.1 M HCl	10				117
95	Methylcysteine	S-Methylcysteine	19.13	543.0	61	300	hF	20	0.1 M HCl	10	HMDB02108	24417		71
96	Methylglutaric acid	3-Methylglutarate	12.93	510.1	311	282	PB	40	DI water	10	HMDB00752	12284		33
97	Methylmalonic acid	Methylmalonate	7.54	482.0	283	438	PB	40	DI water	10	HMDB00202	487	C02170	8
98	Methylsuccinic acid	Methylsuccinate	10.38	496.0	297	268	PB	40	DI water	50	HMDB01844	10349	C08645	18
99	N-Acetyl-aspartic acid	N-Acetylaspartate	18.38	539.0	270	312	hF	200	0.1 M HCl	50	HMDB00812	65065	C01042	66
100	Nicotinic acid	Nicotinate	12.17	305.0	106	78	hF	40	0.1 M HCl	10	HMDB01488	938	C00253	28
101	Ornithine	Ornithine	27.25	766.1	296	256	hF	40	0.1 M HCl	50	HMDB00214	6262	C00077	109
102	Oxalic acid	Oxalate	5.38	454.0	113	183	PB	200	DI water	10	HMDB02329	971	C00209	2
103	Oxoglutaric acid	2-Ketoglutarate-2 (80%)	14.6	510.0	283	284	PB	200	DI water	100	HMDB00208	51	C00026	42b
104	Palmitic acid	Palmitate	28.91	438.2	255	438	PB	20	ethanol	50	HMDB00220	985	C00249	121
105	Phenylacetic acid	Phenylacetate	12.65	318.0	91	318	PB	20	ethanol	10	HMDB00209	999	C07086	31
106	Phenylalanine	Phenylalanine	23.39	573.1	91	330	hF	40	0.1 M HCl	100	HMDB00159	6140	C00079	98
107	Phenyllactic acid	3-Phenyllactate	20.92	574.0	330	131	PB	20	DI water	10	HMDB00779	3848	C01479	84
108	Phenylpyruvic acid	Phenylpyruvate	22.84	572.0	118	329	PB	40	ethanol	10	HMDB00205	997	C00166	92
109	Phthalic acid	Phthalate	17.79	530.0	331	332	PB	20	DI water	10	HMDB02107	1017	C01606	61
110	Pimelic acid	Pimelate	17.09	524.1	296	325	PB	20	ethanol	10	HMDB00857	385	C02656	56
111	Proline	Proline	16.84	523.0	296	297	hF	40	0.1 M HCl	10	HMDB00162	145742	C00148	55
112	Prolylhydroxyproline	Prolylhydroxyproline	34.6	862.1	296	297	hF	100	0.1 M HCl	10	HMDB06695	11902892		142
113	Propionylglycine	Propionylglycine	15.55	313.1	57	56	hF	100	DI water	10	HMDB00783	98681		48
114	Propyl pentanoate	2-Propylvalerate	8.61	326.1	255	284	PB	40	DI water	10	HMDB40296	67328		10

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115	Pyroglutamic acid	Pyroglutamate	18.9	537.0	310	84	hF	200	DI water	100	HMDB00267	7405	C01879	69
116	Salicylic acid	Salicylate	19.41	546.0	120	303	PB	20	DI water	10	HMDB01895	338	C00805	73
117	Salicylic acid	2-Hydroxyhippurate	28.82	603.0	120	403	hF	40	0.1 M HCl	10	HMDB00840	10253	C07588	120
118	Sarcosine	Sarcosine	12.07	497.0	270	226	hF	20	0.1 M HCl	10	HMDB00271	1088	C00213	25
119	Sebacic acid	Sebacate	23.16	566.1	98	367	PB	40	ethanol	10	HMDB00792	5192	C08277	95
120	Serine	Serine	19.6	739.0	268	295	hF	100	DI water	100	HMDB00187	5951	C00065	77
121	Stearic acid	Stearate	32.5	466.3	255	466	PB	20	ethanol	50	HMDB00827	5281	C01530	134
122	Suberic acid	Suberate	19.2	538.1	339	138	PB	20	MeCN	10	HMDB00893	10457	C08278	72
123	Succinic acid	Succinate	10.32	482.0	283	55	PB	40	DI water	100	HMDB00254	1110	C00042	17
124	Thioprolin	Thioprolin	19.46	541.0	314	287	hF	20	0.1 M HCl	10				75
125	Threonine	Threonine-1 (OH)	17.42	527.0	100	283	hF	100	0.1 M HCl	100	HMDB00167	6288	C00188	57a
126	Tiglylglycine	Tiglylglycine	11.27	339.1	83	55	hF	40	DI water	50	HMDB00959	6441567		23
127	Tryptamine	Tryptamine	34.18	612.1	130	386	hF	20	0.1 M HCl	10	HMDB00303	1150	C00398	139
128	Tryptophan	Tryptophan	34.44	612.1	130	131	hF	40	NaOH	100	HMDB00929	6305	C00078	141
129	Tyramine	Tyramine	30.06	589.1	346	333	hF	20	ethanol	10	HMDB00306	5610	C00483	127
130	Tyrosine	Tyrosine	30.58	815.0	333	289	hF	40	0.1 M HCl	100	HMDB00158	6057	C00082	129
131	Urocanic acid	trans-Urocanate	25.39	546.0	347	546	hF	20	DI water	10	HMDB34174	1549103		102
132	Valine	Valine	14.1	525.1	298	283	hF	40	0.1 M HCl	100	HMDB00883	6287	C00183	40
133	Vanillic acid	4-Hydroxy-3-methoxyphenyllactate	30.38	846.0	375	561	PB	20	DI water	10	HMDB00913	160637		128
134	Vanillylmandelic acid	Vanillylmandelate	28.92	832.0	832	377	PB	20	0.1 M HCl	10	HMDB00291	736172	C05584	122

**Table 2.**

GC Injector	Mode	Pulsed splitless (elevated head pressure from 110 to 220 kPa)
	Liner	Sky® 4 mm I.D. cyclo double taper inlet liner (Restek, P/N 23310)
	Temperature	220 °C
	Injection volume	1 µL
	Temperature	220 °C
GC Oven	Initial temperature	60 °C
	Ramp	5 °C/min to 240°C, 20 °C/min to 320°C
	Run time	38 min
GC Column	Capillary column	ZB-XLB type, 30 m x 0.25 mm ID, 0.25 µm film thickness (Phenomenex, P/N 7HG-G019-11)
	Carrier gas	Helium
	Flow rate	1.2 mL/min
	Mode	Constant flow
	Outlet pressure	vacuum
GC-MS Transfer Line	Temperature	250 °C
MS Quadrupole	Ion source temperature	230 °C
	Full scan mode	m/z 40 – 900 Da
	SIM mode	metabolite SIM m/z ions, see <b>Table 1</b>
Software	MSD ChemStation (version E.02, Agilent)	